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MS-Binding Assays: Kinetic, Saturation, and Competitive Experiments Based on Quantitation of Bound Marker as Exemplified by the GABA Transporter mGAT1

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A new kind of binding assay is described in which the amount of a nonlabeled marker bound to the target is quantified by LC–ESI-MS–MS. This new approach was successfully implemented with nonlabeled NO 711 as marker and the GABA transporter subtype mGAT1 as target. The native marker bound to the target was liberated from the receptor protein by methanol denaturation after filtration. A reliable and sensitive LC–ESI-MS–MS method for the quantitation of NO 711 was developed, and data from mass spectrometric detection were analyzed by nonlinear regression. Kinetic MS-binding experiments yielded values for k_{+1} and k_{-1}, while in saturation MS-binding experiments, K_d and B_{max} values were determined. In competitive MS-binding experiments, K_i values were obtained for various test compounds covering a broad range of affinities for mGAT1. All experiments were performed in 96-well plate format with a filter plate for the separation step which improved the efficiency and throughput of the procedure. The method was validated by classical radioligand-binding experiments with the labeled marker [$^3H_2$]NO 711 in parallel. The results obtained from MS-binding experiments were found to be in good agreement with the results of the radioligand-binding assays. The new kind of MS-binding assay presented herein is further adapted to the conventional radioligand-binding assay in that the amount of bound marker is securely quantified. This promises easy implementation in accordance with conventional binding assays without the major drawbacks that are inherent in radioligand or fluorescence binding assays. Therefore, MS-binding assays are a true alternative to classical radioligand-binding assays.

Introduction

Around 1970, research in drug–receptor interactions experienced a substantial leap forward through a study conducted by Pert and Snyder, who perfected the competitive radioligand-binding assay, thus enabling the direct analysis of ligand/drug–receptor interactions in general.[1,2] Of course, the availability of radioligands and the improvement of scintillation counting devices played a fundamental role as well.[3] Radioisotope-labeled and later also fluorophore-labeled ligands offer the high sensitivity and selectivity that is necessary in receptor research.[4] By addressing a pharmacological target of interest with high affinity and selectivity, such labeled ligands are used as markers in radioligand-binding and fluorescence assays to quantitatively trace the binding event.[4]

Despite the vast amount of valuable insight radioligand-binding and fluorescence assays have yielded and still offer in current receptor research,[4–7] these methods have severe drawbacks: both techniques require a specially labeled marker that requires extended synthetic efforts. Additionally, the handling of radioisotope-labeled compounds is complicated and expensive. The introduction of a fluorophore to a molecule to generate a fluorescent marker can change the structure of the ligand significantly and even modify the affinity for the target (i.e. receptor), which necessitates re-optimization of the marker.[6]

Today, a different analytical technique offers the high sensitivity and exceptional selectivity necessary for ligand-binding assays: mass spectrometry (MS). This technique is implemented in all areas of research, including drug discovery and development.[8–14] By exploiting the characteristic mass over charge ratio (m/z) for each compound and the corresponding m/z value for daughter ions after fragmentation in tandem MS techniques, mass spectrometry is a true alternative to scintillation counting and fluorescence measurements. With MS, the quantitation of ligands in binding assays is possible without special labeling.

We introduced the concept of *competitive MS-binding assays*[13,14] in which a native marker, that is, a nonlabeled ligand, and mass spectrometric detection is used to characterize test compounds effectively in a competitive binding assay. The feasibility of this approach has been exemplified with native dopamine D_1 and D_2 receptors from porcine striatal cell

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membranes as targets. In these experiments, we determined the amount of free marker present in the incubation mixture after its separation with centrifugation, by liquid chromatography coupled to electrospray ionization tandem MS (LC–ESI-MS–MS). For studies in which dopamine D_1 was targeted, the supernatant solution was quantified after centrifugation without further sample preparation.[13] However, with MS quantitation techniques, the impact of the buffer system must be considered as well; the buffer system is optimized for receptor–marker binding in the biological assay and is not necessarily compatible with mass spectrometric detection.[11,15] Buffer systems common in cell biology often contain a high ionic load that can affect the quantitation or unnecessarily complicate the analysis if an aliquot is directly injected. An alternative solution to this problem would be the quantitation of the marker bound to the target after the receptor–marker complex has been separated from the incubation buffer (either by centrifugation or filtration). In this way, the MS quantitation would be decoupled from the biological assay; as a consequence it would be possible to choose the incubation buffer independently from the quantitation technique. In a final step, the bound marker would only have to be liberated from the protein, possibly by common protein-denaturing procedures, and the initially bound marker could then be quantified in its free form. This kind of MS-binding assay would follow the same principles as conventionally conducted binding studies in which the amount of bound labeled marker is quantified. Therefore, the implementation of MS-binding assays of this new kind would become more attractive.

The aim of the study presented herein was to further develop MS-binding assays; instead of an assay for the nonbound marker, the goal was to quantify the amount of marker bound to the receptor after separation of the receptor–marker complex from the incubation mixture. While the separation techniques of filtration and centrifugation are common in ligand-binding assays, both have their advantages and disadvantages; filtration is surely still unsurpassed for speed and efficiency.[16] Hence, we aimed to establish a filtration MS-binding assay to avoid the rather labor- and time-intensive centrifugation technique.

To prove the feasibility of this approach, we chose the γ-aminobutyric acid (GABA) uptake transporter GAT1 as a target. Four distinct, high-affinity GABA uptake transporters have been characterized from various species including humans.[17,18] The respective mouse homologues of these transporters are termed mGAT1, mGAT2, mGAT3, and mGAT4.[1] GABA transporters play a pivotal role in the removal of GABA released into the synaptic cleft, thereby terminating the inhibitory action of GABA. They also control the status of GABA in the brain. The four GATs are important targets in the search for new drugs in the treatment of epilepsy, anxiety, sleep disorders, and certain movement disorders.[18–21] For this reason, the development of lead structures and ligands for GABA uptake transporters is also a vital area of research pursued by our group.

Monitoring the uptake of [^{3}H]GABA is a widely applied tool in GABA transporter research. However, classical ligand-binding assays in which the ligand is not transported but instead labels the binding site are rarely employed, although they are generally easier to perform as long as a suitable radioligand is available. Radioligand-binding assays with GAT1 as target and [^{3}H]tiagabine as marker were first described by Braestrup et al.[22] These radioligand-binding assays were established during the development and testing of the anticonvulsant tiagabine (Gabitril).[21] The same radioligand was further used to characterize GABA uptake sites in human frontal cortex.[23,24] In these examples, a non-physiologically high NaCl concentration in Tris–NaCl buffer (50 mM Tris–citrate containing 1 M NaCl, pH 7.1) was used to sufficiently increase the affinity of tiagabine to GAT1.

To establish MS-binding assays, we used HEKmGAT1 cell fragments (membrane preparation) as a target source, as a HEK cell line stably expressing mGAT1 has been established by our group and is routinely employed in [^{3}H]GABA uptake experiments. Another requirement for our endeavor was an appropriate native marker. We chose NO 711 (**1**, Figure 1), a known GAT1-selective inhibitor with high affinity.[25] This lipophilic guvacine derivative is, contrary to tiagabine, commercially available for research purposes. Moreover, radioligands for any of the various GABA transporters are not commercially available. This aspect is another advantage of MS-binding assays, because such binding experiments are especially worthwhile when a radioligand is unavailable or too expensive.

