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Entwicklung und Anwendung von MS-Bindungsassays für die Glycintransportersubtypen 1 (GlyT1) und 2 (GlyT2)

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Eidesstattliche Versicherung:

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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„Wissen gibt Macht,
aber der Charakter verschafft Respekt und Anerkennung.“

Bruce Lee

Diese kumulative Dissertation ist auf folgenden originalen Publikationen aufgebaut:

1. Publikation:

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„MS binding assays for GlyT1 based on Org24598 as nonlabelled reporter ligand“

2. Publikation:

Thomas M. Ackermann, Lars Allmendinger, Georg Höfner und Klaus T. Wanner; *ChemMedChem* **2021**, *16*, 199-215

„MS Binding Assays for Glycine Transporter 2 (GlyT2) Employing Org25543 as Reporter Ligand”

3. Publikation:

Thomas M. Ackermann, Georg Höfner und Klaus T. Wanner; *ChemMedChem* **2021,** *16*, 3094-3104

„Screening for new Inhibitors of Glycine Transporter 1 and 2 by means of MS Binding Assays”

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Thomas M. Ackermann und Klaus T. Wanner

„MS Binding Assays for GlyT1 based on Org24598 as nonlabelled reporter ligand”

Inhaltsverzeichnis

1 Einleitung

Das Nervensystem stellt das komplexeste Organsystem des menschlichen Organismus dar. Es besteht aus Milliarden von Neuronen und umgebenden Gliazellen[1] und ermöglicht uns Menschen durch seine vielfältigen Funktionen wie zum Beispiel Koordination von Bewegungen, Kommunikation, Umweltwahrnehmungen durch unsere Sinne, aber auch Denken, Lernen, Fühlen und Erinnern ein autonomes sowie stetig fortschreitendes Leben. Viele Funktionen des Nervensystems konnten dank jahrelanger Forschung und immer besser werdender Techniken erforscht werden, allerdings „wissen wir aber gerade einmal fünf Prozent von dem, was im Gehirn vor sich geht – vielleicht ist es auch nur ein Prozent“ (Zitat Prof. Dr. Thomas Südhof, Professor für Molekulare und Zelluläre Physiologie an der Stanford University in Kalifornien).[2] Wie Informationen innerhalb des Nervensystems zwischen Nervenzellen weitergeleitet werden, wird jedoch bereits sehr gut verstanden. Hierfür sind sogenannte Synapsen zuständig, hochspezialisierte Strukturen, die Verbindungen zwischen Nervenzellen oder aber auch zum Beispiel zwischen Nerven- und Muskelzellen herstellen.[3] Synapsen können in elektrische und chemische Synapsen unterteilt werden. Elektrische Synapsen ermöglichen eine Weiterleitung der Informationen direkt als elektrisches Signal über Gap Junctions.[3] Bei einer chemischen Synapse hingegen kommen Botenstoffe, sogenannte Neurotransmitter, für die Signalweiterleitung zum Einsatz, die präsynaptisch in Vesikeln gespeichert werden.[3] Durch ein ankommendes elektrisches Signal in der Präsynapse und der damit verbundenen Depolarisation ebendieser kommt es zu einem Ca^+ Einstrom aufgrund der Aktivierung von spannungsabhängigen Ca^+-Kanälen, was eine Fusion der Vesikel mit der Membran der Präsynapse zur Folge hat. Dadurch werden die Neurotransmitter in den synaptischen Spalt freigesetzt und können dann an der Postsynapse an ionotrope oder G-Protein-gekoppelte Rezeptoren binden, wodurch entweder Ionenkanäle geöffnet oder G-Protein-gekoppelte Signalkaskaden ausgelöst werden.[3] Die Neurotransmission im Bereich der Synapsen obliegt einer strengen Regulation. Eine gestörte Neurotransmission zum Beispiel durch fehlerhafte Proteine, hervorgerufen durch Mutationen, oder externe Einflüsse wie Stress oder dem (unbewussten) Einsatz von ZNS-aktiven Substanzen, kann zur Ausbildung von

Krankheiten, die das zentrale Nervensystem betreffen, führen (z.B Morbus Parkinson, Depression, Schizophrenie, etc.).[4]

Glycin, als einer der wichtigsten Neurotransmitter des Zentralnervensystems (ZNS), spielt sowohl in der inhibitorischen glycinergen als auch der exzitatorisch glutamatergen Neurotransmission eine entscheidende Rolle. Die Regulation der Neurotransmission, an der Glycin beteiligt ist, obliegt u.a. den Glycintransportern GlyT1 und GlyT2.[5] Bei einer gestörten glycinergen oder glutamatergen Neurotransmission (in beiden Fällen bei einer verringerten Neurotransmission) können Inhibitoren der Glycintransporter dazu beitragen, die Signaltransduktion zu verlängern, indem sie die Glycinkonzentrationen im synaptischen Spalt erhöhen. Bei Studien zur Wirkung von GlyT-Inhibition auf den Organismus wurden vielversprechende Ergebnisse erhalten. So zeichnen sich neue Strategien zur Behandlung von z.B. der Schizophrenie, affektiver und kognitiver Störungen, der Alkoholsucht, der Schmerztherapie, Epilepsie oder der Hyperekplexie ab.[5,6] Aus diesem Grund, trat in den letzten Jahren die Suche nach Glycintransporter-Inhibitoren verstärkt in den Vordergrund.

Um eine Substanz als Glycintransporter-Inhibitor identifizieren zu können, sind Bindungs- oder Transportstudien das Mittel der Wahl. Die bis dato am häufigsten eingesetzten Methoden sind Bindungs- bzw. Transportstudien und beruhen auf der Verwendung von Radioliganden.[7,8,9,10,11,12,13,14,15,16,17,18] Diese Studien verursachen allerdings aufgrund der Anschaffung und Nutzung radioaktiv markierter Substanzen sowie der Entsorgung hohe Kosten und sie stellen ein erhöhtes gesundheitliches Risiko dar, weshalb besondere Sicherheitsmaßnahmen getroffen werden müssen.[19] Deshalb sind Alternativen zu diesen Assays, bei denen kein radioaktives Material nötig ist, von großem Vorteil. So war es das Ziel dieser Arbeit, für die Neurotransmittertransporter GlyT1 und GlyT2 Bindungsassays als Ersatz für analoge Radioligand-Bindungsassays zu entwickeln. Diese neu zu entwickelnden Assays sollten dabei dem im Arbeitskreis etablierten Konzept der Bindungsstudien folgen, bei dem nicht markierte Substanzen als Reporterliganden verwendet und mittels LC-MS/MS-Analytik quantifiziert werden. Nach der Entwicklung dieser MS-Bindungsassays für GlyT1 bzw. GlyT2 sollte schließlich eine Substanzbibliothek von knapp 2400 Verbindungen auf neue Glycintransporter-Inhibitoren untersucht werden.

1.1 Bildung und Funktionen von Glycin im menschlichen Körper

Glycin stellt die einfachste proteinogene Aminosäure des menschlichen Körpers dar und wird von diesem selbst synthetisiert, weshalb es sich um eine nicht-essenzielle Aminosäure handelt. Für die Biosynthese von Glycin stehen unterschiedliche Möglichkeiten zur Verfügung, wie z.B. aus Cholin über den Weg des Sarkosins, durch Threonin-Abbau, über den Carnitin-Syntheseweg oder die Transaminierung von Glyoxylat.[20] Die größte Bedeutung in der Glycin-Biosynthese spielt allerdings die Gewinnung aus L-Serin.[20] Hierbei wird eine Methylengruppe (C-3 des Serins) mittels des Enzyms Glycinhydroxymethyltransferase (GHMT) im Zusammenspiel mit Pyridoxalphosphat auf das Coenzym Tetrahydrofolat (THF) übertragen. Dadurch entsteht das gewünschte Glycin sowie die Nebenprodukte N^5-N^{10}-Methylen-Tetrahydrofolat und H_2O (**Abb. 1**).[20]

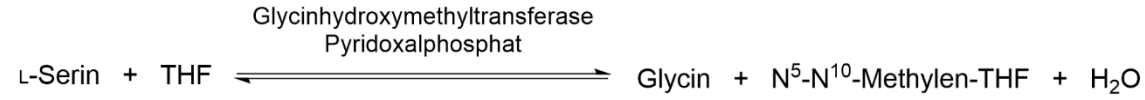


Abb. 1: Glycin-Biosynthese aus L-Serin

Glycin als proteinogene Aminosäure ist ein wichtiger Bestandteil nahezu aller Proteine und spielt im Bereich des Stoffwechsels eine entscheidende Rolle, wie z.B. der Generierung von L-Serin (Reversibilität der GHMT-Reaktion), der Beteiligung zur Bildung von Glutathion oder der Biosynthese von Porphyrinen, Purinen sowie des Kreatinins.[21]

Neben den eben erwähnten Funktionen wird Glycin eine weitere wichtige Funktion zuteil, nämlich als Neurotransmitter im Nervensystem. Hierbei tritt es u.a. als inhibitorischer Neurotransmitter an inhibitorischen glycinergen Synapsen auf. An diesen Synapsen bindet Glycin, welches präsynaptisch in Vesikeln gespeichert und in den synaptischen Spalt freigesetzt wird, an der Postsynapse an der Strychnin-sensitiven Glycin-A-Bindungsstelle des ionotropen Glycinrezeptors (GlyR). Dadurch kommt es zu einem Einströmen von Chlorid in die Postsynapse, was eine Hyperpolarisation zur Folge hat, die zu einer Hemmung des postsynaptischen Neurons führt (**Abb. 2a**).[22]

Des weiteren tritt Glycin, neben L-Glutamat, als Coagonist an ionotropen NMDA (*N*-Methyl-D-Aspartat)-Rezeptoren an exzitatorischen glutamatergen Synapsen auf (**Abb. 2b**). An diesen Rezeptoren bindet es an der Strychnin-insensitiven Glycin-B-

Bindungsstelle der NR1-Untereinheit (**Abb. 2c**) und dient dabei als positiv allosterischer Modulator, wodurch sich die Affinität des L-Glutamats durch eine Konformationsänderung des Rezeptors erhöht und dadurch die Erregbarkeit des Rezeptors erhöht wird. Bei simultaner Bindung von L-Glutamat und Glycin am Rezeptor sowie gleichzeitiger ausreichender Depolarisation der Postsynapse durch die Aktivierung von AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolpropionsäure)-Rezeptoren kommt es zu einer „Öffnung“ des Ionenkanals (Blockade des NMDA-Rezeptors durch Mg^{2+} wird aufgehoben), wodurch Ca^{2+}-Ionen in die Postsynapse einströmen. Dadurch wird eine stärkere Depolarisation der postsynaptischen Membran initiiert und zusätzlich fungiert das einströmende Ca^{2+} als Second Messenger im postsynaptischen Neuron.[6,23]

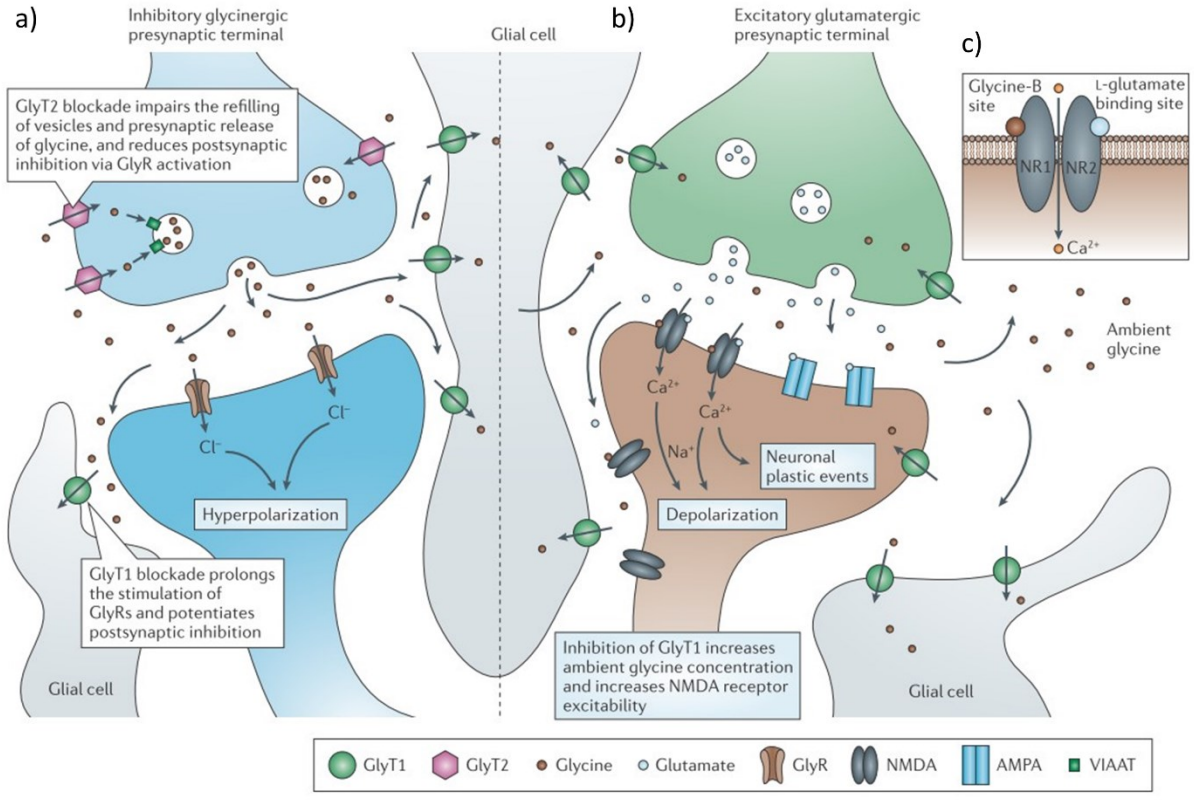


Abb. 2: Schematische Darstellung einer inhibitorisch glycinergen (a) und einer exzitatorisch glutamatergen Synapse (b+c). [6]

1.2 Glycintransporter GlyT1 und GlyT2

Wie bereits erwähnt bedarf es bei der Neurotransmission an chemischen Synapsen einer sehr genauen Regulierung der Neurotransmitter. Im Falle der Neurotransmission, an der Glycin beteiligt ist, geschieht dies durch die

Glycintransporter GlyT1 und GlyT2, welche die extrazelluläre Glycinkonzentration steuern. Diese Transporter sind Teil der Na^+/Cl^--abhängigen Aminosäure-Transporter der SLC6-Neurotransmittertransporter-Familie.[24] Zu dieser Familie zählen unter anderem auch die Transporter der Monoamine Dopamin, Serotonin und Norepinephrin (DAT, SERT und NET) sowie für die γ-Aminobuttersäure (GABA; GAT1, GAT2, GAT3, BGT1). Die beiden Transporter GlyT1 und GlyT2 treten in unterschiedlichen Varianten auf, die durch alternative Promotoren oder durch alternatives Splicing entstehen. Bisher sind von GlyT1 fünf Varianten bekannt (GlyT1a-e) während GlyT2 drei Varianten (GlyT2a-c) aufweist.[6] Obwohl GlyT1 als auch GlyT2 mit Glycin das gleiche Substrat aufweisen, ähneln sich beide „nur“ zu 50 % in ihrer Aminosäuresequenz. Allerdings unterscheiden sie sich auch in ihrer Pharmakologie und damit in ihrer Funktion (siehe Abschnitt 1.2.3).[7]

1.2.1 Der Leucintransporter $LeuT_{Aa}$ des Aquifex aeolicus als Homologiemodel

Für die Erstellung aussagekräftiger Struktur-Aktivitäts-Beziehungen als Grundlage für die Entwicklung hoch affiner Moleküle für definierte Zielproteine ist die Kenntnis der genauen Struktur des jeweiligen Zielproteins unabdingbar. Für die Proteine der SLC6-Neurotransmittertransporter-Familie und damit auch für GlyT1 und GlyT2 diente hierfür lange Zeit der Leucintransporter $LeuT_{Aa}$ als Homologiemodel. Im Jahr 2005 gelang es der Gruppe um Eric Gouaux die 3-dimensionale Struktur dieses Transporters des termophilen Bakteriums Aquifex aeolicus (ebenfalls ein Natrium-getriebener Symporter) durch Röntgenstrukturanalyse zu entschlüsseln.[25] Die Aminosäuresequenz dieses prokaryotischen Transporters stimmt zu 20-25 % mit den eukaryotischen SLC6-Transportern überein, bei Betrachtung des Kernbereichs, welcher für den eigentlichen Transport zuständig ist, kann allerdings eine 55-67 % Übereinstimmung ausgemacht werden.[26] Dadurch kann $LeuT_{Aa}$ als gutes Modell herangezogen werden, um den Transportmechanismus und die Funktionsweise der SLC6-Transporter zu beschreiben.[27] Wie alle SLC6-Transporter ähnelt auch der Aufbau der Glycintransporter dem Aufbau des $LeuT_{Aa}$. $LeuT_{Aa}$ besteht aus zwölf transmembranären α-Helices, auch Transmembrandomänen (TMD) genannt, welche eine V-förmige Struktur ausbilden. Diese V-förmige Struktur wird durch TMD 1-5 sowie TMD 6-10 gebildet, welche einer Pseudosymmetrie folgen, die als „5+5 inverted repeat“ bezeichnet wird. Der innere Kern, der die Substratbindungsstelle S1 und die Natriumbindungsstellen Na1 und Na2 enthält sowie für den Transport zuständig ist,

wird von TMD 1,3,6 und 8 gebildet (**Abb. 3a**). Die Art der Transporterstruktur wurde von Yamashita et al. als „hohles Schnapsglas“ beschrieben, wobei die Öffnung der extrazellulären Seite zugewandt ist, das untere Ende der intrazellulären Seite und der Boden im Inneren des Glases, also der Teil, wo sich die Bindungsstelle des Substrats befindet, sich ungefähr in der Mitte der Zellmembran befindet, was man gut in der Röntgenkristallstruktur in **Abb. 3b** erkennen kann.[25] Zusätzlich zu den transmembranären α-Helices, zeigt der Transporter extrazelluläre Loops (EL), an denen unter anderem Glykosylierungen stattfinden, als auch intrazelluläre Loops (IL) (**Abb. 3a**).

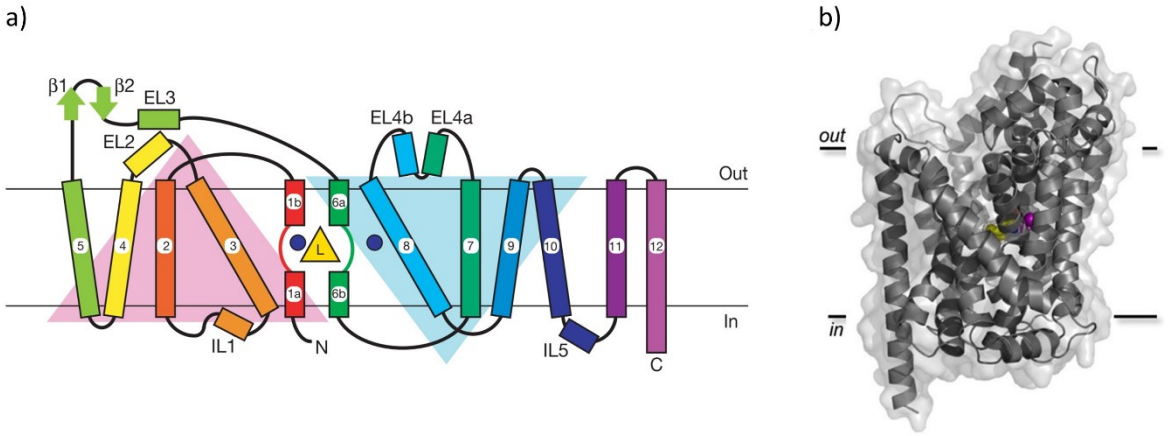


Abb. 3: Topologie und Röntgenstrukturanalyse des $LeuT_{Aa}$. a) Dargestellt wird die Topologie des $LeuT_{Aa}$. TMD 1-5 und TMD 6-10 bilden in ihrer Tertiärstruktur einen V-förmigen Aufbau und stellen damit das „5+5 inverted repeat“ dar, das in der zweidimensionalen Abbildung durch das rosa und blaue Dreieck verbildlicht werden soll. Der innere Kern, der für die Bindung des Leucins und der Natriumionen zuständig ist (dargestellt als gelbes Dreieck und zwei blaue Kugeln), wird von TMD 1,3,6 und 8 gebildet.[25] b) Röntgenkristallstruktur des $LeuT_{Aa}$ in der substratgebundenen, „outward-occluded“ Konformation. Leucin in Gelb und zwei Natriumionen in Lila als Van der Waals Kugeln dargestellt.[26]

Der Transport des Substrats findet u.a. durch eine Konformationsänderung des Transporters statt und wird als „alternating access“ bezeichnet (der Ablauf des Transportmechanismus wird in Abschnitt 1.2.3 genauer erläutert). Dabei spielen TMD 1, 3, 6 und 8 eine maßgebliche Rolle. Ganz allgemein beschreibt der Begriff „alternating access“ einen Substrattransport, bei dem der Transporter zur selben Zeit immer nur zu einer Seite hin, also zur extrazellulären oder interzellulären, geöffnet ist.[26] Mittels Röntgenstrukturanalysen des $LeuT_{Aa}$ konnten drei Zustände beschrieben werden, die diese Transporter dabei annehmen können: „Outward-open“-, „occluded“- und „inward-open“-Konformation (**Abb. 4**).[24] Durch die Bindung des Substrats sowie der Ionen an ihren Bindungsstellen kommt es zunächst zu einer Schließung des sog. extrazellulären „Gates“ und der Transporter befindet sich im „occluded“-Zustand. Danach wird das sog. intrazellulär „Gate“ geöffnet und Substrat und Ionen werden in

das Zytoplasma freigegeben.[26] Bei genauer Betrachtung von TMD 1 und TMD 6 kann man erkennen, dass diese antiparallel angeordnet sind und ungefähr in der Mitte der Membran einen Bruch in ihrer helikalen Struktur aufweisen (**Abb. 3a**). Einerseits bilden diese dadurch eine Bindungstasche, in der der Neurotransmitter und die Ionen durch Ausbildung von H-Brückenbindungen und Ionenkoordinationen mit den Carbonylsauerstoffen und Stickstoffatomen des Proteinrückgrats binden können, andererseits scheinen die Brüche als eine Art Scharnier für den Transportmechanismus zu dienen. TMD 3 und TMD 8 hingegen bilden zusammen mit TMD 6 eine hydrophobe Tasche aus, in die sich hydrophobe Seitenketten von Neurotransmittern hin orientieren, wie z.B. die aliphatische Seitenkette des Leucins.[25]

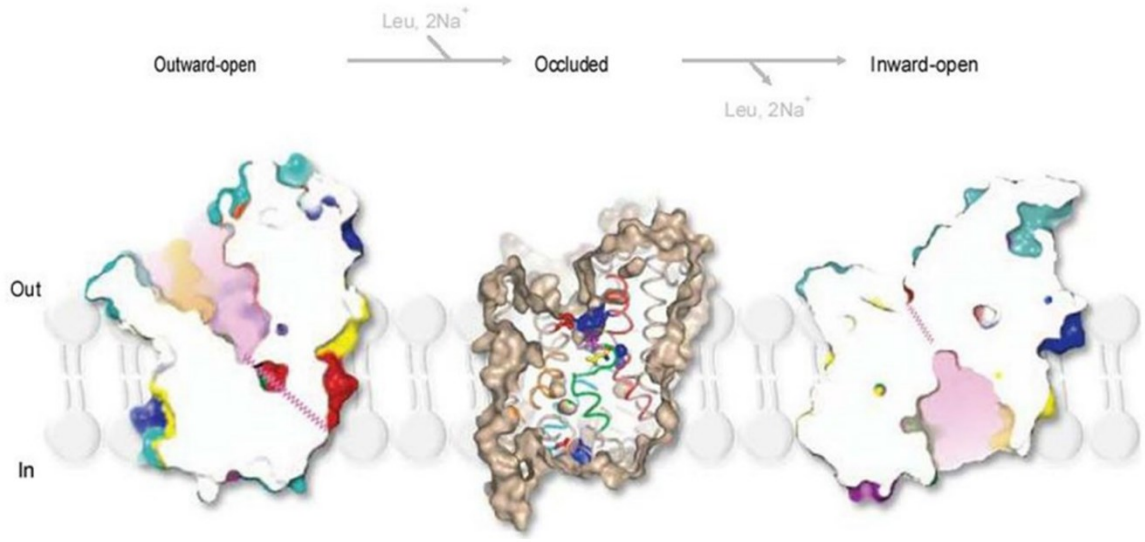


Abb. 4: Konformationen des $LeuT_{Aa}$: Die zur extrazellulären Seite geöffnete „outward-open"-Konformation (links), die zu beiden Seiten geschlossene „occluded"-Konformation (mitte) und die zur intrazellulären Seite geöffnete „inward-open"-Konformation (rechts).[24]

Neben der $LeuT_{Aa}$-Struktur als Basis eines Homologiemodels konnten inzwischen die Strukturen weiterer Transporter bestimmt werden, die $LeuT_{Aa}$ sehr ähnlich sind. Bei einem dieser Transporter handelt es sich um den Dopamintransporter (dDAT) von Drosophila melanogaster,[28] ein zweiter ist der humane Serotonintransporter (hSERT).[29] Speziell die hSERT-Struktur soll als „Blueprint" für die zukünftige Arzneistoffentwicklung für ebendiesen dienen, wie die Autoren schreiben. Die für die Entwicklung neuer GlyT-Inhibitoren derzeit bedeutenste Entdeckung gelang jedoch 2021 Poul Nissen und Mitarbeitern mit der erstmaligen Bestimmung der GlyT1-Struktur.[30] Durch die Bindung eines Inhibitors und eines single-domain Antikörpers (Sybody) war es der Gruppe möglich, den Transporter außerhalb einer Zellmembran zu kristallisieren und dessen Struktur durch Synchrotronkristallographie zu

charakterisieren. Dabei zeigte sich, dass dieser in der inward-open Konformation vorliegt und der bei der Kristallisation eingesetzte Inhibitor im Bereich des intrazellulären „Gates“ des Transporters bindet und sich dessen Bindungsstelle teilweise mit der Glycin-Bindungsstelle überschneidet. Es wird vermutet, dass der Inhibitor den Transporter mit hoher Wahrscheinlichkeit von der cytosolischen Seite blockiert, was bisher noch für keinen anderen Transporter der SLC6-Familie beschrieben wurde. Diese neu gewonnenen Erkenntnisse dürften künftig einen entscheidenden Beitrag zur Entwicklung neuer und besonders potenter GlyT1-Inhibitoren leisten.

1.2.2 Lokalisation

Glycinerge Neurotransmission tritt sowohl an inhibitorisch glycinergen sowie an exzitatorisch glutamatergen Synapsen auf. Es konnte dabei festgestellt werden, dass GlyT1 an beiden Typen von Synapsen vorkommt, während GlyT2 nur an den inhibitorischen gefunden wird.[5] In **Abb. 2** kann man sehr gut die Verteilung der beiden Transporter an den unterschiedlichen Synapsen erkennen, als auch die zelluläre Verteilung ebendieser. GlyT2 ist ausschließlich am präsynapstischen Ende der inhibitorischen Synapse lokalisiert, während sich GlyT1 an der inhibitorischen Synapse nur auf den umliegenden Gliazellen befindet. An den exzitatorischen Synapsen hingegen findet man GlyT1 sowohl auf den prä- und postsynaptischen Enden als auch auf den umliegenden Gliazellen.[6] In ihrer Gewebeverteilung im ZNS zeigen GlyT1 und GlyT2 sowohl Ähnlichkeiten als auch Unterschiede. Beide konnten in den Bereichen des Rückenmarks, Hirnstamms und Kleinhirns ausfindig gemacht werden, GlyT1 jedoch zusätzlich in den Bereichen des Vorderhirns wie dem Cortex oder dem Hippocampus. Vor allem der Cortex und der Hippocampus können mit hohen Konzentrationen an NMDA-Rezeptoren in Verbindung gebracht werden und sind somit Bereiche, in denen eine exzitatorische glutamaterge Neurotransmission stattfindet.[31]

1.2.3 Transportmechanismus

Die Transporter der SLC6-Familie haben alle gemeinsam, dass es sich um Na^+/Cl^--abhängige Transporter handelt. Der Transport des Substrats bedarf keiner direkten Energiequelle, wie z.B. ATP, allerdings wird der Transport durch einen Natriumgradienten getrieben, der wiederum durch eine Na^+-K^+-ATPase aufrechterhalten wird.[24] Die Rolle des Chlorids, das ebenfalls transportiert wird,

konnte noch nicht vollständig geklärt werden.[26] Während des Transports nimmt der Transporter die in Abschnitt 1.2.1 dargestellten Konformationen ein und unterliegt somit dem sogenannten „alternating access“.

Bei Betrachtung der Stöchiometrie der beiden Glycintransporter-Subtypen kann man einen entscheidenden Unterschied feststellen. Während GlyT1 pro Glycinmolekül $2Na^+/Cl^-$ transportiert, werden bei GlyT2 pro Glycinmolekül $3Na^+/Cl^-$ transportiert.[32] Dies hat Auswirkungen auf deren Pharmakologie und damit auf deren Funktion. Aufgrund der geringeren Triebkraft des Natriumgradienten bei GlyT1 hat dieser die Möglichkeit bei einer Änderung des Membranpotentials (Depolarisation) und/oder der Glycinkonzentration extrazellulär bzw. der Na^+/Cl^--Konzentration intrazellulär den Glycintransport umzukehren (reverser GlyT1-Transport) und somit Glycin in den synaptischen Spalt abzugeben (**Abb. 5a**).[33] Dies ermöglicht (vor allem an glutamatergen exzitatorischen Synapsen) eine feine Regulierung von sehr geringen Glycinkonzentration im synaptischen Spalt, die, im Gegensatz zur üblichen Freisetzung von Glycin, Ca^{2+}-unabhängig und nicht-vesikulär stattfindet.[34]

GlyT2 hingegen kann im Vergleich zu GlyT1 aufgrund der gegebenen Stöchiometrie eine stärkere Triebkraft aufbringen und kann damit über einen größeren Konzentrationsbereich wirken. Dies hat zur Folge, dass GlyT2 intrazelluläre Glycinkonzentrationen von 20-40 mM im Bereich der Präsynapse ermöglicht. Diese hohen Konzentrationen sind notwendig, um die präsynaptischen Vesikel über den vesikulären inhibitorischen Aminosäure Transporter (VIAAT), an dem Glycin weder spezifisch transportiert wird noch eine gute Affinität aufweist, wieder mit Glycin befüllen zu können.[33] Ein reverser Transport, wie es GlyT1 unter bestimmten Bedingungen ermöglicht, steht GlyT2 unter physiologischen Bedingungen nicht zur Verfügung (**Abb. 5b**).[33]

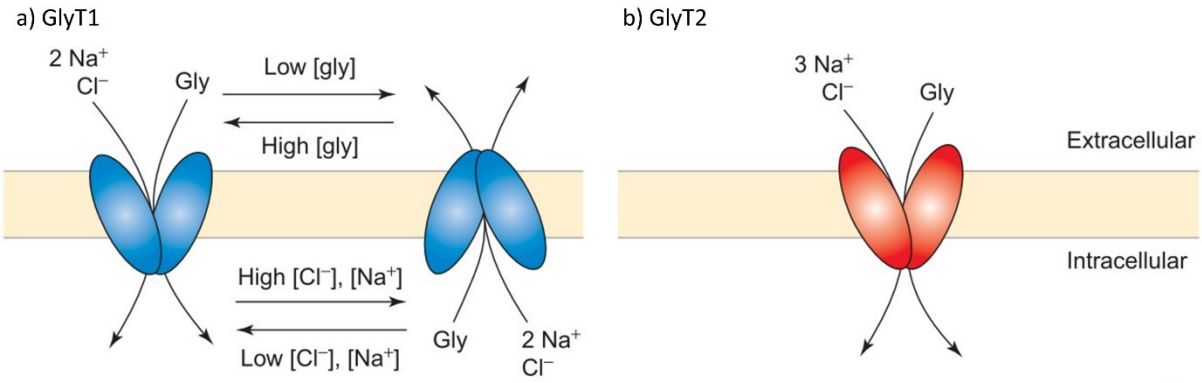


Abb. 5: Transportmechanismus und Stöchiometrien der Glycintransporter: Na^+/Cl^--abhängiger Transport der Glycintransporter GlyT1 und GlyT2. a) GlyT1 benötigt für den Transport eines Glycinmoleküls 2 Na^+ und 1 Cl^-. Je nach extrazellulärer Glycinkonzentration bzw. intrazellulärer Na^+- und Cl^--Konzentration und dem Membranpotential besitzt GlyT1 die Möglichkeit den Transport umzukehren und Glycin nach außen zu transportieren. b) GlyT2 benötigt für den Transport eines Glycinmoleküls 3 Na^+ und 1 Cl^-. Im Gegensatz zu GlyT1 kann GlyT2 Glycin in der Regel nur vom Extra- in den Intrazellulärraum transportieren.[34]

1.2.4 Funktion

Im übergeordneten Sinn ist es die Hauptaufgabe der Glycintransporter GlyT1 und GlyT2 den Neurotransmitter Glycin und somit die Neurotransmission sowohl an inhibitorisch glycinergen als auch an exzitatorisch glutamatergen Synapsen zu regulieren. Allerdings konnte gezeigt werden, dass sich die Transporter in ihren Funktionen unterscheiden. An inhibitorischen Synapsen ist GlyT1 hauptsächlich für die Regulation der Glycinkonzentration im synaptischen Spalt zuständig und beendet die glycinerge Neurotransmission durch den Transport des Neurotransmitters in die umliegenden Gliazellen, wodurch die Aktivität dieser Synapsen verringert wird. Außerdem soll es einen sogenannten „Spillover“ von Glycin zu benachbarten Synapsen verhindern.[6,35] An exzitatorischen Synapsen hingegen soll GlyT1 die Glycinkonzentration im synaptischen Spalt unterhalb der Sättigungskonzentration für die Glycin-B-Bindungsstelle halten, um so einer Überstimulation dieser Synapsen vorzubeugen.[35] Durch den reversen GlyT1 Transport besteht außerdem die Möglichkeit die Glycinkonzentration im synaptischen Spalt zu erhöhen und dadurch die NMDA-Rezeptor-Aktivität zu verstärken.[5]

Der GlyT2-Transporter, welcher nur an inhibitorischen Synapsen vorkommt, besitzt zwei Funktionen: Erstens, GlyT2 besitzt wie GlyT1 die Fähigkeit Glycin aus dem synaptischen Spalt zu entfernen und dadurch die Neurotransmission zu unterbrechen.

Durch die im Gegensatz zu GlyT1 stärkeren Transportkraft sind zudem Glycinkonzentrationen im synaptischen Spalt möglich, die im niedrig nanomolaren Bereich liegen und deshalb sogar „low-level“ GlyR-Aktivitäten unterbinden.[5] Zweitens, hat diese höhere Triebkraft beim Transport zur Folge, dass GlyT2, wie unter Abschnitt 1.2.3 beschrieben, intrazelluläre Glycinkonzentrationen im präsynaptischen Neuron ermöglicht, die notwendig sind, um präsynaptische Vesikel über VIAAT aufzufüllen und somit wiederrum Glycin für eine erneute Freisetzung in den synaptischen Spalt bereitzustellen, wodurch die Neurotransmission aufrechterhalten werden kann. Dies kann als eine Art Recycling-Funktion von GlyT2 angesehen werden.[5]

1.2.5 Inhibition

Ganz allgemein hat die Inhibition von Neurotransmitter-Transportern eine reduzierte Aufnahme der Neurotransmitter in die entsprechenden Zellen zur Folge und ist somit für eine erhöhte Konzentration ebendieser im synaptischen Spalt verantwortlich, was zu einer Verstärkung und/oder Verlängerung des postsynaptischen Signals führt. Im Falle der Glycintransporter GlyT1 und GlyT2 bedeutet dies, dass sowohl an inhibitorisch glycinergen als auch exzitatorisch glutamatergen Synapsen erhöhte Glycinkonzentrationen im synaptischen Spalt generiert werden können. Dieser Umstand wird versucht sich zu Nutze zu machen, um Therapiemöglichkeiten für Krankheiten, die mit einer verringerten glycinergen oder glutamatergen Neurotransmission in Verbindung gebracht werden, zu entwickeln.

1.2.5.1 Inhibition von GlyT1

Die Inhibition von GlyT1 wird vor allem mit der Behandlung der negativen und kognitiven Symptome der Schizophrenie in Verbindung gebracht, für die es bislang keine entsprechenden Behandlungsmöglichkeiten gibt. Als Grundlage dieser Symptome wird eine verringerte glutamaterge Neurotransmission an NMDA-Rezeptoren vermutet, die zu Apathie, motorischer Hemmung, emotionalem Rückzug und kognitiven Defiziten führen kann.[36] Durch die Inhibition von GlyT1 kann die Glycinkonzentration im Bereich dieser Synapsen erhöht werden, wodurch die Glycin-B-Bindungsstelle vermehrt belegt und dadurch die Erregbarkeit des Rezeptors erhöht wird.[37] Dieser Ansatz konnte in mehreren Tier-Modellen erfolgsversprechende Ergebnisse liefern und wurde deshalb bereits in verschiedenen klinischen Studien mit unterschiedlichen Inhibitoren untersucht.[38,39,40,41,42] Bisher konnte allerdings keine

klinische Studie erfolgreich beendet werden. Neben dem Einsatz als mögliche Therapieform für Schizophrenie konnte außerdem gezeigt werden, dass eine Inhibition von GlyT1 unter anderem bei der Behandlung der Alzheimer-Krankheit[41,43] sowie, aufgrund der Lokalisierung an inhibitorisch glycinergen Synapsen, in der Therapie von Drogen- bzw. Alkoholabhängigkeit[44,45,46,47,48,49,50] und chronisch neuropathischen Schmerzen[51,52,53] positive Effekte aufweist.

Der erste Inhibitor, der für GlyT1 entdeckt wurde, war Sarcosin (*N*-Methylglycin; siehe **Abb. 6a**). Dieses stellt ein alternatives Substrat des Transporters dar, welches eine deutliche Selektivität für GlyT1 gegenüber GlyT2 zeigt, wodurch es zur pharmakologischen Unterscheidung der beiden Transporter verwendet werden kann.[54,55] Sarcosin diente als Ausgangspunkt für die Synthese weiterer, hoch affiner Inhibitoren, zu welchen die bekanntesten, nämlich Org24598 und ALX5407, zählen (siehe **Abb. 6a**).[8,56,57] Über die Jahre wurden außerdem weitere GlyT1 Inhibitoren entwickelt, die keine Sarcosin-Partialstruktur aufweisen, wie zum Beispiel Benzoylpiperazin-Derivate.[58] Eine gute Übersicht über bekannte GlyT1-Inhibitoren liefert die Publikation von Dohi et al.[35]

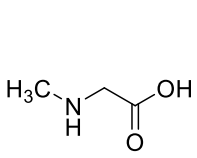
1.2.5.2 Inhibition von GlyT2

Die Inhibition von GlyT2 zeigte vor allem in der Schmerztherapie positive Ergebnisse. In Tier-Modellen konnte durch die Inhibition von GlyT2 mehrfach demonstriert werden, dass chronisch neuropathische Schmerzen, die sich durch spontan auftretende Schmerzen, Hyperalgesie oder Allodynia widerspiegeln, durch eine Verstärkung der inhibitorisch glycinergen Neurotransmission behandelt werden können.[59] Mit den bisher verfügbaren Analgetika und den in der Schmerztherapie eingesetzten Arzneistoffen (z.B. NSAIDs, Opioide, Antikonvulsiva, Antidepressiva, NMDA-Rezeptor-Antagonisten) war dies bisher nur bedingt möglich und war zusätzlich mit Nebenwirkungen verbunden, wie z.B. gastrointestinalen Beschwerden, Toleranzen oder Abhängigkeiten und Missbrauch.[60] Inhibitorisch glycinerge Neurone und damit verbunden GlyT2 kommen hauptsächlich im dorsalen Horn des Rückenmarks vor und nehmen dadurch eine wichtige Funktion in der Schmerzweiterleitung in Richtung Gehirn wahr. Durch die Aktivierung dieser Neurone wird die Schmerzweiterleitung unterbrochen, der empfangene Schmerz lässt nach und hört schließlich auf. Eine Störung dieses Systems führt zu den bereits angesprochenen neuropathischen Schmerzen, da die Schmerzweiterleitung nicht mehr entsprechend gedämpft oder

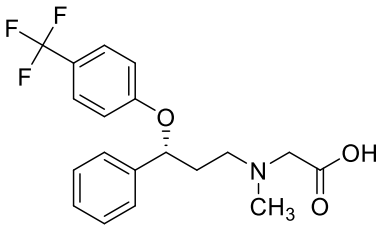
unterbrochen wird. Durch eine Inhibition von GlyT2 kann nun die Glycinkonzentration im synaptischen Spalt gesteigert werden, wodurch es zu einer erhöhten GlyR-Aktivierung kommt und dadurch das inhibitorische postsynaptische Signal verstärkt wird, was zu einer Linderung der Schmerzen führen sollte.[5] Allerdings konnte bereits gezeigt werden, dass eine zu starke Inhibition von GlyT2, z.B. mit Org25543, zu einem gegenteiligen Effekt führen kann. Denn die unter Abschnitt 1.2.4 beschriebene Recycling-Funktion von GlyT2 kann bei einer vollständigen Blockade nicht mehr stattfinden, weswegen eine erneute Glycinfreisetzung aus Mangel an Neurotransmitter in der Präsynapse stark reduziert ist oder ganz ausbleibt. Dies hat zur Folge, dass GlyR nicht mehr ausreichend aktiviert werden können, um das inhibitorische Signal in der notwendigen Stärke aufrechtzuerhalten.[5]

Die bekanntesten GlyT2-Inhibitoren stellen die Verbindungen Org25543 und ALX1393 dar (siehe **Abb. 6b**). Für beide konnte bereits in Tier-Modellen gezeigt werden, dass sie antinozizeptive Eigenschaften aufweisen,[52,61,62,63] allerdings besitzen beide Substanzen auch Nachteile. ALX1393 besitzt eine sehr geringe ZNS-Gängigkeit. So können bei intravenöser Gabe nur 5 % die Blut-Hirn-Schranke überwinden.[64] Die Gabe von Org25543 hingegen führte zu der bereits erwähnten vollständigen Blockade von GlyT2 und Unterbrechung der glycinergen Neurotransmission.[65] Die aktuellste Forschung im Bereich der GlyT2-Inhibitoren zielt auf lipid-basierte Inhibitoren. Hierbei sind vor allem *N*-Arachidonylglycin (NAGly) und *N*-Oleoylglycin (NOGly) zu erwähnen, die zu den ersten Inhibitoren dieser Klasse zählen (siehe **Abb. 6b**). Mit diesen konnte in Studien bereits positive Ergebnisse hinsichtlich der Reduktion neuropathischer Schmerzen bei gleichzeitig geringeren Nebenwirkungen als bei Org25543 erzielt werden.[66,67,68,69] Inzwischen konnten sogar lipid-basierte GlyT2-Inhibitoren synthetisiert werden (*N*-Acyl-Aminosäuren), die eine biologische Aktivität mit IC_{50}-Werten im niedrig nanomolaren Bereich aufweisen, wie z.B. Oleoyl-D-lysin (IC_{50} = 48 nM), und ebenfalls weniger Nebenwirkungen zeigen als Org25543 (siehe **Abb. 6b**).[70] Auf Grundlage von computer-basierten Modellen wird vermutet, dass diese Inhibitoren nicht an die Substratbindungsstelle sondern an eine extrazelluläre allosterische Bindungstasche binden, die sich zwischen den TMDs 1, 5, 7 und 8 sowie EL4 von GlyT2 befindet und hauptsächlich Aminosäuren mit unpolaren Seitenketten enthält.[71] Weitere GlyT2-Inhibitoren sind ebenfalls in der Übersicht von Dohi et al. zu finden.[35]

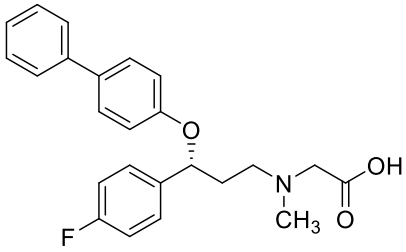
a) GlyT1 Inhibitoren



Sarcosin

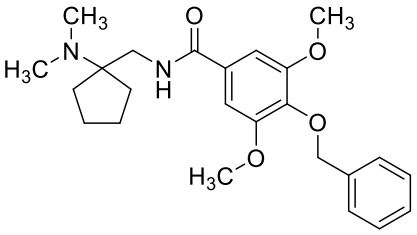


Org24598

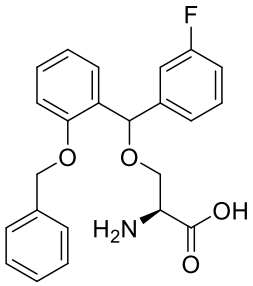


ALX5407

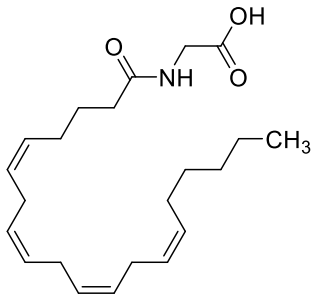
b) GlyT2 Inhibitoren



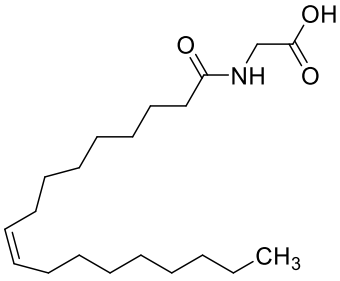
Org25543



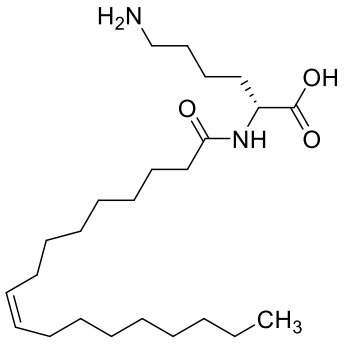
ALX1393



N-Arachidonylglycin
(NAGly)



N-Oleoylglycin
(NOGly)



Oleoyl-D-lysin

Abb. 6: GlyT1- und GlyT2-Inhibitoren.