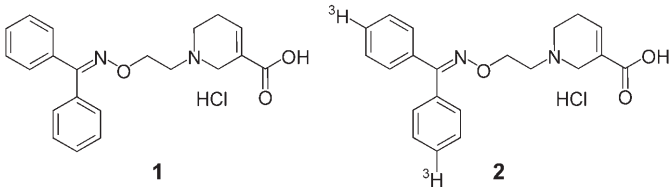


Figure 1. Structures for the native marker NO 711 (**1**) used in MS-binding experiments and for the radioisotope-labeled derivative [$^3H_2$]NO 711 (**2**) used in analogous radioligand-binding experiments.

To demonstrate the reliability of MS-binding assays, we compared the results obtained with this new assay directly with those of classical radioligand-binding assays. Therefore, a radioactively labeled analogue of **1** was required, and the synthesis of the tritiated analogue [$^3H_2$]NO 711, (**2**, Figure 1), which had been selected for this purpose, had to be carried out.

Results and Discussion

As a prerequisite for the planned radioligand-binding assays, a radioactively labeled analogue of NO 711 (**1**) was necessary, and we decided to prepare the dibromo derivative **7a** of **1**. Compound **7a** was expected to give easy access to the tritium-labeled analogue **2**, as a halogen–tritium exchange, rou-

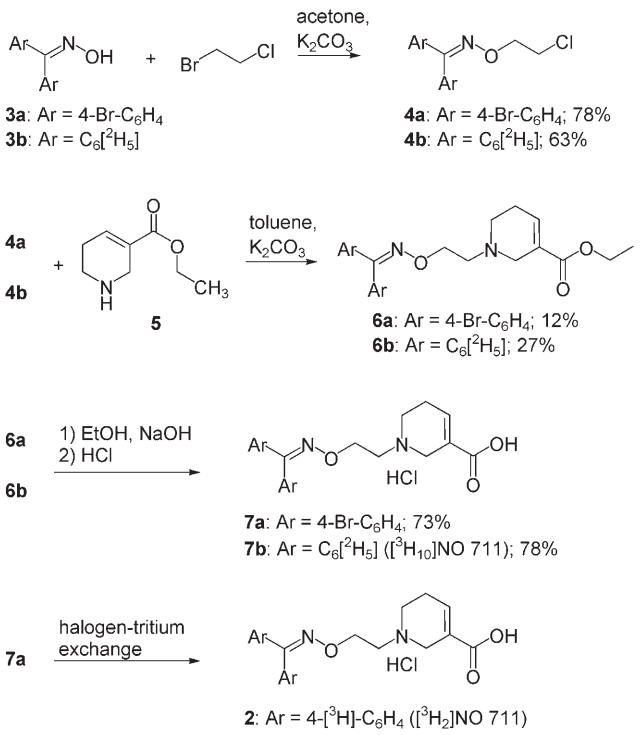
[1] *The nomenclature of species homologues is partially unfortunate as the mouse GAT2 (mGAT2) corresponds to the BGT-1 transporter in other species. For a discussion on nomenclature, see the review by Dalby.*[18]

tinely performed by custom synthesis, was expected to result in the desired radioligand **2**.

We feared that the precision of the LC–ESI-MS–MS quantitation of the native marker might suffer matrix effects from the biological sample (signal suppression or enhancement) and therefore decided to use an internal standard to minimize these problems. The use of a multiply deuterated analogue of the analyte as an internal standard is a well-established procedure in LC–ESI-MS–MS analysis of small molecules.[11] As perdeuterobenzophenone is commercially available, we came to the conclusion that **7b** would be a good candidate as an internal standard, also because its synthesis was easily performed.

Synthesis of [$^2H_{10}$]NO 711 and [$^3H_2$]NO 711

To prepare [$^3H_2$]NO 711 (**2**) and [$^2H_{10}$]NO 711 (**7b**), we decided to closely follow the synthetic procedure described in the literature for the preparation of NO 711.[26] Consequently, the synthesis was performed according to the synthetic plan outlined in Scheme 1.



Scheme 1. Preparation of tritiated and deuterated NO 711 analogues.

The reaction of bis(4-bromophenyl)methanone oxime (**3a**)[27] with 1-bromo-2-chloroethane in the presence of K_2CO_3 provided the oxime ether **4a**. Substitution of **4a** with guvacine ethyl ester (**5**)[28,29] resulted in ester **6a** from which the dibromo analogue **7a** of NO 711 (**1**) was obtained upon subsequent hydrolysis. Compound **7a** was then transformed into the final compound [$^3H_2$]NO 711 (**2**) through a catalytic halogen–tritium exchange performed by custom synthesis[30] following the catalytic Wilzbach procedure. In analogy to the preparation of **7a**, we could also access the [$^2H_{10}$] derivative **7b** of our native MS marker **1** (Scheme 1).

LC–ESI-MS–MS method for the quantitation of native marker NO 711

NO 711 (**1**) showed good ionization properties in ESI-MS–MS analysis (mass transition: 381→180, *m/z*) which was an essential prerequisite to choose this known high-affinity GAT1 ligand as a native maker in MS-binding experiments. It is clear that MS markers must also be securely quantifiable at the low concentrations that are encountered in ligand-binding assays. Of course, this feature strongly depends on the ionization properties of the compound used.

It is still reasonable to minimize matrix suppression or cut off unwanted sample components by chromatographic separation despite the high selectivity of tandem MS.[11] Therefore, an RP HPLC method was developed with isocratic elution (10 mM ammonium formate buffer/acetonitrile/methanol 50:30:20, *v/v/v*) and [$^2H_{10}$]NO 711 as internal standard (mass transition: 391→190, *m/z*) to improve precision. The method was characterized by a short run time (retention time of **1**: 1.4 min; run time: 3.0 min) and allowed the analysis of ≈400 samples overnight (Experimental Section). The lower limit of quantitation was found to be 0.018 nM **1**. In routine assay samples, concentrations ranging from approximately 0.02 nM to 2 nM native marker were found which were all within the tested linear range of the detector response (see Figure 2 for example chromatogram).

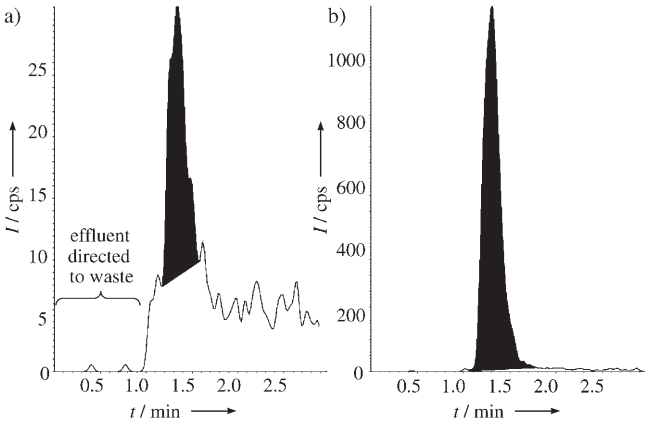


Figure 2. Example of a standard matrix sample. Matrix was obtained by the incubation of HEKmGAT1 membrane preparation in Tris–NaCl buffer (≈10 μg protein content), subsequent filtration, and elution with methanol, as described in the text. This empty matrix was then spiked with NO 711 and [$^2H_{10}$]NO 711 to obtain standard matrix samples for validation of the LC–MS method. a) Trace for 0.015 nM NO 711 (mass transition 381→180, *m/z*); b) trace for 1 nM [$^2H_{10}$]NO 711 (391→190, *m/z*) in the same sample. Effluent was directed to waste from 0 to 1.0 min.

Liberation of bound marker

As discussed above, we aimed for the quantitation of the marker initially bound to the target by LC–ESI-MS–MS in a new type of MS-binding assay. For this, it was necessary to find a

suitable and practical technique for the liberation of the bound marker from the marker–protein complex isolated by filtration and the transfer of the marker into an LC–ESI-MS–MS-compatible sample. It seemed appropriate to liberate receptor-bound marker by denaturation of the target protein trapped on the filter with an organic solvent. Acetone or methanol are commonly used for this purpose.[31] In our case, methanol appeared to be the better choice, as much of the plastic materials prevalent in cell biology and related techniques is not fully solvent compatible.

Indeed, we found 96-well glass fiber filter plates and a corresponding vacuum manifold were well-suited for the separation of bound from free marker. Compared with the cell harvester that is widely used in binding studies, this setup has the advantage that the eluate from the filters can be collected in another 96-deep-well plate in the lower part of the vacuum manifold which was very important for our approach. In conformity with regular practices in binding studies, nonspecific binding of marker to the filters was minimized by rapid subsequent washing of the filters with cold incubation buffer (Tris–NaCl buffer).

We were quite fortunate to discover that bound marker could indeed be liberated by subsequent elution of the filters with methanol. However, in spite of the addition of an internal standard to the denaturing solvent, the variation within triplicate samples was still not acceptable. We then found that a dramatic reduction of the variation within triplicate samples was possible by drying the filter plates (50 °C, 30–60 min) prior to elution with methanol. To explain the observed phenomenon, two possible mechanisms are proposed: First, the filter disks retained slightly different volumes of washing buffer, thereby increasing the variation in the concentration of **1** in the methanol eluate between triplicate samples. ESI is a concentration-dependent process and is therefore susceptible to volume changes.[32] Second, as the water was removed from the trapped protein, denaturation of the receptor–marker complex was further improved in all samples.

Cross-talk between wells was measured in adjacent wells during the elution of a standard sample and turned out to be negligible (<1%). The completeness of elution of bound marker was tested in a series of experiments, and an elution volume of 3×100 μL was found to be sufficient for this purpose. Finally, a defined amount of aqueous buffer (10 mM ammonium formate, pH 7) was added to the methanol eluate, as this improved LC performance (peak shape). The binding assay was carried out in a 96-deep-well plate in all MS-binding experiments. To terminate incubation, an aliquot of the sample suspension was transferred onto the filter plate. Experiments in which the filter plates were used for the incubation were found to be less efficient, as nonspecific binding of the marker was significantly higher in these cases. Figure 3 schematically summarizes the implementation of these MS-binding assays.

Kinetic experiments with native NO 711 and mass-spectrometric quantitation

The time required to establish equilibrium binding had to be defined prior to carrying out any saturation or competitive MS-binding experiments. Our intent was to characterize the binding of NO 711 to HEKmGAT1 membrane fractions in kinetic experiments to determine the equilibrium incubation time, the on- and off-rates of binding, the reversibility of binding, and the equilibrium dissociation constant (K_d) of the binding event calculated from the reverse and forward rates of the binding reaction.

We carried out association and dissociation ligand-binding experiments with the native marker and quantified the bound marker after liberation under the optimized conditions described above. In kinetic experiments, incubation was initiated by the addition of NO 711 to a preincubated protein suspension (HEKmGAT1 membrane preparation in Tris–NaCl buffer). Only a relatively low protein concentration per well (5–20 μg, measured as protein content according to Bradford[33]) was necessary to obtain a sufficiently high signal. Incubation was terminated by rapid filtration at various times with the previously described 96-well filter plate with vacuum manifold.

In association experiments, NO 711 binding reached equilibrium after 20–30 min as depicted in Figure 4. Nonspecific binding, defined as the binding of NO 711 in the presence of GABA (10 mM), was reasonably low and was not further decreased in

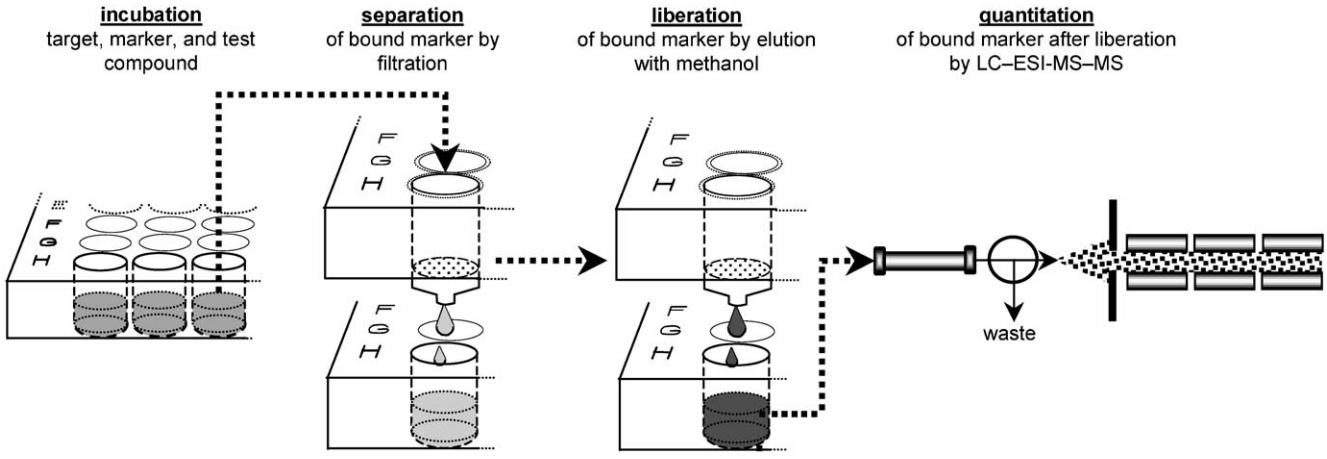


Figure 3. Schematic flowchart of a competitive MS-binding assay including the liberation of bound marker.