1.3 Screening-Methoden als Werkzeuge in der Wirkstoffsuche

Die Wirkstoffsuche und die Weiterentwicklung von Wirkstoffen zur Behandlung pathophysiologischer Veränderungen ist ein immer fortschreitender und vermutlich nie endender Prozess. Während es in der Vergangenheit häufig der Fall war, dass Arzneistoffe zufällig entdeckt wurden, wie es z.B. 1928 Alexander Fleming mit der Entdeckung des Penicillins gelang, werden heutzutage durch den medizinischen und technischen Fortschritt der letzten Jahrzehnte groß angelegte und gut geplante Screening-Kampagnen durchgeführt, die zur Identifizierung neuer Wirkstoffe für genau

definierte Zielstrukturen führen sollen. Zunächst muss aber das Target (z.B. ein Protein), dass mit der Fehlfunktion und dem Auftreten des jeweiligen Krankheitsbilds in Verbindung steht, identifiziert werden. Sobald dies geschehen ist, kann die Suche nach Wirkstoffen beginnen, die diese Fehlfunktion beseitigen und somit als Therapeutika in Betracht kommen. Dafür wird insbesondere nach niedermolekularen Verbindungen (sog. Small Molecules) gesucht, inzwischen aber auch vermehrt nach geeigneten Peptiden bzw. Proteinen wie auch Nukleotiden. Damit Substanzen den gewünschten Effekt hervorrufen können, müssen sie naturgemäß an das jeweilige Target binden und dort die erforderliche Aktivität zeigen.

Um die Bindung von Wirkstoffen an Proteinen untersuchen zu können, wurden über die Jahre unterschiedliche leistungsfähige Screeningmethoden entwickelt. Häufig handelt es sich hierbei um funktionelle Assays, bei denen z.B. die Bildung von second Messengern oder eine Änderung des Membranpotentials gemessen wird. Eine Alternative, die im Bereich der Wirkstoffsuche große Bedeutung erlangt hat, sind Bindungsassays. Hierzu zählen die seit den 1970er Jahren eingeführten Bindungsstudien, in denen radioaktiv markierte Verbindungen eingesetzt werden, um die Bindung eines Liganden an ein Protein zu detektieren. Diese Bindungsstudien erhielten aufgrund der radioaktiv markierten Liganden den Namen „Radioligand-Bindungsassays“ und stellen bis heute wegen der hohen Validität der damit zu erreichenden Daten den Goldstandard der Bindungsassays dar.[72] Insbesondere für Untersuchungen des Bindungsverhaltens von Liganden an membranständige Targets haben Radioligand-Bindungsstudien große Bedeutung erlangt. Der Einsatz von Radioaktivität bringt allerdings auch Nachteile mit sich, weshalb in der Vergangenheit große Anstrengungen unternommen wurden, Alternativen zu entwickeln, die ohne eine radioaktive Markierung auskommen und stattdessen optische (Absorption, Fluoreszenz) oder thermodynamische Eigenschaften (kalorimetrische Assays) ausnutzen.[73] Einige dieser Methoden konnten sich mehr und andere weniger gut durchsetzen, da sie oftmals mit hohem Aufwand und/oder Kosten verbunden sind. Hervorheben lassen sich allerdings Fluoreszenz-basierte Assays, wie z.B. Fluoreszenz-Resonanz-Energie-Transfer (FRET) oder die Fluoreszenz-Polarisation (FP). Fluoreszenzbasierte Methoden weisen im Vergleich zu Szintillationsmessungen bei den Radioligand-Bindungsstudien ähnlich gute Nachweisempfindlichkeiten auf, ermöglichen dabei aber deutlich kürzere Messzeiten.[74,75] Für Fluoreszenzmessungen, müssen Ligand und/oder Target, z.B. ein Zielprotein, jedoch

mit einem Fluorophor modifiziert sein. Eine solche Modifikation wiederum kann zeitaufwendig und mit erhöhten Kosten verbunden sein und kann zusätzlich zu Veränderungen in den Bindungseigenschaften des Liganden zum Target führen. Des Weiteren kann durch auftretende Fluoreszenz-Hintergrundstrahlung die Sensitivität verringert und somit die Messung der Bindungsinteraktion erschwert werden.[76]

In den letzten Jahren hat sich durch die Fortschritte im Bereich der Massenspektrometrie (MS) ein weiterer Weg für die Durchführung von Ligandbindungsassays eröffnet. Als sehr bekannte und erfolgreiche Beispiele können ALIS (automated ligand identification system),[77] ASMS (affinity selection/mass spectrometry)[78] und MASS (multitarget affinity/specificity screening)[79] hervorgehoben werden, die besonders leistungsstarke High-Throughput-Screeningtechniken darstellen. Bei diesen Methoden wird die Zielstruktur mit einer Ligandbibliothek inkubiert und die Liganden, die aufgrund ihrer Affinität an das Ziel binden können, werden mittels MS detektiert und identifiziert. Die identifizierten Verbindungen werden entsprechend als sog. „Hits“ bezeichnet. Jedoch kann bei diesen Techniken nur bedingt eine Aussage über die Affinität der Liganden an der Zielstruktur getroffen werden.

In unserer Arbeitsgruppe wurde eine leistungsfähige Alternative zu den Radioligand-Bindungsassays entwickelt, die ebenfalls auf dem Prinzip der Massenspektrometrie als „read-out“ basiert und unter dem Namen „MS-Bindungsassay“ bekannt ist. Mit dieser können native Verbindungen als Reporterliganden eingesetzt werden, um Affinitäten gegenüber membrangebundenen Proteinen zu bestimmen. Dabei weist diese Methode einen ähnlichen Durchsatz auf wie Radioligand-Bindungsassays und das bei relativ niedrigen Kosten und ohne die Notwendigkeit für besondere Sicherheitsvorkehrungen. Dies konnte bereits an vielen Beispielen, bei denen in der MS-Bindungsstudie unterschiedliche Zielproteine zum Einsatz kamen, gezeigt werden.[80,81,82,83,84,85,86,87,88,89,90] Diese Methodik konnte außerdem erst kürzlich mit der Screeningtechnik ASMS kombiniert werden, wodurch es ermöglicht wurde, Hits aus Bibliotheken zu identifizieren und gleichzeitig eine Aussage über deren Affinität bezüglich des untersuchten Zielproteins zu tätigen.[91,92] In nachfolgendem Abschnitt soll nun näher auf MS-Bindungsassays im Vergleich zu Radioligand-Bindungsassays eingegangen werden.

1.3.1 MS Bindungsassays als Alternative zu Radioligand-Bindungsassays

Radioligand-Bindungsassays wurden in den 1970er Jahren eingeführt und stellen bis heute eine viel genutzte Methode dar, um die Affinität von Liganden zu ihren Zielstrukturen (meist Proteine) zu bestimmen. Die hierbei genutzten Liganden müssen für ihren Einsatz zunächst radioaktiv markiert werden, wofür formal stabile durch radioaktive Isotope in den jeweiligen Radioliganden ersetzt werden müssen. Bei Radioisotopen kommen meist ^{3}H, ^{14}C oder ^{125}I zum Einsatz.[93] Diese Liganden werden Radioliganden oder allgemein Reporterliganden oder einfach nur Marker genannt. Die Quantifizierung findet aufgrund der Radioaktivität mittels Szintillationsmessung statt und erlaubt deshalb eine Quantifizierung bis in sehr niedrige Konzentrationsbereiche (picomolarer Bereich), was auch die Untersuchung von Proteinen ermöglicht, die nur in geringen Mengen vorkommen oder für die eine Anreicherung von ausreichend Material zu aufwendig und teuer ist, wie z.B. bei membrangebundenen Proteinen. Ganz allgemein kann als Quelle für die Zielstruktur eine Präparation des zu untersuchenden Organs verwendet werden, aber auch ganze Zellen oder Membranfragmente. Für die Bestimmung der Bindungsstärke des Reporterliganden zum Zielprotein müssen zunächst beide für eine ausreichend lange Zeit inkubiert werden, um die Bildung des Protein-Ligand-Komplexes entsprechend der Gleichgewichtssituation zu gewährleisten. Danach werden Protein-Ligand-Komplex von der Inkubationslösung, die noch freien in Lösung befindlichen Marker enthält, mittels Filtration oder Zentrifugation abgetrennt. Im letzten Schritt wird der im Protein-Ligand-Komplex gebundene Radioligand mittels Szintillationsmessung bestimmt. Ein allgemeines Schema für die Durchführung kann **Abb. 7** entnommen werden.

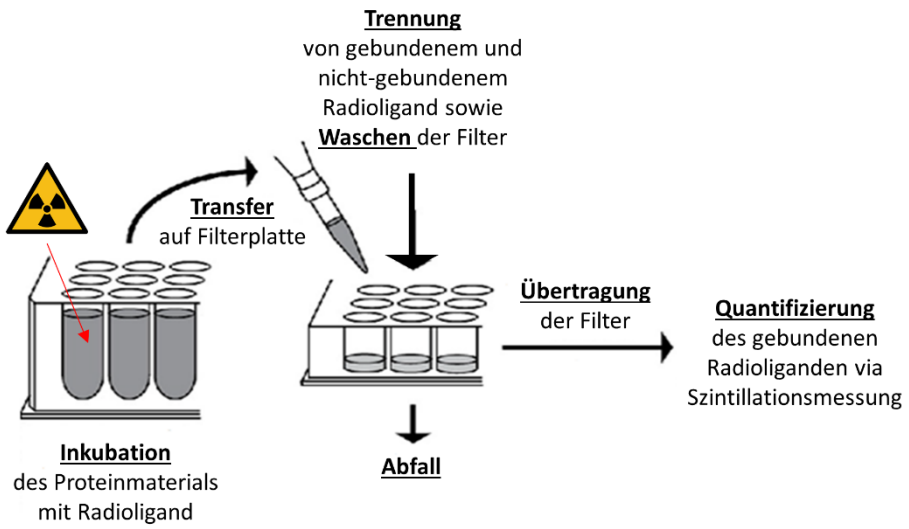


Abb. 7: Schematische Darstellung der Durchführung eines Sättigungsexperiments in Form eines filtrationsbasierten Radioligand-Bindungsassays.

Der Einsatz von Radioaktivität ist allerdings auch mit einer Reihe von Nachteilen verbunden. Zum einen müssen besondere und hohe Sicherheitsvorkehrungen während der Arbeit mit radioaktivem Material getroffen werden,[19] da ein unsachgemäßer Umgang mit Radioaktivität schwere Auswirkungen auf die Gesundheit von Mensch und Tier sowie die Umwelt haben kann. Die Einhaltung der Sicherheitsvorkehrungen wird regelmäßig von staatlichen Behörden überprüft, was mit einem zusätzlichen bürokratischen und zeitlichen Aufwand verbunden ist. Des Weiteren ist der Erwerb von radioaktivem Material sowie die Entsorgung von kontaminiertem Material bzw. Abwasser mit hohen Kosten verbunden, da ein ungewolltes Austreten in die Umwelt vermieden werden muss.

Die in unserer Gruppe entwickelten MS-Bindungsassays stellen deshalb eine vielversprechende Alternative dar, da dadurch die eben erwähnten Nachteile umgangen werden können und trotzdem vergleichbare Ergebnisse erhalten werden und dies ohne den Einsatz radioaktiv markierter Liganden. Native Liganden sind hier völlig ausreichend. Aufbau und Durchführung und damit der Arbeitsaufwand unterscheidet sich bei beiden Methoden jedoch kaum. Der größte Unterschied zwischen den beiden Techniken betrifft den Nachweis und die Quantifizierung des gebundenen Reporterliganden, welche bei MS-Bindungsassays massenspektrometrisch (meist LC-ESI-MS/MS) erfolgt anstatt radiometrisch (Szintillationsmessung), wie das bei den Radioligandbindungsstudien der Fall ist. Durch den technischen Fortschritt der letzten Jahrzehnte konnten Selektivität sowie Sensitivität von

Massenspektrometern so weit verbessert werden, dass sie dem Einsatz von Szintillationsmethoden bei Bindungsassays nicht oder nicht nennenswert nachstehen.[94] MS-Bindungsstudien haben zudem zwei weitere Vorteile: Zum einen wächst das Repertoire an Reporterliganden, da sämtliche Verbindungen, die mittels MS detektierbar sind, als Marker eingesetzt werden können und nicht mehr auf vereinzelt verfügbare oder synthetisch aufwendig neu herzustellende Radioliganden zurückgegriffen werden muss. Allerdings müssen die bei MS-Bindungsstudien zum Einsatz kommenden Reporterliganden eine ausreichend hohe Ionisationseffizienz aufweisen, um die für MS-Bindungsassays erforderliche Empfindlichkeit zu erreichen (dies wird in Abschnitt 1.3.2 näher erläutert). Zum anderen erlaubt die Massenspektrometrie eine simultane Detektion mehrerer Verbindungen in einer Messung, was bei Szintillationsmessungen nur schwer möglich ist. Eine vorherige flüssig-chromatographische Trennung der gleichzeitig eingesetzten Reporterliganden ist bei solchen MS-Bindungsstudien nicht erforderlich. Dies wird durch die Detektion auf Grundlage der Massenübergänge ermöglicht, also der $[M+H]^+$ Mutterionen und den entsprechenden Fragmentionen, die für die meisten Verbindungen unterschiedlich sind (Ausnahmen bilden hier z.B. Stereoisomere). Dadurch entstehen Chromatogramme mit separaten Massenspuren, die für jeden Analyten individuell ausgewertet werden können. Somit besteht die Möglichkeit, verschiedene Reporterliganden für verschiedene Zielproteine in einem Ansatz zu inkubieren, was schließlich eine selektive Quantifizierung eines jeden Liganden für sein entsprechendes Ziel ermöglicht. Dieses Konzept konnte bereits erfolgreich praktiziert werden.[88,90]

Vor der Quantifizierung des gebundenen Markers ist bei MS-Bindungsassays allerdings noch ein weiterer Verfahrensschritt erforderlich. Der in Protein-Ligand-Komplex gebundene Marker muss vor der Quantifizierung erst wieder freigesetzt werden. Dies gelingt durch Denaturierung des Protein-Ligand-Komplexes durch Wärme und/oder Einsatz eines organischen Lösungsmittels. Anschließend wird mittels Filtration oder Zentrifugation der nun wieder freigesetzte Marker von den Membranfragmenten getrennt. Letzteres ist erforderlich, da Membranfragmente sowohl die analytische Trennsäule der LC als auch die Nadel der Elektrospray-Ionisations-Quelle (ESI) verstopfen könnten. Die Durchführung eines filtrationsbasierten MS-Bindungsassays ist am Beispiel eines Sättigungsexperiments schematisch in **Abb. 8** dargestellt.

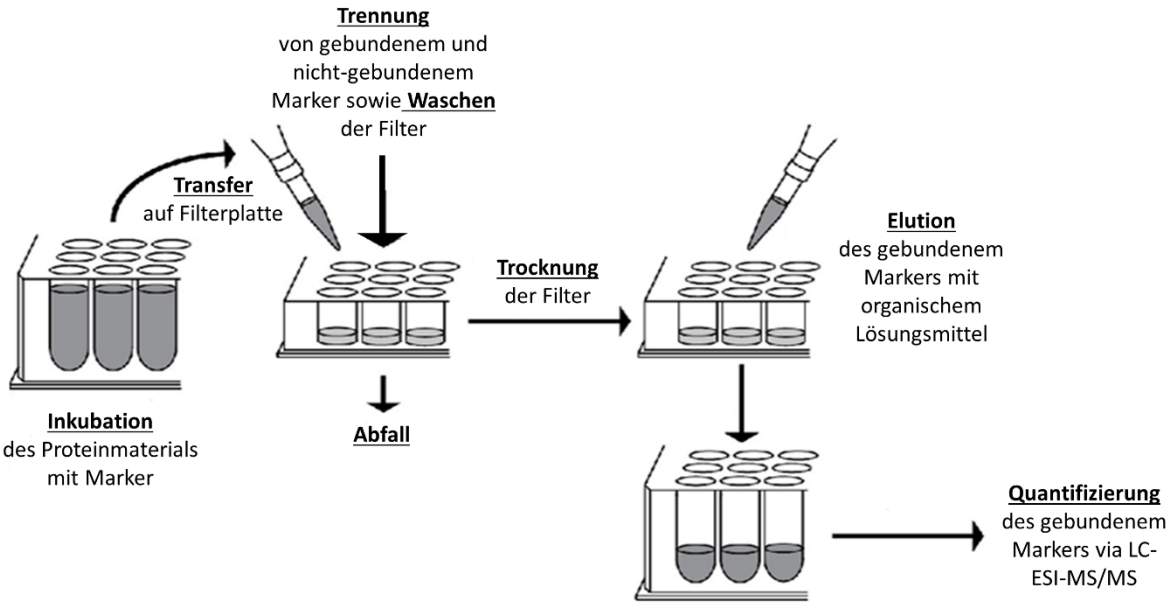


Abb. 8: Schematische Darstellung der Durchführung eines Sättigungsexperiments in Form eines filtrationsbasierten MS-Bindungsassays.

Da sich die beiden Arten von Bindungsassays – MS- und Radioligandbindungsstudien – im Wesentlichen nur in ihrer Art der Detektion des Reporterliganden unterscheiden und sonst einen sehr ähnlichen Ablauf aufweisen, kommen MS-Bindungsassays auch für sämtliche von Radioligand-Bindungsassay bekannten Formen von Bindungsexperimenten in Betracht.

1.3.2 Formen von Bindungsexperimenten

Ganz allgemein geht es um drei Formen von Ligand-Bindungsstudien, die als Sättigungs-, Kinetik- und Kompetitionsexperimente bezeichnet werden. Jedes dieser Experimente liefert hierbei andere Informationen, entweder direkt zum Reporterliganden oder indirekt zu Testverbindungen. Die Vorgehensweise ist für alle drei Formen von Experimenten im Wesentlichen gleich, je nach Experiment müssen jedoch vereinzelt Anpassungen vorgenommen werden. Der Reporterligand wird anfangs entweder in Gegenwart (bei Kompetitionsexperimenten) oder in Abwesenheit (bei Sättigungsexperimenten und kinetischen Experimenten) von Testverbindungen mit dem Zielprotein unter bestimmten Bedingungen (u.a. Inkubationspuffer, Temperatur) inkubiert. Die Inkubation wird dann zu einem bestimmten Zeitpunkt oder zu unterschiedlichen Zeitpunkten (für Kinetikexperimente) beendet, indem Target mit gebundenem von nicht gebundenem Reporterliganden durch Filtration oder Zentrifugation abgetrennt wird. Der Anteil an Reporterligand, der an das Protein

gebunden hat, wird schließlich mit dem entsprechenden Detektionsprinzip, d.h. bei MS-Bindungsassays massenspektrometrisch, quantifiziert.

Für die Auswertung der Experimente ist vor allem der Anteil an Reporterligand von Bedeutung, der spezifisch an der Bindungsstelle des Zielproteins gebundenen hat (spezifische Bindung, SB). Dieser Anteil kann allerdings nicht direkt bestimmt werden, da Reporterliganden stets auch an nicht-spezifische Bindungsstellen binden (nicht-spezifische Bindung, NSB) wie z.B. an Membranfragmente, an anderweitige Proteine, aber auch an Materialien, die mit dem Bindungsprotein in Kontakt kommen wie Kunststoff- oder Filtermaterialien. Dies bedeutet, dass die SB als Summe aus NSB und SB bestimmt werden kann, was als Gesamtbindung (GB) bezeichnet wird. Zusätzlich kann jedoch die NSB durch gezieltes Blockieren der spezifischen Bindungsstelle des Zielproteins durch einen Überschuss eines anderen Liganden dieser Bindungsstelle oder durch Denaturierung des Zielproteins (z.B. durch Hitze, „Heat-Shock“) separat bestimmt werden. Dies ermöglicht schlußendlich eine indirekte Bestimmung der SB durch Differenzbildung aus GB und NSB (siehe Gleichung 1).

$$SB = GB - NSB \qquad \text{(Gleichung 1)}$$

Im Hinblick auf Filtrations-basierte Bindungsassays müssen bei der Auswahl des Reporterliganden zwei Punkte beachtet werden: 1) die Affinität und 2) die Sensitivität des Reporterliganden für die zu verwendende Analytik. Wie bereits erwähnt muss gebundener von nicht-gebundenem Marker getrennt werden, um die Inkubation zu beenden, was unter anderem mittels Filtration passieren kann. Die Protein-Ligand-Komplexe, die sich anschließend auf den Filtern befinden, müssen durch mehrere Waschschritte gereinigt werden, um überschüssigen freien Reporterliganden zu entfernen und somit die bereits angesprochene NSB so niedrig wie möglich zu halten. Hierbei kann es passieren, dass spezifisch gebundener Marker vom Zielprotein dissoziiert. In der Regel bedeutet eine niedrige Affinität auch eine schnelle Dissoziation des Protein-Ligand-Komplexes. Reporterliganden, die eine Affinität im niedrig nanomolaren Bereich aufweisen, sollten allerdings einen Protein-Ligand-Komplex ausbilden, der stabil genug ist und keine nennenswerte Dissoziation zulässt.[95] Eine zu hohe Affinität, also z.B. im mittleren bis niedrig picomolaren Bereich, sollte allerdings auch vermieden werden, da ansonsten womöglich die Bestimmungsgrenze der MS-Methode unterschritten wird (siehe hierzu Gleichung 2).

Des Weiteren muss überprüft werden, ob der Reporterligand in dem erforderlichen Bereich mit der angewandten Methode und der festgelegten Präzision und Richtigkeit quantifiziert werden kann. Die notwendige Bestimmungsgrenze, bei der Reporterligand noch ausreichend quantifiziert werden muss, wird anhand der Affinität des Markers und den allgemeinen Regeln für die Durchführung von Sättigungsexperimenten bestimmt. Vorgeschrieben ist ein Konzentrationsbereich des Reporterliganden von 0,1 x K_d (Gleichgewichtsdissoziationskonstante) bis 10 x K_d sowie eine Zielproteinkonzentration von 0,1 x K_d.[95] Gemäß der Hill-Langmuir-Gleichung (Gleichung 2)

$$[PL] = \frac{[L] \cdot B_{max}}{[L] + K_d}$$

(Gleichung 2)

[PL] ≙ Konzentration Protein-Ligand-Komplex

[L] ≙ Ligand- bzw. Markerkonzentration

B_{max} ≙ maximal verfügbare Zahl der Bindungsstellen

ergibt sich nach Einsetzen der oben erwähnten Grenzen, folgende Bestimmungsgrenze:

$$[PL] = \frac{0{,}1\ K_d \cdot 0{,}1\ K_d}{0{,}1\ K_d + K_d} = 0{,}0091\ K_d$$

Ein Reporterligand muss deshalb bei ca. 1% des K_d-Werts noch richtig und präzise quantifiziert werden können.

In den folgenden Abschnitten soll nun auf die einzelnen Bindungsexperimente näher eingegangen werden.

1.3.2.1 Sättigungsexperimente

Sättigungsexperimente dienen zur Bestimmung der Affinität des Reporterliganden am Zielprotein, die durch die Gleichgewichtsdissoziationskonstante K_d angegeben wird. Für dessen Bestimmung wird eine feste Konzentration an Protein mit steigenden Konzentrationen Reporterligand inkubiert. Für die Inkubationsdauer ist entscheidend, dass diese für eine Gleichgewichtseinstellung lange genug ist. Wie bereits unter Abschnitt 1.3.2 erläutert, wird für eine Sättigung die Markerkonzentration so gewählt, dass ein Bereich von 0,1 x K_d bis10 x K_d abgedeckt wird, um sichergehen zu können, dass die resultierende Sättigungsisotherme ausreichend Datenpunkte für eine gut

definierte Kurve enthält. Die Konzentration des Proteins soll bei 0,1 x K_d liegen, um eine Markerdepletion so gering wie möglich zu halten. Als Markerdepletion wird eine Verringerung der freien Markerkonzentration durch die Bindung an eine Zielstruktur (oder auch an ungewollte Strukturen) bezeichnet. Diese soll im Falle der Bindungsassays so gering wie möglich gehalten werden, da für die Berechnung der Sättigungsisotherme (siehe Gleichung 2) die freie Markerkonzentration zu verwenden ist. Zur Vereinfachung wird hierfür allerdings in der Praxis die nominell eingesetzte Konzentration des Reporterliganden verwendet. Dies ist aber nur bei vernachlässigbarer Markerdepletion statthaft. Für eine maximal tolerierbare Depletion wird ein Wert von höchstens 10 % angesehen. Bei einer größeren Depletion muss diese miteinberechnet werden, um verlässliche Ergebnisse zu erhalten.[95]

Experimentell werden sowohl GB als auch NSB bestimmt. Aus theoretischen Überlegungen sollte die NSB linear mit der Markerkonzentration ansteigen, was in der Regel auch der Fall ist. So kann sie mittels linearer Regression mathematisch erfasst werden, während die GB bereits eine gewisse Ähnlichkeit zum Kurvenverlauf einer Sättigungsisotherme aufweist, ohne sich aber einem Grenzwert zu nähern (siehe **Abb. 9a**). Durch Differenzbildung zwischen GB und NSB wird schließlich die spezifische Bindung erhalten. Wird diese, die SB, gegen die nominell eingesetzte Markerkonzentration aufgetragen, wird eine typische Sättigungsisotherme erhalten, die sich entsprechend Gleichung 2 verhält (siehe **Abb. 9b**). Durch nicht-lineare Regression kann nun sowohl der K_d-Wert des Reporterliganden als auch die Anzahl maximal verfügbarer Bindungsstellen B_{max} ermittelt werden. Der K_d-Wert definiert die Affinität des Markers und ist identisch mit der Konzentration, bei der der Reporterligand die verfügbaren Bindungsstellen des Zielproteins zu 50 % besetzt. B_{max} als Plateau der Sättigungsisotherme definiert die Menge an besetzbaren Bindungsstellen, an die sich die Bindungskurve bei hohen Markerkonzentration asymptotisch annähert.

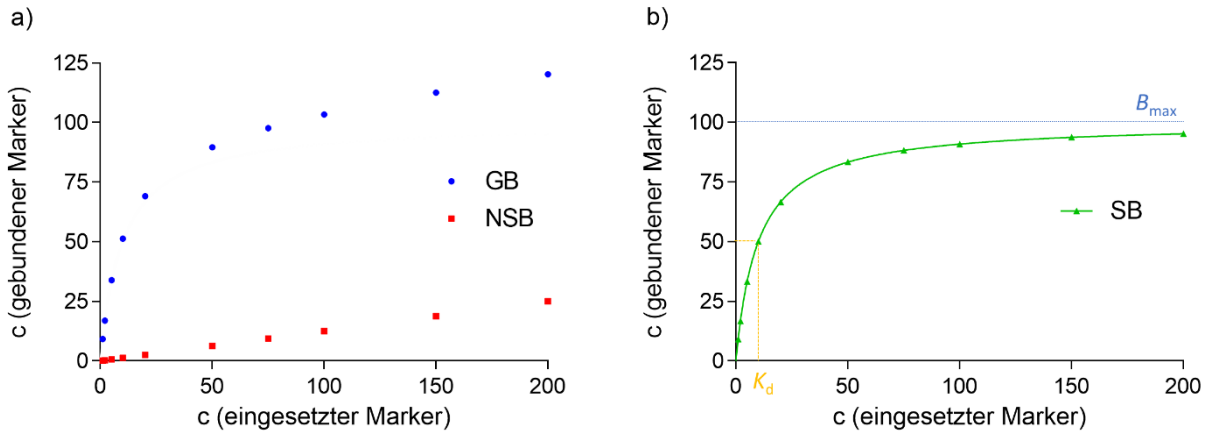


Abb. 9: Exemplarische Darstellung der Ergebnisse eines Sättigungsexperiments. a) Experimentell bestimmte Werte der GB (dunkelblau) und NSB (rot), die gegen die nominell eingesetzten Markerkonzentrationen aufgetragen werden. b) Auftragung der SB (grün; Differenz aus GB und NSB) gegen die nominell eingesetzten Markerkonzentrationen. Mittels nicht-linearer Regression kann eine Sättigungsisotherme erstellt werden, von der sowohl der K_d-Wert (orange) als auch der B_{max}-Wert (hellblau) abgeleitet werden kann.

1.3.2.2 Kinetikexperimente

Kinetikexperimente dienen der Bestimmung der kinetischen Eigenschaften des Liganden, also seiner Dissoziations- und Assoziationsgeschwindigkeit gegenüber dem Zielprotein. Da es für die Ergebnisse von Sättigungs- und Kompetitionsexperimenten entscheidend ist, dass ein Gleichgewicht zwischen Ligand und Zielprotein vorliegt, sind diese Informationen unter anderem wichtig, um die Inkubationszeiten für eben genannte Experimente festlegen zu können.

Durch Dissoziationsexperimente wird die sog. „off-rate“ (k_{off}) des Liganden bestimmt, also die Konstante, die beschreibt, mit welcher Geschwindigkeit der Ligand vom Zielprotein abdissoziiert, woraus sich auch die Halbwertszeit ($t_{1/2}$) des Protein-Ligand-Komplexes ableiten lässt. Für eine Bestimmung von k_{off}, muss zunächst ein geeigneter Anteil des Protein-Ligand-Komplexes vorliegen, was durch eine Vorinkubation des Liganden mit dem Protein erreicht wird. Sodann wird die Dissoziation initiiert, wobei es zwei unterschiedliche Ansätze gibt: Einmal besteht die Möglichkeit einen großen Überschuss eines zweiten Liganden (Kompetitor) der Inkubationslösung zuzugeben („Verdrängungsmethode“), der an die gleiche Bindungsstelle des Proteins wie der zu untersuchende Ligand bindet. Dadurch wird der zu untersuchende Ligand von der Bindungsstelle verdrängt und durch die hohe Konzentration des Kompetitors wird eine erneute Assoziation verhindert. Die zweite Methode besteht darin, die bereits vorhandenen Protein-Ligand-Komplexe stark zu verdünnen, so dass die freiwerdende Konzentration des Reporterliganden vernachlässigbar klein wird, wodurch ebenfalls

eine erneute Assoziation des Markers verhindert wird („Verdünnungs-Methode“).[95] Unabhängig von der Methode, die verwendet wird, ist es wichtig, dass der Verlauf der Dissoziation über die Zeit bestimmt wird. Dafür muss die Dissoziation nach bestimmten Zeitpunkten unterbrochen werden, was erreicht wird, indem das Target einschließlich der Protein-Ligand-Komplexe von den in der Lösung frei vorliegenden Liganden getrennt wird. Da die Trennung mittels Zentrifugation in diesem Fall zu lange dauern würde, kann die Trennung unter diesen Umständen nur mittels Filtration stattfinden. Für die Auswertung wird die verbliebene Menge an gebundenem Marker bestimmt und diese wird gegen die Zeit aufgetragen (siehe **Abb. 10a**). Der Kurvenverlauf verhält sich entsprechend einer Kinetik 1. Ordnung und wird durch Gleichung 3 beschrieben:

$$[PL] = [PL_{equ}] \cdot e^{-k_{off} \cdot t} \qquad \text{(Gleichung 3)}$$

$[PL_{equ}]$ ≙ Konzentration Protein-Ligand-Komplex im Gleichgewichtszustand

t ≙ Zeit

Die Dissoziationskurve wird durch nicht-lineare Regression erstellt, aus der k_{off} und $t_{1/2}$ erhalten werden können.

In Assoziationsexperimenten hingegen kann die sog. „on-rate“ (k_{on}), also die Konstante, die beschreibt, wie schnell sich Protein-Ligand-Komplexe formen, nicht direkt bestimmt werden. Dies liegt daran, dass es bei der Inkubation von Ligand und Protein nie zu einem reinen Assoziationsprozess kommt, sondern dass parallel zur Assoziation des Liganden auch immer dessen Dissoziation auftritt. Deshalb kann experimentell nur eine beobachtbare (engl.: observed) kinetische Konstante bestimmt werden, welche als k_{obs} bezeichnet wird. k_{obs} und k_{on} stehen gemäß Gleichung 4 in Beziehung. Durch Umformung von Gleichung 4, kann schließlich k_{on} berechnet werden (siehe Gleichung 5).

$$k_{obs} = k_{on} \cdot [L] + k_{off} \qquad \text{(Gleichung 4)}$$

$$k_{on} = \frac{(k_{obs} - k_{off})}{[L]} \qquad \text{(Gleichung 5)}$$

Wie aus Gleichung 4 ersichtlich wird, ist k_{obs} abhängig von der eingesetzten Markerkonzentration. Deshalb sollte eine möglichst geringe Konzentration gewählt werden. Andernfalls läuft die Assoziation zu schnell ab und der zeitliche Verlauf ebendieser würde fehlerhaft und nicht ausreichend genau bestimmt werden können. Die Assoziation wird durch die Zugabe von Reporterligand zum Zielprotein in der

Inkubationslösung initiiert und die Inkubation wird schließlich nach genau definierten Zeitpunkten durch Filtration beendet. Sodann wird die Menge an proteingebundenem Marker quantifiziert. Das Auftragen der Menge an gebundenem Marker gegen die Zeit (siehe **Abb. 10b**) ergibt einen Kurvenverlauf der theoretisch Gleichung 6 folgt:

$$[PL] = [PL_{equ}] \cdot (1 - e^{-(k_{on} \cdot [L] + k_{off}) \cdot t}) = [PL_{equ}] \cdot (1 - e^{-k_{obs} \cdot t}) \quad \text{(Gleichung 6)}$$

Mittels nicht-linearer Regression lässt sich aus dieser Kurve k_{obs} bestimmen, welches es ermöglicht, zusammen mit dem Wissen über k_{off} und [L] k_{on} mit Gleichung 5 zu berechnen.

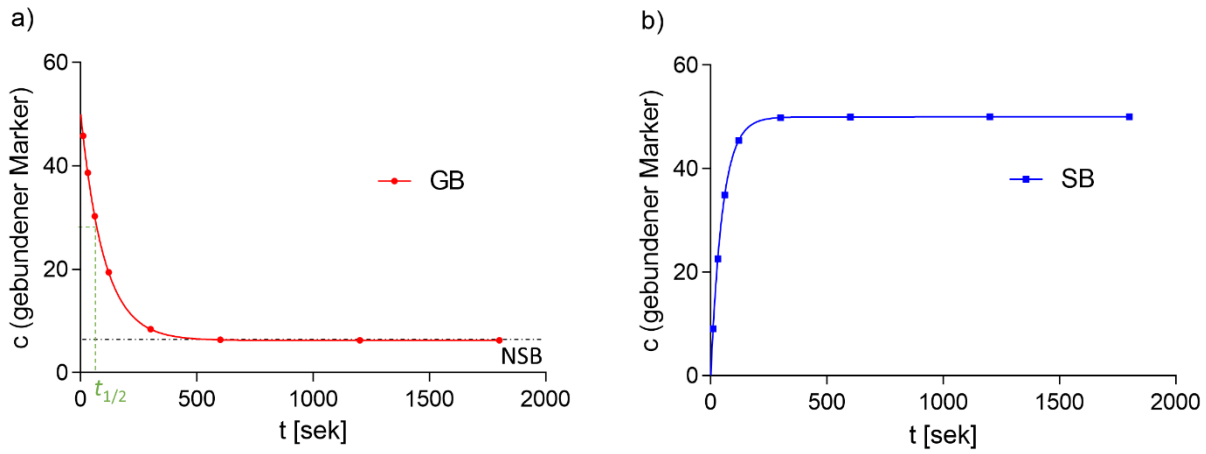


Abb. 10: Exemplarische Darstellung der Ergebnisse von Kinetikexperimenten. a) Dissoziationsexperimente zeigen den zeitlichen Verlauf der Abnahme des gebundenen Markers, was durch die Auftragung der GB gegen die Zeit (rot) dargestellt wird, bis das Level der NSB erreicht ist (schwarz). Mittels nicht-linearer Regression kann schließlich k_{off} sowie $t_{1/2}$ (grün) ermittelt werden. b) Assoziationsexperimente zeigen den zeitlichen Verlauf der Formung von Protein-Ligand-Komplexen bei gleichzeitig stattfindenden Dissoziationsvorgängen. Dies wird durch die Auftragung der SB gegen die Zeit dargestellt (blau). Mittels nicht-linearer Regression kann k_{obs} ermittelt werden, welches anschließend durch das Wissen von k_{off} und [L] für die Berechnung von k_{on} verwendet werden kann.

Wie bereits erwähnt dienen diese Experimente u.a. dazu, die Inkubationszeiten für Sättigungs- und Kompetitionsexperimente festzulegen. Hierfür wurde als Faustregel festgelegt, dass die Inkubationszeit mindestens fünfmal die Dissoziations-Halbwertszeit betragen soll.[95] Außerdem kann durch die Bestimmung von k_{off} und k_{on} zusätzlich der K_d-Wert des eingesetzten Reporterliganden bestimmt werden. Die Beziehung zwischen diesem und den Kinetik-Konstanten wird durch Gleichung 7 beschrieben:[95]

$$K_d = \frac{k_{off}}{k_{on}} \quad \text{(Gleichung 7)}$$

1.3.2.3 Kompetitionsexperimente

Kompetitionsexperimente dienen zur Bestimmung von Affinitäten von Testsubstanzen (auch Kompetitoren genannt) gegenüber dem Zielprotein, die als Inhibitionskonstanten K_i ausgedrückt werden. Diese Affinitäten werden allerdings nicht direkt bestimmt wie bei Sättigungsexperimenten, sondern indirekt über ihr Potential mit dem Reporterliganden, um die Proteinbindungsstelle zu konkurrieren.

Hierfür wird eine genau definierte Menge Protein sowie eine genau definierte Konzentration an Reporterligand (meist in der Größenordnung des K_d-Werts) mit steigenden Konzentrationen an Testsubstanz inkubiert. Sowohl Marker als auch Kompetitor konkurrieren um die Bindungsstelle des Zielproteins. Je nach Affinität des Kompetitors und den Konzentrationen, in denen dieser zum Einsatz kommt, wird der Reporterligand mehr oder weniger stark von der Bindungsstelle verdrängt wird. Wenn nach der Gleichgewichtseinstellung die Inkubation durch Trennung des Protein-Ligand-Komplexes von frei in Lösung befindlichem Reporterligand und Kompetitor durch Filtration oder Zentrifugation beendet wird, werden mit steigenden Kompetitorkonzentrationen abnehmende Konzentrationen an gebundenem Marker beobachtet. Die Menge an gebundenem Marker kann so bis auf das Level der NSB verringert werden. Da in diesen Experimenten die SB des Reporterligand auf die spezifische Bindung in Abwesenheit eines Kompetitors normalisiert wird, stellt die NSB die 0 %-Grenze dar, während die SB in Abwesenheit eines Kompetitors die 100 %-Grenze definiert. Für die Auswertung wird schließlich die Menge an spezifisch gebundenem Reporterligand (in %) gegen die logarithmische Konzentration der Testverbindung aufgetragen, wodurch eine sigmoidale Inhibitionskurve erhalten wird (siehe **Abb. 11**). Diese Kurve leitet sich von der Gaddum-Schild-Gleichung ab (Gleichung 8):

$$[PL] = [PL_0] \cdot \frac{[L]}{K_d \cdot \left(1 + \frac{[I]}{K_i}\right) + [L]}$$

(Gleichung 8)

$[PL_0]$ ≙ Konzentration Protein-Ligand-Komplex in Abwesenheit von Kompetitor

[I] ≙ eingesetzte Kompetitorkonzentration

Der Punkt dieser Kurve, an dem der spezifisch gebundene Marker auf 50 % reduziert wurde, wird als IC_{50}-Wert definiert und kann mittels nicht-linearer Regression ermittelt werden.

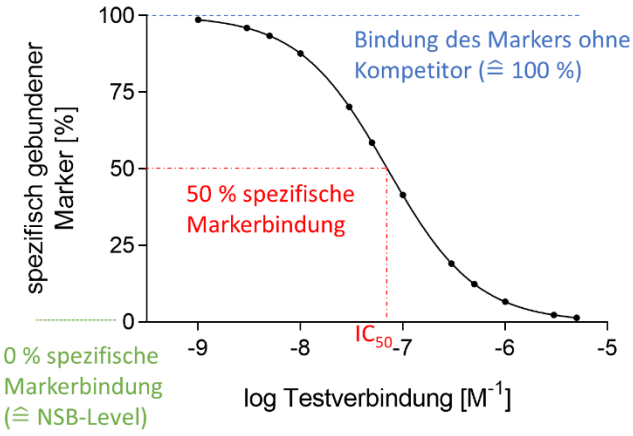


Abb. 11: Exemplarische Darstellung des Ergebnisses eines Kompetitionsexperiments. Aufgetragen wird hierbei die Menge an spezifisch gebundenem Marker (in %) gegen die logarithmische Konzentration der Testverbindung. Das 100 %-Level wird durch die Bindung des Markers ohne Kompetitor und das 0 %-Level durch die NSB bei der eingesetzten Markerkonzentration definiert. Bei 50 % spezifischer Markerbindung befindet sich der sog. IC_{50}-Wert, welcher mittels nicht-linearer Regression ermittelt wird.