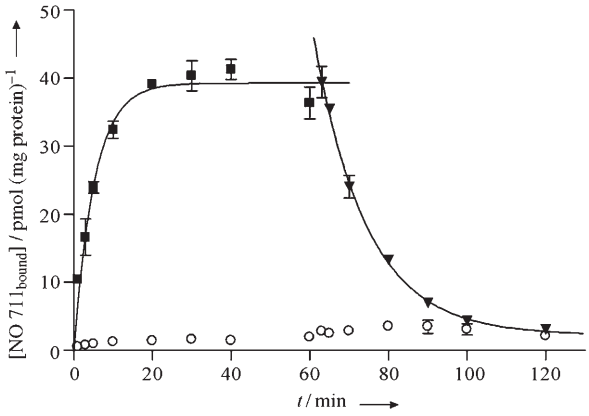


Figure 4. Total binding of NO 711 (**1**) at final concentration of 20 nM in association with (■) and dissociation from (▼) HEKmGAT1 membrane fractions. GABA (10 mM) was added at $t = 60$ min to initiate dissociation. Nonspecific binding (○) was determined as the binding of NO 711 in the presence of 10 mM GABA. Shown are the data for total binding from one representative experiment with determinations in triplicate.

the presence of higher GABA concentrations (100 mM). Binding was stable for up to 120 min (longest incubation time measured, results not shown) indicating that the receptor–marker complex neither denatured nor was otherwise affected by the chosen incubation parameters such as incubation temperature (37 °C). The association rate was determined from nonlinear regression by k_{obs} (Experimental Section) to $k_{+1} = 0.0054 \pm 0.0007$ min^{-1} nM^{-1}.

To determine the dissociation rate constant, NO 711 binding at HEKmGAT1 was allowed to reach equilibrium ($t = 60$ min) before GABA (10 mM) was added as a competitor to suppress the formation of marker–receptor complex (association) and by that to initiate net dissociation of NO 711. The samples were then rapidly filtered after various time intervals to separate bound from free marker as described above. The receptor–ligand complex dissociated completely upon the addition of GABA (10 mM), and the dissociation rate determined by nonlinear regression was found to be $k_{-1} = 0.085 \pm 0.004$ min^{-1}. Calculation of the dissociation equilibrium constant for NO 711 binding from the association (k_{+1}) and dissociation rate (k_{-1}) constants led to a $K_d = (k_{-1}/k_{+1}) = 15.8$ nM.

Saturation MS-binding experiments with native NO 711

The next step to establish MS-binding experiments in quantitation of the bound marker was to determine the K_d value and the maximum amount of binding sites (B_{max}) of the HEKmGAT1 membrane preparation in saturation binding experiments. An incubation time of 40 min was chosen for saturation binding because kinetic experiments had shown that this period of time is adequate for the NO 711–mGAT1 binding interaction to reach equilibrium. Nonspecific binding of NO 711 was defined as the binding of NO 711 in the presence of GABA (10 mM).

We performed a multitude of saturation MS-binding experiments and found a B_{max} value of 34.6 ± 4.02 pmol mg^{-1} protein ($n = 14$, mean ± SEM) for the HEKmGAT1 membrane preparation. A K_d value of 23.4 ± 2.19 nM ($n = 15$, mean ± SEM) was determined. This is in good agreement with the K_d value obtained in the kinetic MS-binding assays described above ($K_d = 15.8$ nM). Figure 5 shows a representative example of saturation curves obtained in the concentration-dependent binding of NO 711 to HEKmGAT1 and indicates that the binding of NO 711 is saturated at a NO 711 concentration of ≈100 nM.

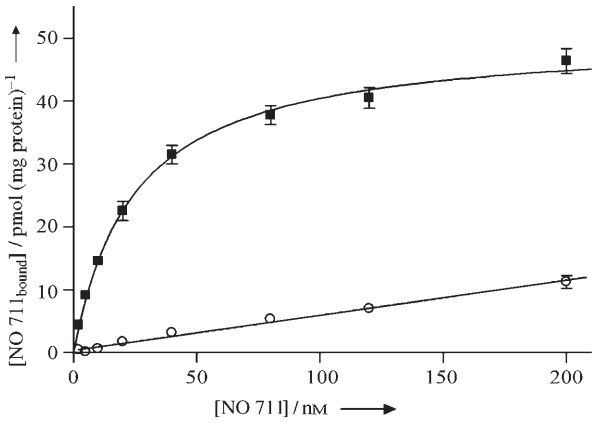


Figure 5. Saturation isotherms of NO 711 (**1**) binding to HEKmGAT1 membrane fractions as measured in MS-binding experiments. One representative experiment from a series of similar assays is shown. Total binding of NO 711 to 10 μg protein (HEKmGAT1 membrane fractions) per well (■). Nonspecific binding (○) was measured as binding of NO 711 in the presence of 10 mM GABA. Each data point depicts the mean ± SEM from triplicate values.

Competitive MS-binding experiments with NO 711 as a native marker

The preceding MS-binding experiments (kinetics and saturation), provided the basic data that are required to develop the desired competitive MS-binding assays with quantitation of initially bound marker. Besides demonstrating that the binding of NO 711 to HEKmGAT1 is a rapid, high-affinity, fully reversible and saturable process, these experiments had further yielded K_d (23.4 ± 2.19 nM) and B_{max} (34.6 ± 4.02 pmol mg^{-1}) values: the knowledge of these constants is a prerequisite to establish competitive binding assays. According to these data, marker concentration was set to 10 nM (marker depletion was negligible, < 10%) and ≈10 μg protein per well were used in a total volume of 300 μL, corresponding to a binding-site concentration of ≈1.15 nM in the assay preparation. Otherwise, the MS-binding experiments were performed under the same conditions that had been successfully implemented in the kinetic and saturation MS-binding experiments (in short: Tris–NaCl buffer, incubation time 40 min, incubation temperature 37 °C, separation by filtration over 96-well glass fiber filter plates, and liberation of bound marker by elution with methanol after drying the filter plates).

To demonstrate the general applicability of the new approach, several different known mGAT1 inhibitors were tested for their affinity toward HEKmGAT1 membrane fractions. Hence, a series of competitive MS-binding experiments was conducted under the above-mentioned conditions. We chose well-known hydrophilic parent structures such as GABA (**8**), gu-

vacine (**13a**), nipecotic acid ((*R*/*S*)-**14a**),[34] and some of their lipophilic derivatives such as CI 966 (**13d**), tiagabine ((*R*)-**14b**), and SKF89976A (**14c**),[34] which cover a broad range of affinities as well. Lipophilic compounds developed by us (compounds (*S*)-**15b** to (2*S*,4*R*)-**18b**,[35,36] see Figure 6) were also included in the study.

Increasing concentrations of the potential mGAT1 inhibitors were tested to determine their IC_{50} values (the concentration of a test compound that decreases the specific binding of the marker by 50%). For each test compound, at least three independent competitive MS-binding experiments were conducted, always in triplicate. In relation to the concentration of the test compound, the data obtained from the quantitation of bound NO 711 using the LC–ESI-MS–MS method described above yielded sigmoidal binding curves. Data were fitted by nonlinear regression (Prism 4.0; one-site competition) and the deduced IC_{50} values were converted into K_i values by the Cheng–Prusoff equation.[37] The K_i values determined for the various compounds are summarized in Table 1. Additionally, Figure 7 shows results obtained for the test compound (*S*)-**15b**, a pyrrolidine-2-acetic acid derivative, as a representative example. This example highlights the good reproducibility observed in competitive MS-binding experiments with quantitation of bound marker.