Die Affinität der Testsubstanz, d.h.der K_i-Wert ebendieser, kann schließlich mit Hilfe der Cheng-Prusoff-Gleichung aus dem IC_{50}-Wert bestimmt werden (Gleichung 9). [96]

$$K_i = \frac{IC_{50}}{1+\frac{[L]}{K_d}} \qquad \text{(Gleichung 9)}$$

Wie man Gleichung 9 entnehmen kann, ist für die Berechnung des K_i-Werts der IC_{50}-Wert der Testsubstanz erforderlich, aber auch die freie Konzentration des Markers (im Falle einer vernachlässigbaren Markerdepletion kann die nominell eingesetzte Markerkonzentration eingesetzt werden) sowie dessen K_d-Wert. Deshalb ist es vor der Durchführung von Kompetitionsexperimenten notwendig, die Affinität des Reporterliganden in Sättigungsexperimenten zu bestimmen. Der IC_{50}-Wert ist unter bestimmten Bedingungen ($[L]/K_d << 1$) dem K_i-Wert sehr ähnlich, stellt aber keine Konstante wie den K_i-Wert dar. Vielmehr ist er von der eingesetzten Markerkonzentration abhängig.

Die Durchführung von Kompetitionsexperimenten hat zwei entscheidende Vorteile. 1) Sie erlauben es, die Affinitäten von unbekannten Testsubstanzen an Zielproteinen zu bestimmen, ohne individuelle Bindungsassays mit der entsprechenden Analytik für jede einzelne Testsubstanz entwickeln und für diese anschließend Sättigungsexperimente für die K_d-Wert-Bestimmung durchführen zu müssen. Dies hat eine deutliche Zeitersparnis sowie geringere Kosten zur Folge. 2) Wie bereits in Abschnitt 1.3.2 beschrieben, ist nicht jede Substanz als Reporterligand in

Sättigungsexperimenten geeignet (speziell für die Durchführung von Filtrations-basierten Assays), da bei zu geringen Affinitäten und damit verbunden einer zu schnellen off-Kinetik ein zu großer Verlust der spezifisch gebundenen Verbindung auftritt. Da in Kompetitionsexperimenten aber die Bindung des Reporterliganden bestimmt wird, ist die Quantifizierung vollkommen unabhängig von der eingesetzten Testsubstanz und somit auch von deren Affinität. Das bedeutet, dass nahezu jede Verbindung hinsichtlich ihres K_i-Werts problemlos untersucht werden kann.

2 Zielsetzung

Da, wie in der Einleitung beschrieben, GlyT1 und GlyT2 vielversprechende Zielproteine für die Entwicklung von Wirkstoffen zur Behandlung von Krankheiten sind, für die es heutzutage noch keine adäquaten Behandlungsmöglichkeiten gibt (z.B. negative und kognitive Symptome der Schizophrenie-Krankheit, Drogenabhängigkeiten oder in der Schmerztherapie), war es das Ziel der vorliegenden Arbeit, Bindungsassays für diese Neurotransmitter-Transporter zu entwickeln. Diese sollten auf dem Prinzip der MS-Bindungsassays basieren, welches in unserer Arbeitsgruppe entwickelt wurde.[97] Die experimentellen Bedingungen für die Durchführung bereits etablierter MS-Bindungsassays für andere Zielproteine sollten dabei als Vorlage dienen.[80,81,82,83,85,86,87,88,89,90] Die zu entwickelnden Assays sollten schließlich dazu genutzt werden, um neue Inhibitoren an GlyT1 und GlyT2 zu identifizieren.

Bei der Entwicklung eines MS-Bindungsassays für GlyT1 konnte, wie schon in anderen Fällen, auf Daten zu publizierten Bindungsassays zurückgegriffen werden. Bei diesen handelt es sich zwar um Radioligand-Bindungsassays,[8,9,11,12,13,40] lieferten aber dennoch wertvolle Informationen, die in die Entwicklung des GlyT1-MS-Bindungsassays einfließen konnten. Für GlyT2 war bis dahin jedoch noch kein Bindungsassay bekannt und somit auch keine hilfreichen Informationen. Der in dieser Arbeit zu entwickelnde GlyT2-MS-Bindungsassay würde somit den ersten Vertreter eines Bindungsassay für dieses Target darstellen. Dieser sollte es dann ermöglichen, Bindungsaffinitäten von Liganden zu GlyT2 zu bestimmen.

Um einen MS-Bindungsassay erfolgreich entwickeln zu können, muss zunächst ein geeigneter Reporterligand ausgewählt werden. Dieser sollte selektiv an das gewünschte Zielprotein mit einer Affinität (K_d) im niedrig nanomolaren Bereich binden sowie eine ausreichend niedrige Bestimmungsgrenze bei der Quantifizierung aufweisen. Dafür sollte ein LC-ESI-MS/MS-Verfahren zum Einsatz kommen, dass sich für die Entwicklung und Durchführung solcher Bindungsstudien als besonders geeignet erwiesen hatten.[84] Durch eine Affinität im niedrig nanomolaren Bereich sollte gewährleistet werden, dass der ausgewählte Reporterligand eine ausreichend langsame off-Kinetik aufweist, um nicht tolerable Verluste des Liganden während der Waschschritte zu vermeiden. Bei einem K_d im niedrig nanomolaren Bereich, wie er hierfür erforderlich ist, war es wie oben bereits beschrieben notwendig, dass die

Nachweisgrenze bis in den niedrig pikomolaren Bereich reicht (siehe hierzu Abschnitt 1.3.2 und 1.3.2.1). Für die Entwicklung der beiden MS-Bindungsassays in dieser Arbeit sollten die selektiven und hoch affinen Inhibitoren Org24598 (GlyT1) und Org25543 (GlyT2) verwendet werden, da diese laut Literaturdaten die zuvor beschriebenen Anforderungen erfüllen sollten.

Im Anschluss an die Auswahl der geeigneten Marker sollten die analytischen Bedingungen für die Quantifizierung der Reporterliganden definiert werden, und zwar hinsichtlich des flüssig-chromatographischen wie auch massenspektrometrischen Teils. Die HPLC-Methoden sollten es ermöglichen, dass die Marker effizient von Matrixbestandteilen abgetrennt und so Matrixeffekte minimiert werden können. Zugleich sollten kurze Analysenzeiten erreicht werden, um im Routinebetrieb einen möglichst hohen Probendurchsatz zu erlauben. Die Quantifizierung sollte nach dem Prinzip des internen Standards durchgeführt werden. Als interne Standards sollten daher Isotopen-markierte Verbindungen der Marker zum Einsatz kommen. Dadurch wäre gewährleistet, dass Marker und interner Standard sehr ähnliche physikochemische Eigenschaften aufweisen und sich deshalb chromatographisch als auch massenspektrometrisch sehr ähnlich verhalten.

Anschließend sollten die Bedingungen für die Durchführung der MS-Bindungsassays definiert werden. Wie bereits erwähnt sollten sich diese an bereits etablierten MS-Bindungsassays und, im Fall von GlyT1, an publizierten Bindungsassays für das entsprechende Zielprotein orientieren. Die Trennung des gebundenen vom nicht-gebundenen Marker sollte mittels Vakuumfiltration erfolgen, da diese Technik bei Bindungsassays einen höheren Durchsatz als der Einsatz der Zentrifugation erlaubt. Als Quelle für die Transporter sollte eine GlyT1 stabil exprimierende CHO-K1- und eine GlyT2 stabil exprimierende HEK293-Zelllinie zum Einsatz kommen. Von diesen Zellen sollten Membranpräparationen als Targetquelle, welche für die Durchführung der Bindungsexperimente verwendet werden sollten, hergestellt werden.

Nachdem die Bedingungen für die Durchführung der MS-Bindungassays festgelegt sind, sollten die finalen LC-ESI-MS/MS-Methoden validiert werden. Diese sollte gemäß den Vorschriften der „CDER Guideline for bioanalytical method validation der FDA“ hinsichtlich Spezifität, Richtigkeit, Präzision, Linearität und lower limit of quantification (LLOQ) geschehen.[98]

Im Anschluss an die Validierungen der entwickelten Methoden sollten in Bindungsexperimenten (Sättigungs- und Kompetitionsexperimenten) die Affinitäten der Marker sowie anderer Inhibitoren bzw. Substrate bestimmt werden. Im Fall des GlyT2-MS-Bindungsassays sollten zusätzlich Kinetikexperimente (Assoziations- und Dissoziationsexperimente) durchgeführt werden, um so erstmals Daten zur Kinetik eines Liganden an diesem Transporter zu bestimmen. In der Literatur wird Org25543 als „tight binder“ und „biologically irreversible inhibitor“ gegenüber GlyT2 beschrieben.[64,70] Mit der Durchführung von Kinetikexperimenten sollte zudem auch dieses Phänomen näher untersucht werden. Für GlyT1 sind bereits Kinetik-Untersuchungen für einen Inhibitor ähnlich zu Org24598 bekannt (ALX5407).[8] Da dabei keine kinetischen Auffälligkeiten beobachtet wurden, wurde eine Durchführung von Kinetikexperimenten an GlyT1 als nicht notwendig erachtet. Die erhaltenen Ergebnisse aus den Bindungsexperimenten sollten schließlich mit den Ergebnissen aus anderen Studien (Bindungs- und Transportstudien) verglichen werden.

Letztendlich sollten die für GlyT1 und GlyT2 entwickelten MS-Bindungsassays zur Identifizierung neuer potenter Inhibitoren verwendet werden. Hierfür sollte eine Substanzbibliothek von ungefähr 2400 Verbindungen in einem Screeningverfahren an beiden Zielproteinen untersucht werden, wobei das Augenmerk auf Substanzen mit Affinitäten im niedrig mikromolaren bis sub-mikromolaren Bereich liegen sollte. Die entsprechenden Verbindungen sollten schließlich in Kompetitionsexperimenten hinsichtlich ihrer Affinität (pK_i) charakterisiert werden.

3 Ergebnisse und Diskussion

3.1 Erste Publikation

MS binding assays for GlyT1 based on Org24598 as nonlabelled reporter ligand

3.1.1 Zusammenfassung der Ergebnisse

Da GlyT1, wie bereits in der Einleitung geschildert, ein vielversprechendes Zielprotein in der Bekämpfung von unterschiedlichen Krankheitsbildern darstellt, wurde, in Anlehnung an frühere Arbeiten,[80,81,82,83,85,86,87,88,89,90] für diesen Neurotransmitter-Transporter ein MS-Bindungsassay entwickelt. Dieser sollte zur Identifizierung neuer GlyT1-Inhibitoren genutzt werden. In den erwähnten Arbeiten konnte bereits für viele unterschiedliche Proteine gezeigt werden, dass MS-Bindungsassays leistungsfähige Alternativen zu den häufig eingesetzten Radioligand-Bindungsassays darstellen, um Ligand-Protein-Interaktion zu untersuchen und Bindungsaffinitäten zu bestimmen.

Als Reporterligand (auch kurz als MS-Marker oder nur Marker bezeichnet) wurde der bereits bekannte selektive und hoch affine GlyT1-Inhibitor Org24598 verwendet. Für diesen wurde eine LC-ESI-MS/MS-Methode entwickelt, welche es erlaubt, den Marker in seiner nativen Form zu quantifizieren. Die entwickelte LC-Methode basierte auf einer isokratischen reversed phase (RP)-HPLC Methode, für die eine Luna C8(2) (50 mm × 2 mm, 3 µm) Säule von Phenomenex als stationäre Phase und ein Fließmittelgemisch aus 5 mM Ammoniumhydrogencarbonat-Puffer pH 7,8 und Acetonitril (55:45; V/V) als mobile Phase verwendete wurde. Dadurch konnte der Analyt von störender Matrix abgetrennt und somit ein möglicher Matrixeffekt umgangen werden. Bei einer Flussrate von 400 µL/min, einer Säulentemperatur von 20 °C und einem Injektionsvolumen von 45 µL konnten chromatographische Laufzeiten von 2,0 min pro injizierte Probe erreicht werden, was einen hohen Probendurchsatz zur Folge hatte. Für die Detektion des Markers wurde ein AB Sciex API 5000 Triple Quadrupol Massenspektrometer mit Elektrospray-Ionisation (ESI) Ionenquelle im multiple reaction monitioring (MRM)-Modus verwendet, womit der Massenübergang *m*/*z* 368,0/102,0 aufgezeichnet wurde. Dadurch konnte ein LLOQ im niedrig picomolaren Bereich erreicht werden. Um eine möglichst robuste Probenauswertung mittels Massenspektrometer gewährleisten zu können, wurde zusätzlich das deuterierte Racemat des Reporterliganden, [$^{2}H_{5}$]Org24461, als interner Standard verwendet, der

über den Massenübergang *m*/*z* 373,0/102,0 detektiert wurde. Seine Synthese gelang in zwei einfachen Schritten aus [$^2H_5$]Fluoxetin.

Vor der Validierung der LC-ESI-MS/MS-Methode musste zunächst der gewünschte Filtrations-basierte MS-Bindungsassay als weiterer Baustein der Gesamtmethode implementiert werden. Dabei wurde auf Bedingungen von bereits vorhandenen MS-Bindungsassays und GlyT1-Radioligand-Bindungsassays zurückgegriffen und diese an die vorliegenden Gegebenheiten angepasst. Der entwickelte Bindungsassay läuft wie folgt ab. Zunächst werden Zielprotein, Reporterligand und, falls notwendig, Kompetitor (z.B. ALX5407 zur Bestimmung der nicht-spezifischen Bindung) in Inkubationspuffer für 1 h bei Raumtemperatur in 96-deepwell Platten inkubiert und anschließend über 96-well Glasfaserfilterplatten filtriert, um gebundenen von ungebundenem Marker zu separieren und somit die Inkubation zu beenden. Die Glasfaserfilter werden vor der Filtration mit einer 1 % (*m/m*) wässrigen Tween20 Lösung vorbehandelt, um die Filterbindung des Reporterliganden zu reduzieren. Die zurückgehaltenen Protein-Ligand-Komplexe auf den Filtern werden nachfolgend mit eisgekühltem Ammoniumacetatpuffer gewaschen und für 1 h bei 50 °C getrocknet. Nach der Trocknung werden durch die Zugabe von Methanol Proteine einschließlich der Transporterproteine denaturiert, wodurch der Marker in Lösung geht, der dann durch Vakuumfiltration in einer 96-deepwell-Platte aufgefangen wird. Die Eluate werden bei 50 °C im N_2-Strom eingedampft, der Rückstand mit mobiler Phase resuspendiert und die Proben werden schließlich mit der entwickelten LC-ESI-MS/MS-Methode analysiert.

Durch die für den MS-Bindungsassay festgelegten Bedingungen, konnten Matrixproben hergestellt werden, die für die Durchführung der Validierung erforderlich waren. Die Validierung der finalen LC-ESI-MS/MS-Methode erfolgte schließlich hinsichtlich Spezifität, Richtigkeit, Präzision, Linearität und lower limit of quantification (LLOQ) angelehnt an die CDER Guideline for bioanalytical method validation der FDA.[98] Die Vorgaben dieser Richtlinie konnte in allen erwähnten Punkten eingehalten werden und es konnte ein Arbeitsbereich von 5 pM (LLOQ) bis 1 nM definiert werden.

Schlussendlich wurden Sättigungs- und Kompetitionsexperimente durchgeführt und die experimentell erhaltenen Ergebnisse mit publizierten Ergebnissen verglichen. Für Org24598 wurde ein K_d-Wert von 16,8 ± 2,2 nM ermittelt, der sehr gut mit K_d- und K_i-Werten aus der Literatur übereinstimmt.[9,40] Außerdem wurden in

Kompetitionsexperimenten zwölf weitere Substanzen untersucht. Diese lieferten K_i-Werte, die im Vergleich mit Resultaten aus literaturbekannten Bindungs- bzw. Transportassays eine überaus befriedigende Korrelation aufweisen. Diese Ergebnisse zeigen somit, dass der entwickelte MS Bindungsassay eine geeignete Alternative zu bereits vorhandenen Radioligand-Bindungsassays für GlyT1 darstellt.

3.1.2 Erklärung zum Eigenanteil

Die Synthese und Analytik des deuterierten internen Standards [$^2H_5$]Org24461 erfolgte durch Dr. Karuna Bhokare. Die Charakterisierung der MRM-Massenübergänge für die verwendeten Analyten, die LC-ESI-MS/MS-Methodenentwicklung und Validierung sowie erste Vorexperimente für den Bindungsassay wurden von mir im Rahmen meiner Masterarbeit mit dem Titel „Entwicklung von MS-Bindungsassays für den Glycintransporter GlyT1“ durchgeführt. Die Hauptarbeit zur Optimierung des MS-Bindungsassays und seine Anwendung in Sättigungs- und Kompetitionsexperimenten waren Gegenstand meiner Tätigkeit als Doktorand. Das Manuskript wurde unter Mithilfe von Dr. Georg Höfner von mir verfasst. Tabellen und Abbildungen habe ich alle selbst erstellt. Das Manuskript wurde von Prof. Dr. Klaus T. Wanner korrigiert.

3.1.3 Manuskript der ersten Publikation

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MS binding assays for GlyT1 based on Org24598 as nonlabelled reporter ligand

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HIGHLIGHTS

- GlyT1 binding assays with nonlabelled Org24598 as reporter ligand were developed.
- Org24598 binding at GlyT1 could be monitored by means of LC-ESI-MS/MS.
- Binding of Org24598 at GlyT1 was characterized in saturation experiments.
- Determined affinities for known GlyT ligands are in agreement with literature.
- Established GlyT1 MS Binding Assays can substitute radioligand binding assays.

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ABSTRACT

In this study an alternative to radioligand binding assays addressing the glycine transporter 1 (GlyT1) based on quantification of a nonlabelled reporter ligand by means of mass spectrometry (MS) is presented. The established MS Binding Assays employ the GlyT1 inhibitor Org24598 as reporter ligand for which a highly sensitive LC-ESI-MS/MS (liquid chromatography electrospray ionization tandem mass spectrometry) method was developed. A validation of this LC-ESI-MS/MS method with respect to selectivity, linearity, accuracy and precision according to the FDA guidance demonstrated its reliability for quantification of Org24598 in binding experiments. For the implementation of GlyT1 binding experiments conditions in accordance to known GlyT1 radioligand binding assays and already known filtration based MS Binding Assays were chosen. In saturation experiments the affinity of Org24598 towards GlyT1 could be characterized with an equilibrium dissociation constant (K_d) of 16.8 ± 2.2 nM that is well in agreement with the affinity determined in radioligand binding assays. Finally, several known GlyT ligands were studied in competition experiments and the determined inhibition constants (K_i) compared with results from radioligand binding and uptake assays. The almost perfect correlation of the affinities obtained in the MS based binding experiments with results from literature clearly indicates that the established GlyT1 MS Binding Assays are a powerful substitute for the GlyT1 radioligand binding assays so far used for affinity profiling and screening.

This article is part of the issue entitled 'Special Issue on Neurotransmitter Transporters'.

1. Introduction

Glycine (Fig. 1; **1**) has two pivotal functions as neurotransmitter in the central nervous system (CNS). Firstly, it can act as an inhibitory neurotransmitter at inhibitory glycinergic synapses where it binds to the strychnine-sensitive glycine-A binding site on glycine receptors (GlyR) at the postsynapse, which leads to an inward current of chloride and results in a hyperpolarization of the neuron (Cascio, 2006). Secondly, glycine acts as a co-agonist next to the neurotransmitter L-glutamate at NMDA (*N*-methyl-D-aspartate) receptors of excitatory glutamatergic synapses where it binds to the strychnine-insensitive glycine-B binding site. When glycine as well as L-glutamate are simultaneously bound at the NMDA receptor and the postsynaptic membrane is depolarized sufficiently, its channel can be passed by cations, whereby the influx of Ca^{2+} is particularly noteworthy, as the latter acts as a second messenger in the postsynaptic neuron. Furthermore, binding of glycine at the strychnine-insensitive glycine-B binding site results in a positive allosteric effect, that increases the affinity for L-glutamate and, therefore, leads to an enhanced excitation of NMDA receptors (Zafra et al., 2017).

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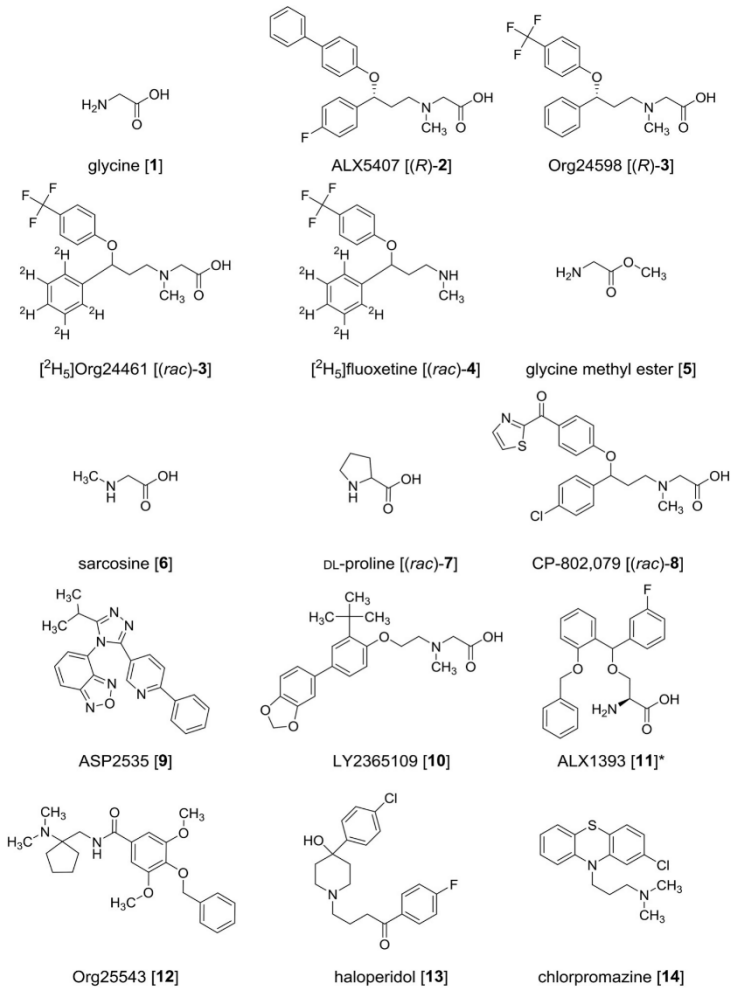

Fig. 1. Structures of GlyT ligands, Org24598 used as GlyT1 marker and [$^2H_5$]Org24461 used as internal standard together with [$^2H_5$]fluoxetine as precursor for synthesis of [$^2H_5$]Org24461. * Stereochemistry not unequivocally characterized neither by literature nor by manufacturer or vendor.

Essential for control of the glycinergic neurotransmission is the regulation of the extracellular glycine concentration which is mainly achieved by glycine transporters (GlyT). There are two GlyT subtypes known, referred to as GlyT1 and GlyT2, both occurring in different variants as a result of alternative promotor usage and splicing (GlyT1a-e and GlyT2a-c) (Harvey and Yee, 2013). Both subtypes are members of the Na^+/Cl^--dependent solute carrier 6 (SLC6) family and show a similarity at the amino acid sequence level of 48% (López-Corcuera et al., 2001). As the family name indicates, the transport of glycine is coupled to a co-transport of Na^+ and Cl^- and driven by a concentration gradient of Na^+ (stoichiometry for GlyT1: 2 Na^+: 1 Cl^-: 1 glycine; GlyT2: 3 Na^+: 1 Cl^-: 1 glycine) (Supplission and Roux, 2002).

The glycine transporter subtypes exhibit different distribution patterns and functions. GlyT1 occurs at inhibitory glycinergic synapses, where it is preferentially located on glial cells. Here it regulates the glycine concentration in the synaptic cleft and terminates glycinergic neurotransmission. Additionally, GlyT1 is found at excitatory glutamatergic synapses, where it is located on glial cells as well as on pre- and postsynaptic terminals. There its main function is to keep the glycine concentration under the level required for saturation at the strychnine-insensitive glycine-B site of NMDA receptors. The occurrence of GlyT2 is, in contrast to GlyT1, restricted to presynapses of inhibitory glycinergic synapses, where it is primarily responsible for termination of glycinergic neurotransmission together with GlyT1 and for recycling of glycine (Dohi et al., 2009; Harvey and Yee, 2013; Vandenberg et al., 2014).

NMDA receptors at excitatory glutamatergic synapses are known to play an important role for learning, memory and developmental plasticity but also for the pathology of several diseases such as schizophrenia or Alzheimer's disease (Zafra et al., 2017). It is hypothesized that their hypofunction is responsible for symptoms like apathy, motor retardation, emotional withdrawal and cognitive deficits, which are

typically associated with schizophrenia (Javitt, 2007). According to this hypothesis, an amplification of NMDA receptor function is assumed to have beneficial effects for the treatment of schizophrenia. Unfortunately, direct activation of NMDA receptors with NMDA agonists or glycine agonists is of limited therapeutic value due to problems such as neurotoxic effects or seizures and poor blood-brain-barrier penetration. As inhibition of GlyT1 at glutamatergic synapsis results in an increased glycine concentration in the synaptic cleft, and thereby, in the end also in an increased excitation of NMDA receptors, it is hypothesized to be an alternative to the use of NMDA and glycine agonists. Hence this approach moved into the focus for treatment of schizophrenia (Lindsley et al., 2006). Several studies confirmed the antipsychotic activities of GlyT1 inhibitors in animal models, especially in the case of treating negative and cognitive symptoms of schizophrenia (Alberati et al., 2012; Boulay et al., 2008; Chaki et al., 2015; Depoortère et al., 2005; Harada et al., 2012). Apart from their potential for the treatment of schizophrenia, GlyT1 inhibitors may additionally be suited for the therapy of drug addiction (e.g. alcohol dependence) or in therapy of neuropathic chronic pain (Danysz and Parsons, 1998; Harvey and Yee, 2013; Zafra et al., 2017). The therapeutic potential of GlyT1 inhibitors already demonstrated *in vivo* and the lack of antipsychotics for treatment of negative and cognitive symptoms of schizophrenia led to an increased interest for this target in pharmaceutical companies, which resulted in several clinical studies since 2008 (Singer et al., 2015). Although no GlyT1 inhibitor has been approved for the above mentioned indications so far, the interest in GlyT1 inhibitors is still present today. Just recently, Boehringer Ingelheim announced that the potent and selective GlyT1 inhibitor BI 425809 (structure not published so far), passed phase I clinical studies for the treatment of schizophrenia and additionally for cognitive impairment in Alzheimer's disease (Moschetti et al., 2018). Right now, BI 425809 resides in phase II clinical studies for both indications and currently participants are recruited for successive studies (ClinicalTrials.gov: NCT02788513, NCT02832037, 2018).

Due to the therapeutic potential of GlyT1 inhibitors, numerous drug screening campaigns have been started - and are still going on - which aimed at the identification of compounds with biological activity at or affinity for this drug target. For characterization of biological activity at GlyT1, transport assays based on radiolabelled glycine as substrate are employed, in case a considerable throughput is required (Brown et al., 2001; Kopec et al., 2009; Santora et al., 2018). When only single compounds are to be characterized regarding their inhibitory activity at GlyT1 electrophysiological studies may be suitable as well (Mezler et al., 2008). For identification of GlyT1 ligands or for determination of their affinity radioligand binding assays are used (Amberg et al., 2018; Mezler et al., 2008; Wang et al., 2018). Both techniques, i.e. transport and binding assays, are exclusively based on the use of radioisotopes such as ^{3}H and ^{14}C. As the use of radioisotopes is, however, inherently coupled to several serious drawbacks, such as safety issues, restrictions set by authorities and problems with remaining waste, there is a strong need for label-free assay techniques addressing GlyT1. For GlyT1 transport assays based on radiolabelled glycine already a fluorescence based alternative has been reported, recording changes in the membrane potential as a consequence of the net inward flux of a single positive charge for every glycine molecule transported by the transporter (Allan et al., 2006). With respect to binding assays addressing GlyT1, however, to the best of our knowledge, up to now no alternative to radioligand binding assays has been described. Furthermore, all of the radioligands used for this purpose so far are not commercially available, as they have been synthesized in-house by pharma companies for their own purposes. Due to this unsatisfactory situation we decided to establish an alternative to GlyT1 radioligand binding assays by applying the concept of MS Binding Assays recently introduced by our group (Grimm et al., 2015a, 2015b; Hess et al., 2011a, 2011b; Neiens et al., 2015, 2018a, 2018b; Schuller et al., 2017; Zepperitz et al., 2006, 2008). MS Binding Assays are based on the use of a nonlabelled reporter ligand instead of a ligand labelled with a radioisotope, the former of which is therefore termed “native marker”, “MS Marker” or simply “marker” that is quantified by means of mass spectrometry (MS). Binding experiments following this strategy can be performed as simple as radioligand binding experiments by incubation of the target with the marker (together with test compounds if necessary). MS Binding Assays require – like radioligand binding assays – for termination of the binding experiment a separation of the formed target-marker-complexes from nonbound marker which is typically achieved by filtration. In MS Binding Assays, subsequently to this separation step, the formerly bound marker is finally quantified by LC-MS after its liberation and elution from the target-marker-complexes remaining on the filter with an organic solvent, whereas in radioligand binding experiments in contrast the bound marker remaining on the filter employed for separation is quantified by LSC (liquid scintillation counting).

For the establishment of GlyT1 MS Binding Assays, at first, an appropriate marker has to be identified. As several tritium labelled GlyT1 inhibitors have already proved their suitability as reporter ligands in filtration based binding experiments (Alberati et al., 2012; Herdon et al., 2010; Mallorga et al., 2003; Mezler et al., 2008; Zhang et al., 2011), we decided to start with a nonlabelled GlyT1 inhibitor already employed as radioligand. Therefore, the commercially available GlyT1 selective inhibitors ALX5407 (Fig. 1; (*R*)-**2**) and Org24598 (Fig. 1; (*R*)-**3**) were selected for this purpose. For none of both compounds a method for a highly sensitive quantification has been described so far. Hence, at first such a method using liquid chromatography electrospray ionization with tandem mass spectrometry (LC-ESI-MS/MS), a technique assumed to be best suited for this task, had to be established (Höfner and Wanner, 2015). The analytical range required for marker quantification can be estimated from the equilibrium dissociation constant (K_d) of the marker towards GlyT1 taking into account the commonly accepted directives for radioligand binding assays suggesting examination of nominal marker concentrations in saturation experiments between 0.1 K_d and 10 K_d while the target concentration should not exceed 0.1 K_d to avoid marker depletion (Bylund et al., 2004; Hulme, 1992). Hence, as a rule of thumb, at least a marker concentration of about 0.01 K_d should be quantifiable (Grimm et al., 2015b). Taking into account the K_d values described for ALX5407 and Org24598 ranging from 4 to 21 nM (Alberati et al., 2012; Mallorga et al., 2003; Zhang et al., 2011) it was aspired to reach a lower limit of quantification (LLOQ) in the lower pM range. After the final selection of the marker, i.e. whether ALX5407 or Org24598 should be used, based on its suitability for LC-MS quantification, the developed LC-ESI-MS/MS method should be validated according to the FDA guidance for bioanalytical method validation (FDA, 2018) to guarantee its reliability for marker quantification in binding experiments. Subsequently, binding experiments should be performed in analogy to known GlyT1 radioligand binding assays and MS Binding Assays established for other neurotransmitter transporters of the SLC6 family (Alberati et al., 2012; Grimm et al., 2015a, 2015b; Herdon et al., 2010; Hess et al., 2011a, 2011b; Mallorga et al., 2003; Mezler et al., 2008; Neiens et al., 2018a, 2018b; Zepperitz et al., 2006, 2008; Zhang et al., 2011). Thereby, the affinities of the marker and known GlyT ligands (see Fig. 1) towards GlyT1 should be characterized in saturation and competition experiments, respectively. Finally, the results obtained with the established GlyT1 MS Binding Assays should be compared with those from literature to prove their reliability.

2. Results and discussion

2.1. LC-ESI-MS/MS method

As already discussed in the introduction, the first important step in the development of an MS Binding Assay is to establish an LC-ESI-MS/MS method which allows the quantification of the marker with sufficient sensitivity. For this purpose, a triple quadrupole mass

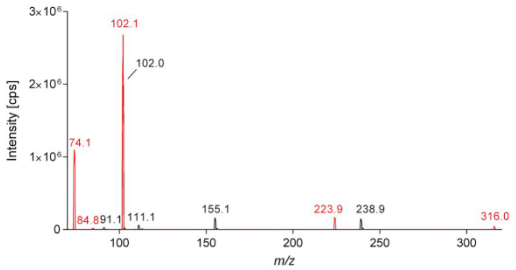

Fig. 2. Product ion scans for the $[M+H]^+$ parent ions of ALX5407 (m/z 394.1; red) and Org24598 (m/z 368.0; black) with the five most intensive product ions.

spectrometer coupled to a pneumatically assisted electrospray ionization (ESI) source and operated in the multi-reaction monitoring (MRM) mode to reach the required selectivity and sensitivity was employed as successfully done in former related studies (Grimm et al., 2015a, 2015b; Hess et al., 2011a, 2011b; Neiens et al., 2015, 2018a, 2018b; Schuller et al., 2017; Zepperitz et al., 2006, 2008). As we aimed at a substitute for commonly used GlyT1 radioligand binding assays, we started with studying the nonlabelled species of two of the most popular radioligands that are commercially available, namely ALX5407 and Org24598 as already mentioned in the introduction. ESI-MS/MS mass spectrometry data had not been described so far for these compounds. When determined by us we found the expected $[M+H]^+$ parent ions with m/z 394.1 and 368.0 for ALX5407 and Org24598, respectively, and the following fragment ions: m/z 316.0, 223.9, 102.1, 84.8 as well as 74.1 for ALX5407 and m/z 316.0, 223.9, 102.0, 84.8 as well as 74.1 for Org24598 (Fig. 2). The mass transitions with the highest intensities (ALX5407 → m/z 394.1/102.1 and Org24598 → m/z 368.0/102.0) were selected for further method development.

After the mass spectrometer's compound-dependent parameters had been optimized (see Material and methods), an LC method had to be established which was intended to be based on reversed-phase (RP) chromatography as this chromatographic approach was expected to require the least efforts due to the following reasons. At first, it facilitates a straightforward method development due to its simple retention principle. Secondly, the marker to be quantified should be separated as far as possible from matrix components, generated during the whole procedure of the binding assay, by means of chromatography. As the majority of matrix components is – according to our experience – highly polar, we aimed to develop an RP chromatography method characterized by a capacity factor for the marker in the range from 1 to 2, to achieve, on the one hand, separation of the analyte from interfering matrix components and to ensure, on the other hand, favorable peak shapes as well as short chromatographic cycle times. A Luna C8(2) (50 mm × 2 mm, 3 µm) column in combination with a mobile phase consisting of 5 mM ammonium bicarbonate buffer (pH 7.8) and acetonitrile (55:45, *v/v*) appeared to fulfil these requirements. For ALX5407 as well as for Org24598 capacity factors (k) of 2.12 for ALX5407 and 1.32 for Org24598 were achieved, respectively, and the chromatographic cycle time amounted to only 2.0 min at a flowrate of 400 µL min^{-1}, which is short enough to guarantee a considerable throughput. Remarkably, peak intensities in preliminary MRM chromatograms displayed by Org24598 were at least three times higher than those of ALX5407, as shown in Fig. 3a. Hence, we decided to continue the development of an MS Binding Assay for GlyT1 with Org24598 as marker. Lastly, after all chromatographic conditions had been defined, the source-dependent parameters of the mass spectrometer were optimized (see Material and methods).

Quantification of an analyte by means of LC-ESI-MS/MS in a biological matrix is commonly based on calibration using an external or an

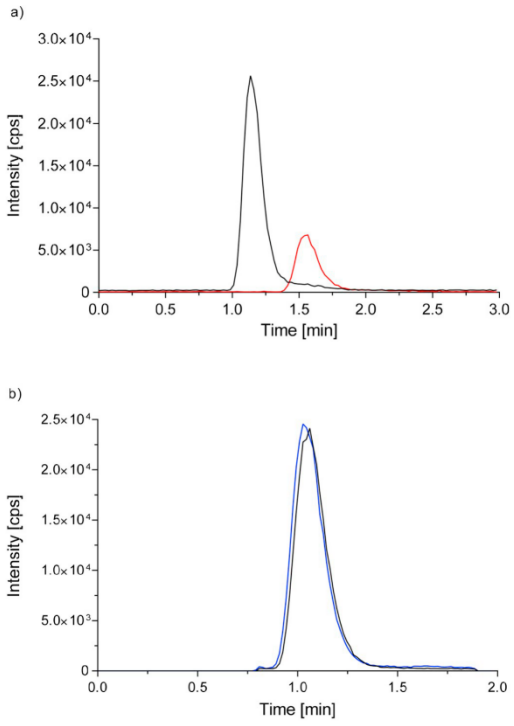

Fig. 3. MRM chromatograms of a) solvent containing 100 pM Org24598 (m/z 368.0/102.0, black) and 100 pM ALX5407 (m/z 394.1/102.1, red) and b) matrix standard containing 100 pM Org24598 (m/z 368.0/102.0, black) and 100 pM [$^2H_5$]Org24461 (m/z 373.0/102.0, blue). For LC a Luna 3µ C8(2) (50 mm × 2 mm, 3 µm) column was used as stationary phase in combination with a mobile phase consisting of ammonium bicarbonate buffer (5 mM, pH 7.8)/acetonitrile (55:45, *v/v*) at a flow rate of 400 µL min^{-1}. In routine LC-ESI-MS/MS runs the eluent was directed to waste from 0.0 to 0.8 min and from 1.9 to 2.0 min as recognizable in b).

internal standard. Both approaches are also applicable for marker quantification in MS Binding Assays (Höfner and Wanner, 2015; Massink et al., 2015). It is commonly accepted that the latter approach provides a distinctly higher degree of robustness, especially when internal standard and analyte coeluate (Höfner und Wanner, 2015). In particular, isotopically labelled analogs of the analyte by exhibiting almost identical physicochemical properties are considered best suited as internal standards in LC-ESI-MS/MS quantifications. With respect to the validity of the data, which should be as high as possible, for the MS Binding Assay under discussion, we decided to perform quantitation of the MS Marker by means of an internal standard. Due to its simple access, we aimed at a deuterated analog of Org24598 [(*R*)-**3**] for this purpose. Even though not commercially available, it appeared to be easily accessible in a two-step synthesis starting from fivefold deuterated fluoxetine [(*rac*)-**4**], closely related to Org24598 [(*R*)-**3**] and still in stock from a recent project (Hess et al., 2011a, 2011b). Indeed, the preparation of [$^2H_5$]Org24461 [(*rac*)-**3**] could be smoothly performed as described below. In this context it should be, however, emphasized that an isotopically labelled internal standard is not a prerequisite for marker quantification in MS Binding Assays and it is worth mentioning that even ALX5407 proved to be a well suited alternative during LC-MS method development (data not shown).

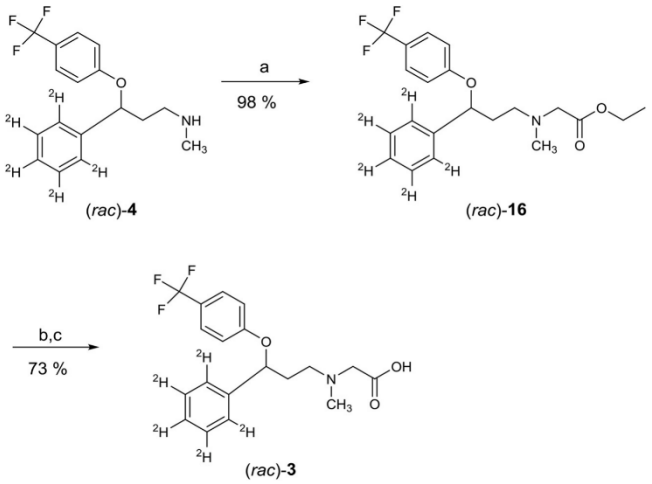

Fig. 4. Synthesis of [$^2H_5$]Org24461. Reagents and conditions: a) K_2CO_3, ethyl 2-bromoacetate, CH_3CN, rt, 18 h; b) 2 M NaOH, MeOH, rt, 4 h; c) 2 M HCl, CH_2Cl_2, extracted.

2.2. Synthesis of the internal standard [$^2H_5$]Org24461

For the synthesis of [$^2H_5$]Org24461 (Fig. 4) [$^2H_5$]fluoxetine was reacted with ethyl 2-bromoacetate according to Ognyanov et al. (2001) providing (*rac*)-**16** in a yield of 98%. Upon subjection of the carboxylic ester (*rac*)-**16** to hydrolysis under basic conditions (NaOH) [$^2H_5$] Org24461 was obtained in a yield of 73% (see Material and methods).

2.3. Considerations and preliminary experiments regarding the general setup and the conditions for the GlyT1 MS binding assays to be established

Before applying of the established LC-ESI-MS/MS method for marker quantification in GlyT1 MS Binding Assays it was deemed necessary to validate it according to the FDA guidance for bioanalytical method validation (FDA, 2018) in order to guarantee its reliability. For this validation the generation of the matrix of the final sample subjected to LC-ESI-MS/MS analysis had to be exactly defined. Therefore, the general setup and the conditions under which the MS Binding Assays is run had to be defined. At first, a suitable incubation buffer had to be selected. We choose a buffer composed of 10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (pH 7.5), which has already been reported in literature to be used in radioligand binding assays for GlyT1 (Mezler et al., 2008). An essential part of an MS Binding Assay is the separation of bound from nonbound marker after incubation. This was intended to be done via filtration over glass fiber filters in this case, followed by washing of these filters to remove residual nonbound marker from target-marker-complexes. As the washing buffer also contributes to the composition of the final sample instead of the incubation buffer, with a high load of nonvolatile components, for this purpose a 154 mM ammonium acetate buffer (pH 7.4) as in successful previous approaches should be used (Grimm et al., 2015a). The pretreatment of filters with solvents or reagents to reduce filter binding of the reporter ligand is often indispensable to limit non-specific binding in filtration based binding assays (i.e. in MS Binding Assays as well as in radioligand binding assays), but may lead to unfavorable contributions to the matrix of the final samples obtained after elution of the bound marker (Neiens et al., 2018a, 2018b). As filter binding of Org24598 was observed to be significant in preliminary experiments, the influence of several filter pretreatment reagents reported by Scott et al. (1995) on filter binding was investigated. From the studied pretreatment conditions (for details see Supplementary Information Fig. S1), an aqueous 1% (*m/m*) Tween20 solution was found most efficient with respect to reduction of filter binding. Finally, the elution step, in which bound marker is liberated and eluted from target-marker-complexes remaining on the filters, was studied. For this purpose, the organic solvents methanol and acetonitrile had already been shown to be suited in former MS Binding Assays (Grimm et al., 2015a, 2015b; Hess et al., 2011a, 2011b; Neiens et al., 2015, 2018a, 2018b; Schuller et al., 2017; Zepperitz et al., 2006, 2008). Preliminary experiments clearly revealed that methanol was distinctly more efficient in eluting the target-bound marker from the filters than acetonitrile (data not shown). Unfortunately, methanol as major solvent constituent of the final sample subjected to LC-MS tended to broaden and split the peaks obtained for Org24598 in MRM chromatograms. Nevertheless, we decided – due to its higher efficiency – to use methanol for elution of the target-bound marker. To avoid the chromatographic problem, i.e. the unfavorable peak shapes caused by methanol in the sample, we decided to evaporate the sample solvent and to reconstitute the resulting residues with mobile phase (5 mM ammonium bicarbonate buffer pH 7.8 and acetonitrile; 55:45, *v/v*). Examination of the recovery of Org24598 in different samples spiked before or after evaporation of methanol demonstrated that no significant loss occurred during this step (for details see Supplementary Information Fig. S2).