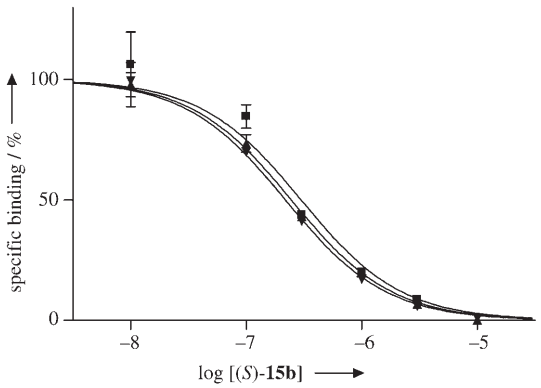


Figure 7. Results from a competitive MS-binding experiments in which the pyrrolidine-2-acetic acid derivative (*S*)-**15b** was tested (three independent experiments). Data points represent specific binding of NO 711 (mean ± SEM from triplicate values), and the binding curves were generated by nonlinear regression.

Direct comparison of results from MS-binding experiments with those of radioligand-binding assays

To validate the results obtained in our MS-binding experiments, we also conducted conventional radioligand-binding assays (saturation and competition experiments) with [$^3H_2$]NO 711 (**2**) as the marker. Incubation parameters were chosen according to the MS-binding assay described above, except that filtration was carried out by using a Brandel MLXR 96TI cell harvester equipped with glass fiber filters instead of the 96-well-filter plate. The resulting receptor–marker complexes trapped on the filter were finally subjected to scintillation counting to determine the amount of bound marker. Saturation and competition curves were generated from the data obtained as described for the MS-binding assays including their analysis with respect to K_d, B_{max}, IC_{50}, and K_i.

Under these conditions, a large number of saturation radioligand-binding experiments were performed. Herein, we acquired a B_{max} value of 26.0 ± 2.64 pmol mg^{-1} protein (n = 22, mean ± SEM) and a K_d value of 35.9 ± 1.81 nM (n = 19, mean ± SEM). Both values agree well with results from MS-binding experiments (B_{max} = 34.6 ± 4.02 pmol mg^{-1} protein and K_d = 23.4 ± 2.19 nM).

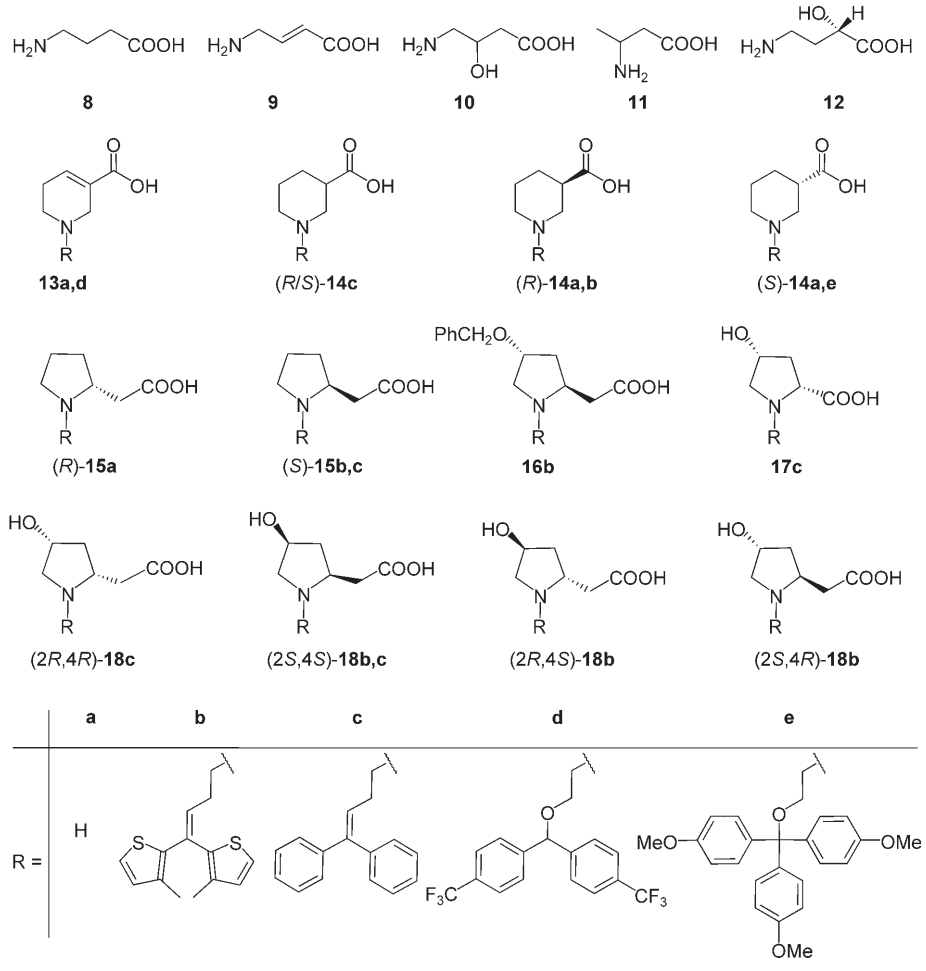


Figure 6. Structures of test compounds.

Table 1. Affinity toward HEKmGAT1 membrane preparations

Compound	K_i [μM][a] MS-binding	Radioligand Binding
GABA (**8**)[34]	83.2 ± 8.9	115 ± 4.9
trans-4-aminocrotonic acid (TACA, **9**)[39]	221 ± 7.5	233 ± 25
DL-4-amino-3-hydroxy butyric acid (**10**)[39]	3719 ± 279	5280 ± 294
DL-3-amino-*n*-butyric acid (**11**)[40]	1043 ± 266	1341 ± 48.8
(*S*)-4-amino-2-hydroxy butyric acid (**12**)[41]	251 ± 28	236 ± 34
guvacine (**13a**)[34]	146 ± 16	157 ± 21
Cl 966 (**13d**)[34]	0.245 ± 0.024	0.180 ± 0.018
SKF89976A ((*R*/*S*)-**14c**)[34]	0.192 ± 0.007	0.186 ± 0.008
(*R*)-nipecotic acid ((*R*)-**14a**)[34]	32.3 ± 3.3	24.5 ± 4.2
tiagabine ((*R*)-**14b**)[34]	0.041 ± 0.009	0.059 ± 0.009
(*S*)-nipecotic acid ((*S*)-**14a**)[34]	1049 ± 116	1324 ± 280
(*S*)-SNAP 5114 ((*S*)-**14e**)[42]	27.4 ± 1.1	34.3 ± 2.0
(*R*)-homoproline ((*R*)-**15a**)[38]	444 ± 85	528 ± 13.5
(*S*)-**15b**[35]	0.179 ± 0.012	0.212 ± 0.003
(*S*)-**15c**[35]	0.075 ± 0.006	0.082 ± 0.001
16b[36]	55.1 ± 3.93	32.7 ± 1.02
17c[36]	6.96 ± 0.321	6.70 ± 0.85
(2*R*,4*R*)-**18c**[36]	3.53 ± 0.39	3.45 ± 0.22
(2*S*,4*S*)-**18b**[36]	0.517 ± 0.065	0.421 ± 0.05
(2*S*,4*S*)-**18c**[36]	1.13 ± 0.28	1.07 ± 0.18
(2*R*,4*S*)-**18b**[36]	0.954 ± 0.07	1.44 ± 0.08
(2*S*,4*R*)-**18b**[36]	1.20 ± 0.26	0.921 ± 0.11

[a] Measured in competitive binding assays with NO 711 (**1**) for competitive MS-binding and [$^3H_2$]NO 711 (**2**) in competitive radioligand binding (in each case, reported as the mean ± SEM, $n = 3$).