Based on the above described results and by considering the conditions used for already established MS Binding Assays (Grimm et al., 2015a, 2015b; Hess et al., 2011a, 2011b; Neiens et al., 2015, 2018a, 2018b; Schuller et al., 2017; Zepperitz et al., 2006, 2008) and GlyT1 radioligand binding assays (Alberati et al., 2012; Herdon et al., 2010; Mallorga et al., 2003; Mezler et al., 2008; Zhang et al., 2011) allowed to define the conditions and setup for GlyT1 MS Binding Assays employing Org24598 as marker as follows. Crude membrane fractions (as common in radioligand binding assays) prepared from CHO-K1 cells stably expressing the human glycine transporter 1 serving as target source are incubated with Org24598 in the incubation buffer (mentioned above) at room temperature for 1 h in 96-deepwell plates, in a total volume of 250 µL per well. Termination of the binding experiment is accomplished by transferring aliquots of 210 µL of the corresponding binding samples to a 96-well glass fiber filter plate (pretreated with Tween20, for details

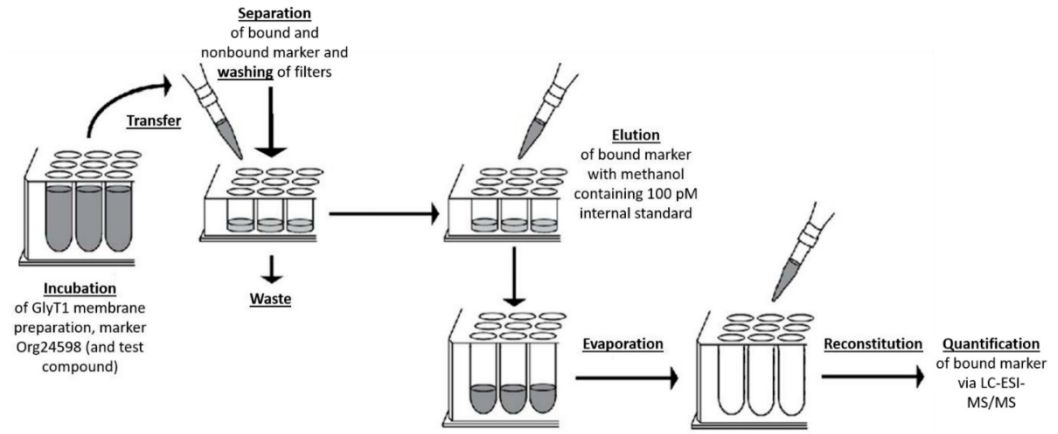

Fig. 5. Workflow of the developed GlyT1 MS Binding Assays.

see Material and methods), their filtration and subsequent washing (three times) with 200 µL ammonium acetate buffer (154 mM, pH 7.4). The filter plates (with the target-marker-complexes remaining on the filter) are then dried at 50 °C for 1 h, and eluted with 3 × 70 µL of a methanolic solution containing the internal standard [$^2H_5$]Org24461 in a concentration of 100 pM into another 96-deepwell plate. Finally, after removing the solvent of the samples at 50 °C under a nitrogen stream, remaining residues are reconstituted in 210 µL of the solvent of the mobile phase and subjected to LC-ESI-MS/MS analysis (45 µL injection volume) for the quantitation of Org24598 by the developed method. A pictorial description of the technical setup and the performance of this GlyT1 MS Binding Assay is given in Fig. 5.

2.4. LC-ESI-MS/MS method validation

With the matrix generation of the samples subjected to LC-MS being defined, the validation of the above described LC-ESI-MS/MS method according to the FDA guidance for bioanalytical method validation was performed (FDA, 2018). For examination of selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision in a range from 5 pM to 1 nM a blank matrix was prepared following the procedure of the MS Binding Assays described above, except that the incubation was performed without marker or any other GlyT ligand. From this blank matrix, five sets of matrix standards and quality control (QC) samples, matrix blanks and zero samples were prepared which contained (as far as necessary) Org24598 in a concentration range from 5 pM to 1 nM as well as the internal standard [$^2H_5$]Org24461 in a fixed concentration of 100 pM (for details see Material and methods). The thus established sets of calibration standards, blanks, zero samples and quality control samples were analyzed with the developed method at different days as described in detail in Material and methods. The validation results obtained are given in Supplementary Information (Table S1), representative chromatograms for Org24598 at the LLOQ and a matrix blank as well as the linear calibration function deduced from a single set of calibration standards are depicted in Fig. 6. In total, these results can be summarized as following. Regarding linearity in a concentration range of 5 pM (LLOQ) to 1 nM the developed LC-ESI-MS/MS method is in full agreement with the criteria from the FDA guidance. The same is true for intra- and inter-batch precision and accuracy as determined for QC samples at three different concentration levels (15 pM, 250 pM, 750 pM; defined criteria for linearity, intra- and inter-batch precision and accuracy as given in the FDA guidance; see Material and methods). As confirmed by injecting six individually prepared matrix blanks, per validation series, which did not exhibit any interfering signals for the chosen mass transitions, also the criteria of selectivity of this LC-ESI-MS/MS method is fulfilled. Thus, with all results being in accordance with the FDA guidance, the developed LC-ESI-MS/MS method has been demonstrated to be reliable for the quantification of Org24598 as marker in MS Binding Assays.

2.5. GlyT1 MS binding assay – saturation experiments

After successful validation of the developed LC-ESI-MS/MS method, the binding of Org24598 towards GlyT1 in saturation assays was characterized. In saturation experiments total and non-specific binding at different marker concentrations (typically in a range between 0.1 K_d and 10 K_d) are determined to gain access to the specific binding of the marker. While total binding is determined in the absence of any competitor, non-specific binding is commonly determined in the presence of a high excess of competitor which almost completely impedes binding of the marker to its specific binding site. To obtain the amount of specific marker binding, non-specific binding has to be subtracted from total binding. From a saturation isotherm constructed from these specific binding data the marker's K_d at the target as well as the maximum density of binding sites (B_{max}) of the target may be obtained. To determine total binding of Org24598, the GlyT1 membrane fractions were incubated with the native marker Org24598 in eight different concentrations (1 nM - 200 nM), for determination of non-specific binding the membrane fractions were incubated with the same concentrations of Org24598 as in total binding samples but in presence of the GlyT1 inhibitor ALX5407 in a concentration of 20 µM that could be assumed to be high enough to block the GlyT1 binding sites almost completely. Quantification of Org24598 for total binding samples was possible even at the lowest nominal concentration of 1 nM Org24598. Quantification of non-specific binding was also possible down to nominal marker concentrations of about 10 nM. At lower concentrations, however, the concentration of Org24598 in corresponding samples fell below the LLOQ. As non-specific binding can be assumed to increase linearly with the nominal marker concentration (Davenport and Russel, 1996), data points below this concentration were calculated by extrapolation after modeling the data points for non-specific binding at nominal marker concentrations from 10 nM to 200 nM by linear regression (see also 4.10. Saturation experiments in Material and methods).

In Fig. 7 a representative example for a saturation experiment showing total, non-specific and specific binding is depicted. From data points representing specific binding a saturation isotherm was

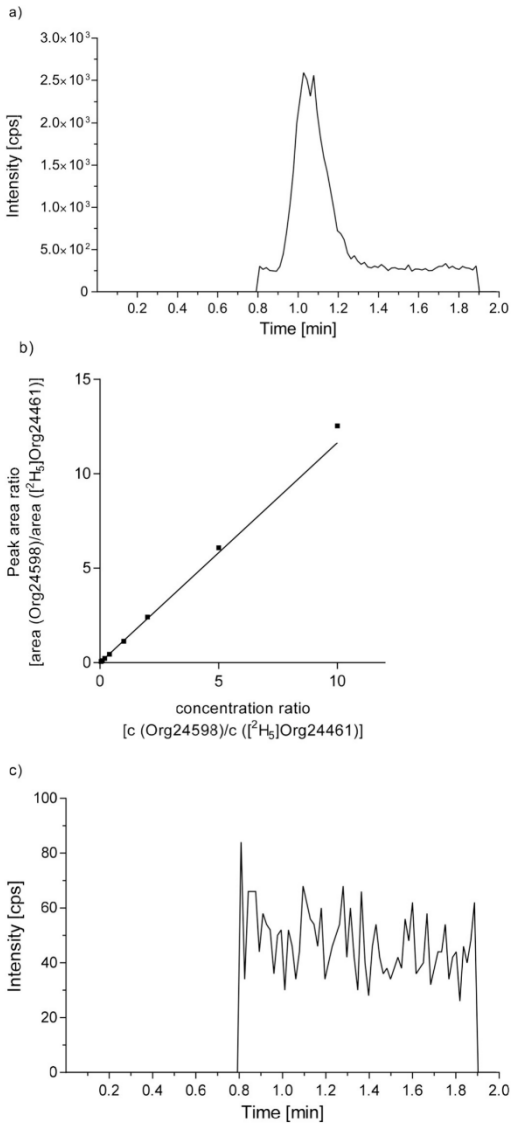

Fig. 6. Validation of the GlyT1 LC-ESI-MS/MS method. a) MRM chromatogram for Org24598 at the LLOQ of 5 pM. b) Representative calibration for Org24598 in a range from 5 pM to 1 nM employing [$^2H_5$]Org24461 as internal standard. The peak area ratio of marker and internal standard was plotted against the concentration ratio of marker and internal standard. The corresponding calibration function obtained by linear regression was y = 1,164x + 0,003127; R^2 = 0,9932. c) MRM chromatogram for a matrix blank (for demonstration of selectivity).

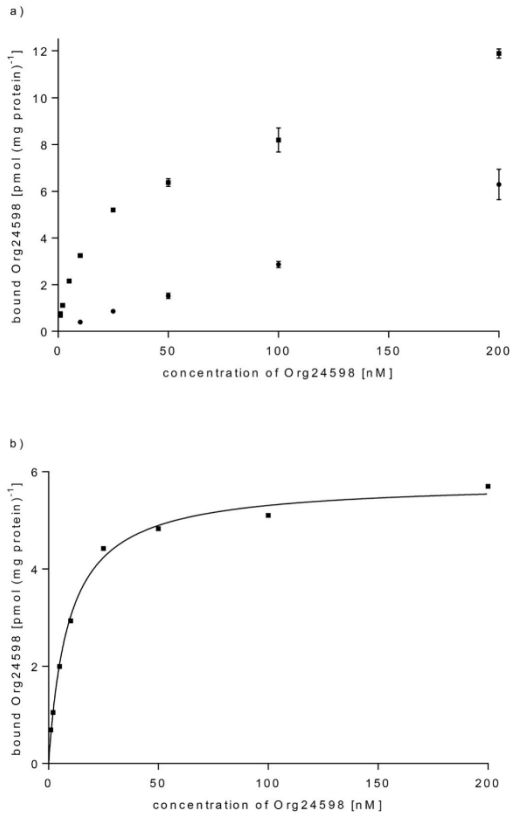

Fig. 7. Representative saturation experiment showing total, non-specific and specific binding of Org24598 at GlyT1. To determine total binding GlyT1 was incubated with increasing concentrations of Org24598 (1–200 nM). Non-specific binding was determined in the same way as total binding but in presence of 20 µM ALX5407. a) Experimental data (mean ± SD, n = 3) for total (■) and non-specific (●) binding. Non-specific binding for Org24598 concentrations less than 10 nM was extrapolated after modeling of experimentally determined non-specific binding at concentrations of Org24598 ≥ 10 nM by linear regression. b) Specific binding (means, pmol (mg protein)$^{-1}$) calculated as the difference of total binding and non-specific binding from a) and saturation isotherm generated by non-linear regression.

generated by means of non-linear regression. Thereby, a K_d value of 16.8 ± 2.2 nM and a maximum density of binding sites (B_{max}) of 6.86 ± 1.33 pmol (mg protein)$^{-1}$ (mean ± SEM, n = 11), respectively, could be calculated for the binding of Org24598 at GlyT1. In literature K_d values of 5.8–7.4 nM are described for [^{3}H]Org24598 binding determined in a radioligand binding assay for different GlyT1 sources (Alberati et al., 2012). Unfortunately, the experimental conditions for the determination of these K_d values are not sufficiently specified in this study. Therefore, a comparison of these K_d values, with that determined in our MS Binding Assays is not very meaningful. In another GlyT1 radioligand binding assay, however, based on [^{3}H]CHIBA-3007 as selective GlyT1 reporter ligand performed under conditions similar to those used by us, a K_i value of 16.9 ± 3.1 nM for Org24598 at GlyT1 was found, which matches exactly the K_d value determined in our MS Binding Assay (Zhang et al., 2011).

2.6. GlyT1 MS binding assays – competitive experiments

Competition experiments are by far the most often applied type of binding experiments as they provide affinity measures for any

compound deduced from their capability to compete with the reporter ligand for the target binding site. In this study, we used competition experiments to determine the affinities for glycine [**1**] as well as other well-known GlyT ligands (ALX5407 [(*R*)-**2**], glycine methyl ester [**5**], sarcosine [**6**], DL-proline [(*rac*)-**7**], CP-802,079 [(*rac*)-**8**], ASP2535 [**9**], LY2365109 [**10**], ALX1393 [**11**], Org25543 [**12**], haloperidol [**13**], chlorpromazine [**14**]; for structures see Fig. 1) in order to compare them with those from literature (Caulfield et al., 2001; Harada et al., 2012; López-Corcuera et al., 1998, Martina et al., 2004; Williams et al., 2004; Zhang et al., 2011) and hence to prove the reliability of the established MS Binding Assays. The conditions for competition experiments were basically the same as described above for saturation experiments but instead of incubating the target with varying marker concentrations the concentration of Org24598 was set to 15 nM (i.e. close to the determined K_d value as it is typically done in radioligand binding assays) (Hulme, 1992). In this way the above mentioned reference compounds were studied at seven concentration levels (covering about three concentration log units) in competitive binding experiments, quantifying marker binding in presence of the test compounds by LC-ESI-MS/MS. Subtraction of non-specific binding (again determined in the presence of 20 µM ALX5407) from the obtained Org24598 concentrations in the binding samples (total binding), led to specific binding which was normalized (i.e. 100% equivalent to specific binding of Org24598 without any inhibitor and 0% to non-specific binding) and plotted as percentage (y-axis) against the logarithm of the competitor concentration (x-axis). Subsequent generation of corresponding competition curves by means of nonlinear regression analysis revealed half maximal inhibitory concentration (IC_{50}), and finally, K_i values applying the Cheng-Prusoff equation. Representative sigmoidal competition curves showing the competition of Org24598 in the presence of glycine and the selective GlyT1 inhibitor ALX5407, respectively, are depicted in Fig. 8. Table 1 lists all determined pK_i values for the investigated reference compounds in comparison with results reported in literature. In this context it has to be mentioned that for some of these compounds affinity data are lacking, presumably due to the fact that radioligands for GlyT1 are commercially not available, as already described above. Therefore, for these compounds the inhibitory potencies assessed in uptake assays were used for the comparison (Caulfield et al., 2001; Harada et al., 2012; López-Corcuera et al., 1998; Martina et al., 2004; Williams et al., 2004). With respect to the GlyT2 selective inhibitor Org25543 a reasonable comparison was not possible, as its inhibitory potency at GlyT1 has not been exactly described so far in literature (IC_{50} value > 100 µM), nevertheless it could be confirmed that it shows only poor affinity for GlyT1. With respect to all other reference compounds, however, it could be shown that the rank order of affinity for the reference compounds obtained in MS Binding Assays (ordered by increasing affinities: DL-proline < glycine methyl ester < glycine < sarcosine < chlorpromazine < haloperidol < ALX1393 < ASP2535 < CP-802,079 < LY2365109 < ALX5407) matches exactly the rank order of affinity/inhibitory potency deduced from radioligand binding experiments and uptake assays reported in literature. Next we tried to examine the correlation of the pK_i values determined in the GlyT1 MS Binding Assays with results from literature despite the difficulties mentioned before. Due to the different origin of literature results (i.e. from radioligand binding or uptake assays, respectively) the reference compounds were split into two different sets. The first set contains compounds studied in radioligand binding assays and the second one compounds studied in uptake assays. Since only percentages of inhibition at a defined concentration for glycine methyl ester and DL-proline but no detailed K_i or IC_{50} values are published, these compounds were excluded from this correlation. To explore this correlation, experimental results from MS Binding Assays (i.e. pK_i values) were plotted for both sets against the corresponding results from radioligand binding assays and uptake assays (i.e. corresponding pK_i and pIC_{50} values), respectively. To this end, all K_i and IC_{50} values from literature were transformed into pK_i or pIC_{50} values by taking the negative decadal logarithm (whereby the latter - taking into account the Cheng-Prusoff equation - can be assumed not to deviate distinctly from their corresponding pK_i values as the substrate concentration in the uptake experiments was always low in comparison to the K_m value of glycine). For the first set, the correlation of the pK_i values from MS Binding Assays with those from radioligand binding experiments can be described with a straight line characterized by $y = 0.878x + 0.581$ and $R^2 = 0.965$ (Fig. 9a). Analogously, for the second set, the correlation of pK_i values from MS Binding Assays with pIC_{50} values from uptake can be described with a straight line characterized by $y = 0.912x + 0.328$ and $R^2 = 0.999$ (Fig. 9b). Even the combination of both sets results in a good correlation as indicated by the straight line characterized with $y = 0.886x + 0.516$ and $R^2 = 0.973$ (Fig. 9c). The results obtained in the described competition experiments clearly indicate the reliability of the established MS Binding Assays and thus unequivocally demonstrate their suitability for affinity profiling at GlyT1.

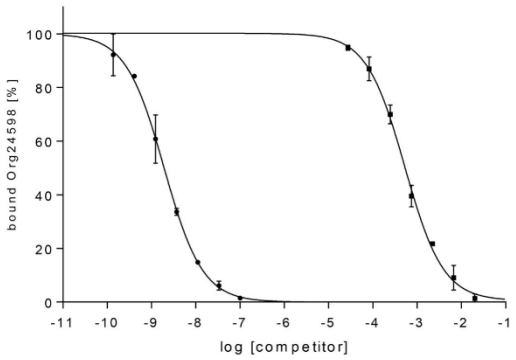

Fig. 8. Representative competition curves obtained in GlyT1 MS Binding Assays for ALX5407 (●) and glycine (■). The experimental data represent specific binding (mean ± SD, n = 3) of Org24598 at varying concentrations of ALX5407 and glycine. 100% binding was equivalent to specific binding of Org24598 without any competitor and 0% to non-specific binding.

3. Conclusion

In this study the first binding assays based on a nonlabelled reporter ligand addressing GlyT1 are described. Following the concept of MS Binding Assays recently introduced in our group, binding of the well-known GlyT1 inhibitor Org24598 employed as reporter ligand towards GlyT1 could be monitored using LC-MS for quantification. For this purpose, a highly sensitive LC-ESI-MS/MS method was developed that enables reliable quantification of Org24598 in binding experiments in a range from 5 pM to 1 nM using the five times deuterated racemic species of the marker, [$^2H_5$]Org24461, as internal standard. The use of a deuterated internal standard might give rise to the assumption that the established GlyT1 MS Binding assays employing Org24598 as non-labelled marker do not really represent a label-free assay technique and furthermore, that the access to this internal standard may be also somehow restricted. However, in this context it should be mentioned that deuterated compounds can be employed without any safety or environmental concern or restriction in every lab and that the two step synthesis of [$^2H_5$]Org24461 described in this study is based on commercially available compounds and does not require skills beyond basic synthetic chemistry. The most important argument in the discussion of this issue is, however, that the use of a deuterated internal standard is not essential for marker quantification by means of LC-ESI-MS/MS in MS Binding Assays as demonstrated in former studies (Massink et al., 2015; Neiens et al., 2015; Schuller et al., 2017). In this project during

Table 1
Affinities of known GlyT ligands determined in GlyT1 MS Binding Assays (mean ± SEM, n = 3–5) with a comparison of affinities determined in radioligand binding assays or inhibitory potencies determined in [^{3}H] or [^{14}C]glycine uptake assays published in literature. * Glycine uptake in presence of 1 mM competitor (% of control). **[a]** pK_i values. **[b]** pIC_{50} values. [1] Zhang et al. (2011); [2] López-Corcuera et al. (1998); [3] Martina et al. (2004); [4] Harada et al. (2012); [5] Caulfield et al. (2001); [6] Williams et al. (2004).

Compound	pK_i (GlyT1 MS Binding Assays)	K_i (literature, binding) [μM]	IC_{50} (literature, uptake) [μM]	pK_i[a] or pIC_{50}[b] (literature; calculated)
glycine [**1**]	3.47 ± 0.04	288 ± 27[1]		3.54[1,a]
ALX5407 [(*R*)-**2**]	8.89 ± 0.04	0.004 ± 0.001[1]		8.39[1,a]
glycine methyl ester [**5**]	1.97 ± 0.07		55 ± 3%*[2]	
sarcosine [**6**]	4.32 ± 0.05	103 ± 11[1]		3.99[1,a]
DL-proline [(*rac*)-**7**]	1.94 ± 0.03		76 ± 4%*[2]	
CP-802,079 [(*rac*)-**8**]	8.22 ± 0.03		0.016[3]	7.79[3,b]
ASP2535 [**9**]	7.01 ± 0.06		0.170 ± 0.021[4]	6.77[4,b]
LY2365109 [**10**]	8.46 ± 0.02	0.016 ± 0.004[1]		7.79[1,a]
ALX1393 [**11**]	5.49 ± 0.06	0.852 ± 0.056[1]		6.07[1,a]
Org25543 [**12**]	4.33 ± 0.02		> 100 μM[5]	
haloperidol [**13**]	5.22 ± 0.01		9 ± 2[6]	5.05[6,b]
chlorpromazine [**14**]	4.80 ± 0.01		19 ± 3[6]	4.72[6,b]

method development, it has been found, for example, that also another commercially available GlyT1 inhibitor, namely ALX5407, can be used for this purpose.

After validation of the developed LC-ESI-MS/MS method for quantification of Org24598 it was used as readout for filtration based MS Binding Assays performed in analogy to known GlyT1 radioligand binding assays and MS Binding Assays recently established for other neurotransmitter transporters. In saturation experiments, the affinity of Org24598 at GlyT1 could be characterized with a K_d of 16.8 nM. The developed MS Binding Assays were also used to determine the affinities of known GlyT ligands at GlyT1 in competition experiments. A comparison of the affinities obtained in MS Binding Assays with results from literature showed an almost perfect match thus demonstrating the reliability of the established GlyT1 MS Binding Assays.

Due to the importance of GlyT1 as a drug target - particularly for schizophrenia but also for other CNS disorders - techniques for identification of GlyT1 ligands and determination of their affinities towards this target are of high value for drug development in this field. The radioligand binding assays employed so far for this task have two remarkable disadvantages. At first, all the problems associated with the use of radioactivity itself have to be considered and restrict in the end substantially the possibility to perform radioligand binding assays. Secondly, GlyT1 radioligands are not commercially offered, meaning that even their access is distinctly restricted which is quite astonishing. Therefore, it can be assumed that the established GlyT1 MS Binding Assays will be of great interest to a wide group of potential users facing so far severe obstacles to perform radioligand binding assays. Furthermore, the GlyT1 MS Binding Assays described in this study, may be an appealing alternative, to substitute screening techniques based on the usage of radioisotopes in order to avoid the inherent drawbacks associated with them.

4. Material and methods

4.1. 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). LY2365109 (*N*-[2-[4-(1,3-Benzodioxol-5-yl)-2-(1,1-dimethylethyl)phenoxy]ethyl]-*N*-methylglycine) and ASP2535 (4-[3-(1-Methylethyl)-5-(6-phenyl-3-pyridinyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole) were part of the Tocriscreen Plus library from Tocris (Bristol, UK). CP-802,079 (*N*-[3-(4-Chlorophenyl)-3-[4-(2-thiazolylcarbonyl) phenoxy]propyl]-*N*-methyl-glycine) as hydrochloride (purity ≥ 98%), ALX1393 (*O*-[(2-Benzyloxyphenyl-3-flurophenyl)methyl]-L-serine) (purity ≥ 98%), haloperidol, chlorpromazine as hydrochloride (purity ≥ 98%, TLC) were purchased from Sigma-Aldrich (Steinheim, Germany). [$^2H_5$] fluoxetine hydrochloride was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Glycine (purity ≥ 99%) and DL-proline (purity ≥ 99%) was from Acros Organics (Geel, Belgium), glycine methyl ester as hydrochloride (purity ≥ 99%) and sarcosine as hydrochloride were from Merck KGaA (Darmstadt, Germany) and ICN Biomedicals (Irvine, California, US), respectively. 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 and for the elution of marker from target-marker-complexes. LC-MS grade acetonitrile from VWR Prolabo (Darmstadt, Germany) was used for the mobile phase in LC-MS. All other chemicals were purchased in analytical grade. For cell culture, Nutrient Mixture F-12 Ham from Sigma-Aldrich (Steinheim, Germany) was employed, fetal bovine serum, penicillin, streptomycin and trypsin-EDTA were from BioWest (Nuaillé, France) and G418 from Merck KGaA (Darmstadt, Germany), respectively.

4.2. LC-ESI-MS/MS instrumentation

For LC-ESI-MS/MS an API5000 triple quadrupole mass spectrometer with a TurboV-ESI source (AB Sciex, Darmstadt, Germany) was used. It was coupled to an Agilent 1200 Series HPLC system (vacuum degasser G1379B, binary pump G1312B, oven G1316B, Agilent, Waldbronn, Germany) and a HTS-PAL auto sampler (CTC-Analytics, Zwingen, Switzerland) with a 50 μL syringe and a 50 μL sample loop. The hardware components were all controlled by Analyst v. 1.6.1 software (AB Sciex, Darmstadt, Germany).

4.3. Compound-dependent MS parameters for precursor and fragment ions of ALX5407, Org24598 and [$^2H_5$]Org24461

20 nM solutions of ALX5407, Org24598 and [$^2H_5$]Org24461 in 0.1% (*v/v*) aqueous formic acid/methanol (50:50, *v/v*) were infused into the ESI source via an external syringe pump (Harvard Apparatus, Holliston, Massachusetts, US) at a flow rate of 10 μL min^{-1}. With the manual tuning mode of the Analyst software *m/z* 394,1, *m/z* 368.0 and *m/z* 373.0 were identified as $[M+H]^+$ parent ions for ALX5407, Org24598 and [$^2H_5$]Org24461, respectively. The compound optimization mode of the Analyst software was used to identify the most intense fragment ions (*m/z* 102.1 for ALX5407 and *m/z* 102.0 for Org24598 and [$^2H_5$]Org24461) as well as to optimize the compound-dependent parameters for the precursor ions, which are listed below: ALX5407: Declustering potential (DP) 101 V, entrance potential (EP) 10 V,

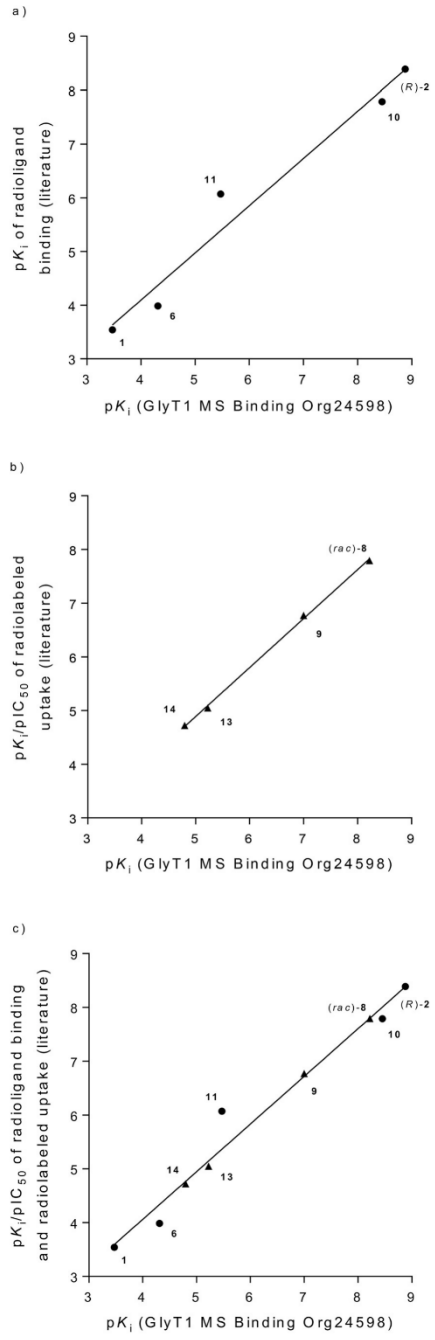

Fig. 9. Correlation of pK_i values from GlyT1 MS Binding Assays with results from literature. Data points represent means of pK_i values determined in this study (n = 3–5) and pK_i/pIC_{50} values calculated from affinities determined in radioligand binding assays (K_i) or inhibitory potencies determined in [^{3}H] or [^{14}C]glycine uptake assays (IC_{50}) published in literature. a) Correlation for compounds, with known affinities determined in radioligand binding assays (y = 0.878x + 0.581; R^2 = 0.965). b) Correlation for compounds not characterized in radioligand binding assays but with known inhibitory potencies determined in [^{3}H] or [^{14}C]glycine uptake assays (y = 0.912x + 0.328; R^2 = 0.999). c) Correlation for compounds with known affinities determined in radioligand binding assays and compounds not characterized in radioligand binding assays but with known inhibitory potencies determined in [^{3}H] or [^{14}C] glycine uptake assays (y = 0.886x + 0.516; R^2 = 0.973). (●) data points from a) (▲) data points from b).

collision energy (CE) 21 V, cell exit potential (CXP) 16 V; Org24598: DP 71 V, EP 10 V, CE 23 V, CXP 16 V; [$^2H_5$]Org24461: DP 60 V, EP 10 V, CE 23 V, CXP 16 V.

4.4. Chromatography

The chromatography was performed under isocratic conditions. As mobile phase a mixture of ammonium bicarbonate buffer (5 mM, pH 7.8) and acetonitrile (55:45, *v/v*) was used at a flow rate of 400 µL min^{-1} at 20 °C. A Luna 3µ C8(2) column (50 mm × 2 mm, 3 µm, Phenomenex, Aschaffenburg, Germany) was used as stationary phase. The column was protected with a SecurityGuard C8 column (4 mm × 2 mm, Phenomenex, Aschaffenburg, Germany) and two in-line filters (0.5 and 0.2 µm, Idex, Oak Harbor, Washington, US).

4.5. LC-ESI-MS/MS

The mass transitions of *m/z* 368.0/102.0, *m/z* 373.0/102.0 and *m/z* 394.1/102.1 were used to analyze Org24598, [$^2H_5$]Org24461 and ALX5407, respectively. Q1 and Q3 were operated under unit resolution for dwell times of 500 ms under the conditions mentioned in *Compound-dependent MS parameters for precursor and product ions of ALX5407, Org24598 and [$^2H_5$]Org24461*. The optimized source-dependent parameters were determined after the selection of Org24598 as marker using the flow injection analysis tool of the Analyst software. Therefore 10 µL of a solution containing 500 pM Org24598 and 100 pM [$^2H_5$] Org24461 were injected. Thereby, the following optimized parameters were obtained: source temperature 600 °C, ion-spray voltage 2000 V, curtain gas (N_2) 15 psi, auxiliary gas (N_2) 60 psi, nebulizing gas (N_2) 40 psi and collision gas (N_2) 7 psi. For quantification of Org24598 the sample volume injected to LC-MS was 45 µL. Data acquisition was restricted to the time period of 0.8–1.9 min after injection. To protect the mass spectrometer during routine measurements an external switching valve (Valco Instruments Co. Inc., Houston, Texas, US) was integrated to direct the eluent to waste from 0.0 to 0.8 min and from 1.9 to 2.0 min.

4.6. Synthesis of [$^2H_5$]Org24461

To a stirred solution of ethyl 2-bromoacetate (6.07 mg, 0.0356 mmol) in acetonitrile (0.7 mL) K_2CO_3 (19.7 mg, 0.143 mmol) and dropwise a solution of [$^2H_5$]fluoxetine ((*rac*)-**4**, 25,0 mg, 0.0713 mmol) in acetonitrile (0.5 mL) were added at rt. After stirring for 18 h the reaction mixture was filtered and evaporated to dryness. The residue was chromatographed on a silica gel column with a mobile phase consisting of ethyl acetate and *i*-hexane (40:60, *v/v*) and evaporated to dryness to afford 28.0 mg (yield: 98%) of ethyl *N*-methyl-*N*-(3-([$^2H_5$]phenyl)-3-(4-(trifluoromethyl)phenoxy)propyl) glycinate ((*rac*)-**16**).

To a solution of (*rac*)-**16** (22.8 mg, 0.057 mmol) in methanol (2 mL) aqueous NaOH (2 M, 4 mL) was added and stirred at rt for 4 h. The

reaction mixture was concentrated to half volume, acidified with aqueous HCl (2 M) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The resulting solid was washed with diethyl ether in *i*-hexane (10%) and subsequently dried *in vacuo* to afford 15.5 mg (yield: 73%) of [$^2H_5$]Org24461 ((*rac*)-3).

^{1}H NMR (500 MHz, methanol-d_4) δ 7.51–7.45 (m, 2H, 2 × CF_3CCH), 7.06–7.00 (m, 2H, 2 × CF_3CCHCH), 5.53 (dd, J = 8.6/3.9 Hz, 1H, CHO), 4.01 (d, J = 18.1 Hz, 1H, CH_2C=O), 3.98 (d, J = 18.1 Hz, 1H, CH_2C=O), 3.53–3.38 (m, 2H, NCH_2CH_2), 2.98 (s, 3H, NCH_3), 2.53–2.42 (m, 1H, $CHOCH_2$), 2.39–2.30 (m, 1H, $CHOCH_2$). ^{13}C NMR (126 MHz, methanol-d_4) δ 168.8 (C=O), 161.5 (q, $J_{C\text{-}F}$ = 1.5 Hz, OC_{arom}), 140.72 (C_{arom}, *ipso*), 129.6 (t, $J_{C\text{-}D}$ = 23.7 Hz, 2 × [^{2}H]C_{arom}, *meta*), 129.0 (t, $J_{C\text{-}D}$ = 23.7 Hz, [^{2}H]C_{arom}, *para*), 127.8 (q, $J_{C\text{-}F}$ = 3.9 Hz, 2 × CF_3CC_{arom}), 126.7 (t, $J_{C\text{-}D}$ = 23.7 Hz, [^{2}H]C_{arom}, *ortho*), 125.8 (q, $J_{C\text{-}F}$ = 269.3 Hz, CF_3), 124.3 (q, $J_{C\text{-}F}$ = 33.0 Hz, CF_3C_{arom}), 117.4 (2 × CF_3CCHC_{arom}), 78.6 (CHO), 58.0 (NCH_2C=O), 55.4 (NCH_2CH_2), 42.2 (CH_3), 34.1 ($CHOCH_2$). MS HRMS (EI): $M^{+\cdot}$ calcd. for $C_{19}H_{16}[^2H_5]F_3NO_3$ 373.1709; found. 373.1781.

4.7. Validation of the LC-ESI-MS/MS method

For validation of the LC-ESI-MS/MS method spiked matrix samples were used to examine linearity, precision, accuracy and selectivity according to the FDA guidance for bioanalytical method validation (FDA, 2018). The spiked matrix samples were obtained in the same way as described for preparation of binding samples in *MS Binding Assay – general procedure*, except for incubation which was carried out without marker (or test compound). The validation was performed with five series of samples (matrix calibration standards and quality control (QC) samples, matrix blanks and zero samples) on different days with different GlyT1 membrane preparations. Quality control (QC) samples, which were used for examination of accuracy and intra- and inter-batch precision, were prepared independently from each other in different wells of a 96-well plate (1.2 mL well volume, Sarstedt, Nümbrecht, Germany). For every of the five validation series six replicate QC samples at each of the three concentration levels (15 pM, 250 pM, 750 pM) were prepared. Calibration standards were studied at eight concentration levels and for every concentration level three replicates were prepared, except for the lower limit of quantification (LLOQ), for which six replicates were prepared. According to the FDA guidance the response of the LLOQ should be at least five times the response of the noise of a matrix blank, with an accuracy between 80% and 120% and a precision characterized by a relative standard deviation of less than 20%, while accuracy and precision of all other calibration standards and QC samples should be between 85% and 115% and exhibit a relative standard deviation of not more than 15%. Linearity was studied after linear regression of the data obtained for the calibration standards. For this purpose, the peak area ratios of analyte vs. internal standard (y) were plotted against the concentration ratios of analyte vs. internal standard. For calculation of calibration curves a weighting factor of $1/x^2$ was used in all cases. To prove selectivity for marker and internal standard, six matrix blanks were injected in every series.

4.8. Glycine transporter 1 (GlyT1) membrane preparations

For preparation of GlyT1 membrane preparations CHO-K1 cells, stably expressing the hGlyT1c with a confluence ≥ 90%, were used. This cell line was kindly provided by AbbVie (Wiesbaden, Germany). The cells were cultivated in dishes (143 cm^2) at 37 °C and 8% CO_2 in Nutrient Mixture F-12 Ham containing 10% fetal bovine serum (*m/v*), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.7 mg mL^{-1} G418. For detachment of the cells the medium was aspirated and the dishes were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4). After incubation with 4 mL 0.05% trypsin (*m/v*) and 0.02% EDTA (*m/v*) for 5 min at 37 °C, 8 mL medium were added per dish. Subsequently, the cells were washed twice with PBS (5 min, 1600 rpm, Biofuge Stratos, rotor: #3047, Heraeus, Hanau, Germany) and afterwards homogenized in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) using a Polytron PTA 10 S (Kinematica Polytron, Littau-Luzern, Switzerland). After homogenization aliquots of about 1 mg protein were frozen and stored at −80 °C. The amount of protein in the membrane preparation (after treatment with 100 mM NaOH for 1 h) was determined according to Bradford (1976) using bovine serum albumin standards for calibration. At the day of the assay the membrane preparation was rapidly thawed, diluted in 20 mL incubation buffer and centrifuged at 4 °C for 20 min at 20500 rpm (Sorvall Evolution RC, SS34, Thermo. Electron, Hanau, Germany). Finally, the resulting pellet was resuspended in 6 mL incubation buffer yielding a protein concentration of ~0.1 mg mL^{-1}.

4.9. MS binding assay – general procedure

Membrane preparations (~15 µg protein) in presence of the marker (and other compounds as far as required) were incubated in triplicates in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) in polypropylene 96-well plates (1.2 mL well volume, Sarstedt, Nümbrecht, Germany) in 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. 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). Before their use, the glass fiber filters were washed with 3 × 200 µL water and subsequently with 3 × 200 µL methanol per well, incubated with 200 µL 1% (*m/m*) aqueous Tween20 solution per well at room temperature for 2 h, and finally, exempted from the Tween20 solution by vacuum filtration. After transfer of 210 µL aliquots of the binding samples by means of a 12-channel pipette onto the filter plates (transferring at first all 96 samples) and subsequent application of vacuum (i.e. concurrently to all transferred samples in the filter plate), the remaining membrane fractions with the bound marker were rapidly washed row per row (12-channel pipette) with 3 × 200 µL ice-cold washing buffer (154 mM ammonium acetate, pH 7.4). After drying of the filter plates for 1 h at 50 °C, the bound marker was eluted with 3 × 70 µL (per well) of a methanolic solution containing the internal standard at a concentration of 100 pM into a 96-well plate (1.2 mL well volume, Sarstedt, Nümbrecht, Germany; filtration via vacuum application after exposure of the filters of all wells to methanol for ~ 10 s; 12-channel pipette). The obtained 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 mM ammonium bicarbonate buffer, pH 7.8/acetonitrile (55:45, *v/v*)). The 96-well plates containing the eluted marker were sealed with aluminum foil and centrifuged 10 min at 2500 rpm (Biofuge Stratos, rotor: #3048, Heraeus, Hanau, Germany). Finally, the samples were subjected to LC-ESI-MS/MS quantification (see above) without further sample preparation. In the same way non-specific binding was determined by adding ALX5407 (20 µM) to the incubation samples. Marker depletion was negligible (< 10%) in all experiments.

4.10. Saturation experiments

For saturation experiments GlyT1 membrane preparations were incubated with eight different concentrations of Org24598 in a concentration range of 1 nM–200 nM. The following steps after incubation were performed as described in *MS Binding Assay – general procedure*. Non-specific binding was determined in the same way as total binding but in the presence of 20 µM ALX5407 for the same marker

concentrations. When non-specific binding of Org24598 fell below the LLOQ of the calibration curve (generally at a nominal marker concentration of < 10 nM), linear regression for the data points of non-specific binding higher than the LLOQ were performed, and the non-specific binding for data points below the LLOQ was calculated by extrapolation of the linear function using Prism v. 6.07 (GraphPad Software, San Diego, California, US).

4.11. Competition experiments

For competition experiments GlyT1 membrane preparations were incubated with Org24598 in a concentration of 15 nM and in presence of test compounds (at least seven concentrations). Additionally, control samples were prepared 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.

4.12. Data analysis

All results of the binding experiments (K_d, B_{max}, K_i) 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. Based on the obtained calibration functions the bound marker concentrations were determined with Analyst v. 1.6.1 Software. Specific binding was calculated as difference between total binding and non-specific binding. Data from the binding experiment were analyzed by means of non-linear regression with Prism v. 6.07 (GraphPad Software, San Diego, California, US). For saturation experiments the *One site – specific binding* non-linear regression tool was used to obtain saturation isotherms with calculated K_d (equilibrium dissociation constants) and B_{max} (maximum density of binding sites) values. For competition experiments the *One Site – Fit K_i* non-linear regression tool was used 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%. From the concentration, at which a test compound inhibited 50% of specific marker binding (IC_{50} value) K_i values (inhibition constant of the test compound) were calculated using the Cheng-Prusoff equation.

Conflicts of interest

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2019.03.004.

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SUPPLEMENTARY INFORMATION

METHODS

Investigating filter binding of Org24598 on 96-well glass fiber filter plates

The investigation of filter binding of Org24598 was performed analog to *MS Binding Assay - general procedure* described in **Materials and methods**, except no GlyT1 membrane preparations were used for incubation. At first, the filters of 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 µm, 350 µL; Pall Corporation, Port Washington, New York, US) were washed three times with 200 µL water and 200 µL methanol and subsequently incubated for 2 h at room temperature with different aqueous preincubation solutions (*m*/*m*): 200 µL of 0.5 % polyethylenimine (PEI), 1 % polyvinylpyrrolidone (PVP), 1 % Tween20, 0.5 % PVP-0.2 % Tween20 and 0.5 % L-lysine were used as preincubation solutions (triplicates), as control filters without incubation and pretreated filters with water were used (triplicates). During the incubation of the filters 50 nM Org24598 were incubated for 1 h in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) in polypropylene 96 well plates (1.2 mL well volume, Sarstedt, Nümbrecht, Germany) in total volume of 250 µL at 22 °C in a Stuart® Microtitre Plate Shaker Incubator SI505 (Bibby Scientific Limited, Staffordshire, Great Britain). After incubation of filters and incubation samples, the preincubation solutions of the filters were removed by vacuum filtration and 210 µL aliquots of the incubation samples were transferred on the filters and were removed by vacuum filtration as well. The filters were washed three times with 200 µL of 154 mM ammonium acetate buffer (pH 7.4) and were dried at 50 °C for 1 h. After that the remaining Org24598 on the filters were washed down with 3 x 70 µL methanol, the methanol was 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 mM ammonium bicarbonate buffer (pH 7.8)/acetonitrile (55:45, *v*/*v*)) containing 100 pM [$^2H_5$]Org24461. Finally, the samples were subjected to LC-ESI-MS/MS quantification without further sample preparation. The peak area ratios of Org24598 and [$^2H_5$]Org24461 were plotted against the different types of filter preincubations in a bar chart.