In addition to K_i values from competitive MS-binding experiments, Table 1 also lists results from competitive radioligand-binding assays in which the same potential mGAT1 inhibitors were tested. It is evident that the results of the radioligand-binding experiments are in good accord with those obtained with mass spectrometric quantitation. Results deviate only marginally and indicate a high correlation between K_i values obtained from the two different methods. The difference between K_i values from the MS-binding assays in comparison with the radioligand-binding assays is $< 40\%$ in all cases. Only the K_i value for compound **16b** represents an exception, with a difference of $< 70\%$, which is still in the range one would expect for biological assays.

Conclusions

The concept of MS-binding assays was further adapted to the classical and widely accepted approach of radioligand-binding assays insofar as the amount of marker bound to the receptor is now quantified by LC–ESI-MS–MS after filtration. Initially bound marker is liberated from the receptor–marker complex by simple solvent denaturation. As the buffer solution is separated from the receptor–ligand complex, the mass spectrometric quantitation is decoupled from the biological assay, and the impact of the ionic load on the MS analysis is reduced. This allows free choice of incubation buffer in the biological assay and therefore ensures optimum binding conditions for the receptor under investigation.

The principle of the MS-binding assay with quantitation of bound marker was applied to mGAT1 by using NO 711 as a nonlabeled marker. Kinetic, saturation and competitive MS-binding experiments were implemented successfully in a 96-well plate format. The binding of NO 711 was characterized, and a range of potential mGAT1 inhibitors, widely varying in their affinity, were assayed for their K_i values with this novel MS-binding approach. Results obtained in MS-binding assays were validated in radioligand-binding assays using a tritium-labeled NO 711 derivative ([$^3H_2$]NO 711) and found to be in excellent accord with the former.

As exemplified by NO 711 binding to mGAT1, the new MS-binding assay with quantitation of bound marker is a straightforward and true alternative to binding assays that depend on markers labeled with radioisotopes or fluorescent groups. Additionally, the results of the work reported herein suggest that MS-binding assays have the potential for reasonably high-throughput characterization of new drug candidates, yielding results equivalent to those of radioligand-binding assays.

Experimental Section

Chemistry: Solvents used were analytical reagent grade and freshly distilled before use. Purchased reagents were used without further purification. TLC plates were made from silica gel 60 F_{254} on glass (Merck). Flash chromatography (CC) was carried out with Merck silica gel 60 (mesh 0.040–0.063 mm). Melting points (uncorrected) were determined with a Büchi 512 melting point apparatus. ^{1}H NMR spectra were recorded at room temperature with a JNMR-GX (JEOL, 400 or 500 MHz) using TMS as an internal standard and were integrated with the NMR software Nuts (2D Version 5.097, Acorn NMR, 1995). IR spectroscopy was carried out with an FTIR Spectrometer 410 (Jasco); samples were measured as KBr pellets. Mass spectrometry was performed on a Hewlett Packard 5989 A with 59,980 B particle beam LC–MS interface; analysis was carried out by using chemical ionization (CH_5^+). High-resolution mass spectrometry was conducted with a JEOL MS-Station JMS-700, FAB (Xenon, 6 kV, MBA, reference: PEG).

*Synthesis of [$^3H_2$]NO 711 (**2**) and [$^2H_{10}$]NO 711 (**7a**)*:Bis(4-bromophenyl)methanone oxime (**3a**): Bis(4-bromophenyl)methanone (2.5 g, 7.35 mmol) was suspended in EtOH (5 mL). After the addition of hydroxyamine hydrochloride (0.8 g, 11.5 mmol) and H_2O (0.54 mL), pulverized NaOH (1.48 g, 37 mmol) was added in small portions. The mixture was heated at reflux for 5 min and after cooling to room temperature, it was poured into ice-cold HCl (1.5 M, 31 mL). The resulting precipitate was filtered and washed with H_2O. Yield: 2.3 g (89%); colorless crystals; mp: 138–143 °C; TLC: $R_f = 0.62$ (*n*-heptane/EtOAc 6:4); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.24$–7.28 (m, 2H, $H_{aromatic}$), 7.30–7.34 (m, 2H, $H_{aromatic}$), 7.49–7.45 (m, 2H, $H_{aromatic}$), 7.59–7.62 (m, 2H, $H_{aromatic}$), 7.82–7.85 ppm (br s, 1H, OH); MS: *m*/*z* (%) [*M*+1]: 356 (100), 340 (72), 198 (14).

Bis(4-bromophenyl)methanone *O*-(2-chloroethyl)oxime (**4a**): Dried K_2CO_3 (3.45 g, 25 mmol) and 1-bromo-2-chloroethane (8.95 g, 62.4 mmol) were added to a solution of bis(4-bromophenyl)methanone oxime (2.215 g, 6.24 mmol) in freshly distilled acetone (18.7 mL). After heating at reflux for 90 h, solids were filtered off and washed with plenty of acetone. The combined organic extracts were evaporated, and the resulting oil was purified by CC (*n*-hep-

tane/EtOAc 95:5, increasing polarity to 70:30). Yield: 2.04 g (78%); colorless crystals; mp: 48–52 °C; TLC: R_f = 0.73 (*n*-heptane/EtOAc = 7:3); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.77 (t, J = 5.8, 2H, CH_2Cl), 4.38 (t, J = 5.8, 2H, OCH_2), 7.23–7.27 (m, 2H, $H_{aromatic}$), 7.31–7.34 (m, 2H, $H_{aromatic}$), 7.45–7.49 (m, 2H, $H_{aromatic}$), 7.56–7.59 ppm (m, 2H, $H_{aromatic}$).

Ethyl 1-[2-({[bis(4-bromophenyl)methylene]amino}oxy)ethyl]-1,2,5,6-tetrahydropyridine-3-carboxylate (**6a**): Guvacine ethyl ester[28,29] (**5**) (300 mg, 1.9 mmol) and dried K_2CO_3 (520 mg, 3.8 mmol) were added to a solution of **4a** (793 mg, 1.9 mmol) in toluene (25 mL). The reaction mixture was heated at reflux for 120 h. After cooling to room temperature, filtration and evaporation provided a slightly yellow oil which was purified by CC (cyclohexane/EtOAc 9:1, increasing polarity to 1:1). Yield: 120 mg (12%); colorless oil; TLC: R_f = 0.16 (EtOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.28 (t, J = 7.2 Hz, 3H, CH_3), 2.31 (m, 2H, $CHCH_2$), 2.57 (t, J = 5.6, 2H, $CHCH_2CH_2$), 2.85 (t, J = 6 Hz, 2H, $NOCH_2CH_2$), 3.26 (m, 2H, $CH_2CCOOEt$), 4.19 (q, J = 7.2 Hz, 2H, CH_2CH_3), 4.37 (t, J = 6 Hz, 2H, $NOCH_2$), 6.96–7.02 (m, 1H, CH), 7.22–7.27 (m, 2H, $H_{aromatic}$), 7.31–7.35 (m, 2H, $H_{aromatic}$), 7.44–7.48 (m, 2H, $H_{aromatic}$), 7.54–7.57 ppm (m, 2H, $H_{aromatic}$).

1-[2-({[Bis(4-bromophenyl)methylene]amino}oxy)ethyl]-5-carboxy-1,2,3,6-tetrahydropyridinium chloride (**7a**): NaOH (10 M, 80 μL, 0.8 mmol) was added to a cooled (0 °C) solution of **6a** (120 mg, 0.22 mmol) in EtOH (400 μL). The reaction mixture was stirred for 6.5 h at room temperature, the solvent was then evaporated, the residue was dissolved in H_2O (2 mL) and the solution was adjusted to pH 2 with HCl (2 M). The resulting colorless crystals were filtered and washed with toluene. Yield: 74 mg (73%); colorless crystals; mp: 250 °C (decomp.); TLC: R_f = 0.67 (*n*-heptane/EtOAc 7:3); IR (KBr): $\tilde{\nu}$ = 2926, 2706, 2659, 2602, 1733, 1668, 1068, 832 cm^{-1}; ^{1}H NMR (400 MHz, [D$_4$]methanol): δ = 2.57–2.64 (m, 2H, $CHCH_2$), 3.36 (t, J = 5.9 Hz, 2H, $CHCH_2CH_2$), 3.60–3.66 (m, 2H, $NOCH_2CH_2$), 3.96–4.03 (m, 2H, CH_2CCOOH), 4.55–4.61 (m, 2H, $NOCH_2CH_2$), 7.08–7.14 (m, 1H, CH), 7.25–7.31 (m, 2H, $H_{aromatic}$), 7.37–7.42 (m, 2H, $H_{aromatic}$), 7.52–7.57 (m, 2H, $H_{aromatic}$), 7.63–7.69 ppm (m, 2H, $H_{aromatic}$); MS: *m/z* (%) [M^+]: 509 (4), 340 (38), 183 (100); HRMS (FAB) for $C_{21}H_{21}Br_2N_2O_3$: calcd: 508.9900, found: 508.9908.

1-[2-({[Di([^{3}H$_2$]phenyl)methylene]amino}oxy)ethyl]-5-carboxy-1,2,3,6-tetryhydropyridinium chloride (**2**): The catalytic halogen–tritium exchange was carried out by Hartmann Analytic, Braunschweig, Germany.[30]

Di([^{2}H$_5$]phenyl)methanone oxime (**3b**): Hydroxyamine hydrochloride (508 mg, 7.3 mmol) and H_2O (2.3 mL) were added to a solution of di([^{2}H$_5$]phenyl)methanone (890 mg, 4.63 mmol) in EtOH (4 mL) followed by pulverized NaOH (943 mg, 23.6 mmol) in small portions. The mixture was then heated at reflux for 5 min. After cooling to room temperature, it was poured into ice-cold HCl (1 M, 35 mL). The resulting precipitate was filtered and washed with H_2O. Yield: 851 mg (89%); colorless crystals; mp: 130–135 °C; TLC: R_f = 0.62 (*n*-heptane/EtOAc 6:4); ^{1}H NMR (500 MHz, CD_2Cl_2): 7.72–7.74 ppm (m, 1H, OH); MS: *m/z* (%) [M+1]$^+$: 208 (100), 190 (40), 109 (4).

Di([^{2}H$_5$]phenyl)methanone *O*-(2-chloroethyl)oxime (**4b**): Dried K_2CO_3 (1.18 g, 8.55 mmol) and 1-bromo-2-chloroethane (6.28 g, 43.8 mmol) were added to a solution of **3b** (0.89 mg, 4.32 mmol) in freshly distilled acetone (13 mL). After heating at reflux for 90 h, solids were filtered off and washed with acetone. The combined organic extracts were evaporated, and the resulting oil was purified by CC (*n*-heptane/EtOAc 95:5, increasing polarity to 70:30). Yield: 737 mg (63%); colorless crystals; mp: 40–45 °C; TLC: R_f = 0.56 (*n*-heptane/EtOAc 8:2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.79 (t, J = 6.1 Hz, 2H, CH_2Cl), 4.38 ppm (t, J = 6.1 Hz, 2H, OCH_2); recovered starting material (**3b**): 26%.

Ethyl 1-[2-({[di([^{2}H$_5$]phenyl)methylene]amino}oxy)ethyl]-1,2,5,6-tetrahydropyridine-3-carboxylate (**6b**): Guvacine ethyl ester (**5**) (212 mg, 1.1 mmol) and dried K_2CO_3 (307 mg, 2.2 mmol) were added to a solution of **4b** (300 mg, 1.1 mmol) in toluene (15 mL), and the reaction mixture was heated at reflux for 90 h. After cooling to room temperature, filtration and evaporation provided a slightly yellow oil which was purified by CC to yield **6b** (cyclohexane/EtOAc 9:1, increasing polarity to 1:1). Yield: 116 mg (27%); colorless oil; TLC: R_f = 0.17 (*n*-heptane/EtOAc 6:4); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.27 (t, J = 7.1 Hz, 3H, CH_3), 2.27–2.33 (m, 2H, $CHCH_2$), 2.58 (t, J = 5.6 Hz, 2H, $CHCH_2CH_2$), 2.88 (t, J = 6.0 Hz, 2H, $NOCH_2CH_2$), 2.72 (m, 2H, $CH_2CCOOEt$), 4.19 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.38 (t, J = 6.0 Hz, 2H, $NOCH_2$), 6.97–7.01 ppm (m, 1H, CH).

1-[2-({[Di([^{2}H$_5$]phenyl)methylene]amino}oxy)ethyl]-5-carboxy-1,2,3,6-tetrahydropyridinium chloride (**7b**): NaOH (10 M, 0.27 mL, 2.7 mmol) was added to a cooled (0 °C) solution of **6b** (112 mg, 0.287 mmol) in ethanol (6.53 mL). The reaction mixture was stirred for 4.5 h at room temperature, the solvent was then evaporated, the residue was dissolved in H_2O (1.57 mL), and the solution was adjusted to pH 2 with HCl (2 M). After extraction with CH_2Cl_2 and evaporation of the solvent, colorless crystals were obtained. Yield: 89 mg (78%), colorless crystals; mp: 205–207 °C; TLC: R_f = 0.65 (*n*-heptane/EtOAc 7:3); IR (KBr): $\tilde{\nu}$ = 2942, 2876, 2544, 1714, 1660, 1217, 968, 730, 701 cm^{-1}; ^{1}H NMR (400 MHz, [D$_4$]methanol): δ = 2.54–2.63 (m, 2H, $CHCH_2$), 3.37 (t, J = 5.9 Hz, 2H, $CHCH_2CH_2$), 3.61–3.68 (m, 2H, $NOCH_2CH_2$), 3.95–4.07 (m, 2H, CH_2CCOOH), 4.55–4.61 (m, 2H, $NOCH_2$), 7.08–7.14 ppm (m, 1H, CH); MS: *m/z* (%) [M^+]: 361 (2), 192 (100), 145 (17); HRMS (FAB) for $C_{21}H_{11}D_{10}N_2O_3$: calcd: 361.2336, found: 361.2318.