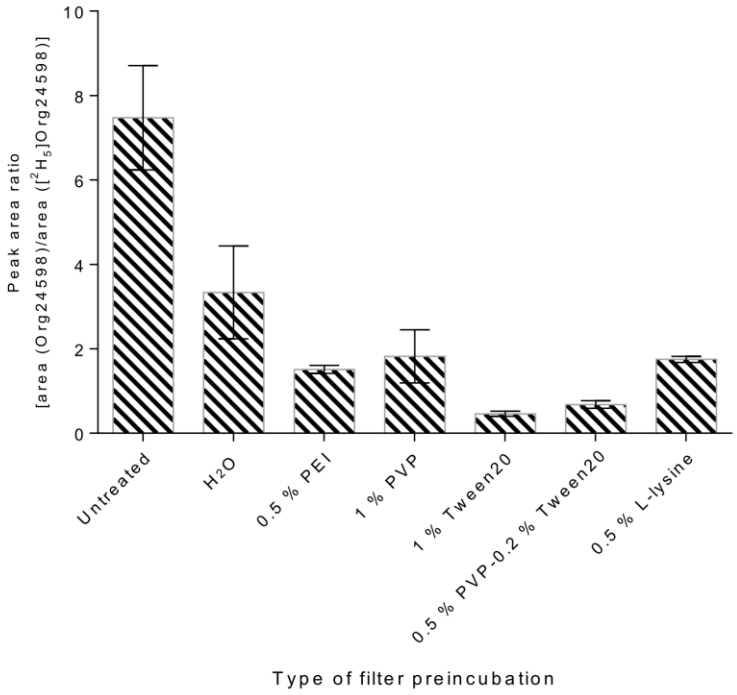
Investigating the recovery of Org24598 after evaporation of methanol subsequently to the elution step

The investigation of the recovery of Org24598 after evaporation of methanol was performed analog to *MS Binding Assay - general procedure* described in **Materials and methods**, except GlyT1 membrane preparations were incubated without Org24598 or any other test compound. All in all, twelve incubation samples were prepared and treated the same way until the elution was performed. For the elution all twelve samples were eluted at the same time by means of a 12-channel pipette

with 3 x 70 µL methanol and collected in a 96-well plate (1.2 mL well volume, Sarstedt, Nümbrecht, Germany). Six of those samples were spiked with 21 µL of a 5 nM Org24598 acetonitrile solution. After that the solvent of all samples was evaporated at 50 °C under N_2 flow by means of a MiniVap Microevaporator (Porvair Sciences Limited, Norfolk, UK). The residues of the six samples, which were spiked before evaporation, were reconstituted with 210 µL of mobile phase (5 mM ammonium bicarbonate buffer (pH 7.8)/acetonitrile (55:45, *v*/*v*)) containing 100 pM [$^2H_5$]Org24461 and the residues of the six samples which were not spiked before evaporation were reconstituted with 189 µL 5 mM ammonium bicarbonate buffer (pH 7.8)/acetonitrile (65:35, *v*/*v*) containing 111 pM [$^2H_5$]Org24461 and spiked with 21 µL of the same 5 nM Org24598 acetonitrile solution than before. In the end, all samples consisted of the same sample solvent and obtained the same amount of marker and internal standard. Finally, the samples were subjected to LC-ESI-MS/MS quantification without further sample preparation. The peak area ratios of Org24598 and [$^2H_5$]Org24461 of the samples spiked with marker before and after evaporation of methanol were plotted in a bar chart and compared to each other.

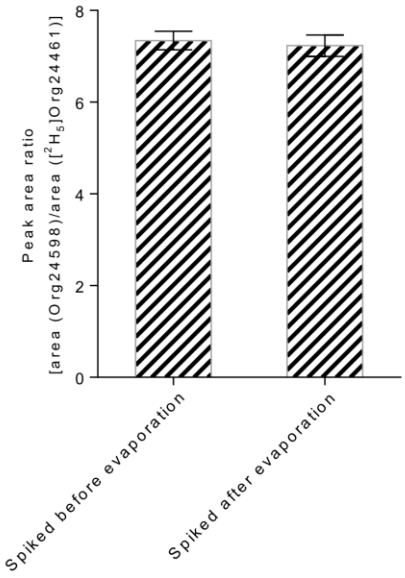
RESULTS

Fig. S1. Investigating filter binding of Org24598 on 96-well glass fiber filter plates



The results are given as mean ± standard deviation (n = 3). The preincubation solution with the smallest peak area ratio shows to the lowest filter binding of Org24598. 1 % Tween20 was identified as the best solution to reduce the filter binding of Org24598, so it was chosen as preincubation solution for the filters of the 96-well filter plates.

Fig. S2. Investigating the recovery of Org24598 after evaporation of methanol subsequently to the elution step



The results are given as mean ± standard deviation (n = 6). The deviation of the peak area ratios of the samples spiked before and after the evaporation is not significant (t (10) = 0.884; p = 0.397).

Table S1. Validation Results of LC-ESI-MS/MS method for Org24598

intra-batch accuracy and precision															
Samples (n)	**Series 1**			**Series 2**			**Series 3**			**Series 4**			**Series 5**		
	M	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**
Kal 5 pM (6)	5.170	103.4	6.5	5.162	103.2	4.7	5.110	102.2	4.8	5.165	103.3	6.6	5.165	103.3	6.5
Kal 10 pM (3)	9.043	90.4	0.6	9.290	92.9	4.3	9.630	96.3	2.8	9.260	92.6	3.7	9.113	91.1	4.4
Kal 20 pM (3)	18.77	93.8	2.0	18.20	91.0	2.2	18.50	92.5	1.4	18.07	90.3	3.0	18.73	93.7	0.3
Kal 40 pM (3)	37.97	94.9	0.4	37.33	93.3	2.0	38.17	95.4	0.4	38.20	95.5	1.6	38.27	95.7	1.2
Kal 100 pM (3)	97.77	97.8	0.5	99.73	99.7	1.4	95.67	95.7	1.4	95.53	95.5	2.1	96.60	96.6	0.8
Kal 200 pM (3)	207.0	103.5	1.0	208.7	104.3	1.1	200.7	100.3	5.2	206.7	103.3	0.7	204.0	102.0	0.5
Kal 500 pM (3)	522.3	104.5	0.5	524.0	104.8	2.2	537.0	107.4	1.0	547.3	109.5	0.6	535.7	107.1	1.8
Kal 1000 pM (3)	1080	108.0	0.9	1073	107.3	1.9	1080	108.0	1.9	1067	106.7	0.5	1073	107.3	1.4
Equation of calibration*	y = 1.164 x + 0.00313 (r = 0.9966)			y = 1.176 x + 0.00197 (r = 0.9966)			y = 1.020 x + -0.00299 (r = 0.9971)			y = 1.021 x + 0.00120 (r = 0.9957)			y = 1.037 x + -0.00637 (r = 0.9965)		
QC 15 pM (6)	16.10	107.3	4.3	15.88	105.9	3.2	15.88	105.9	1.7	15.83	105.6	2.5	16.03	106.9	2.2
QC 250 pM (6)	261.0	104.4	1.0	259.8	103.9	1.2	263.5	105.4	1.0	266.5	106.6	1.0	264.8	105.9	0.9
QC 750 pM (6)	825.0	110.0	1.1	827.3	110.3	0.9	820.5	109.4	1.2	819.5	109.3	0.3	816.0	108.8	0.5

inter-batch accuracy and precision			
	M	**A**	**P**
QC 15 pM	15.95	106.3	2.8
QC 250 pM	263.13	105.3	1.0
QC 750 pM	821.67	109.6	0.8

M Mean; **A** Accuracy; **P** Precision; * Weighting factor: $1/x^2$

3.2 Zweite Publikation

MS Binding Assays for Glycine Transporter 2 (GlyT2) Employing Org25543 as Reporter Ligand

3.2.1 Zusammenfassung der Ergebnisse

In zahlreichen Tierstudien konnte bereits gezeigt werden, dass die Inhibition des Neurotransmitter-Transporters GlyT2 ein vielversprechender Therapieansatz zur Behandlung von Schmerzzuständen, vor allem von chronischen Schmerzen begleitet von Hyperalgesie und Allodynie, sein kann, für welche es in der heutigen Medizin keine adäquate Behandlungsmöglichkeit gibt. Für die Identifizierung von GlyT2-Inhibitoren waren bis dahin ausschließlich funktionelle Assays eingesetzt worden, Bindungsassays waren nicht bekannt. Somit beschreibt diese Publikation die Entwicklung des ersten GlyT2-Bindungsassays, wobei dieser dem Prinzip der MS Bindungsassays folgt. Aufbauend auf den in Abschnitt 3.1 dargestellten GlyT1-MS-Bindungsassays wurde der nachfolgend beschriebene GlyT2-MS-Bindungsassays entwickelt.

Als Reporterligand diente der selektive und hoch affine GlyT2-Inhibitor Org25543. Für eine robuste Quantifizierung des Analyten, wurde auch in diesem Fall ein interner Standard eingesetzt. Dazu diente der siebenfach deuterierte Marker [$^2H_7$]Org25543. Dieser wurde ausgehend von Methyl-4-hydroxy-3,5-dimethoxybenzoat und [$^2H_7$]Benzylchlorid in einer vierstufigen Synthese dargestellt. Wie schon für den GlyT1-MS-Bindungsassay gezeigt, war es auch diesmal notwendig zunächst eine geeignete LC-ESI-MS/MS-Methode zu entwickeln. Für die Detektion und Quantifizierung wurde ein AB Sciex 5000 Triple-Quadrupol-Massenspektrometer mit Elektrospray-Ionisation (ESI) Ionenquelle im multiple reaction monitioring (MRM)-Modus verwendet. Als Massenübergänge wurden *m*/*z* 413,2/368,3 für Org25543 und *m*/*z* 420,3/375,4 für [$^2H_7$]Org25543 aufgezeichnet. Dadurch konnte eine hohe Selektivität und ein LLOQ der Analyten im niedrig pikomolaren Bereich erreicht werden. Für die Abtrennung der Analyten von etwaigen störenden Matrixkomponenten wurde eine geeignete RP-HPLC-Methode entwickelt. Diese beruht auf einer Luna C8(2) (50 mm × 2 mm, 3 µm) Säule von Phenomenex als stationärer Phase. Als mobile Phase wurde ein Fließmittelgemisch aus 5 mM Ammoniumhydrogencarbonat-Puffer pH 7,8 und Acetonitril (20:80; V/V) verwendet. Eine Flussrate von 600 µL/min bei einer

Säulentemperatur von 20 °C und ein Injektionsvolumen von 45 µL erlaubten Laufzeiten von 2,0 min pro injizierte Probe, was einen hohen Probendurchsatz ermöglicht.

Vor einer Validierung der entwickelten LC-ESI-MS/MS-Methode mussten zunächst die Bedingungen des MS-Bindungsassays festgelegt werden. Dazu durchgeführte Vorversuche zeigten, dass bei diesen GlyT2-MS-Bindungsassays ähnlich wie bei bekannten vorgegangen werden kann. Allerdings traten auch spezifische Probleme auf. Unter anderem war ein starker Adsorptionseffekt des Reporterliganden Org25543 an Polypropylen-Gefäße (z.B. Eppendorf-Caps) in wässrigen Lösungen zu beobachten. Aufgrund dieses Effekts konnten zunächst keine geeigneten Kalibriergeraden für die Quantifizierung des Analyten erstellt werden. Diesem Problem konnte jedoch entgegengewirkt werden, indem für die Herstellung der Verdünnungsreihe 10 % (*V*/*V*) *N,N*-Dimethylacetamid (DMA) in Wasser anstatt reinen Wassers als Lösungsmittel verwendet wurde. Außerdem gelang es nicht die nicht-spezifischen Bindung mittels eines Überschusses eines Kompetitors, der ein Derivat des Reporterliganden darstellte, zu bestimmen, da dadurch auch die Filterbindung des MS-Markers, Org25543, reduziert wurde. Dies hatte eine Diskrepanz zwischen der experimentell ermittelten nicht-spezifischen Bindung und dem Anteil der NSB in den Gesamtbindungsproben zur Folge, was unweigerlich zu falschen Ergebnissen in Sättigungsexperimenten führte. Aufgrund des Mangels von für diesen Zweck geeigneter alternativer Kompetitoren, wurde dieses Problem gelöst, indem anstelle der Kompetitor- die heat-shock-Methode für die Bestimmung der nicht-spezifischen Bindung verwendet wurde (erhitzen der Proteine auf 60 °C für 1 h vor der Inkubation).

Nach umfangreichen Untersuchungen konnten für die Durchführung der GlyT2-MS-Bindungsassays die folgenden Bedingungen als besonders geeignet ermittelt werden: Reporterligand, Proteinmaterial und, falls notwendig, Kompetitor (z.B. in Kompetitionsexperimenten) werden in Inkubationspuffer für 1 h bei 37 °C im Wasserbad in 96-deepwell-Platten inkubiert. Diese Inkubationslösung wird anschließend über 96-well-Glasfaserfilterplatten filtriert, um gebundenen von ungebundenem Marker zu trennen und die Inkubation zu beenden. Um die Filterbindung des Reporterliganden zu reduzieren, werden die Filter zuvor mit einer wässrigen 0,5 % (*m*/*m*) Polyethylenimin-Lösung vorbehandelt. Für die Entfernung von restlichem ungebundenem Marker, wird der Rückstand auf den Filtern mit

eisgekühltem Ammoniumacetatpuffer gewaschen und die Filter werden für 1 h bei 50 °C getrocknet. Nach der Trocknung werden durch die Zugabe von Acetonitril die Transporterproteine denaturiert, wodurch der Marker in Lösung geht und dann durch Vakuumfiltration in einer 96-deepwell Platte aufgefangen wird. Den Eluaten wird anschließend ein geeignetes Volumen 5 mM Ammoniumhydrogencarbonat pH 7,8 hinzugefügt, um ein Probenlösungsmittel zu erzeugen, das der mobilen Phase entspricht. Anschließend wird der Markergehalt der Proben mit der entwickelten LC-ESI-MS/MS-Methode quantifiziert.

Anhand von Matrixproben konnte die entwickelte LC-ESI-MS/MS-Methode schließlich erfolgreich in Anlehnung an die CDER Guideline for bioanalytical method validation der FDA hinsichtlich Spezifität, Richtigkeit, Präzision, Linearität und lower limit of quantification (LLOQ) validiert werden.[98] Alle Parameter entsprachen den Vorgaben der Guideline für den für die Untersuchungen erforderlichen Arbeitsbereich von 5 pM (LLOQ) bis 1 nM.

In Kinetikexperimenten (Assoziation und Dissoziation) konnte für Org25543 ein k_{off} von $7{,}07 \pm 0{,}26 \cdot 10^{-3}$ s^{-1} und ein k_{on} von $1{,}01 \cdot 10^{6}$ M^{-1} s^{-1} bestimmt werden. Die daraus berechnete Gleichgewichtsdissoziationskonstante K_d (siehe Gleichung 7 in Abschnitt 1.3.2.2) ergab einen Wert von 6,99 nM. Dieser Wert stimmte sehr gut mit dem experimentell ermittelten K_d-Wert aus den ebenfalls durchgeführten Sättigungsexperimenten überein ($K_d = 7{,}45 \pm 0{,}55$ nM). In Kompetitionsexperimenten wurden 19 bekannte GlyT-Liganden auf ihre Affinität bezüglich GlyT2 untersucht. Von diesen wies ein Großteil Affinitäten auf, die vergleichbar zu bereits publizierten IC_{50}-Werten aus unterschiedlichen funktionellen Assays sind. In einigen Fällen wurden aber auch deutliche Unterschiede zwischen den Bindungsaffinitäten und den Ergebnissen der funktionellen Assays festgestellt. Dies war zum Beispiel bei Glycin, dem Substrat der Transporter, bei ZINC6865169 und den Lipid-basierten GlyT2-Inhibitoren der Fall. Die genauen Ursachen für diese Diskrepanzen konnten jedoch nicht identifiziert werden. Jedoch ist zu bedenken, dass anders als bei Bindungsassays in funktionellen Assays viele zusätzliche Faktoren wie etwa alternative Bindungsstellen das Ergebnis beeinflussen können.

Gleichwohl stellen die in dieser Studie entwickelten Bindungsassays auf MS-Basis eine wertvolle Alternative zu funktionellen Assays dar, die es nun erstmals erlauben die Bindungsaffinitäten von GlyT2-Inhibitoren auf schnelle und einfache Weise zu

bestimmen und die Informationen bezüglich der inhibitorischen Wirksamkeit aus funktionellen Assays sinnvoll zu ergänzen.

3.2.2 Erklärung zum Eigenanteil

Die Synthese und Analytik des deuterierten internen Standards [$^{2}H_{7}$]Org25543 wurde von Dr. Lars Allmendinger durchgeführt. Alle Arbeiten zur Charakterisierung der MRM-Massenübergänge der verwendeten Analyten, zur LC-ESI-MS/MS-Methodenentwicklung und Validierung, zur Optimierung des MS-Bindungsassays sowie zur Durchführung der Kinetik-, Sättigungs- und Kompetitionsexperimente wurden von mir selbst vorgenommen. Das Publikationsmanuskript einschließlich aller Tabellen und Abbildungen wurde von mir unter Anleitung sowie Mithilfe von Dr. Georg Höfner und Prof. Dr. Klaus T. Wanner erstellt und von Prof. Dr. Klaus T. Wanner überarbeitet und korrigiert.

3.2.3 Manuskript der zweiten Publikation

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MS Binding Assays for Glycine Transporter 2 (GlyT2) Employing Org25543 as Reporter Ligand

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This study describes the first binding assay for glycine transporter 2 (GlyT2) following the concept of MS Binding Assays. The selective GlyT2 inhibitor Org25543 was employed as a reporter ligand and it was quantified with a highly sensitive and rapid LC-ESI-MS/MS method. Binding of Org25543 at GlyT2 was characterized in kinetic and saturation experiments with an off-rate of $7.07\times10^{-3}\,s^{-1}$, an on-rate of $1.01\times10^{6}\,M^{-1}s^{-1}$, and an equilibrium dissociation constant of 7.45 nM. Furthermore, the inhibitory constants of 19 GlyT ligands were determined in competition experiments. The validity of the GlyT2 affinities determined with the binding assay was examined by a comparison with published inhibitory potencies from various functional assays. With the capability for affinity determination towards GlyT2 the developed MS Binding Assays provide the first tool for affinity profiling of potential ligands and it represents a valuable new alternative to functional assays addressing GlyT2.

1. Introduction

Today the treatment of chronic pain is a widespread problem in pain therapy. Patients who are suffering from chronic pain do not just have to deal with normal pain sensation but also with hyperalgesia (amplified pain sensation to a noxious stimulus) and/or allodynia (pain sensation after an innocuous stimulus like a light touch) especially when the reason for pain is a nerve injury in the peripheral or central nervous system (neuropathic pain).[1] Furthermore, this can lead to a worse quality of life due to physical, psychological and social problems like decreased physical activity, sleep disturbances, anxiety, depression, decreased social interactions with family and friends and inability to work.[2] Currently, patients with chronic pain are treated with the typical analgesics like opioids or nonsteroidal anti-inflammatory drugs or with anticonvulsants or tricyclic antidepressants (especially for neuropathic pain) but these therapies are often not very satisfying as the pain relief effect is to low and the drugs are associated with unwanted side effects.[1] Thus there is a strong need for new strategies for the treatment of chronic pain.

The dorsal horn of the spinal cord acts as a control center for the transmission of painful information between periphery and brain. Part of this control center are glycinergic neurons with glycine (Figure 1; **1**) as inhibitory neurotransmitter. These glycinergic neurons can inhibit the transmission of incoming information from the periphery by activating glycine receptors (GlyR) on the postsynaptic neuron due to glycine binding which triggers a Cl^- influx and leads to a hyperpolarization. This mechanism was installed from nature as control mechanism to distinguish between noxious and innoxious information.[1] A decreased function of this inhibitory mechanism, for example after inhibition of glycine receptors with strychnine or nerve injury, leads to a loss of this ability which results in hyperalgesia and allodynia.[3] Thus an increased inhibitory glycinergic neurotransmission could reduce the symptoms of these diseases. One strategy to increase the inhibitory neurotransmission is to inhibit the reuptake of glycine in presynaptic neurons and glial cells mediated by the corresponding neurotransmitter transporters, that is, glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2).[4] Although the inhibition of GlyT1 is showing antinociceptive effects[5–7] this work will just concentrate on GlyT2. GlyT2 is a member of the Na^+/Cl^--dependent solute carrier 6 (SLC6) family and occurs mainly at the presynaptic terminal of inhibitory glycinergic synapses. There it has two main functions: First, in a co-transport with Na^+ and Cl^- (stoichiometry: 3 Na^+ + 1 Cl^- + 1 glycine) glycine is removed from the synaptic cleft of inhibitory glycinergic synapses into the presynaptic neuron to terminate the glycinergic neurotransmission. This leads to a low-nanomolar concentration of glycine in the synaptic cleft, which is insufficient for a significant GlyR activation. Second, GlyT2 has a recycling function for glycine. Due to the transport back into the presynaptic neuron the glycine concentration increases there to ~10–20 mM which is then high enough to refill synaptic vesicles via the vesicular inhibitory amino acid transporter (VIAAT) with glycine whereupon it can be released again into the synaptic cleft. So, the glycinergic neurotransmission can be upheld.[1]

By increasing glycine concentration in the synaptic cleft and thus glycinergic neurotransmission, GlyT2 inhibition can be expected to be active against pain, which could be demonstrated in animal models. Org25543 (Figure 1; **2**) and ALX1393 (Figure 1; **3**) represent the two so far best characterized GlyT2 inhibitors, which were shown to be able to reduce allodynia in

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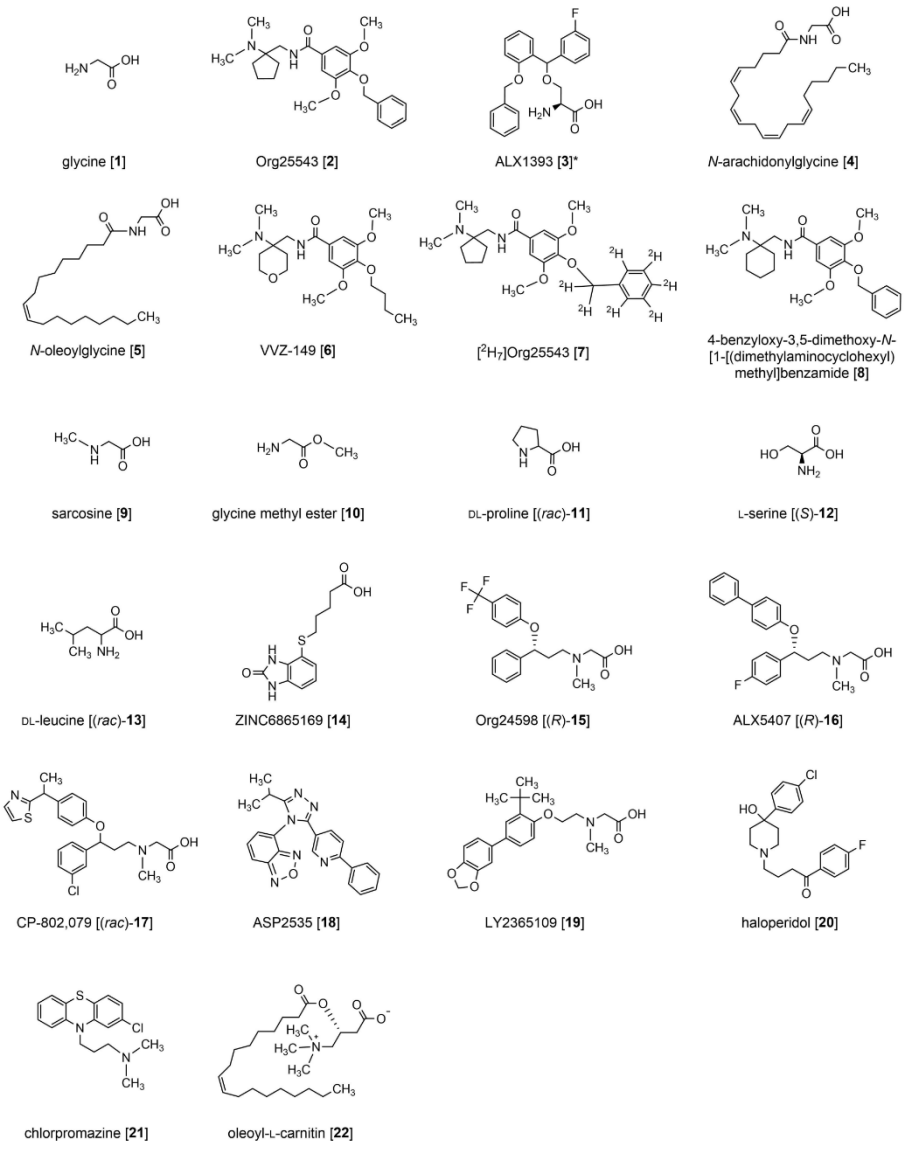

Figure 1. Structures of Org25543, used as a GlyT2 marker, [$^2H_7$]Org25543, used as internal standard, and GlyT ligands. * Stereochemistry not unequivocally characterized either by literature or by manufacturer or vendor.

animals due to their ability to inhibit the glycine transport into the presynaptic neuron.[7–10] Unfortunately, both compounds exhibit considerable disadvantages. ALX1393 shows an insufficient availability in the CNS after intravenous injection (only 5% cross the blood-brain-barrier)[11] whereas a prolonged exposure of Org25543 to GlyT2 leads to a reduced glycinergic neurotransmission. The latter is presumably due to the reduction of glycine transport in the glycinergic neuron.[12] When GlyT2 is completely blocked, the glycine concentration in the presynaptic neuron cannot reach the required concentration level to

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refill the synaptic vesicles with glycine and thus no new glycine can be released into the synaptic cleft, which results in a decreased glycinergic neurotransmission. Despite these disadvantages researchers are still looking for new GlyT2 inhibitors with better and safer pharmacological profiles. Right now clinical trials for the GlyT2 inhibitor VVZ-149 (Figure 1; **6**) from Vivozone, which additionally acts as antagonist at the 5-HT_{2A} and P2X3 receptors, for the treatment of postoperative pain are in progress.[13–16] Furthermore, most recent research aims at the development of lipid-based GlyT2 inhibitors like *N*-arachidonylglycine (NAGly; Figure 1; **4**) or *N*-oleoylglycine (NOGly; Figure 1; **5**),[1,17–21] which show positive effects in neuropathic pain models but fewer side effects.

To find new inhibitors for GlyT2 efficient screening tools are necessary. Until now all methods (to the best of our knowledge), which have been developed for the screening of new inhibitors, are assays aiming for the identification of compounds with a biological activity at GlyT2 like radioisotope labeled glycine uptake assays,[22–26] electrophysiological assays[11,18–21] or fluorescent imaging plate reader (FLIPR) membrane potential assays.[27] But there is no assay measuring the binding affinities of compounds towards GlyT2 so far. As the determination of binding affinities is in general (e.g., no living cells are needed) less elaborate than the performance of functional assays and binding affinities by being direct proportional to the Gibbs free energy ($\triangle G^0$) are required as basis for molecular modelling studies, we aimed at the development of a binding assay addressing GlyT2. To this end we intended to use the concept of MS Binding Assays, which was established in our group during the last years and was employed already for GlyT1 and several other target proteins.[28–39] MS Binding Assays (as described in Section 2.3.) are performed similar to radioligand binding assays but instead of using a radioisotope labeled reporter ligand an unlabeled reporter ligand, which is also termed "native marker", "MS Marker" or simply "marker", is employed. The incubation step and the separation step of unbound marker and target-marker complexes (either by filtration or centrifugation) are the same for both techniques, however, the quantification of the bound reporter ligand differs. In MS Binding Assays the bound marker is quantified typically after its liberation from the target-marker-complex with an organic solvent by LC-MS, whereas in radioligand binding assays the target-bound marker that remains on the filters after the separation step is quantified directly by liquid scintillation counting (LSC).

For the development of the desired MS Binding Assay a reporter ligand was needed, which shows an appropriate affinity to GlyT2 (preferably in the low-nanomolar range) as well as a good capability for the quantification by LC-ESI-MS/MS (liquid chromatography coupled to an electrospray ionization interface and following quantification by tandem mass spectrometry), which commonly proves to be well suited for this purpose.[28] Unfortunately, the number of commercially available GlyT2 inhibitors, which show a biological activity at GlyT2 in the low-nanomolar range, is very low. To be more precise, only two compounds, that is, Org25543 and ALX1393 with reported IC_{50} values from 16 to 100 nM in functional assays, appeared suitable for this task.[11,23] As no LC-ESI-MS/MS method has been described for either GlyT2 inhibitor so far, it was necessary to develop a method which allows a highly sensitive quantification of these compounds. Taking into account that the compounds have to be quantifiable down to about 0.01 K_d, as a rule of thumb,[29,31] the developed LC-ESI-MS/MS method should be able to quantify the reporter ligand in the low-picomolar range. On the basis of the results obtained during the method development process, the compound that appears to be better suited for this task, that is, either Org25543 or ALX1393 by showing better LC-MS properties, should be established as reporter ligand. For the chosen reporter ligand, the developed LC-ESI-MS/MS method should be validated regarding linearity, lower limit of quantification (LLOQ), accuracy, precision and selectivity. After that the kinetics (off- and on-rates) as well as the affinity of the chosen reporter ligand at GlyT2 should be examined in dissociation, association and saturation experiments and, subsequently, known GlyT ligands should be characterized in competition experiments. In the end, the results of these competition experiments should be compared to those from functional GlyT2 assays from literature as far as such data for the used competitors are available for GlyT2. Although other data from neurotransmitter transporters show that the direct comparability of affinities and potencies from functional assays is limited, such a comparison should at least enable to estimate the plausibility of the determined binding affinities.

2. Results and Discussion

2.1. LC-ESI-MS/MS method

For the first step in the development of the desired MS Binding Assay a reliable LC-ESI-MS/MS method had to be established to which end a triple quadrupole mass spectrometer coupled to a pneumatically assisted electrospray ionization (ESI) source

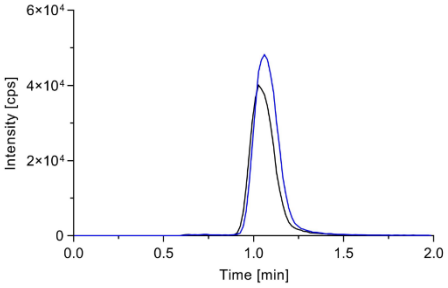

Figure 2. MRM chromatogram of a matrix standard containing 100 pM Org25543 (*m/z* 413.2/368.3, blue) and 100 pM [$^2H_7$]Org25543 (*m/z* 420.3/375.4, black). For LC a Luna 3 µ C8(2) (50 mm × 2 mm, 3 µm) column was used as stationary phase in combination with a mobile phase consisting of ammonium bicarbonate buffer (5 mM, pH 7.8)/acetonitrile (20:80, *v/v*) at a flow rate of 600 µL min^{-1}. In routine LC-ESI-MS/MS runs, the eluent was directed to waste from 0.0–0.6 min.

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should be used. Thereby, the mass spectrometer should be operated in the multi-reaction monitoring (MRM) mode to achieve the required selectivity and sensitivity to analyze binding samples. For ALX1393 (IC_{50} of 25–100 nM[11,40]), one of the two possible reporter ligands, neither the $[M+H]^+$ parent ion nor the fragment ions had been described for ESI-MS/MS so far, whereas for Org25543 (IC_{50} of 16–31 nM[23,27]) the $[M+H]^+$ is known (*m/z* 413.3[23]) but the fragment ions are not. Our experiments revealed the expected $[M+H]^+$ parent ions with *m/z* 396,1 for ALX1393 and *m/z* 413.2 for Org25543 as well as following fragment ions for ALX1393: *m/z* 291.3, 185.1, 184.8, 165.1, 91.1 and Org25543: *m/z* 368.3, 271.2, 243.2, 162.1, 134.1, 98.2, 91.2, 81.2, respectively (see Figure S1 in the Supporting Information). The mass transitions with the highest intensities (ALX1393→*m/z* 396.1/291.3 and Org25543→*m/z* 413.2/368.3) were selected for further method development.

Next, after ALX1393 and Org25543 had been characterized by ESI-MS/MS and the compound-dependent parameters of the mass spectrometer had been optimized, the LC method should be developed. For this purpose, we decided to use a method running under reversed-phase (RP) conditions. RP-HPLC does not just enable an easy and fast method development due to its simple retention principle but it also has the advantage to separate the analyte from matrix components effectively which is important to avoid matrix effects potentially disturbing analyte detection. According to our experience most of the matrix components generated during the assay procedure are highly polar and will thus by being hardly retained elute close to the void time. Due to this it was our aim to achieve a capacity factor (*k*) of the analyte between 1 and 2, which should be large enough to avoid significant matrix effects. Hence, several RP stationary phases and different mobile phases consisting of acetonitrile or methanol as organic component and an aqueous buffer made from volatile buffer salts like ammonium formate or ammonium bicarbonate in various ratios, were tested to identify conditions suitable for this purpose (data not shown). During this method development ALX1393 was found to exert much lower intensities compared to Org25543, its suitability for LC-ESI-MS/MS detection thus being worse than that of Org25543, but still high enough to guarantee a quantification of ALX1393 down to picomolar concentration levels (Figure S2). The following two concerns, however, clearly indicated, that ALX1393 was not an ideal candidate to be used as reporter ligand in binding assays. First, the stereochemistry of ALX1393, which is likely to have distinct influence on its binding affinity, is not defined for one of the two chiral centers. Therefore, commercially available ALX1393 can even not be expected to be just a single stereoisomer with unknown configuration at one chiral center but a mixture of diastereomers, possibly varying in their ratio when different batches from the same or even more from different vendors are used. Secondly, despite its described nanomolar potency in functional assays Table 1 we determined an affinity characterized by a K_i value of about 1 μM at GlyT2 in our competition experiments (see Section 2.7.). Compounds in this affinity range are poorly suited for filtration-based binding assays, as a substantial loss of bound reporter ligand during the washing step after separation of bound from unbound reporter ligand is likely to occur. Therefore, we focused on Org25543 as reporter ligand addressing GlyT2, for which finally a LC method that meets the required needs, could be developed. It is based on a Luna C8(2) (50 mm × 2 mm, 3 μm) column as well as 5 mM ammonium bicarbonate buffer (pH 7.8) and acetonitrile (20 : 80, *v/v*) as mobile phase leading to the desired retention behavior for Org25543, with the capacity factor (*k*) amounting to 1.69. The mentioned conditions enable to run the method under acceptable back pressures (~120 bar) with a flow rate of 600 μL min^{-1} which makes chromatographic cycle times of only 2.0 min possible and guarantees therefore a considerable throughput.

Finally, to improve the robustness of the quantification method being developed for Org25543 an internal standard should be employed.[28] As such the seven times deuterated form of Org25543 (Figure 1; **7**) should be used, which was synthesized in-house (see Section 2.2). Still, as already discussed by us,[29] it is not mandatory to use an isotopically labeled form of the reporter ligand as internal standard. Any other compound which coelutes with the marker and which can be well quantified via LC-ESI-MS/MS under the same conditions as the analyte is in principle appropriate for this use, too.

A representative chromatogram of a matrix standard containing 100 pM Org25543 and 100 pM [$^2H_7$]Org25543, which was acquired under the above described LC-ESI-MS/MS conditions, is depicted in Figure 2. As can be seen from it the signal intensity for Org25543 for this low concentration is still high, the retention time is short and analyte and internal standard co-elute, the method thus fulfilling the conditions required for a sufficiently sensitive and robust quantification of the reporter ligand and a reasonable throughput.

2.2. Synthesis of the internal standard [$^2H_7$]Org25543

For the synthesis of [$^2H_7$]Org25543 (**7**; Scheme 1) we started from methyl syringate [**23**] and [$^7H_2$]benzyl chloride [**24**], both of which are commercially available. By subjecting them to a Williamson ether synthesis, ether **25** was obtained in 93% yield. Hydrolysis of the ester function of **25** under mild basic conditions with LiOH furnished acid **26** (yield: 95%). Subsequent reaction of carboxylic acid **26** with 1-(aminomethyl)-*N*,*N*-dimethylcyclopentane-1-amine after prior activation with 3-(ethyliminomethyleneamino)-*N*,*N*-dimethylpropan-1-amine (EDCI) and benzotriazole-1-ol (HOBt) in the presence of NEt_3 (in CH_2Cl_2, RT, 18 h), delivered the desired deuterated analogue [$^2H_7$]Org25543 [**7**] in 70% yield. Upon treatment of the free base **7** with HCl (4 M) in dioxane the hydrochloride **7**·HCl was obtained (yield 91%, 64% over 2 steps).

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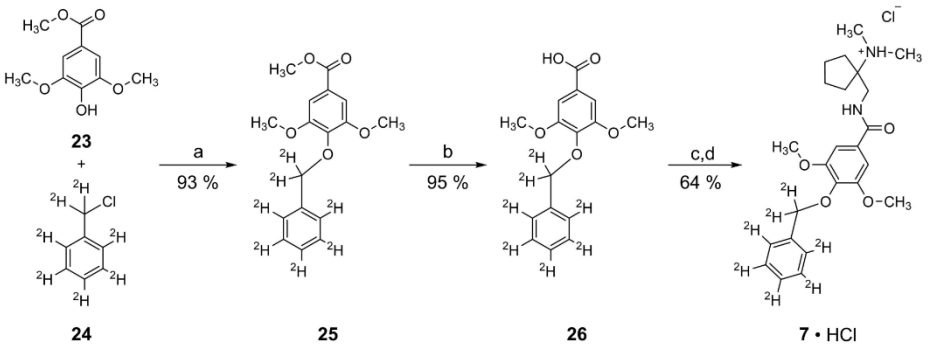

Scheme 1. Synthesis of [$^2H_7$]Org25543. a) K_2CO_3, KI, DMF, 80 °C, 24 h; b) LiOH, THF/H_2O, RT, 68 h; c) 1-(aminomethyl)-*N*,*N*-dimethylcyclopentane-1-amine,[41] HOBt, EDCI, NEt_3, CH_2Cl_2, RT, 18 h; d) 4 M HCl, dioxane, 0 °C.

2.3. Preliminary experiments and considerations for the development of GlyT2 MS Binding Assays regarding its general setup and conditions

So far, no binding assay for GlyT2 has been described in literature and thus no data as initial guide for experimental conditions exist. Therefore, the experimental conditions established for the GlyT1 MS Binding Assay should serve as such.[29] As 10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ with pH 7.5 proved to be suitable for GlyT1 binding experiments, we reasoned that the same should be true for GlyT2. Vacuum filtration over glass fiber filters should be used after incubation, to separate target-bound-marker from unbound marker. To remove residual unbound marker in the filters after the separation step, they later have to be washed several times, for which purpose, a 154 mM ammonium acetate washing buffer (pH 7.4) should be used here for GlyT2 as for GlyT1 before.[29] As buffer components from the washing procedure will remain on the filters and elute from the filters during the elution of the marker, they will contribute to the final sample composition. Ammonium acetate buffer has been found to be favorable for ESI-MS/MS measurements due to its volatile character. Another important issue concerning the quality of the results of the binding assay is related to nonspecific binding of the MS marker to the glass fiber filters. This should be limited to a minimum by means of a pretreatment with suitable compounds. Since such compounds contribute to the final sample composition and matrix effects as well, suitable filter pretreatment reagents and conditions had to be evaluated. Preliminary experiments revealed that Org25543 shows very high filter binding, in case filters are not pretreated. Thus, several pretreatment agents described by Scott et al.[42] were tested from which an aqueous 0.5 % (*m*/*m*) polyethylenimine (PEI) and an aqueous 1 % (*m*/*m*) Tween20 solution

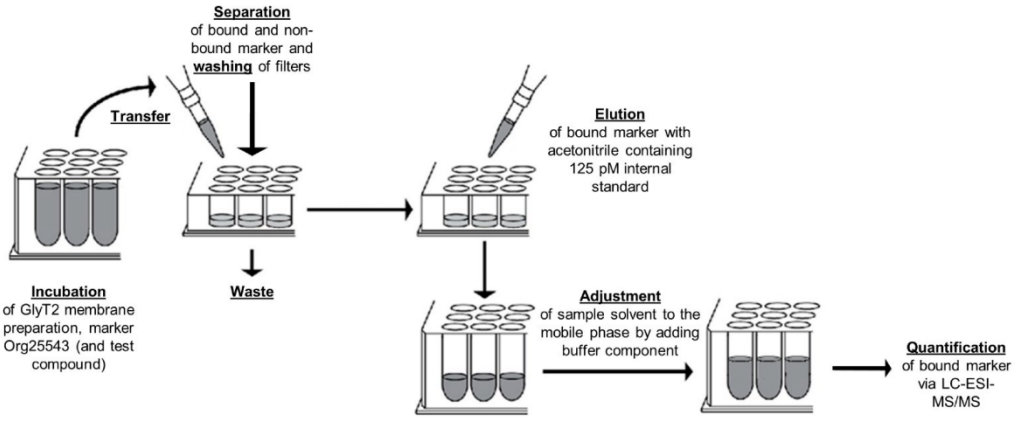

Figure 3. Workflow of the developed GlyT2 MS Binding Assays

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exhibited best and almost similar results in reducing the filter binding of Org25543 (for details, see Figure S3). Finally, 0.5% PEI was selected as pretreatment agent for the reduction of filter binding, since a higher matrix effect was observed when filters were pretreated with Tween20 compared to the ones pretreated with PEI. In the last step of the binding experiment, the target-bound-marker remaining on the filters has to be liberated and eluted. In former MS Binding Assays the solvents acetonitrile and methanol have already been shown to be suitable for this task.[30–39] We decided to select acetonitrile as elution solvent due to the fact that it is advantageous for the chromatography, especially for peak shapes, when the composition of the sample solvent of the final sample is identical with that of the mobile phase of the employed LC-ESI-MS/MS method which in the present case consists of a mixture of acetonitrile and 5 mM ammonium bicarbonate buffer in a ratio of 80:20 (*v/v*). For the final sample this solvent composition can be achieved by just adding a defined small volume of 5 mM ammonium bicarbonate buffer (pH 7.8). This leads only to a slight marker dilution from the original concentration to 80% which can be easily taken into account in the quantification procedure.

During method development it was further recognized that Org25543 is prone to extensive adhesion to container materials when it is dissolved in pure water, especially to polypropylene surfaces of tubes, which leads to a decrease of the free concentration of the analyte as compared to the nominal concentration. This phenomenon has already been observed during method development in other studies.[35,43] To solve this problem we used, as successfully done in former cases, *N,N*-dimethylacetamide (DMA) as organic co-solvent. A concentration of 10% DMA (*v/v*) in water was already enough to gain satisfying results. Thus, all working solutions of Org25543 were prepared in 10% aqueous DMA (Figure S4). As a consequence thereof, DMA was also contained in the final binding samples of the MS Binding Assays. Though this could have influenced ligand affinities, DMA was found to not effect Org25543 binding up to concentrations of 2% (≙ 215 mM; Figure S5). As DMA concentration in binding samples was limited to 0.4%, a negative effect could be clearly excluded.

Another important question was how to determine non-specific binding of Org25543 in binding experiments. To this end, three different approaches were investigated which are commonly applied for this purpose. Firstly, nonspecific binding was evaluated over a concentration range from 0.4 to 150 nM (according to the concentrations in saturation experiments), by applying an excess of the cyclohexane analogue **8** of Org25543 as competitor (Figure 1, **8**), which is a known GlyT2 inhibitor (IC_{50} = 84 nM[23]), too. Secondly, it was studied with GlyT2 membrane fragments, which had been treated at 60 °C for 1 h in a water bath so that the proteins can be expected to be denatured ("heat-shock") and, thirdly, with membrane fragments of non-transfected HEK293 cells. Comparing the results of the three afore described approaches it turned out that using **8** leads to a lower nonspecific binding than using heat-shocked target material or empty HEK293 cells, which was found to be due to the fact that **8** also reduced filter binding of Org25543 to some extent (for details, see Figure S6). For the heat-shocked target material and the empty HEK293 cells, nonspecific binding was identical. Clearly, the use of the same membrane preparation for determination of total and nonspecific binding as it is the case in the heat-shock approach appears more appropriate than using different membrane fragments, that is, from transfected and non-transfected HEK293 cells. As the former approach has already been used successfully in another project,[44] we decided to apply this method for determination of nonspecific binding of Org25543 here, too.

Based on the above-mentioned findings and on data already published for MS Binding Assays, the following conditions were established for the performance of the first binding assay addressing GlyT2 (see the scheme describing the workflow depicted in Figure 3). For binding Org25543 as reporter ligand is incubated with crude membrane fractions of HEK293 cells expressing GlyT2 for 1 h at 37 °C in 96-deepwell plates in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$; pH 7.5) in a total volume of 250 μL. After the incubation aliquots of 210 μL of the corresponding binding samples are transferred to 96-well filter plates (pretreated with PEI; for further information, see the Experimental Section) for the separation of target-marker complexes from the liquid part of the incubation mixture and contained unbound marker by vacuum filtration. For the removal of remaining unbound reporter ligand, the filters are washed subsequently to the filtration with ice-cold aqueous ammonium acetate buffer (4×150 μL; 154 mM; pH 7.4). After the filter plates with the remaining target-marker complexes have been dried at 50 °C for 1 h, bound Org25543 is released from the target-marker complex by eluting it with a [$^2H_7$] Org25543 solution in acetonitrile (3×70 μL; 125 pM) into a 96-deepwell receiver plate by vacuum filtration. To adjust the sample solvent to the mobile phase, aqueous ammonium bicarbonate buffer (52.5 μL; 5 mM; pH 7.8) is added to each sample leading to the same solvent composition as in the mobile phase (buffer/acetonitrile 20:80, *v/v*). The thus obtained samples are finally subjected to an LC-ESI-MS/MS analysis according to the developed LC-ESI-MS/MS method (45 μL injection volume) for the quantification of the reporter ligand Org25543.

2.4. LC-ESI-MS/MS method validation

After the technical details for the performance of the MS Binding Assays had been established, including the generation of a defined matrix the developed LC-ESI-MS/MS method was ready to be validated for linearity, lower limit of quantification (LLOQ), accuracy, precision and selectivity according to the recommendation of the FDA guidance for bioanalytical method validation.[45] For this purpose at first a blank matrix was prepared according to the above described procedure for the binding experiments and the corresponding blanks, standards and quality controls (QC) created therefrom were used to examine the developed LC-ESI-MS/MS method regarding the mentioned parameters. For preparing the blank matrix the

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incubation of target and buffer was performed without reporter ligand or any other test compound. To investigate the validation parameters, matrix calibration standards and quality control samples, matrix blanks and zero samples were prepared on different days with different GlyT2 membrane preparations in five sets, which contained Org25543 in a concentration range from 5 pM to 1 nM (except for matrix blanks and zero samples) as well as the internal standard [$^2H_7$]Org25543 in a fixed concentration of 100 pM (except for matrix blanks, for details see the Experimental Section). The established sets of calibration standards, quality control samples, blanks and zero samples were analyzed by using the developed LC-ESI-MS/MS method. The validation results showed that this method is in full agreement with the criteria from the FDA guidance regarding linearity in a range from 5 pM to 1 nM, intra- and inter-batch precision and accuracy for QC samples at four different concentration levels (LLOQ, 15, 300, and 800 pM; defined criteria for linearity, intra- and inter-batch precision and accuracy as given in the FDA guidance; see the Experimental Section). Overall, in five validation series we determined for the calibration standards accuracies between 94.2–108.6% whereas the QC samples exhibited accuracies and precisions (expressed in relative standard deviations) between 96.3–110.0% and 0.7–5.3% for intra-batch and 100.8–103.9% and 3.4–5.2% for inter-batch, respectively. Selectivity was examined by injecting six individually prepared matrix blanks per validation series to the LC-MS/MS. Thereby, no interfering signals were detected for the chosen mass transitions of the reporter ligand and the internal standard, thus the criteria of selectivity is fulfilled. The results of all sets obtained during the validation regarding linearity, precision and accuracy are summarized in Supporting Information (Table S1). Representative chromatograms for Org25543 at the LLOQ and a matrix blank and the linear calibration function received from one set of calibration standards are depicted in Figure 4. The here presented results are all in accordance with the defined criteria, thus it was demonstrated that the developed LC-ESI-MS/MS method is reliable for the quantification of Org25543 as reporter ligand in MS Binding Assays.

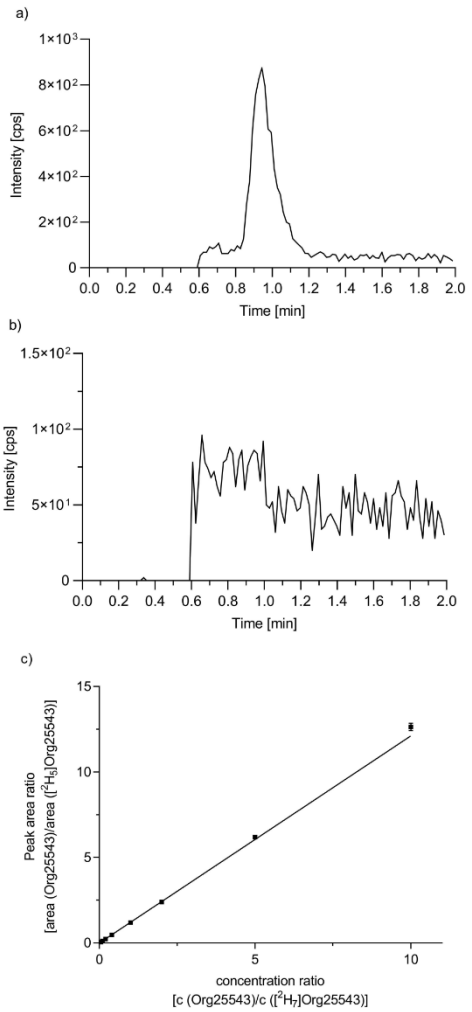

Figure 4. Validation of the developed LC-ESI-MS/MS method. a) MRM chromatogram for Org25543 at the LLOQ of 5 pM. b) MRM chromatogram for a matrix blank (for demonstration of selectivity). c) Representative calibration for Org25543 over a range from 5 pM to 1 nM employing [$^2H_7$] Org25543 (100 pM) as internal standard. The peak area ratio of marker and internal standard (mean ± SD, $n = 3$) was plotted against the concentration ratio of marker and internal standard. The corresponding calibration function obtained by linear regression was $y = 1.212x - 0.008975$; $R^2 = 0{,}9990$.

2.5. GlyT2 MS Binding Assays: Kinetic experiments

Next, the validated LC-ESI-MS/MS method should be employed for quantification of Org25543 in binding experiments. For the generation of reliable results in saturation and competitive experiments it has, however, to be warranted that the reporter ligand reaches equilibrium binding. Since no information about binding kinetics for any GlyT2 inhibitor are available so far, we decided to start our GlyT2 MS Binding Assays experiments with the determination of the off- and on-rate (k_{off} and k_{on}) of Org25543 at GlyT2 in dissociation and association experiments. That way also the equilibrium dissociation constant (K_d) can be obtained, that is, by calculation from the kinetic rate constants. Furthermore, dissociation experiments were expected to shed some light on the binding characteristics of Org25543. In electrophysiological oocyte glycine current assays, it had namely been found that GlyT2 function cannot be restored completely, when cells are washed for several minutes to remove Org25543, which points towards a tight binding of Org25543 to GlyT2 resulting in a very slow off-rate.[11,21] Hence, we first performed dissociation experiments and after that association experiments.

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The kinetic experiments were performed under conditions described above (see Section 2.3.) except that the incubation was done in bulk form. For further information about the setup of the kinetic experiments, see Experimental Section.

For the determination of the dissociation rate constant of Org25543 the so-called displacer method was applied. Thus, after the target protein GlyT2 had been incubated with the reporter ligand Org25543 at a defined concentration (final concentration: 10 nM) and the binding allowed to reach equilibrium (1 h) dissociation was initiated by addition of **8** (final concentration: 30 μM). Overall, 12 samples were taken, the first after 15 s and the last after 3 h, and processed as described for competitive binding experiments. The same way nonspecific binding was determined except that the target protein had been subjected to a heat-shock before. Plotting of specific binding calculated from total and nonspecific binding against time yielded the respective dissociation curves. Therefrom, by means of nonlinear regression analysis a k_{off} of $7.07 \pm 0.26 \times 10^{-3}$ s^{-1} (mean ± SEM, $n = 3$) and a half-life ($t_{1/2}$) of 98.4 ± 3.5 s (mean ± SEM, $n = 3$) could be deduced. In Figure 5a, a representative dissociation curve of the specific binding of Org25543 is depicted. The half-life of Org25543 at 37 °C showed that it is long enough to avoid unwanted dissociation effects during the washing procedure in the workflow of the GlyT2 MS Binding Assays. Furthermore, these results clearly demonstrate that Org25543 is a fully reversible ligand at GlyT2. No extremely tight binding or slow off-rate towards GlyT2 can be observed. In this context, however, it is worth mentioning that the existence of covalent binding of Org25543 at GlyT2 to some extent, though very unlikely, cannot be absolutely excluded, as the amount of covalent binding that would have to be quantified by the established MS Binding Assays might be beyond its capability.

Next, for the determination of the association kinetics of Org25543 association experiments had to be performed. By the knowledge of [L] and k_{off}, from k_{obs} as the primary result of such experiments, finally, the derived rate constant k_{on} becomes accessible by using the relation given at Equation 1, the transformed form of which is Equation 2.

$$k_{obs} = k_{on} \times L + k_{off} \quad (1)$$

$$k_{on} = (k_{obs} - k_{off})/L \quad (2)$$

For the determination of k_{obs} Org25543 was added to the target protein, to start the association process, which was then stopped at eleven different time points (15 s–2 h), to measure the amount of bound marker at these defined time points. The nonspecific binding was determined for the same time points like for total binding samples but utilizing target material that had been subjected to a heat-shock before. The concentration of the reporter ligand Org25543 was set to a value of 10 nM. This value was low enough to ascertain an association kinetic, that was slow enough to be reliably monitored by the applied technique. For the establishment of association curves specific binding calculated from total binding and nonspecific binding was plotted against time. By means of nonlinear regression analysis of these curves a k_{obs} value of $1.72 \pm 0.01 \times 10^{-2}$ s^{-1} (mean ± SEM, $n = 3$) was obtained. Therefrom, with the k_{off} value determined before and the known concentration of Org25543 that had been applied, a k_{on} of 1.01×10^{6} $M^{-1} s^{-1}$ has been calculated. A representative association curve of the specific binding of Org25543 to GlyT2 is depicted in Figure 5b.

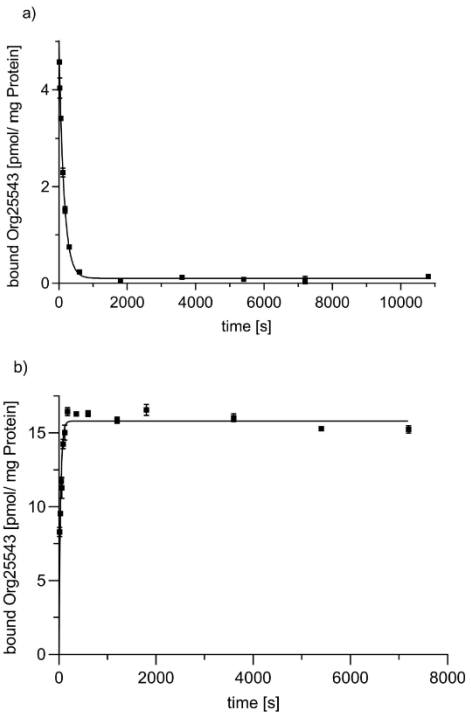

Figure 5. Representative kinetic experiments showing the time dependence of dissociation and association of Org25543 at GlyT2. a) Dissociation kinetics of the target–marker complex determined for Org25543 at GlyT2 after displacement by an excess of **8**. Specific binding (obtained after subtraction of nonspecific binding from total binding) plotted against time (mean ± SD, $n = 3$). b) Association kinetics determined for Org25543 at GlyT2. Specific binding (obtained after subtraction of nonspecific binding from total binding) was plotted against time (mean ± SD, $n = 3$).

The determined dissociation and association rate constants were finally used to calculate the equilibrium dissociation constant (K_d) of Org25543 at GlyT2 according to the equation $K_d = k_{off}/k_{on}$ which yielded a value of 6.99 nM.

As this is the first binding assay for GlyT2 no binding affinities from other binding assays are available for comparison purposes. So far Org25543 has been characterized only with IC_{50} values of 16 ± 1.9 nM in a [^{3}H]glycine uptake assay[23] and 31 ± 6 nM in a FLIPR membrane potential assay.[27] Although these assays are based on distinctly different principles and the inhibitory potencies obtained thereby cannot be expected to be identical with the affinity constant from a binding assay, that

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is, the K_d value of Org25543 found in this GlyT2 MS Binding Assays, these data still clearly indicate that the K_d value found in this study is in a plausible order of magnitude.

2.6. GlyT2 MS Binding Assays: Saturation experiments

For the further characterization of the binding of Org25543 to GlyT2 saturation experiments were performed. To determine total binding of Org25543 GlyT2 membrane fractions were incubated with the reporter ligand in eight different concentrations (0.4–150 nM) and the samples analyzed after the general workup by LC-ESI-MS/MS as described above. Nonspecific binding was obtained from experiments that were completely analogous to the former except that protein subjected to a prior heat-shock was used. Quantification of Org25543 in total binding samples could be realized down to the lowest nominal concentration level (0.4 nM). For nonspecific binding samples the quantification was possible down to 2 nM nominal marker concentration. For nominal marker concentrations lower than 2 nM the concentrations of Org25543 lay below the LLOQ. As it is generally acknowledged that nonspecific binding is related to nominal marker concentration in a linear fashion,[46] data points below this concentration were calculated by extrapolation after linear regression analysis of the data points for nonspecific binding at nominal marker concentrations from 2 to 150 nM.

A representative curve obtained from a saturation experiment with Org25543 is depicted in Figure 6. It shows total and nonspecific binding as well as the specific binding of Org25543 towards GlyT2. By means of nonlinear regression analysis a saturation isotherm was generated from the data points representing specific binding, which revealed a K_d value of 7.45 ± 0.55 nM (mean $\pm$ SEM, $n = 3$) and a maximum density of binding sites (B_{max}) of 26.15 ± 1.03 pmol (mg protein)$^{-1}$ (mean $\pm$ SEM, $n = 3$). The thus obtained K_d value is clearly in excellent accord with the K_d value obtained for Org25543 from the kinetic experiments (7.45 vs. 6.99 nM).

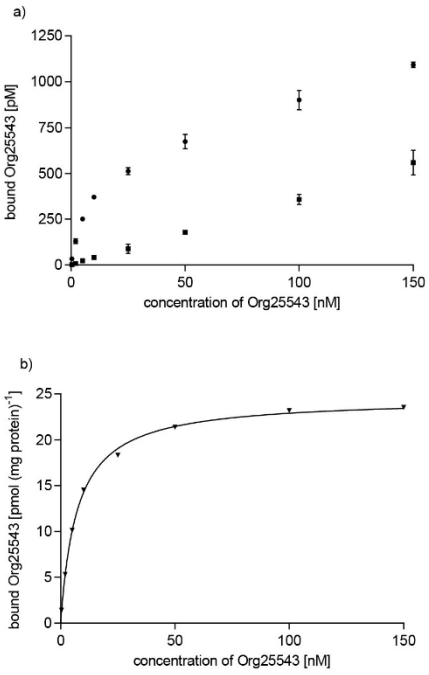

Figure 6. Representative saturation experiment showing total, nonspecific and specific binding of Org25543 at GlyT2. a) Experimental data (mean $\pm$ SD, $n = 3$) for total (■) and nonspecific (●) binding. Nonspecific binding for nominal concentrations of Org25543 less than 2 nM was extrapolated after modeling of experimentally determined nonspecific binding at concentrations of Org25543 $\geq$ 2 nM by linear regression. b) Specific binding (▼; means, pmol (mg protein)$^{-1}$) calculated as the difference of total binding and nonspecific binding from a) and saturation isotherm generated by nonlinear regression.

2.7. GlyT2 MS Binding Assays: Competition experiments

As already mentioned above, there is a strong need for competitive binding assays addressing GlyT2 as a tool for the identification of new GlyT2 inhibitors as well as the characterization of the binding affinities of the respective ligands. For the development of the desired competitive MS Binding Assay, the general concept of this kind of binding experiments was followed, in which the affinity of a test compound is delineated from its potency to compete out a reporter ligand, in our case Org25543, from its target binding site. Accordingly, the target protein was incubated with a defined concentration of the reporter ligand and increasing concentration of the individual test compound. In detail, the competitors were studied at seven different concentration levels, which covered about three concentration log units, whereas the concentration of Org25543 was fixed to 10 nM. Thereafter, via the developed LC-ESI-MS/MS method the amount of bound marker (total binding) was determined for the samples obtained from the individual incubation mixtures by the common workup for MS Binding Assays. Nonspecific binding was again determined in a set of analogous experiments in which GlyT2 membrane fractions were replaced by material subjected to a heat-shock before. The resulting amount of Org25543 binding was defined as 0% level of specific reporter ligand binding. Plotting the percentage of bound marker (*y*-axis) against the logarithm of the competitor concentration (*x*-axis) yielded the corresponding sigmoidal competition curves, from which by means of nonlinear regression analysis the IC_{50} values of the studied test compounds could be obtained. The thus determined IC_{50} values were finally used to calculate the corresponding K_i values by means of the Cheng-Prusoff equation.

In total 19 compounds were investigated including glycine, glycine derivatives and a series of small amino acids already characterized at GlyT1 and GlyT2 in other biological assays.

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Table 1. Affinities of known GlyT ligands determined in GlyT2 MS Binding Assays (mean ± SEM, $n = 3–5$) with a comparison of biological activities determined in [^{3}H]glycine uptake or oocyte glycine current assays published in literature.

Compound	Affinity, pK_i (GlyT2 MS Binding Assays)	Biological activity, IC_{50} ([µM], literature)	pIC_{50} (literature; calcd)
glycine [**1**]	1.98 ± 0.08	6–1801[11,18,22,27,47–51][b]	5.22–2.74
ALX1393 [**3**]	5.93 ± 0.03	0.026–0.100[40,11]	7.59–7.00
N-arachidonylglycine [**4**]	5.40 ± 0.09	5.1 ± 3.1[52]	5.29
N-oleoylglycine [**5**]	5.63 ± 0.06	0.500[19]	6.30
4-benzyloxy-3,5-dimethoxy-*N*-[1-[(dimethylaminocyclohexyl) methyl]benzamide [**8**]	7.38 ± 0.01	0.084 ± 0.003[23]	7.08
sarcosine [**9**]	2.2 mM, 98 ± 6 %[a] (< 3.03)	> 1000[22]	< 3
glycine methyl ester [**10**]	1.11 ± 0.06	84 ± 5 %[47],[c]	
DL-proline [(*rac*)-**11**]	1.50 ± 0.11	95 ± 5 %[47],[c]	
L-serine [(*S*)-**12**]	180 mM, 97 ± 4 %[a] (< 1.11)	89 ± 7 %[47],[c]	
DL-leucine [(*rac*)-**13**]	10 mM, 99 ± 3 %[a] (< 2.37)		
ZINC6865169 [**14**]	600 µM, 77 ± 6 %[a] (< 3.59)	0.518 ± 0.066[40]	6.29
Org24598 [(*R*)-**15**]	300 µM, 101 ± 5 %[a] (< 3.89)	> 100[53]	< 4
ALX5407 [(*R*)-**16**]	600 µM, 90 ± 5 %[a] (< 3.59)	> 100[54]/1.8[40]	< 4/5.74
CP-802,079 [(*rac*)-**17**]	4.64 ± 0.001	> 10[55]	< 5
ASP2535 [**18**]	5.99 ± 0.11	4.6 ± 0.290[56]	5.34
LY2365109 [**19**]	10 µM, 94 ± 10 %[a] (< 5.37)	> 30[57]	< 4.52
haloperidol [**20**]	4.90 ± 0.06	13 ± 2[58]	4.89
chlorpromazine [**21**]	4.55 ± 0.01	21 ± 4[58]	4.68
oleoyl-L-carnitine [**22**]	5.58 ± 0.06	0.340[18]	6.47

[a] remaining Org25543 binding (% of specific binding) at the highest compound concentration [b] K_m and EC_{50} values. [c] Glycine uptake in presence of 1 mM competitor (% of control).

Furthermore, a set of commercially available GlyT2 inhibitors, including representatives of a new class of lipid-based GlyT2 inhibitors, as well as selective GlyT1 inhibitors and antipsychotics the characterization of which at GlyT2 has been reported in literature, have been studied for their ability to inhibit Org25543 binding. All these compounds are shown in Figure 1. The results of the competitive experiments are listed in Table 1, representative competition curves are depicted in Figure 7. As no data from other binding assays for GlyT2 is available so far, the obtained results were compared as far as possible with biological activities/potencies published for functional assays in literature.

For the glycine derivatives sarcosine [**9**] and glycine methyl ester [**10**] and for the amino acids DL-proline [(*rac*)-**11**], L-serine [(*S*)-**12**] and DL-leucine [(*rac*)-**13**] results were obtained which are largely in agreement with potencies determined in [^{3}H] glycine uptake assays at GlyT2.[22,47] For these compounds either no inhibition of Org25543 binding to more than 50 % could be found up to mM concentrations of the compounds (sarcosine, L-serine, DL-leucine) or the determined K_i value lies in the high-millimolar range (glycine methyl ester pK_i = 1.11 ± 0.06; DL-proline pK_i = 1.50 ± 0.11). However, for glycine [**1**], the actual substrate of GlyT2, the determined K_i value of about 10 mM (pK_i = 1.98 ± 0.08) found in our GlyT2 MS Binding Assays is noticeable high. In literature a broad range of Michaelis-Menten constants (K_m) and EC_{50} values have been published (6–1801 µM),[11,18,22,27,47–51] reflecting that GlyT2 mediated transport of glycine may strongly depend on the chosen experimental conditions in the respective functional experiments. Though no satisfactory explanation for the rather low affinity determined for glycine at GlyT2 in the MS Binding Assay can be given, it should still be mentioned that a similar tendency (i. e., K_i in binding experiments > K_m) has also been observed for GAT1

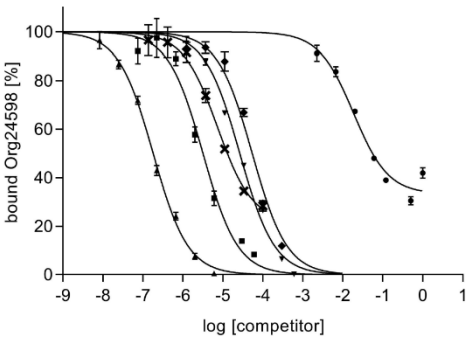

Figure 7. Representative competition curves obtained in GlyT2 MS Binding Assays for **8** (▲), ALX1393 (■), glycine (●), CP-802,079 (♦), haloperidol (▼) and *N*-oleoylglycine (**x**). The experimental data represent specific binding (mean ± SD, $n = 3$) of Org25543 (10 nM) at various concentrations of the competitors. 100 % binding was equivalent to specific binding of Org25543 without any competitor and 0 % to nonspecific binding. For compounds, which are exhibiting incomplete inhibition, the bottom level was not constrained to the nonspecific binding level (0 %).

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and GlyT1 in previous studies.[38,29] However, in preliminary experiments, in which saturation of Org25543 at GlyT2 was studied in the presence of glycine, we obtained results that point to a competitive binding behavior between Org25543 and glycine (Figure S7 and Table S2). Possibly, Org25543 binds to a binding site different from the substrate binding site that is addressed by glycine as well, but with low affinity only. This could explain the difference that has been observed between potency and affinity of glycine at GlyT2. Nevertheless, MS Binding Assays are a valuable tool for the determination of binding affinities for test compounds towards GlyT2, the purpose they have been developed for, though they are not able to provide detailed information about the binding sites or binding mechanisms of test compounds. Another interesting aspect when looking at the inhibition curve of glycine is that it cannot completely displace Org25543 from its binding site even at the highest investigated concentration of 1 M. This can also be seen for DL-proline (Figure S8). One possible explanation for this phenomenon could be that Org25543 binds to two different binding sites, of which only one can be addressed by glycine. However, neither in saturation nor in kinetic experiments any indications for a biphasic nature of the respective curves could be noted. Still, it has to be considered that to distinguish between different binding sites of the reporter ligand at GlyT2 in the generated binding curves were only possible, if the corresponding affinities or the kinetic behavior were markedly different. Another explanation for the observed phenomenon might be, that the high concentrations of glycine (or of structurally related compounds) of about 1 M cause unexpected effects by a so far unclear mechanism, which mediated an increase of nonspecific binding. In this context it has to be noted, that the bottom level of inhibition curves was in this case (and also in similar ones) due to the incomplete inhibition of Org25543 binding not constrained to the level of nonspecific binding as can easily be seen in Figure 7.

When the GlyT2 inhibitors ALX1393 [**3**], **8** and ZINC6865169 [**14**] were studied for ALX1393 a pK_i of 5.93 was found. This pK_i differs at least one order of magnitude from the pIC_{50} values reported in glycine uptake studies.[11,40] Compound **8**, however, the analogue of Org25543 [**2**], exhibits a pK_i of 7.38 which lies in the same range as the published biological activity. Whereas **8** and Org25543 can be expected to have the same binding site due to their structural analogy, the binding site of ALX1393 could differ slightly from that of Org25543. Nevertheless, both inhibitors exhibited reasonable affinities in comparison to their potencies found in literature. More surprising are the findings for ZINC6865169, a recently described GlyT2 inhibitor with a structure significantly different from that of Org25543 and ALX1393. In glycine uptake studies an IC_{50} value of 518 nM was determined[40] whereas the IC_{50} value in our GlyT2 MS Binding Assays was >600 μM (77% remaining binding of Org25543 at 600 μM of ZINC6865169). Thus, for this compound there is a substantial difference between potency and affinity of more than three orders of magnitude, which could also argue for different bindings sites.

For the GlyT1 selective inhibitors Org24598 [(*R*)-**15**], ALX5407 [(*R*)-**16**], CP-802,079 [(*rac*)-**17**], ASP2535 [**18**] and LY2365109 [**19**] the results are in good agreement with published potencies. CP-802,079 and ASP2535 exhibit pK_i values of 4.64 and 5.99, respectively, whereas Org24598 and LY2365109 show hardly any affinity towards the Org25543 binding site on GlyT2 up to concentrations of 300 and 10 μM, respectively. For ALX5407 two different inhibitory potencies have been reported in literature, i.e. an IC_{50} value >100 μM[54] and an IC_{50} value of 1.8 μM.[40] In the GlyT2 MS Binding Assay ALX5407 showed hardly a reduction of Org25543 binding up to 600 μM indicating its very low affinity. Though, it is not clear which of the two IC_{50} values of ALX5407 reported in literature is more reliable, the low affinity found for this compound in our MS Binding Assay ($IC_{50}>600$ μM) fits at least quite well to the higher IC_{50} value, arguing for the validity of the result obtained in the MS Binding Assay.

The antipsychotics haloperidol [**20**] and chlorpromazine [**21**] are exhibiting moderate pK_i values of 4.90 and 4.55, respectively. These results are in very good agreement with the results published in literature (pIC_{50} of 4.89 and 4.68).

Finally, the most well-known lipid-based GlyT2 inhibitors *N*-arachidonylglycine [**4**], *N*-oleoylglycine [**5**] and oleoyl-L-carnitine [**22**] were tested in competition experiments. As already mentioned before, in the last years GlyT2 research was heading for inhibitors with better and safer pharmacological profiles. Thereby lipid-based compounds were found, which are able to inhibit GlyT2 in a fully reversible manner and are partial inhibitors of this transporter, the latter of which is thought to positively contribute to the pharmacological profile of these compounds.[17–21] The affinities that were found in MS Binding Assays for the herein examined lipid-based compounds are in the same range for all three substances (pK_i of 5.40, 5.63 and 5.58). Compared to their inhibitory potencies only *N*-arachidonylglycine exhibits a pK_i value that is similar to the published pIC_{50} value (calculated from IC_{50}), whereas the pK_i values of *N*-oleoylglycine and oleoyl-L-carnitine are slightly lower than the pIC_{50} values calculated from the published potencies (less than one order of magnitude). So far, it is still a matter of discussion how these lipid-based inhibitors are interacting with GlyT2. It is assumed that they either interact directly with GlyT2 at extracellular loops or at the interface between GlyT2 and the surrounding lipid membrane[18,59] or that they are inhibiting GlyT2 indirectly by perturbing the biophysical properties of the bilayer surrounding GlyT2.[60] Due to the difference of their structure in comparison to Org25543 it does not appear unlikely that these compounds bind to a binding site different from that of Org25543, which would explain why the determined binding affinities are different from the published potencies. Additionally, another point worth mentioning in this context, are the critical micelle concentrations (CMC) of the lipid-based compounds. For *N*-arachidonylglycine [**4**] and oleoyl-L-carnitine [**22**] CMCs of >100[61] and 7.4 μM[18] were published, respectively, whereas for *N*-oleoylglycine [**5**] to the best of our knowledge no CMC has been described so far. Taking the knowledge about the CMCs into account the result obtained for oleoyl-L-carnitine [**22**] should be handled with care as the concentrations in competitive experiments are higher than the respective CMC. Possibly, the obtained binding data do not only reflect pure

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competitive interactions but also effects that result from micelle formation. Micelle formation might lead to a decrease of the free concentration of these lipid-based compounds or formed micelles might have a direct effect on the GlyT2 protein to some extent. Since the CMC for *N*-oleoylglycine [**5**] is not known, the same could be true for it, as well. A decreased free concentration of *N*-oleoylglycine [**5**] due to micelle formation, for example, could be an explanation for the incomplete displacement of the reporter ligand Org25543 [**2**] in the competition experiment performed with **5** (Figure 7). For *N*-arachidonylglycine [**4**], the highest employed concentrations were still below the mentioned CMC. Thus, in this case the formation of micelles in the binding assay is to be excluded, which might explain why binding data from the MS Binding Assay and data from functional assays (from literature) are in good accord.

Summarizing the findings resulting from the discussion above, it can be concluded that the affinities determined in the competition experiments are in fair to good agreement with inhibitory potencies obtained in various functional assays for the majority of the investigated compounds, but that there are also distinct discrepancies between affinity and inhibitory potency as exemplified for the GlyT2 inhibitor ZINC6865169. This outcome is in the end not too surprising, as the affinities measured in the established GlyT2 MS Binding Assays only record direct (i.e., competitive) and maybe additionally indirect (i.e., allosteric) interactions at the binding site labeled by Org25543, whereas inhibition in the various functional assays can furthermore be due to inhibition of binding sites not addressed by Org25543 or even by other mechanisms such as changes in protein expression or internalization – to name just a few.

3. Conclusion

The presented study describes the establishment of the first binding assays for the neurotransmitter transporter GlyT2. They are following the concept of MS Binding Assays and use the selective GlyT2 inhibitor Org25543 as unlabeled reporter ligand. For quantification of Org25543 in binding experiments a rapid and highly sensitive LC-ESI-MS/MS method was established. Validation of this method indicated that Org25543 can be accurately and precisely quantified in the matrix resulting from binding experiments in a range from 5 pM to 1 nM.

Based on the workflow of filtration-based MS Binding Assays already established for other neurotransmitter transporters, binding experiments could be performed to characterize the binding behavior of Org25543 towards GlyT2 in kinetic, saturation and competitive experiments. In dissociation and association experiments the off- and on-rate of Org25543 could be characterized with a k_{off} of $7.07 \times 10^{-3}\ s^{-1}$ and a $t_{1/2}$ of 98.4 s and with a k_{obs} of $1.72 \times 10^{-2}\ s^{-1}$ resulting in a k_{on} of $1.01 \times 10^{6}\ M^{-1}\ s^{-1}$, respectively. Furthermore, these experiments revealed full reversibility of Org25543 binding towards GlyT2 with an off-rate that is not exceedingly slow. This result is noticeable as based on electrophysiological experiments with GlyT2 expressing oocytes Org25543 [**2**] was claimed to exhibit tight binding toward GlyT2 and to act like an irreversible inhibitor.[11,21] Calculating the equilibrium dissociation constant from k_{off} and k_{on} yielded a value of 6.99 nM which matches perfectly the K_d of 7.45 nM determined in saturation experiments and which is roughly in agreement with potencies observed in functional assays. In competition experiments 19 known GlyT ligands were characterized for their affinity at the Org25543 binding site of GlyT2. For most of the investigated compounds affinities were determined that are largely in accordance with inhibitory potencies found in various functional assays. For few of the investigated compounds, inhibition of Org25543 binding did not parallel the inhibition described in functional assays. Besides differences in experimental conditions of the different assay techniques, possible explanations must remain speculative without extensive investigations. So far, it cannot be excluded, that occupation of different binding sites at GlyT2 contributes to this phenomenon, in particular when considering the novel extracellular allosteric modulator site for lipid-based GlyT2 inhibitors just recently identified by Mostyn et al.[62] in some cases, at very high concentrations of test compounds (e.g., for glycine) also solubility issues or formation of micelles (e.g., for lipid-based GlyT2 inhibitors) might play a role. Apart from that it has to be pointed out, that the results from these binding experiments cannot be expected to be strictly and completely in agreement with inhibitory effects observed in various functional assays recording substrate transport, translocation of charges or membrane depolarization, as inhibition of functional effects recorded in all these assay types does not necessarily require occupation of the GlyT2 binding site addressed by Org25543.

In the context of this discussion, the established GlyT2 MS Binding Assays as well as all the different functional assay principles should be considered as valuable alternatives but not as absolutely equivalent substitutes, all of them possessing specific strengths and weaknesses. The strengths of binding assays are for example their conceptual simplicity, their robust read out and the possibility to derive structure-activity relationships based on determined affinities in a very straightforward way. Therefore, the presented GlyT2 MS Binding Assays can be assumed to fill a gap and will hopefully contribute to facilitate screening for new GlyT2 inhibitors and furthermore, to assign GlyT2 inhibitors to different categories according to their binding behavior.

Experimental Section

Chemicals

4-Benzyloxy-3,5-dimethoxy-*N*-[1-[(dimethylaminocyclohexyl)methyl] benzamide was synthesized in-house according to Caulfield et al.[23] Org25543 (*N*-[[1-(dimethylamino)cyclopentyl]methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide) as hydrochloride (purity ≥99%, HPLC), 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),

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and *N*-arachidonylglycine (NAGly, *N*-(1-oxo-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenyl)glycine; purity ≥98%, HPLC) were purchased from Tocris (Bristol, UK). LY2365109 (*N*-[2-[4-(1,3-benzodioxol-5-yl)-2-(1,1-dimethylethyl)-phenoxy]ethyl]-*N*-methylglycine) and ASP2535 (4-[3-(1-methylethyl)-5-(6-phenyl-3-pyridinyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole) were part of the Tocriscreen Plus library from Tocris (Bristol, UK). CP-802,079 (*N*-[3-(4-chlorophenyl)-3-[4-(2-thiazolylcarbonyl)phenoxy]propyl]-*N*-methyl-glycine) as hydrochloride (purity ≥98%), ALX1393 (*O*-[(2-benzyloxyphenyl-3-flurophenyl) methyl]-L-serine) (purity ≥98%), haloperidol, chlorpromazine as hydrochloride (purity ≥98%, TLC) and ZINC6865169 (5-((8-hydroxy-9*H*-purin-6-yl)thio)pentanoic acid) were purchased from Sigma-Aldrich. Glycine (purity ≥99%), DL-proline (purity ≥99%) and DL-leucine (purity ≥99%) were obtained from Acros Organics, glycine methyl ester as hydrochloride (purity ≥99%) and L-serine (purity ≥99%) were from Merck and sarcosine as hydrochloride was from ICN Biomedicals (Irvine, CA, USA). Oleoyl-L-carnitine (OLCarn; purity >99%, TLC) and *N*-oleoylglycine (NOGly, *N*-(1-oxo-9-octadecenyl)-(*Z*)-glycine; purity ≥98%) were received from Avanti Polar Lipids and Cayman Chemical Company (Ann Arbor, MI, USA). Water was exclusively obtained from a Sartorius arium pro ultrapure water system (Sartorius, Göttingen, Germany). HPLC and LC-MS grade methanol from VWR Prolabo (Darmstadt, Germany) was used for washing the glass fiber filters and for determination of compound-dependent MS parameters, respectively. LC-MS grade acetonitrile from VWR Prolabo (Darmstadt, Germany) was used for elution of marker from target-marker-complexes and for the mobile phase in LC-MS. All other chemicals were purchased in analytical grade. For cell culture, Dulbecco's modified Eagle's medium (DMEM) was bought from Sigma-Aldrich), fetal bovine serum, penicillin and streptomycin were from BioWest (Nuaillé, France) and hygromycin B (Hygromycin B Gold) was obtained from InvivoGen.

LC-ESI-MS/MS instrumentation

An API5000 triple quadrupole mass spectrometer with a TurboV-ESI source (AB Sciex, Darmstadt, Germany) was used for the LC-ESI-MS/MS. As HPLC system an Agilent 1200 Series HPLC system (vacuum degasser G1379B, binary pump G1312B, oven G1316B, Agilent, Waldbronn, Germany) and a HTS-PAL auto sampler (CTC-Analytics, Zwingen, Switzerland) with a 50 µL syringe and a 50 µL sample loop was coupled to the mass spectrometer. For controlling the hardware components, the Analyst v. 1.6.1 software (AB Sciex, Darmstadt, Germany) was integrated.

Compound-dependent MS parameters for precursor and fragment ions of ALX1393, Org25543 and [$^2H_7$]Org25543

An external syringe pump (Harvard Apparatus, Holliston, MA, USA) set to a flow rate of 10 µL min^{-1} was used to infuse 20 nM solutions of ALX1393, Org25543 and [$^2H_7$]Org25543 in a mixture of methanol (LC-MS grade) and 0.1% (*v/v*) formic acid (LC-MS grade; 50:50, *v/v*) into the ESI source. With the manual tuning mode of the Analyst software *m/z* 396,1, *m/z* 413.2 and *m/z* 420.3 were identified as [*M* + H]$^+$ parent ions for ALX1393, Org25543 and [$^2H_7$]Org25543, respectively. To identify the most intense fragment ions (*m/z* 291.3 for ALX1393, *m/z* 368.3 for Org25543 and *m/z* 375.4 for [$^2H_7$] Org25543) as well as to optimize the compound-dependent parameters for the precursor ions the compound optimization mode of the Analyst software was used. The optimized parameters are listed below: ALX1393: Declustering potential (DP) 66 V, entrance potential (EP) 10 V, collision energy (CE) 15 V, cell exit potential (CXP) 28 V; Org25543: DP 106 V, EP 10 V, CE 25 V, CXP 14 V; [$^2H_7$]Org25543: DP 91 V, EP 10 V, CE 27 V, CXP 14 V.

Chromatography

An isocratic RP-LC method was developed for chromatography, which used a mobile phase consisting of ammonium bicarbonate buffer (5 mM, pH 7.8) and acetonitrile (20:80, *v/v*) at a flow rate of 600 µL min^{-1} at 20 °C. As stationary phase a Luna 3 µ C8(2) column (50 mm × 2 mm, 3 µm, Phenomenex, Aschaffenburg, Germany) was integrated into the system. A SecurityGuard C8 column (4 mm × 2 mm, Phenomenex, Aschaffenburg, Germany) and two in-line filters (0.5 and 0.2 µm, Idex, Oak Harbor, WA, USA) are installed before the column for protection reasons. The injection volume was set to 45 µL.

LC-ESI-MS/MS

To analyze ALX1393, Org25543 and [$^2H_7$]Org25543 the mass transitions of *m/z* 396.1/291.3, *m/z* 413.2/368.3 and *m/z* 420.3/375.4 were used, respectively. Both mass selectors, Q1 and Q3, were operated under unit resolution for dwell times of 500 ms under the conditions mentioned in Experimental Section *Compound-dependent MS parameters for precursor and fragment ions of ALX1393, Org25543 and [$^2H_7$]Org25543*. After Org25543 was selected as reporter ligand the optimized source-dependent parameters were determined by using the flow injection analysis (FIA) tool of the Analyst software. For that a solution containing 100 pM of Org25543 and [$^2H_7$]Org25543 was injected (10 µL) to the LC-MS/MS which resulted in following optimized parameters: source temperature 600 °C, ion-spray voltage 2500 V, curtain gas (N_2) 15 psi, auxiliary gas (N_2) 40 psi, nebulizing gas (N_2) 60 psi and collision gas (N_2) 7 psi.

Synthesis of methyl 3,5-dimethoxy-4-(((2,3,4,5,6-[$^2H_5$])phenyl)[$^2H_2$]methoxy)benzoate (25)

[$^2H_7$]benzyl chloride (**24**; 862 µL, 7.48 mmol) was added dropwise to the suspension of methyl syringate (**23**; 1.47 g, 6.80 mmol), K_2CO_3 (1.14 g, 8.16 mmol) and KI (1.13 g, 6.80 mmol) in DMF (10 mL) at RT. The reaction mixture was stirred at 80 °C for 24 h. After 24 h the reaction mixture was cooled to RT, then added to H_2O (50 mL) and extracted with ethyl acetate (4×50 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL) then dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford 1.95 g (93%) methyl 3,5-dimethoxy-4-(((2,3,4,5,6-[$^2H_5$])phenyl)[$^2H_2$]methoxy)benzoate (**25**) as colorless solid. ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.29 (s, 2H, 2×HC_{arom}), 3.88 (s, 3H, CH_3OC=O), 3.87 (s, 6H, 2×CH_3OC_{arom}). ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 167.06 (C=O), 153.86 (2×CH_3OC_{arom}), 141.49 (CH_3OCCO), 137.98 (C[$^2H_2$]), 128.8–127.6 (m, *5* C, 5×C[^2H]$_{arom}$), 125.94 (CC=O), 107.19 (2×HC_{arom}), 74.51 (quint, $J_{C\text{-}D}$ = 22.3 Hz, C[$^2H_2$]), 56.69 (2×CH_3OC_{arom}), 52.57 (CH_3OC=O). IR (KBr): $\tilde{\nu}$ = 3400, 3101, 2980, 2949, 2874, 2844, 2638, 2270, 2207, 2120, 1950, 1931, 1711, 1591, 1499, 1467, 1437, 1414, 1332, 1227, 1185, 1129 cm^{-1}. MS HRMS (EI): $[M]^{+\cdot}$ calcd. for $C_{17}H_{11}[^2H_7]O_5$ 309.1594; found: 309.1588.

Synthesis of 3,5-dimethoxy-4-(((2,3,4,5,6-[$^2H_5$])phenyl)[$^2H_2$] methoxy)benzoic acid (26)

To a stirred solution of **25** (890 mg, 2.88 mmol) in a mixture of THF (6 mL) and H_2O (2 ml) was added LiOH (352 mg, 14.4 mmol) at RT. After 68 h the reaction mixture was poured onto H_2O (20 mL) followed by the addition of HCl (1 M, 15 mL). The acidic aqueous solution (pH 1–2) was extracted with ethyl acetate (5×30 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford 808 mg (95%) 3,5-dimethoxy-4-(((2,3,4,5,6-[$^2H_5$])phenyl)[$^2H_2$]methoxy)benzoic acid (**26**) as a colorless solid. ^{1}H

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NMR (400 MHz, CD_2Cl_2): $\delta = 7.37$ (s, 2H, 2×HC_{arom}), 3.89 (s, 6H, 2×CH_3OC_{arom}). ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta = 172.31$ (C=O), 153.95 (2×CH_3OC_{arom}), 142.45 (CH_3OCCO), 137.88 (*C*C[$^2H_2$]), 128.49 (t, $J_{C\text{-}D} = 24.2$ Hz, 2×[^{2}H]C_{arom}), 128.22 (t, $J_{C\text{-}D} = 24.2$ Hz, 2×[^{2}H]C_{arom}), 128.03 (t, $J_{C\text{-}D} = 24.2$ Hz, [^{2}H]C_{arom}), 124.68 (*C*C=O), 107.87 (2×HC_{arom}), 74.58 (quint, $J_{C\text{-}D} = 22.1$ Hz, C[$^2H_2$]), 56.74 (2×CH_3OC_{arom}). IR (KBr): $\tilde{\nu} = 3445$, 2997, 2962, 2836, 2271, 2208, 2162, 2120, 1682, 1589, 1500, 1450, 1416, 1327, 1278, 1231, 1200, 1184, 1128 cm^{-1}. MS HRMS (EI): $[M]^{+\bullet}$ calcd. for $C_{16}H_9[^2H_7]O_5$ 295.1437; found: 295.1436.

Synthesis of [$^2H_7$]Org25543·HCl (7·HCl)

To a stirred solution of 1-(aminomethyl)-*N*,*N*-dimethylcyclopentane-1-amine[41] (353 mg, 1.49 mmol) in CH_2Cl_2 (20 mL) was added **26** (400 mg, 1.35 mmol) at RT followed by the addition of benzotriazole-1-ol (HOBt; 187 mg, 1.35 mmol), 3-(ethyliminomethyleneamino)-*N*,*N*-dimethylpropan-1-amine (EDCI; 265 mg, 1.35 mmol) and NEt_3 (137 mg, 1.35 mmol). The resulting reaction mixture stirred at RT for 18 h then poured on H_2O (50 mL) followed by the extraction with ethyl acetate (2×50 mL). The combined organic layers were washed with HCl (1 M, 10 mL), H_2O (10 mL) and brine (10 mL), then dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford 400 mg (70%) [$^2H_7$]Org25543 (**7**) as a yellowish oil. Finally, to a stirred solution of **7** in diethyl ether HCl (4 M, 250 µL) in dioxane was added at 0 °C under gently shaking. The resulting colorless precipitate was filtered, washed with diethyl ether and dried *in vacuo* to obtain 395 mg (91%) **7**·HCl. ^1H NMR (500 MHz, [D_4]methanol): $\delta = 7.26$ (s, 2H, 2×HC_{arom}), 3.89 (s, 6H, 2×CH_3O), 3.76 (s, 2H, NCH_2), 2.97 (s, 6H, 2×CH_3N), 2.11–1.80 (m, 8H, 8×cyclopentyl-H). ^{13}C NMR (126 MHz, [D_4]methanol): $\delta = 170.90$ (C=O), 154.80 (2×CH_3OC_{arom}), 141.10 (CH_3OCCO), 138.45 (*C*C[$^2H_2$]), 130.02 (*C*C=O), 129.24 (t, $J_{C\text{-}D} = 24.0$ Hz, 2×[^{2}H]C_{arom}), 128.64 (t, $J_{C\text{-}D} = 24.4$ Hz, 2×[^{2}H]C_{arom}), 128.60 (t, $J_{C\text{-}D} = 24.2$ Hz, [^{2}H]C_{arom}), 106.30 (2×HC_{arom}), 77.35 ($NCCH_2$), 75.11 (quint, $J_{C\text{-}D} = 22.1$ Hz, C[$^2H_2$]), 56.90 (2×CH_3O), 43.31 (*C*H_2NC=O), 40.07 (2×CH_3N), 33.53 (2×*C*H_2CN), 25.37 (2×CH_2*C*H_2CN). IR (film): $\tilde{\nu} = 3434$, 3234, 2994, 2964, 2876, 2842, 2668, 2595, 2516, 2478, 2279, 2201, 2117, 1669, 1587, 1531, 1496, 1414, 1332, 1272, 1225, 1194, 1167, 1124 cm^{-1}. MS HRMS (ESI): $[M+H]^+$ calcd. for $C_{24}H_{25}[^2H_7]N_2O_4$ 420.2880; found: 420.2876.

Validation of the LC-ESI-MS/MS method

The validation of the developed LC-ESI-MS/MS method was oriented towards the FDA guidance for bioanalytical method validation.[45] For this purpose, spiked matrix samples were used to examine the validation parameters linearity, LLOQ, precision, accuracy and selectivity. As described in Experimental Section *MS Binding Assay: General procedure* spiked matrix samples are obtained in the same way as binding samples are prepared, except for incubation which was carried out without marker or test compound and the elution step was performed with acetonitrile containing 125 pM [$^2H_7$]Org25543 and Org25543 in different concentrations. Those elution solutions were prepared from 125-fold Org25543 and [$^2H_7$]Org25543 solutions in 10% aqueous *N*,*N*-dimethylacetamide (DMA) which were two times diluted 1:10 in acetonitrile (final DMA concentration 0.2%). Five series of samples (matrix calibration standards and quality control (QC) samples, matrix blanks and zero samples) were prepared on different days with different GlyT2 membrane preparations. Calibration standards were studied at eight different concentration levels (5 pM to 1 nM). Every concentration level above the lower limit of quantification (LLOQ) was prepared in three replicates, whereas the concentration level of the LLOQ (5 pM) was prepared in six replicates. According to the FDA guidance the response of the LLOQ should be at least five times the response of the noise of a matrix blank, with an accuracy between 80 and 120% and a precision characterized by a relative standard deviation of less than 20%, whereas the accuracy of all other calibration standards and QC samples should be between 85 and 115% and the precision of QC samples exhibit a relative standard deviation of not more than 15%. The data for the calibration standards were obtained by plotting the peak area ratios of analyte vs. internal standard (*y*-axis) against the concentration ratios of analyte vs. internal standard (*x*-axis). Finally, linearity was studied after linear regression analysis of these data. For calculation of calibration curves a weighting factor of $1/x^2$ was used in all cases. For examination of accuracy and intra- and inter-batch precision QC samples were prepared independently from each other in different wells of a 96-well plate (1.2 mL well volume, Sarstedt, Nümbrecht, Germany). Each series investigated four concentration levels (LLOQ, 15, 300, 800 pM) in six replicate QC samples. Selectivity of marker and internal standard was proven by injecting six matrix blanks in every series.

Glycine transporter 2 (GlyT2) membrane preparations

For preparation of GlyT2 membrane preparations HEK293 cells, stably expressing the hGlyT2 with a confluence ≥90%, were used (kindly provided by AbbVie (Wiesbaden, Germany)). As cell culture medium DMEM containing 10% fetal bovine serum (*m*/*v*), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 100 mg mL^{-1} hygromycin B was used and the cells were cultivated in dishes (143 cm^2) at 37 °C and 8% CO_2. The cells were detached from the dish by aspirating the medium, adding 12 mL cell culture medium without hygromycin B and pipetting the medium several times until the cell lawn was completely detached. Subsequently, the cells were washed twice with PBS (5 min, 1600 rpm, Biofuge Stratos, rotor: #3047, Heraeus, Hanau, Germany) and afterwards homogenized in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) using a Polytron PTA 10 S (Kinematica Polytron, Littau-Luzern, Switzerland). After homogenization aliquots of about 1 mg protein were frozen and stored at −80 °C. To determine the amount of protein in the membrane preparation (after treatment with 100 mM NaOH for 1 h) the protein determination according to Bradford[63] was applied, using bovine serum albumin as standard for calibration. At the day of the assay the membrane preparation was rapidly thawed, diluted in 20 mL incubation buffer and centrifuged at 4 °C for 20 min at 20500 rpm (Sorvall Evolution RC, rotor: SS34, Thermo. Electron, Hanau, Germany). Finally, the resulting pellet was resuspended in 6 mL incubation buffer yielding a protein concentration of ~0.1–0.2 mg mL^{-1}.

MS Binding Assay: General procedure

In polypropylene 96-well plates (1.2 mL well volume, Sarstedt) membrane preparations (~5–10 µg protein) were incubated in the presence of the reporter ligand and, if required, test compounds in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) 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 (every sample was prepared in triplicates). After the incubation 210 µL aliquots of the binding samples were transferred by means of a 12-channel pipette onto 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 µm, 350 µL; Pall Corporation, Port Washington, NY, USA) where the incubation was terminated by vacuum filtration (the samples were transferred and the incubation was terminated row after row; Multi Well Plate Vacuum Manifold, Pall, Dreieich, Germany). Before the binding samples were filtered, the glass fiber filters were washed with water (3×200 µL per well) and methanol (3×200 µL per well), incubated with 0.5%

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(*m*/*m*) aqueous polyethylenimine solution (PEI; 200 µL per well) at room temperature for 2 h and, finally, exempted from the PEI solution by vacuum filtration. After the filtration of the binding samples, the filters with remaining target-marker complexes on it were rapidly washed concurrently (12-channel pipette) with ice-cold washing buffer (4×150 µL 154 mM ammonium acetate, pH 7.4) and the filter plates were dried at 50 °C for 1 h. Subsequently, the bound marker was eluted with acetonitrile containing 125 pM [$^2H_7$]Org25543 and 0.2% DMA (3×70 µL per well; in accordance to the calibration standards and QC samples; see Section 4.9.) into a 96-well filter plate (1.2 mL well volume, Sarstedt; filtration via vacuum application after exposure of the filters of all wells to acetonitrile for ~10 s; 12-channel pipette). After the elution 52.5 µL ammonium bicarbonate buffer (5 mM, pH 7.8) were added per well (12-channel pipette) to the eluate to adjust the composition of the sample solvent to the mobile phase (ammonium bicarbonate buffer (5 mM, pH 7.8) and acetonitrile 20:80, *v*/*v*). This means that the injected samples were diluted to 80% of their actual concentrations. The 96-well plates containing the eluted marker were sealed with aluminum foil and centrifuged 10 min at 2500 rpm (Biofuge Stratos, rotor: #3048, Heraeus). Finally, the samples were subjected to LC-ESI-MS/MS quantification (see above) without further sample preparation. In the same way nonspecific binding was determined by adding GlyT2 membrane fractions to the incubation samples, which were heat-shocked before for 1 h at 60 °C in a water bath. Marker depletion was negligible (< 10%) in all experiments. DMA concentrations in final binding samples were always ≤ 1%.

Saturation experiments

For saturation experiments GlyT2 membrane preparations were incubated with eight different concentrations of Org25543 in a concentration range of 0.4 to 150 nM. The following steps after incubation were performed as described in Experimental Section *MS Binding Assay: General procedure.* Nonspecific binding was determined in the same way as total binding but GlyT2 membrane fractions were used, which were heat-shocked for 1 h at 60 °C, for the same marker concentrations. When nonspecific binding of Org25543 fell below the LLOQ of the calibration curve (generally at a nominal marker concentration of < 2 nM), a straight line for the data points of nonspecific binding higher than the LLOQ was generated by using linear regression analysis and the nonspecific binding for data points below the LLOQ was calculated by extrapolation of the linear function using Prism v. 6.07 (GraphPad Software).

Competition experiments

For competition experiments GlyT2 membrane preparations were incubated with Org25543 in a concentration of 10 nM and in presence of test compounds (at least seven concentrations). Additionally, control samples were prepared to define total binding of Org25543 in absence of any competitor and nonspecific binding was defined as remaining binding in presence of GlyT2 membrane preparations, which were heat-shocked for 1 h at 60 °C. The top and bottom level of competition curves, which were showing a complete displacement of Org25543, were constrained to 100 and 0%, respectively, whereas in competition curves, which were exhibiting incomplete inhibitions, only the top level was set to 100% and the bottom-level was not constrained.

Kinetic studies: Dissociation experiments

Dissociation experiments via displacing Org25543 from GlyT2 with an excess of competitor were performed in 25 mL round bottom flasks made of glass (Duran Wheaton Kimble Life Sciences GmbH, Wertheim, Germany). For determining dissociation rate constants by the displacer approach GlyT2 membrane fractions were pre-incubated with Org25543 at a nominal concentration of 10 nM at 37 °C in a water bath for 1 h in a total volume of 24.85 mL. The solution and the water bath were stirred constantly with magnetic stir bars at a frequency of ~500 rpm. After 1 h of incubation dissociation was initiated by adding 150 µL of **8** resulting in 12 concentrations of 30 µM. After defined time points (15 s–3 h, twelve dissociation time points, samples for each time point were prepared in triplicates) dissociation was terminated via vacuum filtration and the samples were treated the same way as described in Experimental Section *MS Binding Assay: General procedure.* Non-specific binding was determined at the same time points as for the dissociation samples but incubating 10 nM Org25543 in the presence of 30 µM **8** and heat-shocked GlyT2 membrane preparations (triplicate).

Kinetic studies: Association experiments

Association experiments were performed in 25 mL round bottom flasks made of glass (Duran Wheaton Kimble Life Sciences GmbH, Wertheim, Germany). For determining association rate constants Org25543 were added to the incubation buffer containing GlyT2 membrane preparations which results in a final concentration of 10 nM Org25543 and in a total volume of 25 mL. The binding solution was incubated at 37 °C in a water bath and both, binding solution and water bath, are stirred with magnetic stir bars at a frequency of ~500 rpm. The incubation was terminated via vacuum filtration after defined time points (15 s–2 h, eleven association time points, samples for each time point were prepared in triplicates). The filtrated samples were treated the same way as described in Experimental Section *MS Binding Assay: General procedure.* Nonspecific binding at a marker concentration of 10 nM Org25543 was determined for the same time points as for the association samples but in the presence of heat-shocked GlyT2 membrane preparations (triplicates).

Data analysis

All results of the binding experiments (K_d, B_{max}, K_i, k_{off}, k_{obs}) 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. They were generated by means of linear regression analysis with a $1/x^2$ weighting in all cases. Based on the obtained calibration functions the bound marker concentrations were determined with Analyst v. 1.6.1 Software. Due to the dilution step of bound marker to 80% after elution the concentrations have to be back calculated for final evaluation to 100%. Specific binding was calculated as difference between total binding and nonspecific binding. Data from the binding experiment were analyzed by means of nonlinear regression analysis with Prism v. 6.07 (GraphPad Software). For saturation experiments the *One site-specific binding* nonlinear regression tool was used to obtain saturation isotherms with calculated K_d (equilibrium dissociation constants) and B_{max} (maximum density of binding sites) values. For competition experiments the *One Site-Fit* K_i nonlinear regression tool was used to obtain sigmoidal competition curves. The top level (total binding in absence of test compound) was set to 100% and the bottom level (nonspecific binding) was set to 0%. For competition curves with incomplete inhibition of Org25543 only the top level was set to

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100% and the bottom level was not constrained. The concentration, at which a test compound inhibited 50% of specific marker binding (IC_{50} value), was transferred into K_i values (inhibition constant of the test compound) using the Cheng-Prusoff equation. To obtain k_{off} (dissociation rate constants), k_{obs} (observed association rate constants) and dissociation $t_{1/2}$ (half-life) from kinetic experiments the nonlinear regression tools *Dissociation-One phase exponential decay* and *One-phase association* were used. Actual k_{on} (association rate constant) was calculated using following equation: $k_{on} = (k_{obs} - k_{off})/L$ (with L being the used Org25543 concentration in the association experiment in [M]).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: binding assay · glycine transporter 2 · liquid chromatography · mass spectrometry · neurotransmitter

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Supporting Information

MS Binding Assays for Glycine Transporter 2 (GlyT2) Employing Org25543 as Reporter Ligand

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METHODS

Investigating filter binding of Org25543 on 96-well glass fiber filter plates

The investigation of filter binding of Org25543 was performed analog to *MS Binding Assay - general procedure* described in **Materials and methods**, except no GlyT1 membrane preparations were used for incubation. At first, the filters of 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 µm, 350 µL; Pall Corporation, Port Washington, NY, USA) were washed with water (3 x 200 µL) and methanol (3 x 200 µL) and subsequently incubated for 2 h at room temperature with different aqueous preincubation solutions (*m*/*m*): 200 µL of 0.5 % polyethylenimine (PEI), 1 % polyvinylpyrrolidone (PVP), 1 % Tween20, 0.5 % PVP-0.2 % Tween20 and 0.5 % L-lysine were used as preincubation solutions (triplicates), as control filters without incubation and pretreated filters with water were used (triplicates). During the incubation of the filters 50 nM Org25543 were incubated for 1 h in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 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 shaking water bath. After incubation of filters and incubation samples, the preincubation solutions of the filters were removed by vacuum filtration and 210 µL aliquots of the incubation samples were transferred on the filters and were removed by vacuum filtration as well. The filters were washed with ammonium acetate buffer (3 x 200 µL, 154 mM, pH 7.4) and were dried at 50 °C for 1 h. After that the remaining Org25543 on the filters was washed down with acetonitrile (3 x 70 µL) and 52.5 µL ammonium bicarbonate buffer (5 mM, pH 7.8) was added to the samples to adjust the sample solvent to the mobile phase (5 mM ammonium bicarbonate buffer (pH 7.8)/acetonitrile (20:80, *v*/*v*)). Finally, the samples were subjected to LC-ESI-MS/MS quantification without further sample preparation. The peak areas of Org25543 were plotted against the different types of filter preincubations in a bar chart (Figure S3).

Investigating adsorption of Org25543 to polypropylene container material

To investigate the adsorption ability of Org25543 to polypropylene container material dilution series of Org25543 were prepared in reaction tubes (1.5 mL, PP, Sarstedt, Nümbrecht, Germany) using different solvents or solvent mixtures, respectively (H_2O, acetonitrile, H_2O/DMSO (80:20, *v/v*), H_2O/*N,N*-dimethylacetamide (DMA; 80:20, 90:10 and 95:5 *v/v*)). At least five concentration levels (10-fold concentration; 10 nM – 625 pM) were prepared in each solvent and these concentration levels were diluted again 1:10 (final concentrations of Org25543: 1 nM – 62.5 pM) in a mixture of ammonium bicarbonate buffer (5 mM, pH7.8) and acetonitrile containing 111 pM [$^2H_7$]Org25543 (final concentration: 100 pM) that the final sample solvent equals the mobile phase. Then the samples were subjected to LC-ESI-MS/MS quantification and the obtained peak area ratios of Org25543 and [$^2H_7$]Org25543 were plotted against the concentration of Org25543. By using linear regression analysis calibration curves were obtained for all dilution series and were compared to each other. The calibration curves are depicted in Figure S4.

Investigating the influence of *N,N*-dimethylacetamide (DMA) on the binding of Org25543 towards GlyT2 in competition experiments

This experiment was performed in the same way as it is described in the **Material and methods** part (see *4.13. Competition experiments)* except that Org25543 was stored in 100 % H_2O working solutions instead of 10 % aqueous DMA working solutions and that DMA was used as competitor in seven different concentrations (the concentration range of DMA goes from 6.0 % - 0.82 % which equals 647 mM – 888 µM (100 % DMA ≙ 10.79 M DMA)). The results are depicted in Figure S5.

Investigating the influence of 4-benzyloxy-3,5-dimethoxy-N-[1-[(dimethylaminocyclohexyl)methyl]benzamide (8) on the filter binding of Org25543

The investigation of the influence of **8** (see Figure 1) on the filter binding of Org25543 was performed analog to *MS Binding Assay - general procedure* described in **Materials and methods**, except no GlyT1 membrane preparations were used for incubation. At first, the filters of 96-well glass fiber filter plates (AcroPrep Advance,

glass fiber, 1.0 µm, 350 µL; Pall Corporation, Port Washington, NY, USA) were washed with water (3 x 200 µL) and methanol (3 x 200 µL) and subsequently pre-incubated for 2 h at room temperature with 0.5 % polyethylenimine (PEI). During the pre-incubation of the filters increasing concentrations of Org25543 (0.4 – 150 nM; triplicates) were incubated either in the absence of **8** or in the presence of three different concentrations of **8** (3, 30, 300 µM) for 1 h in incubation buffer (10 mM HEPES, 120 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.5) in polypropylene 96 well plates (1.2 mL well volume, Sarstedt, Nümbrecht, Germany) in total volume of 250 µL at 37 °C in a shaking water bath. After the incubation times for the filters and incubation samples had been expired, PEI was removed from the filters by vacuum filtration and 210 µL aliquots of the incubation samples were transferred on the filters and were removed by vacuum filtration as well. The filters were washed with ammonium acetate buffer (3 x 200 µL, 154 mM, pH 7.4) and were dried at 50 °C for 1 h. After that the remaining Org25543 on the filters were washed down with acetonitrile (3 x 70 µL) containing [$^2H_7$]Org25543 (125 pM) and 52.5 µL ammonium bicarbonate buffer (5 mM, pH 7.8) was added to the samples to adjust the sample solvent to the mobile phase (5 mM ammonium bicarbonate buffer (pH 7.8)/acetonitrile (20:80, *v*/*v*)). Finally, the samples were subjected to LC-ESI-MS/MS quantification without further sample preparation. The bound Org25543 on the filters (in [pM]) were plotted against the nominal concentration of Org25543 in the incubation samples (in [nM]) (Figure S6).

Investigating the inhibition mode of glycine in preliminary Org25543 saturation experiments

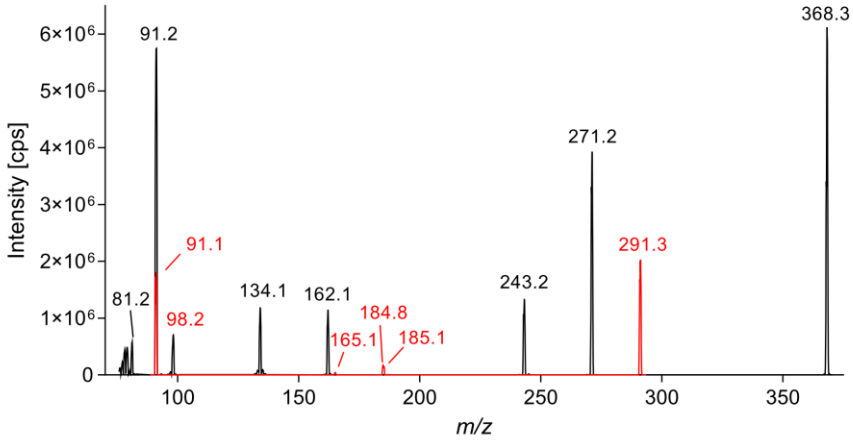
In these preliminary experiments saturation experiments were performed in the same way as it is described in the **Material and methods** part except that Org25543 and GlyT2 were incubated either in the absence of glycine or in the presence of 2, 20 or 200 mM glycine. The saturation isotherms of a representative experiment are depicted in Figure S7. K_d and B_{max} value determined without glycine were normalized to 100 % (control) in all experiments and K_d and B_{max} values determined in the presence of the three glycine concentrations were seen in relation to the control values (in [%]). The results are compiled in Table S2.

GlyT2 MS Binding Assay – Competition experiment using glycine, DL-proline and *N*-oleoylglycine as test compound

These competition experiments were performed in the same way as it is described in the **Material and methods** part. The results are depicted in Figure S8.

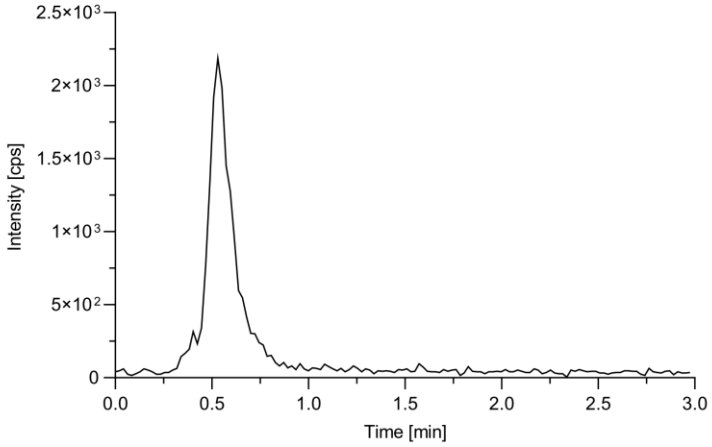
RESULTS

Figure S1. Product ion scans for the $[M+H]^+$ parent ions of ALX1393 and Org25543 with the most intensive product ions.



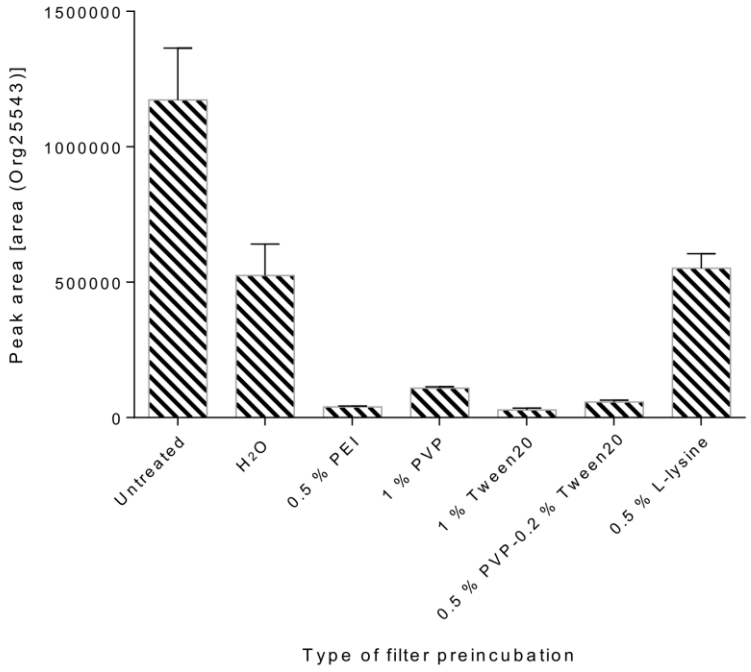
Depicted are the most intensive product ions of ALX1393 (m/z 396.1) in red and Org25543 (m/z 413.2) in black.

Figure S2. Representative LC-ESI-MS/MS chromatogram of ALX1393



MRM chromatogram of 100 pM ALX1393 (*m*/*z* 396.1/291.0) diluted in ammonium bicarbonate buffer (5 mM, pH 7.8)/acetonitrile (40:60, *v*/*v*). For LC a Luna 3µ C8(2) (50 mm x 2 mm, 3 µm) column was used as stationary phase in combination with a mobile phase consisting of ammonium bicarbonate buffer (5 mM, pH 7.8)/acetonitrile (40:60, *v*/*v*) at a flow rate of 600 µL min^{-1}. Injection volume was set to 45 µL and temperature to 25 °C. A capacity factor *k* of 0.35 was achieved for ALX1393.

Figure S3. Investigating filter binding of Org25543 on 96-well glass fiber filter plates



The results are given as mean ± standard deviation (n = 3). The preincubation solution with the smallest peak area shows to the lowest filter binding of Org25543. 0.5 % PEI and 1 % Tween20 were identified as the best solutions to reduce the filter binding of Org25543.

Figure S4. Investigating adsorption of Org25543 to polypropylene container material

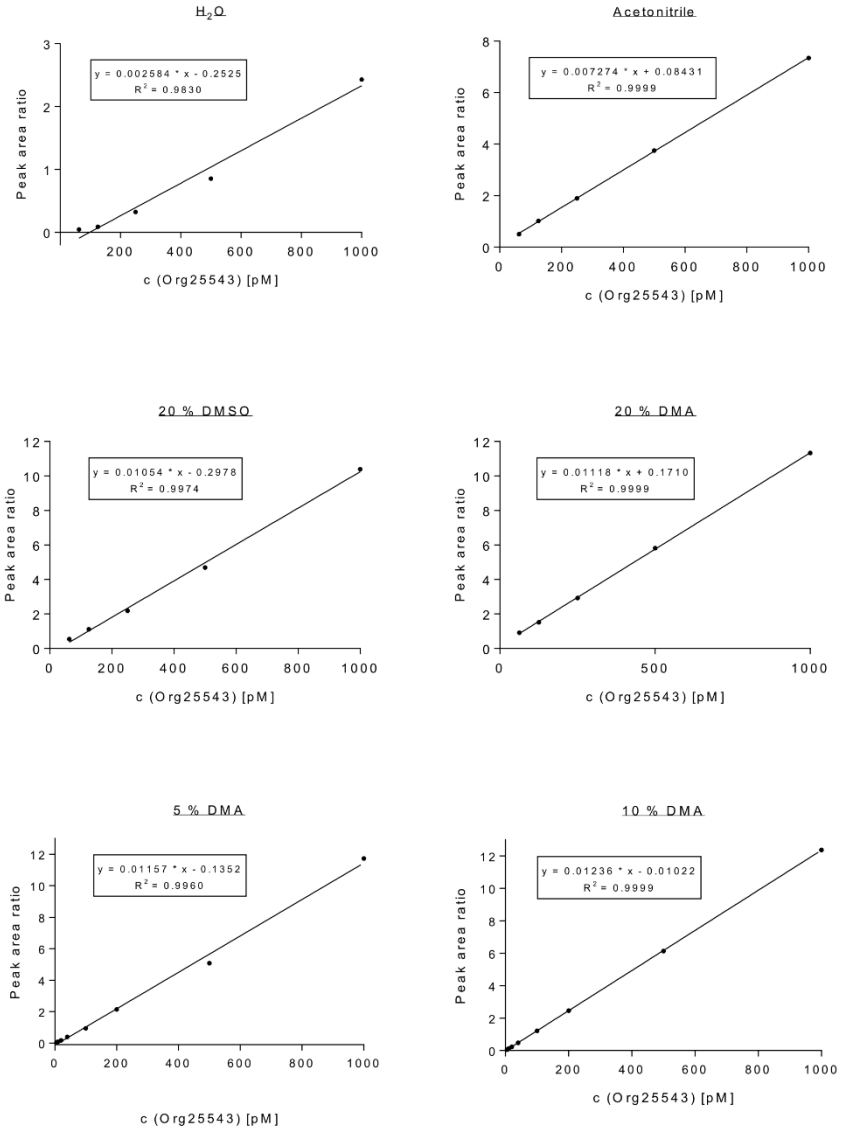
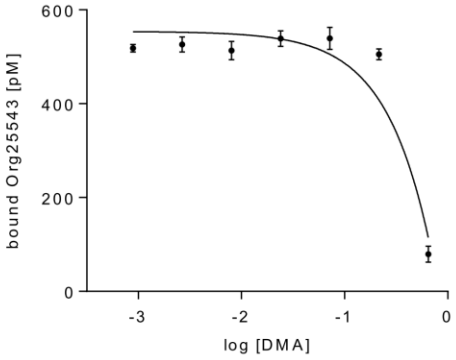


Figure S4 shows that the quality of calibration curves as well as the amount of measured Org25543 increases in the presence of an organic solvent. Since we were already experienced in our group with DMA as additive, we decided to continue with it.

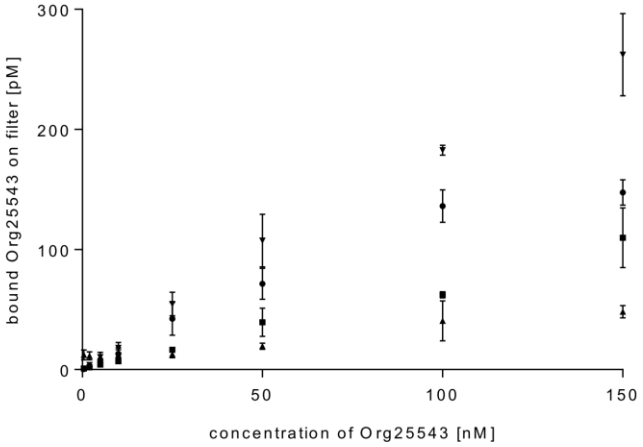
As it can be seen in the bottom row a concentration of 5 % DMA in water already improves the quantification of Org25543 compared to pure water dilutions and 10 % DMA in water is enough to obtain satisfying calibration curves, so following solutions of Org25543 were all prepared in 10 % aqueous DMA.

Figure S5. Investigating the influence of *N,N*-dimethylacetamide (DMA) on the binding of Org25543 towards GlyT2 in competition experiments



The results are given as mean ± standard deviation (n = 3). This competition experiment shows that DMA has no influence on the Org25543 binding at GlyT2 up to a concentration of 2 % (≙ 215 mM). Since the DMA concentration in binding samples was in all experiments 0.4 %, it should not have influenced the results of the performed experiments.

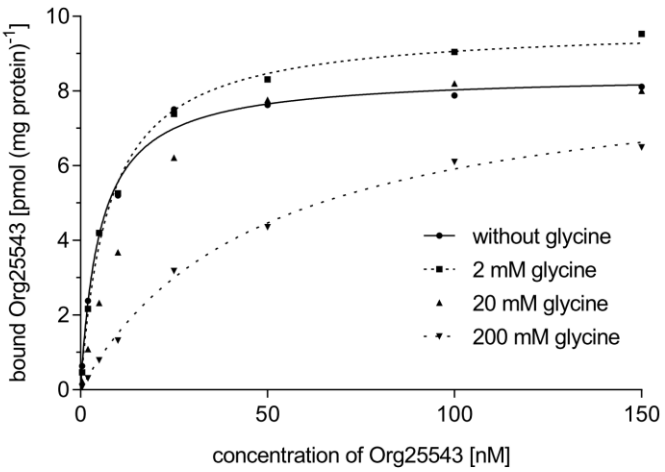
Figure S6. Investigating the influence of 4-benzyloxy-3,5-dimethoxy-N-[1-[(dimethylaminocyclohexyl)methyl]benzamide (8) on the filter binding of Org25543



The results are given as mean ± standard deviation (n = 3). Figure S6 shows the filter binding of Org25543 in the absence of **8** (▾) and in the presence of 3 µM (•), 30 µM (▪) and 300 µM (▴) of **8**. As it can be seen the filter binding of Org25543 decreases in the presence of increasing concentrations of **8**, whereas in the absence of **8** the concentration of filter bound Org25543 was the highest. As filter binding is a part of the non-specific binding determined in binding experiments, the decreased filter binding is problematic especially for high concentrations in saturation experiments, in which the determined non-specific binding is lower due to the presence of **8** than the non-specific binding that is included in the total binding due to the absence of **8**. This means that specific binding still contains a small percentage of non-specific binding after subtracting the determined non-specific binding from total binding. However, the lower the Org25543 concentration gets, the more negligible is this effect. In competition experiments, for example, in which Org25543 is used in a concentration of 10 nM, the concentration of bound reporter ligand (total binding) lies between 350 and 400 pM, whereas the difference between non-specific binding of Org25543 determined with heat-shocked GlyT2 membrane fragments and non-specific binding determined with **8** lies between 5-8 pM in all cases we compared them to each other. This difference accounts just for 1-2 % of total binding which has hardly any effect on the result of a

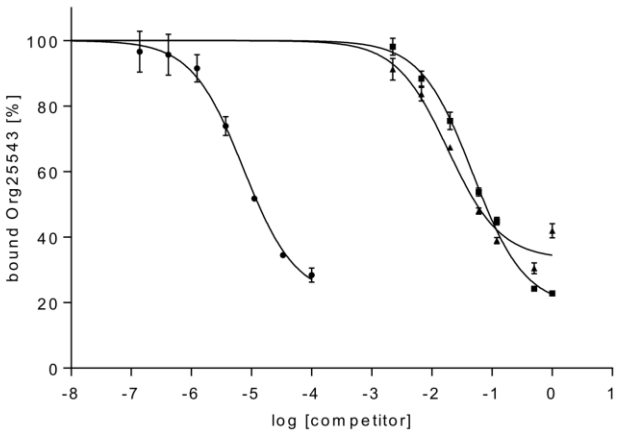
competition experiment. But especially for saturation experiments, we decided to determine the non-specific binding without **8** as competitor and used heat-shocked GlyT2 membrane fragments, so that Org25543 is not able to bind to the target protein any more due to denaturation of it.

Figure S7. Investigating the inhibition mode of glycine in preliminary Org25543 saturation experiments



Representative preliminary Org25543 saturation experiment in absence of glycine and presence of three different glycine concentrations (2, 20, 200 mM). Depicted is the specific binding of every incubation condition resulting in four saturation isotherms which were determined by means of non-linear regression analysis. Without glycine a K_d = 5.18 nM and a B_{max} = 8.45 pmol (mg protein)$^{-1}$, for the incubation in the presence of 2 mM glycine a K_d = 7.56 nM and a B_{max} = 9.75 pmol (mg protein)$^{-1}$, for the incubation in the presence of 20 mM glycine a K_d = 13.55 nM and a B_{max} = 9.24 pmol (mg protein)$^{-1}$ and for the incubation in the presence of 200 mM glycine a K_d = 47.84 nM and a B_{max} = 8.74 pmol (mg protein)$^{-1}$ were found. These results indicate increasing K_d values of Org25543 for GlyT2 when it is incubated with increasing concentrations of glycine whereas B_{max} stays nearly the same.

Figure S8. GlyT2 MS Binding Assay – Competition experiment using glycine, DL-proline and *N*-oleoylglycine as test compound



The results are given as mean ± standard deviation (n = 3). In all three competition curves it can be seen that the competitors glycine (▲), DL-proline (▪) and *N*-oleoylglycine (•) are not able to displace Org25543 completely from GlyT2 up to the highest concentration investigated, so the curves do not reach the non-specific binding level (0 %).

Table S1. Validation Results of LC-ESI-MS/MS method for Org25543

intra-batch accuracy and precision															
Samples (n)	**Series 1**			**Series 2**			**Series 3**			**Series 4**			**Series 5**		
	M	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**	**M**	**A**	**P**
Kal 5 pM (6)	5.054	101.1	1.7	5.078	101.6	3.3	5.102	102.0	2.6	4.929	98.6	3.5	5.027	100.5	5.8
Kal 10 pM (3)	9.789	97.9	1.0	9.594	95.9	4.0	9.421	94.2	0.5	10.62	106.2	4.3	10.54	105.4	4.1
Kal 20 pM (3)	19.69	98.5	1.5	19.48	97.4	2.1	19.40	97.0	1.8	19.98	99.9	1.1	21.30	106.5	2.0
Kal 40 pM (3)	38.78	96.9	1.0	39.01	97.5	0.6	38.65	96.6	3.3	39.71	99.3	0.7	42.64	106.6	2.2
Kal 100 pM (3)	99.20	99.2	0.5	99.13	99.1	0.5	97.60	97.6	1.4	99.70	99.7	2.2	105.2	105.2	2.5
Kal 200 pM (3)	197.9	99.0	0.5	194.8	97.4	1.9	204.9	102.4	1.1	195.5	97.7	1.6	211.1	105.5	1.8
Kal 500 pM (3)	511.5	102.3	0.9	521.2	104.2	0.9	523.2	104.6	0.4	500.6	100.1	1.3	532.7	106.5	3.0
Kal 1000 pM (3)	1043	104.3	1.6	1055	105.5	1.3	1035	103.5	1.3	1001	100.1	0.8	1086	108.6	1.6
Equation of calibration*	y = 1.212 x + -0.00898 (r = 0.9990)			y = 1.150 x + -0.00333 (r = 0.9976)			y = 1.153 x + -0.00196 (r = 0.9975)			y = 1.218 x + 0.00562 (r = 0.9983)			y = 1.304 x + 0.00192 (r = 0.9983)		
QC 15 pM (6)	15.74	107.3	2.4	15.41	102.7	3.7	16.32	108.8	1.6	14.45	96.4	3.7	15.23	101.6	5.3
QC 300 pM (6)	304.0	104.4	0.9	306.4	102.1	0.9	323.4	107.8	0.7	300.7	100.2	1.2	324.1	108.0	0.9
QC 800 pM (6)	820.0	110.0	1.2	830.7	103.8	0.9	868.9	108.6	0.7	770.3	96.3	0.7	813.4	101.7	1.2

inter-batch accuracy and precision			
	M	**A**	**P**
LLOQ 5 pM	5.038	100.8	3.6
QC 15 pM	15.43	102.9	5.2
QC 300 pM	311.7	103.9	3.4
QC 800 pM	820.7	102.6	4.0

M Mean [pM]; **A** Accuracy [%]; **P** Precision [%]; * Weighting factor: $1/x^2$

Table S2. Investigating the inhibition mode of glycine in preliminary Org25543 saturation experiments

	Incubation conditions		
Parameters	**2 mM glycine**	**20 mM glycine**	**200 mM glycine**
K_d[a] **[%]**	129 ± 24	222 ± 56	814 ± 154
B_{max}[a] **[%]**	106 ± 13	101 ± 12	---[b]

[a]K_d and B_{max} are seen in relation to the control values determined without glycine which were normalized to 100 %. [b]B_{max} was not included due to Org25543 concentrations which are not high enough to determine reliable results. All results are presented as mean ± SD from two independently performed experiments.

3.3 Dritte Publikation

Screening for new Inhibitors of Glycine Transporter 1 and 2 by means of MS Binding Assays

3.3.1 Zusammenfassung der Ergebnisse

Der Zweck der in der Publikation beschriebenen Studie war es, mittels der neu entwickelten MS-Bindungsassays für GlyT1 und GlyT2 neue Inhibitoren ebendieser Transporter mit möglichst hohen Affinitäten, die sich zumindest im niedrig mikromolaren bis sub-mikromolaren Bereich bewegen sollten, zu identifizieren. Für die entsprechenden Screeningversuche sollte hierfür eine 2439 Verbindungen umfassende arbeitsgruppeninterne Substanz-Bibliothek verwendet werden. Der Großteil dieser Verbindungen war in unserer Arbeitsgruppe als potenzielle Inhibitoren für verschiedene Neurotransmitter-Transporter der SLC6-Transporterfamilie synthetisiert worden. Zudem enthält diese Bibliothek eine Reihe einschlägiger Synthesezwischen- und nebenprodukte. Die in dieser Substanz-Bibliothek enthaltenen α-Aminosäuren (insgesamt 170 Verbindungen) wurden von den übrigen Verbindungen separat betrachtet. Dies geschah aus der Überlegung heraus, dass bei diesen Verbindungen, aufgrund ihrer Ähnlichkeit zum natürlichen Substrat Glycin, die Hit-Rate besonders hoch sein könnte. Die restlichen 2269 Verbindungen bildeten eine eigene Gruppe und wurden in Unterbibliotheken aus jeweils 16 Verbindungen zusammengefasst (insgesamt 142 Unterbibliotheken).

In Screening-Experimenten wurden die Einzelsubstanzen bzw. Unterbibliotheken zunächst in Kompetitionsexperimenten bei einer definierten Konzentration, nämlich 10 µM, untersucht. Wenn eine Einzelsubstanz oder Unterbibliothek die Reporterligandbindung unter ein bestimmtes Limit reduzierte (50 % Reporterligandbindung an GlyT1 und GlyT2), wurde diese erneut, aber nun bei 1,0 µM gescreent. Als Auswahlkriterium für die weitere Untersuchung gilt auch hier das Erreichen der 50%-Grenze. Einzelsubstanzen, soweit sie das Auswahlkriterium erfüllten, konnten direkt als Hit-Verbindungen identifiziert werden. Die Unterbibliotheken, welche das Selektionskriterium erfüllt hatten, mussten hingegen noch einer Dekonvolution unterzogen werden, um die Verbindungen herauszufiltern, die für die Reduktion der Reporterligandbindung maßgeblich verantwortlich waren. Bei den Dekonvolutionsexperimenten wurde analog zu den Screeningexperimenten bei

den Unterbibliotheken vorgegangen, nur dass in diesem Fall die einzelnen Verbindungen zum Einsatz kamen. Die identifizierten Hit-Verbindungen wurden schließlich in Kompetitionsexperimenten vollständig hinsichtlich ihrer Affinität für den jeweiligen Transporter charakterisiert.

Für GlyT1 konnten die Verbindungen **48**, **SC121 - d**, **SC121 - f**, **SC121 - n** und **SC140 - a** als Inhibitoren identifiziert werden, die dem gesuchten Affinitätsprofil, also mindestens eine Affinität bezüglich des Targets im niedrig mikromolaren Bereich aufweisen, entsprechen (Strukturformeln sind in Abbildung 2 im Abschnitt 3.3.3 des Publikationsmanuskripts zu finden). Die zwei potentesten Verbindungen, nämlich **48** und **SC121 - n** wiesen deutlich höhere Affinitäten als die übrigen Hit-Verbindungen auf. Deshalb wurden nur diese in Kompetitionsexperimenten hinsichtlich ihrer Affinität charakterisiert. Dabei konnten pK_i-Werte von 7,00 ± 0,01 für **48** und 6,85 ± 0,03 für **SC121 - n** ermittelt werden. Beide Verbindungen waren ursprünglich als potenzielle GABA-Transporter-Inhibitoren entwickelt worden.[99,100] Besonders **SC121 - n** besitzt eine für GlyT1-Inhibitoren so noch nicht bekannte Struktur mit einer Allenuntereinheit. *N*-Substituierte Proline, zu welchen Verbindung **48** ebenfalls zählt, sind bereits bekannt als GlyT1 Inhibitoren,[101] allerdings wurden diese bisher nicht in Kombination mit einem Trityl-Rest beschrieben, weshalb **48** einen durchaus interessanten Anhaltspunkt für die Entwicklung weiterer GlyT1-Inhibitoren darstellt.

Für GlyT2 wurden die Verbindungen **SC70 - o**, **SC70 - p**, **SC71 - a**, **SC71 - f**, **SC71 - m** und **SC107 - f** mit pK_i-Werten von 5,42 ± 0,01, 6,40 ± 0,07, 6,18 ± 0,05, 5,94 ± 0,08, 5,46 ± 0,03 und 6,67 ± 0,03 identifiziert (Strukturformeln sind in Abbildung 4 im Abschnitt 3.3.3 des Publikationsmanuskripts zu finden). Bei den Verbindungen **SC70 - o**, **SC70 - p**, **SC71 - a**, **SC71 - f** und **SC71 - m** handelt es sich ebenfalls um Substanzen, die als potenzielle GABA-Transporter-Inhibitoren entwickelt worden waren.[102] Das Besondere an den Strukturen dieser fünf Verbindungen ist deren 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacen-Rest (BODIPY-Rest), der hervorragende Fluoreszenzeigenschaften aufweist. Dadurch könnten diese Substanzen als mögliche Liganden in Fluoreszenz-basierten Bio-Assays eingesetzt werden. Da bisher keine Fluoreszenzmarker für diesen Transporter beschrieben wurden, könnten diese Verbindungen ein großes Interesse im Bereich der zukünftigen GlyT2-Forschung erfahren. Substanz **SC107 - f** stellte die potenteste Verbindung der im GlyT2-Screening identifizierten Verbindungen dar. Bei ihr handelt es sich um einen bereits

bekannten GlyT2-Inhibitor, der aufgrund seiner 4-(Benzyloxy)benzamid Partialstruktur strukturelle Ähnlichkeit zum verwendeten Reporterliganden Org25543 aufweist.[15]

Mit den MS-Bindungsassays für GlyT1 und GlyT2 konnte somit das Ziel, neue Inhibitoren für GlyT1 und GlyT2 mit Affinitäten im niedrig mikromolaren bis sub-mikromolaren Bereich zu identifizieren, erreicht werden. Zusätzlich konnte gezeigt werden, dass MS-Bindungsassays einfach durchführbar sind und einen hohen Durchsatz erlauben und so für die Untersuchung von Substanzbibliotheken von mittlerer Größe sehr gut geeignet sind.

3.3.2 Erklärung zum Eigenanteil

Sowohl für GlyT1 als auch GlyT2 wurden die Screeningexperimente, deren Auswertung sowie die nachfolgenden Kompetitionsexperimente mit den identifizierten Hit-Verbindungen von mir durchgeführt. Das Manuskript wurde ebenfalls von mir mit Unterstützung von Dr. Georg Höfner verfasst und von Prof. Dr. Klaus T. Wanner korrigiert.

3.3.3 Manuskript der dritten Publikation

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European Chemical Societies Publishing

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 (*Ki*) 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).

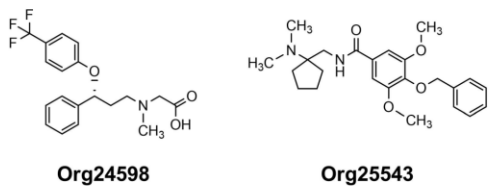

Figure 1. Structures of Org24598 and Org25543 used as reporter ligands in the GlyT1 and GlyT2 MS Binding Assays, respectively.

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

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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.

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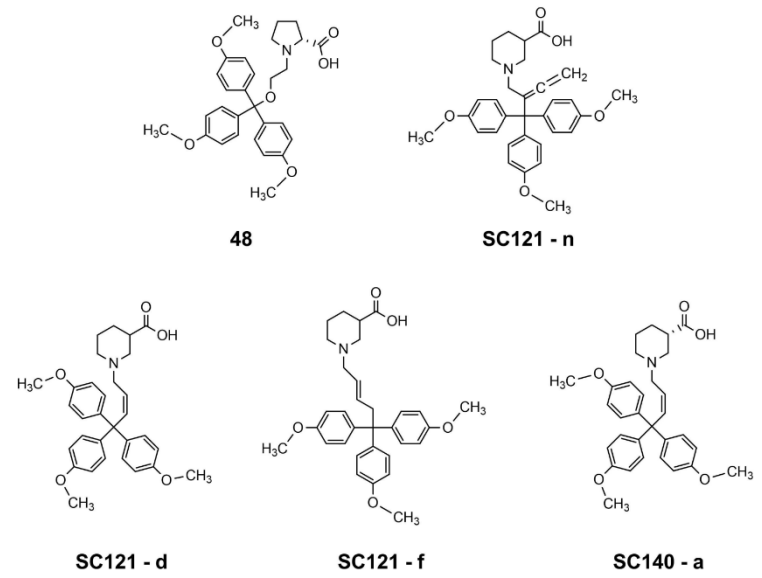

Figure 2. Hit compounds of the GlyT1 MS Binding Assay screening

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.

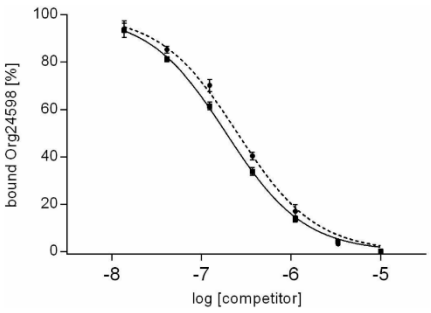

Figure 3. Representative inhibition curves of α-amino acid **48** (•, solid line) and compound **SC121–n** (●, dashed line). Data represent remaining specific binding of Org24598 to GlyT1 in the presence of increasing concentrations of competitor and are given as mean ± SD of triplicates. Resulting IC_{50} 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

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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

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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

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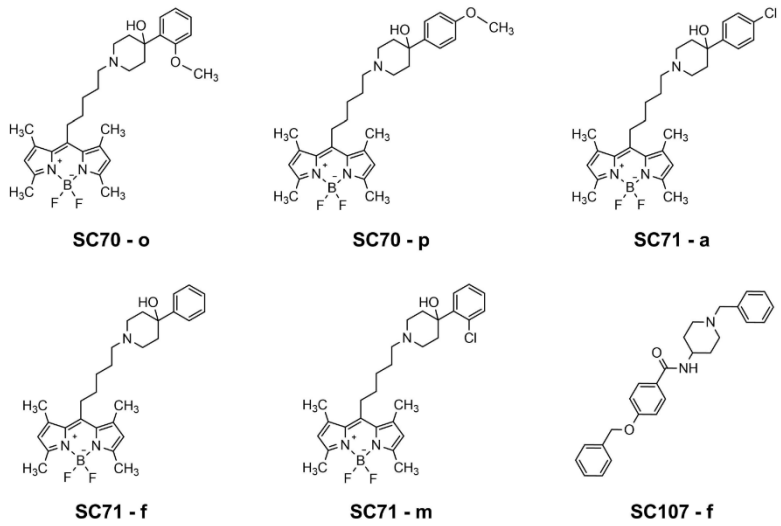

Figure 4. Hit compounds of the GlyT2 MS Binding Assay screening

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.

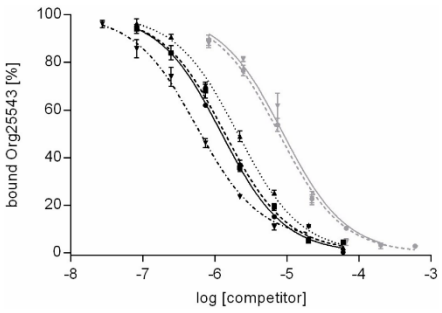

Figure 5. Representative inhibition curves of compounds **SC70–o** (▼, solid line), **SC70–p** (●, solid line), **SC71–a** (▪, dashed line), **SC71–f** (▲, dotted line), **SC71–m** (●, dashed line) and **SC107–f** (▼, dash-dotted line). Data represent remaining specific binding of Org25543 to GlyT2 in 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

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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)

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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

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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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Supporting Information

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

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Supporting Information

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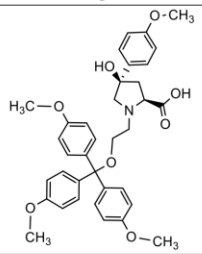
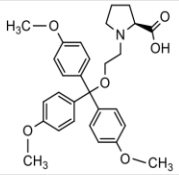
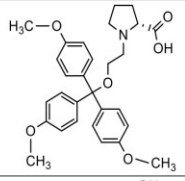
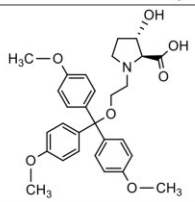
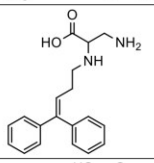
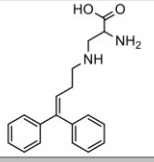
Results:

- Table S1

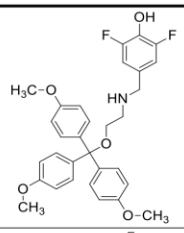
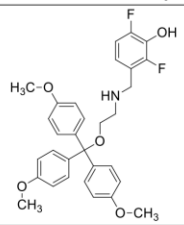
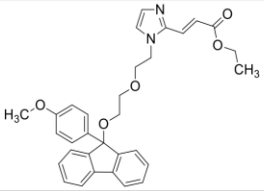
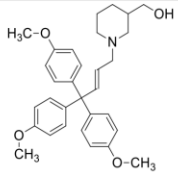
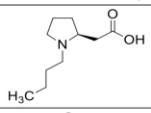
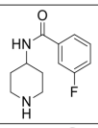
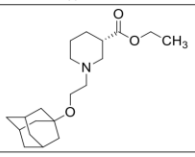
Table S1. GlyT1 MS Binding Assay screening results – Chemical structures and screening results of α-amino acids 1, 2, 30, 47, 48, 131, 149 and 165 at 10 µM and 1 µM test concentration

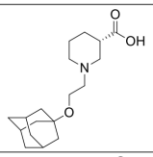
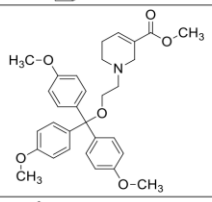
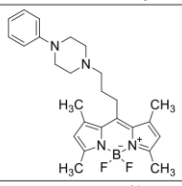
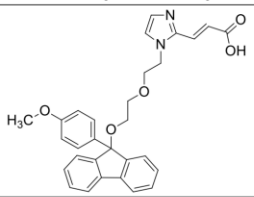
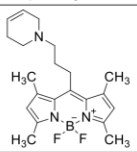
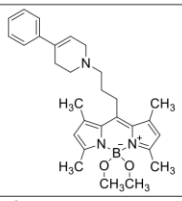
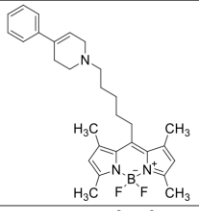
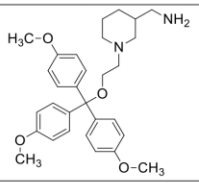
Compound	Structure*	Remaining specific binding of Org24598 at **10 µM** [%]	Remaining specific binding of Org24598 at **1 µM** [%]
1		27.8 ± 4.2	---
2		43.7 ± 14.7	---
30		42.0 ± 4.3	---
47		7.0 ± 1.0	57.0 ± 6.2

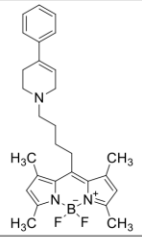
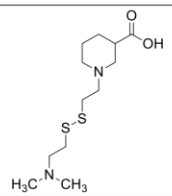
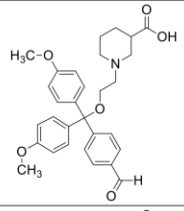
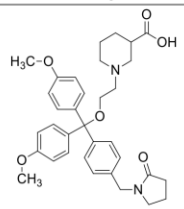
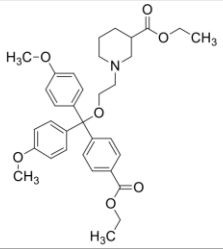
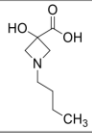
48		2.7 ± 0.8	18.2 ± 1.6
131		28.3 ± 0.6	---
149		45.3 ± 3.6	---
165		39.0 ± 9.8	---
* Chiral compounds without specification of their stereochemistry represent racemates.			

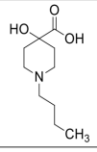
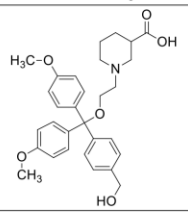
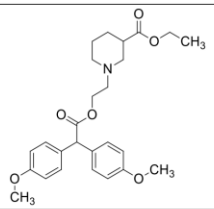
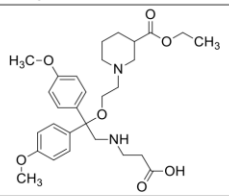
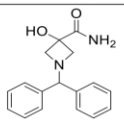
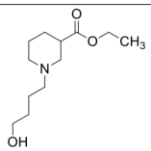
- Table S2

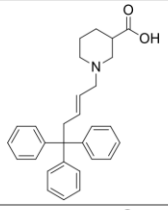
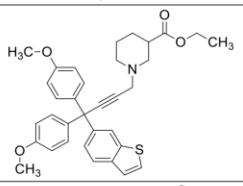
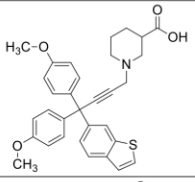
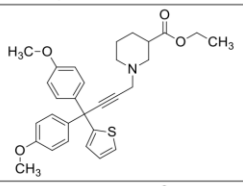
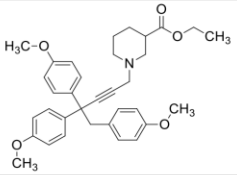
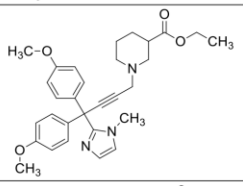
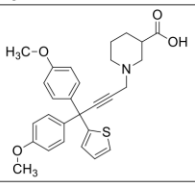
Table S2. GlyT1 MS Binding Assay deconvolution experiments – Chemical structures and screening results of the constituents of sublibraries 73, 116, 120, 121 and 140 at 1 µM test concentration

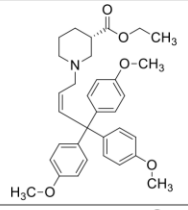
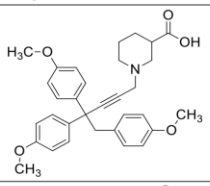
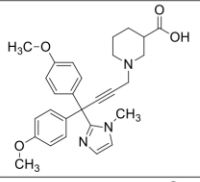
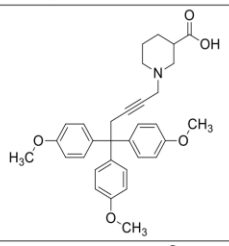
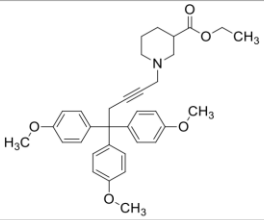
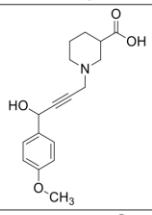
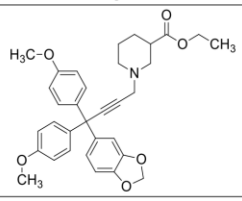
Compound	Structure*	Remaining specific binding of Org24598 at **1 µM** [%]
SC73 - a		96.7 ± 3.5
SC73 - b		104.1 ± 2.3
SC73 - c		85.3 ± 10.7
SC73 - d		83.8 ± 9.2
SC73 - e		101.5 ± 4.7
SC73 - f		105.4 ± 7.7
SC73 - g		107.7 ± 2.5

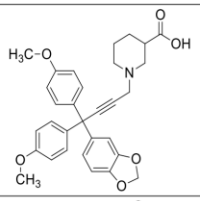
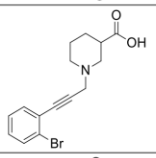
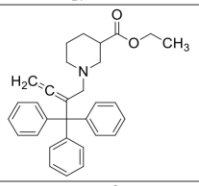
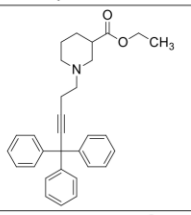
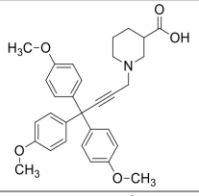
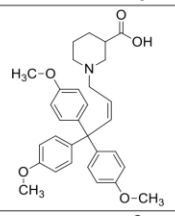
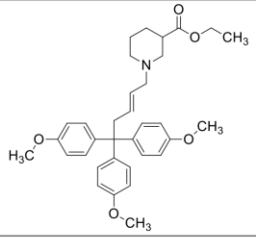
SC73 - h		101.5 ± 4.5
SC73 - i		76.0 ± 5.1
SC73 - j		105.1 ± 3.4
SC73 - k		99.2 ± 3.9
SC73 - l		96.4 ± 7.4
SC73 - m		84.0 ± 0.5
SC73 - n		84.8 ± 7.3
SC73 - o		78.9 ± 2.8

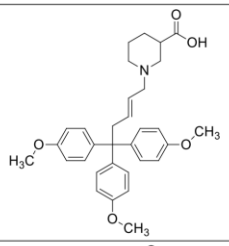
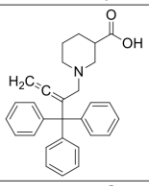
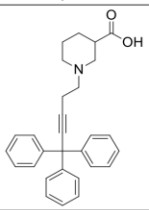
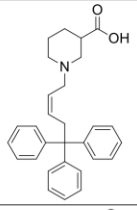
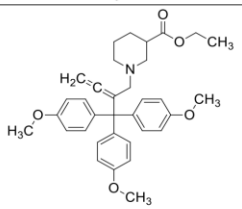
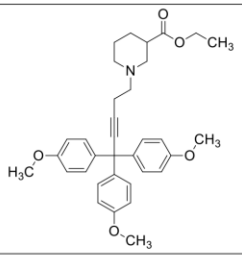
SC73 - p		82.7 ± 2.7
SC116 - a	Structure confidential	79.4 ± 4.4
SC116 - b		85.1 ± 3.4
SC116 - c		73.5 ± 2.1
SC116 - d		57.0 ± 2.5
SC116 - e	Structure confidential	89.9 ± 7.6
SC116 - f		91.8 ± 5.1
SC116 - g		81.7 ± 5.2

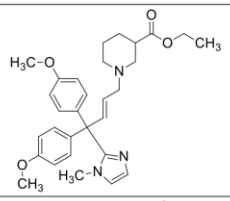
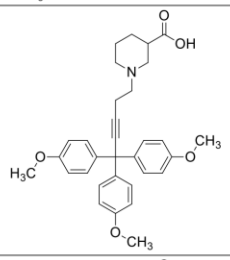
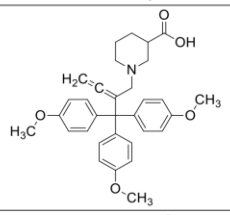
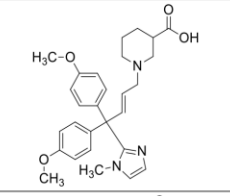
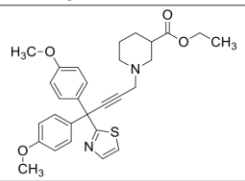
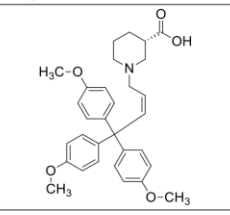
SC116 - h		86.4 ± 8.8
SC116 - i		78.1 ± 3.1
SC116 - j	Structure confidential	88.6 ± 7.3
SC116 - k		80.9 ± 10.8
SC116 - l		86.1 ± 2.9
SC116 - m		97.7 ± 6.6
SC116 - n	Structure confidential	91.5 ± 3.9
SC116 - o		90.5 ± 9.9
SC116 - p	Structure confidential	85.3 ± 2.0

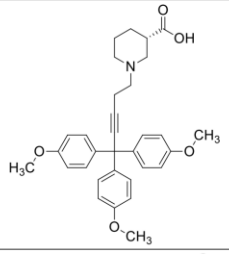
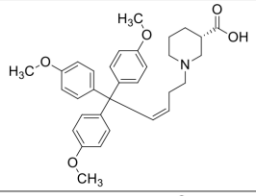
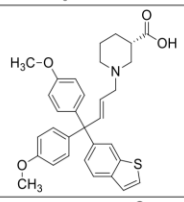
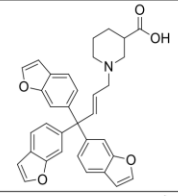
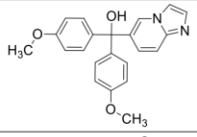
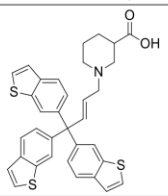
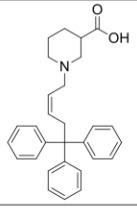
SC120 - a		96.1 ± 11.8
SC120 - b		90.2 ± 16.3
SC120 - c		81.2 ± 1.4
SC120 - d		89.4 ± 5.5
SC120 - e		91.5 ± 9.1
SC120 - f		89.7 ± 7.9
SC120 - g		78.9 ± 9.4

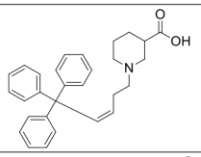
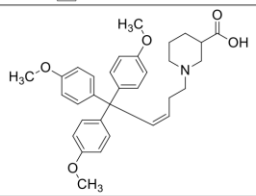
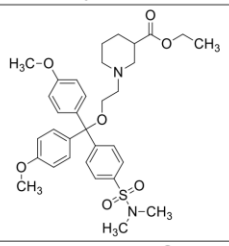
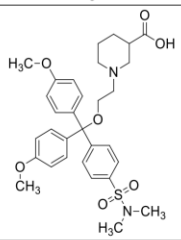
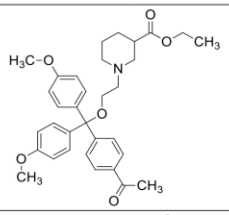
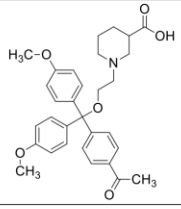
SC120 - h		85.8 ± 6.3
SC120 - i		92.8 ± 2.7
SC120 - j		86.6 ± 4.7
SC120 - k		59.5 ± 11.6
SC120 - l		87.9 ± 7.2
SC120 - m		89.7 ± 7.9
SC120 - n		96.9 ± 0.9

SC120 - o		88.9 ± 3.6
SC120 - p		92.0 ± 2.8
SC121 - a		100.5 ± 5.0
SC121 - b		94.1 ± 10.4
SC121 - c		77.2 ± 3.6
SC121 - d		42.1 ± 3.1
SC121 - e		71.4 ± 1.4

SC121 - f		49.8 ± 6.5
SC121 - g		93.5 ± 5.9
SC121 - h		100.2 ± 0.0
SC121 - i		92.0 ± 14.1
SC121 - j		78.0 ± 3.5
SC121 - k		85.7 ± 6.3

SC121 - l		104.4 ± 6.0
SC121 - m		98.4 ± 4.7
SC121 - n		22.4 ± 2.3
SC121 - o		104.4 ± 5.1
SC121 - p		103.2 ± 10.0
SC140 - a		38.9 ± 1.0

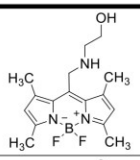
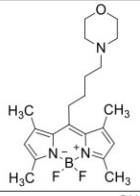
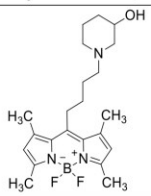
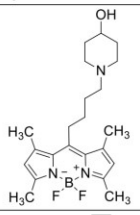
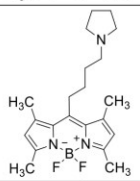
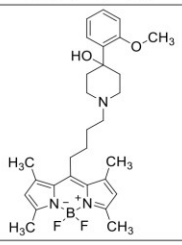
SC140 - b		89.9 ± 5.5
SC140 - c		78.4 ± 3.4
SC140 - d		98.0 ± 3.2
SC140 - e		85.7 ± 3.6
SC140 - f		98.0 ± 5.5
SC140 - g		82.5 ± 4.8
SC140 - h		104.1 ± 5.5

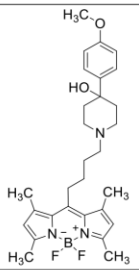
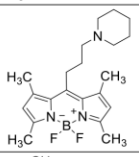
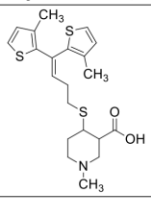
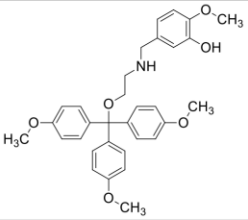
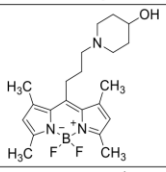
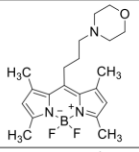
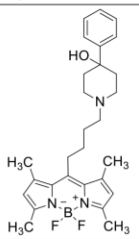
SC140 - i		100.7 ± 5.0
SC140 - j		66.9 ± 2.8
SC140 - k		100.4 ± 6.1
SC140 - l		92.6 ± 12.8
SC140 - m		91.1 ± 8.7
SC140 - n		81.6 ± 5.1
SC140 - o	Structure confidential	90.8 ± 3.7
SC140 - p	Structure confidential	92.0 ± 3.2

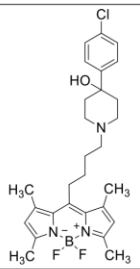
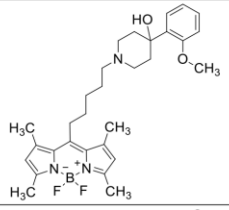
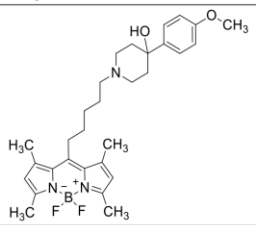
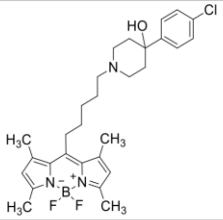
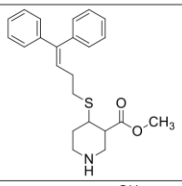
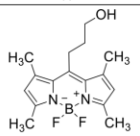
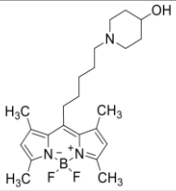
* Chiral compounds without specification of their stereochemistry represent racemates.

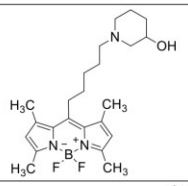
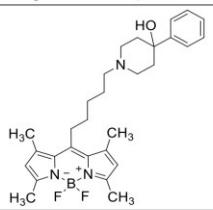
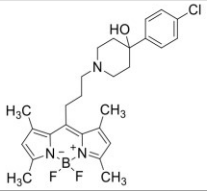
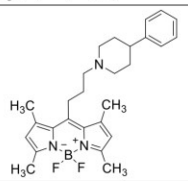
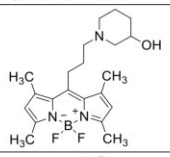
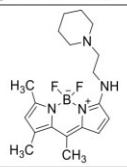
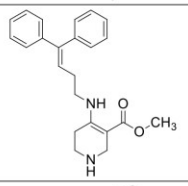
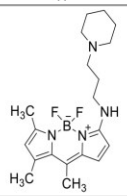
- Table S3

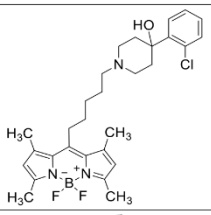
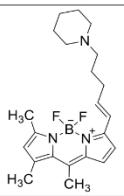
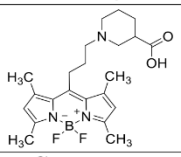
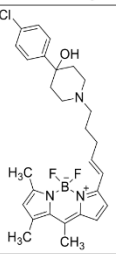
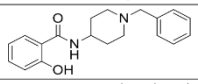
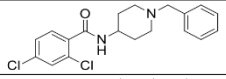
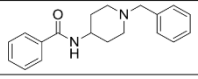
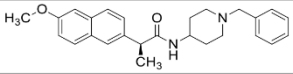
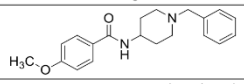
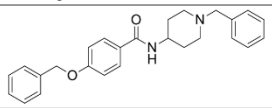
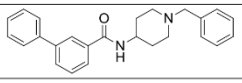
Table S3. GlyT2 MS Binding Assay deconvolution experiments – Chemical structures and screening results of the constituents of sublibraries 70, 71 and 107 at 10 µM test concentration

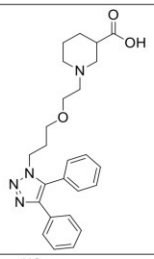
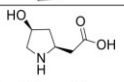
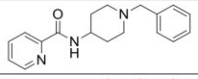
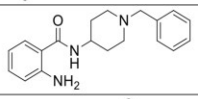
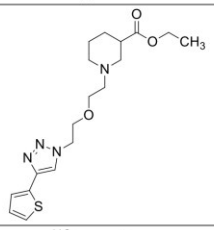
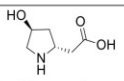
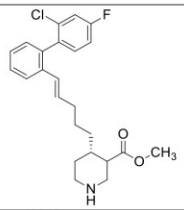
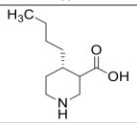
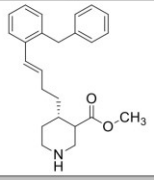
Compound	Structure*	Remaining specific binding of Org25543 at **10 µM** [%]
SC70 - a		91.4 ± 4.3
SC70 - b		88.9 ± 2.1
SC70 - c		85.7 ± 4.6
SC70 - d		83.8 ± 1.8
SC70 - e		92.3 ± 6.4
SC70 - f		69.6 ± 6.1

SC70 - g		63.8 ± 4.9
SC70 - h		89.5 ± 8.2
SC70 - i		109.8 ± 5.9
SC70 - j		78.9 ± 7.4
SC70 - k		89.7 ± 2.8
SC70 - l		96.9 ± 4.1
SC70 - m		64.7 ± 1.4

SC70 - n		95.5 ± 3.8
SC70 - o		48.9 ± 5.5
SC70 - p		10.4 ± 0.4
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
SC107 - a		77.7 ± 1.4
SC107 - b		83.4 ± 4.5
SC107 - c		72.7 ± 14.9
SC107 - d		94.2 ± 3.7
SC107 - e		94.3 ± 5.3
SC107 - f		23.8 ± 1.3
SC107 - g		92.2 ± 3.5

SC107 - h		98.9 ± 3.0
SC107 - i	 And Enantiomer	96.3 ± 4.2
SC107 - j		93.5 ± 1.8
SC107 - k		94.9 ± 3.5
SC107 - l		105.1 ± 6.8
SC107 - m	 And Enantiomer	104.0 ± 6.5
SC107 - n		71.2 ± 2.1
SC107 - o		99.1 ± 6.6
SC107 - p		111.5 ± 2.0
* Chiral compounds without specification of their stereochemistry represent racemates.		

4 Zusammenfassung der Dissertation

Wissenschaftliche Studien der letzten Jahre haben gezeigt, dass die Glycin-Transporter GlyT1 und GlyT2 der SLC6-Transporterfamilie potenzielle Zielproteine zur Behandlung von Krankheiten darstellen, für die es bislang keine oder nur unzureichende Therapiemöglichkeiten gibt, wie zum Beispiel zur Therapie von negativen Symptomen einer Schizophrenieerkrankung, Drogenabhängigkeiten (z.B. Alkoholismus) oder neuropathisch chronischen Schmerzen. Dabei steht die Inhibition der beiden Transporter durch den Einsatz von niedermolekularen Verbindungen (engl.: small molecules) im Fokus, wodurch eine gesteigerte Glycin-Konzentration im Bereich von glycinergen bzw. glutamatergen Neuronen mit einem günstigen therapeutischen Effekt bewirkt werden kann. Daher bedarf es allerdings der Identifizierung von potenten und, wenn möglich, selektiven Inhibitoren, wofür es unterschiedliche Möglichkeiten im Bereich der Bio-Assays gibt. In den letzten Jahren hat sich mit den MS-Bindungsassays eine durchaus erfolgsversprechende Alternative zu den Radioligand-Bindungsassays ergeben, die genau zuvor Erwähntes, durch eine einfache und schnelle Identifizierung neuer Inhibitoren in Screening-Verfahren sowie die Bestimmung der Affinitäten an den Zielproteinen, ermöglichen.

Deshalb war es das Ziel dieser Arbeit zunächst MS-Bindungsassays für die Transporter GlyT1 und GlyT2 zu entwickeln und mit diesen als weitere Validierungsmaßnahme die Bindungsaffinitäten von bereits bekannten GlyT-Inhibitoren zu bestimmen. Anschließend sollten die entwickelten MS-Bindungsassays dazu eingesetzt werden, um in einer aus knapp 2400 Verbindungen bestehenden Substanzbibliothek neue potente Inhibitoren für GlyT1 und GlyT2 zu identifizieren und hinsichtlich ihrer Bindungsaffinität zu charakterisieren.

Für die Entwicklung der MS-Bindungsassays war es zunächst notwendig geeignete Reporterliganden auszuwählen. Diese sollten idealerweise eine Affinität im niedrig nanomolaren Bereich aufweisen und für die Quantifizierung mittels LC-ESI-MS/MS geeignet sein. Der selektive GlyT1-Inhibitor Org24598 als auch der selektive GlyT2-Inhibitor Org25543 stellten sich in Vorversuchen als geeignete Reporterliganden für den jeweiligen Transporter heraus.

Um diese Reporterliganden quantifizieren zu können, war es nötig für sie jeweils eine geeignete LC-ESI-MS/MS-Methode zu entwickeln. Für die Detektion dieser Marker

mittels MS mussten zuallererst deren Massenübergänge im MS bestimmt und die für eine Quantifizierung am besten geeigneten ermittelt werden. Für die massenspektrometrische Anwendung wurde ein AB Sciex 5000 Triple Quadrupol Massenspektrometer mit Elektrospray-Ionisations-Einheit (ESI) eingesetzt. Die Quantifizierung der Reporterliganden erfolgte schließlich im multiple reaction monitioring (MRM)-Modus, der durch als besonders geeignet identifizierte Massenübergänge Bestimmungsgrenzen im erforderlichen niedrig picomolaren Bereich ermöglichte. Anschließend wurden sowohl für Org24598 als auch für Org25543 geeignete HPLC-Methoden entwickelt, die auf reversed-phase- (RP) Bedingungen basierten. Für beide Methoden wurde eine stationäre Phase bestehend aus einem C8-Säulenmaterial eingesetzt sowie eine mobile Phase bestehend aus Acetonitril und 5 mM Ammoniumhydrogencarbonat-Puffer pH 7.8 jedoch in unterschiedlichen Lösungsmittelverhältnissen (45:55 für GlyT1 vs. 80:20 für GlyT2). Diese HPLC-Methoden ermöglichten es, die Marker effizient von Matrixbestandteilen abzutrennen, wodurch ungewollte Matrixeffekte vermieden werden konnten. Sie lieferten zusätzlich kurze Laufzeiten (jeweils 2,0 min), die einen hohen Durchsatz zuließen. Da LC-MS-Methoden oftmals für Matrixeffekte anfällig sind oder unterschiedliche Ionisationseffizienzen zeigen, wurde die Quantifizierung auf Basis von Isotopen-markierten Reporterliganden als internen Standards durchgeführt. Als solche wurden für den GlyT1-MS-Bindungsassay [$^2H_5$]Org24461, das deuterierte Racemat von Org24598, und für den GlyT2-MS-Bindungsassay [$^2H_7$]Org25543 eingesetzt.

Nachdem in Vorversuchen der Ablauf der beiden MS-Bindungsassays optimiert worden war (z.B. Adsorptionseffekte von Org25543 oder Bestimmung der nicht-spezifischen Bindung im GlyT2 MS Bindungsassay) und somit die Bedingungen der jeweiligen MS Bindungsassays für GlyT1 und GlyT2 festgelegt werden konnten, wurden schließlich beide Methoden erfolgreich in Anlehnung an die CDER Guideline for bioanalytical method validation der FDA hinsichtlich Spezifität, Richtigkeit, Präzision, Linearität und lower limit of quantification (LLOQ) validiert.

Mit den validierten GlyT1- und GlyT2-MS-Bindungsassays konnten die Bindungsaffinitäten von Org25498 und Org25543 in Sättigungsexperimenten bestimmt werden, die vergleichbar mit bereits publizierten Werten sind [K_d (Org25498) = 16,8 ± 2,2 nM; K_d (Org25543) = 7,45 ± 0,55 nM]. Zusätzlich war es möglich die on- und off-

Kinetik von Org25543 an GlyT2 zu ermitteln, welche einen berechneten K_d-Wert lieferte, der sehr gut mit dem in Sättigungsexperimenten bestimmten experimentellen Wert übereinstimmt (k_{off} = 7,07 ± 0,26 · 10^{-3} s^{-1}; k_{on} = 1,01 · 10^6 M^{-1} s^{-1}; K_d = 6,99 nM). Die Ergebnisse aus Kompetitionsexperimenten sowohl für den GlyT1- als auch den GlyT2-MS-Bindungsassay zeigten für die untersuchten Kompetitoren durchweg gute bis sehr gute Übereinstimmungen mit Werten aus der Literatur. Allerdings konnten auch einzelne Abweichungen zu Literaturwerten festgestellt werden, vor allem im Rahmen der GlyT2-Experimente, die womöglich auf die grundsätzlichen Unterschiede zwischen Bindungsassays, hier dem GlyT2-MS-Bindungsassay, und funktionellen Assays – wie die für GlyT2 in der Literatur beschriebenen – zurückgehen. Alles in allem konnte aber gezeigt werden, dass die entwickelten MS-Bindungsassays zuverlässige Ergebnisse liefern und als Alternativen zu bereits bestehenden Bio-Assays bestens geeignet sind.

Mit Hilfe der beiden MS-Bindungsassays wurde schließlich eine Substanzbibliothek mit 2439 Verbindungen gescreent, um neue Inhibitoren zu identifizieren, deren Affinität möglichst hoch sein sollte, d.h. mit pK_i-Werten im niedrig mikromolaren bis sub-mikromolaren Bereich. Für das Screening wurde die Gesamtbibliothek in zwei Gruppen unterteilt, bestehend aus allen α-Aminosäuren (170 Verbindungen) und allen übrigen Substanzen (2269 Verbindungen). Während die α-Aminosäuren einzeln an den beiden Zielproteinen getestet wurden, wurden die übrigen in Unterbibliotheken von jeweils 16 Verbindungen zusammengefasst. Anhand der vorgegebenen Regeln zur Identifizierung einer Einzelsubstanz bzw. Unterbibliothek als Hit (≤ 50 % Reporterligandbindung bei Testkonzentrationen von 10 µm bzw. 1,0 µM) konnten für GlyT1 fünf Verbindungen (**48**, **SC121 - d**, **SC121 - f**, **SC121 - n** und **SC140 - a**) und für GlyT2 sechs Verbindungen (**SC70 - o**, **SC70 - p**, **SC71 - a**, **SC71 - f**, **SC71 - m** und **SC107 - f**) identifiziert werden, die potente Binder für das jeweilige Protein darstellen. In nachfolgenden Kompetitionsexperimenten wurden schließlich die Affinitäten (pK_i-Werte) der Hit-Substanzen bestimmt, wobei für GlyT1 nur Verbindung **48** und **SC121 - n** untersucht wurden, da alle anderen als Hit identifizierten Verbindungen beim Screening deutlich schlechtere Werte ergeben hatten. Hierbei konnten für **48** und **SC121 - n** pK_i-Werte von 7,00 ± 0,01 bzw. 6,85 ± 0,03 an GlyT1 und für **SC70 - o**, **SC70 - p**, **SC71 - a**, **SC71 - f**, **SC71 - m** und **SC107 - f** pK_i-Werte von 5,42 ± 0,01, 6,40 ± 0,07, 6,18 ± 0,05, 5,94 ± 0,08, 5,46 ± 0,03 und 6,67 ± 0,03 gefunden werden. Somit konnte das Ziel erreicht werden, neue Inhibitoren für beide Transporter

mit hohen Affinitäten, die wenigstens im niedrig mikromolaren bis sub-mikromolaren Bereich lagen, ausfindig zu machen.

Schlussendlich kann festgehalten werden, dass mit dieser Arbeit für die Transporter GlyT1 und GlyT2 zwei neue MS-Bindungsassays erfolgreich etabliert werden konnten, von denen jener für GlyT2 sogar als erster für dieses Target dem Prinzip der Ligandbindungsassays folgt. Die für GlyT1 und GlyT2 entwickelten Bindungsassays stellen aufgrund ihrer Zuverlässigkeit, ihrer Einfachheit bei der Durchführung sowie des relativ hohen Durchsatzes, den sie erlauben, ein überaus nützliches Werkzeug dar, um neue Inhibitoren, bei der Untersuchung von Einzelverbindungen oder Substanzbibliotheken, zu identifizieren, wie auch zur exakten Bestimmung von Bindungsaffinitäten einzelner Testverbindungen.

5 Abkürzungsverzeichnis

ALIS	automated ligand identification system
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolpropionsäure
ASMS	affinity selection mass spectrometry
BGT	Betain-GABA-Transporter
B_{max}	Maximal verfügbare Bindungsstellen (engl.: maximum amount of binding sites)
CDER	Center for drug evaluation and research
DAT	Dopamintransporter
DMA	*N,N*-Dimethylacetamid
EL	Extrazellulärer Loop
ESI	Elektronenspray-Ionisation
FDA	Food and drug administration
FP	Fluoreszenz-Polarisation
FRET	Fluoreszenz-Resonanz-Energie-Transfer
GABA	γ-Aminobuttersäure
GAT	GABA-Transporter
GB	Gesamtbindung
GHMT	Glycinhydroxymethyltransferase
GlyR	Glycinrezeptor
GlyT	Glycintransporter
HPLC	High-performance liquid chromatography
IC_{50}	Halbmaximale inhibitorische Konzentration (engl.: half maximal inhibitory concentration)
IL	Intrazellulärer Loop
K_d	Gleichgewichtsdissoziationskonstante

K_i	Inhibitionskonstante
k_{obs}	Beobachtete (engl.: observed) Geschwindigkeitskonstante
k_{off}	Off-rate
k_{on}	On-rate
LC	Flüssigchromatographie (engl.: liquid chromatography)
$LeuT_{Aa}$	Leucintransporter des Aquifex aeolicus
LLOQ	Lower Limit of Quantification
MASS	multitarget affinity/specificity screening
MRM	multiple reaction monitoring
MS	Massenspektrometer / Massenspektrometrie
NAGly	*N*-Arachidonoylglycin
NET	Norepinephrintransporter
NMDA	*N*-Methyl-D-Aspartat
NOGly	*N*-Oleoylglycin
NSAID	non-steroidal anti-inflammatory drug
NSB	Nicht-spezifische Bindung
RP	Umkehrphase (engl.: reversed-phase)
SB	Spezifische Bindung
SERT	Serotonintransporter
SLC	Solute carrier
SPR	Surface Plasmon Resonance
$t_{1/2}$	Halbwertszeit
THF	Tetrahydrofolat
TMD	Transmembrandomäne
VIAAT	vesikulären inhibitorischen Aminosäure Transporter
ZNS	Zentrales Nervensystem

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