Analytical quantitation: Solvents used for chromatographic separation were HPLC grade. Ammonium formate buffer (10 mM) was made from ammonium formate (>99% for mass spectrometry) and was adjusted to pH 7.0 with an aqueous solution of ammonia (25%).

LC–MS–MS Analysis of NO 711 and [^{2}H$_{10}$]NO 711: Quantitation of NO 711 was carried out on an Agilent 1100 HPLC system (pump, degasser, and column oven: Agilent, Waldbronn, Germany) with a SIL-HT(A) autosampler (Shimadzu, Duisburg, Germany) coupled to an API 2000 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (ESI, in positive ionization mode) and a Valco six-port switching valve (Applied Biosystems, Darmstadt, Germany). For chromatographic separation, a Purospher STAR RP 18 column (2×55 mm, 3 μm; Merck KGaA, Darmstadt, Germany) with a Security Guard RP 18 guard column (2×4 mm, Phenomenex, Aschaffenburg, Germany) was used. The column oven temperature was set to 20 °C. The mobile phase consisted of acetonitrile/methanol/10 mM ammonium formate buffer (30:20:50 *v/v/v*, pH 7) and an isocratic elution profile was run with a flow rate of 350 μL min^{-1}. From the sample solution obtained after liberation of the marker, volumes of 50 μL were directly introduced onto the column. For routine quantitation, the effluent in the time interval of 0–1.0 min was directed to waste through the Valco valve to protect the MS instrument.

Compound-dependent parameters for the MS detector in MRM mode were set as follows: source temperature 450 °C, ion-spray voltage 2200 V, collision energy 25 V, nitrogen was supplied as the curtain gas (30 psi), as the nebulizing gas (30 psi), as the auxiliary gas (60 psi), and as the collision gas (12 psi). The transitions for the marker compound and the internal standard were 381→180 and

391→190, respectively (all *m/z*). Quadrupoles Q1 and Q3 were set to low resolution, and dwell time was set to 500 ms. Data were collected and quantified with Analyst 1.4 (Applied Biosystems).

Binding assays: All buffers were prepared from deionized water, which was freshly distilled prior to use. For the determination of protein content, a UV/Vis spectrophotometer was used (Ultraspec plus; cuvette length: 1 cm, Pharmacia LKB GmbH, Freiburg, Germany).

mGAT1 membrane preparation: Membrane preparations from HEKmGAT1 were prepared according to the following protocol: cells stably expressing mGAT1 were cultivated and isolated from dishes (145 cm^2) with a confluence $>85\%$. Cells were washed in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4) and were resuspended in 0.32 M sucrose with a Polytron PT A7 (Kinematica Polytron, Littau-Luzern, Switzerland) and aliquots of this suspension, corresponding to $\approx 4\ mg\ mL^{-1}$ protein,[33] were stored at $-80\,^{\circ}C$ and defrosted as needed.

Final preparation of HEKmGAT1 membrane preparation for binding assays: An aliquot of the HEKmGAT1 membrane preparation was rapidly defrosted and diluted in a tenfold volume of cold Tris–NaCl buffer. After centrifugation (Kendro Sorvall, SS34 rotor, 50000 g, $4\,^{\circ}C$, 20 min), the pellet was resuspended in 50 mM Tris–citrate buffer containing 1 M NaCl, pH 7.1 (Tris–NaCl buffer) resulting in a protein content of $\approx 0.2\ mg\ mL^{-1}$. The suspension was stirred on ice for the duration of the experiment.

The amount of protein in all suspensions was estimated according to the method of Bradford with Bovine Serum Albumin (BSA) for external standard calibration.[33] Membrane proteins were solubilized by incubation with 10 mM sodium hydroxide (1 h) prior to the Bradford assay.

MS-binding assays: Tris–NaCl buffer was pipetted into the wells of a 96-deep-well plate (polystyrene, 1 mL well volume, Nunc, Wiesbaden, Germany). For saturation MS-binding experiments, increasing concentrations of NO 711 were added (1–200 nM). NO 711 was used at concentrations of 20 and 10 nM in kinetic and competitive MS-binding experiments, respectively. Only in competitive MS-binding experiments were increasing concentrations of test compound further added to the assay. In saturation and competitive MS-binding assays, incubation was started by the addition of the membrane preparation (50 μL, equivalent to 5–20 μg protein per well), whereas in kinetic experiments, protein suspension was added to the buffer and pre-equilibrated (10 min, $37\,^{\circ}C$) before the experiment was started through the addition of the marker solution. In dissociation experiments, marker binding to the protein was allowed to equilibrate for 40 min before the addition of GABA (10 mM) to start dissociation. Total assay volume was 300 μL in all experiments. The whole incubation plate was carefully vortexed and incubated in a shaking water bath ($37\,^{\circ}C$). Incubation was terminated after 40 min in saturation and competitive MS-binding experiments, and after varying time intervals in kinetic MS-binding experiments. This was done by the transfer of an aliquot of assay suspension (250 μL) onto the 96-well filter plate and rapid vacuum filtration, followed by washing of the filters with ice-cold Tris–NaCl buffer (5×150 μL). The filter plate was then dried (30–60 min, $50\,^{\circ}C$) and after cooling to room temperature, it was eluted with 3×100 μL methanol (containing 1 nM [$^2H_{10}$]NO 711). Finally, to improve LC–MS–MS performance, ammonium formate buffer (100 μL, 10 mM, containing 1 nM [$^2H_{10}$]NO 711) was added to each well. After careful vortexing, the plate was sealed with aluminum foil (Corning, New York, USA) and analyzed with LC–ESI-MS–MS as described above.

Radioligand-binding assays: Tris–NaCl buffer was pipetted into a 96-well plate (polystyrene, 350 μL well volume, Sarstedt AG & Co., Nümbrecht, Germany). Marker solution was then added: increasing concentrations of [$^3H_2$]NO 711/NO 711 (1:3, 1–120 nM) in saturation binding assays and 10 nM [$^3H_2$]NO 711 in competitive radioligand-binding assays. In competitive radioligand-binding assays, increasing concentrations of test compounds were further added. Incubation was started by the addition of 50 μL membrane preparation (equivalent to 5–20 μg protein per well). Total assay volume was 200 μL. After 40 min incubation time (for both saturation and competitive radioligand-binding assays) in a shaking incubator ($37\,^{\circ}C$; Certomat IS, B. Braun Biotech International, Melsungen, Germany), samples were filtered over Whatman GF/C filters with a Brandel MLXR 96TI cell harvester. After washing (≈ 4–5×300 μL), filters were removed from the harvester. Filters loaded with receptor–radioligand complex were then dried ($50\,^{\circ}C$, 60 min) and treated with MeltiLex A (Perkin–Elmer, Wallac, Turku, Finland). Filter sheet and melt-on scintillator were brought into close contact and treated for 2 min in an oven at $95\,^{\circ}C$. After cooling to room temperature, the sheet was put into an adequate cassette (1450–104 Filtermat 96) and analyzed with a Wallac MicroBeta TriLux (Perkin–Elmer LAS, Jügesheim, Germany) with the corresponding MicroBeta software for Windows Workstation Version 4.0.

Data analysis: In all experiments, specific binding was defined as the difference between total binding and nonspecific binding (NO 711 binding in the presence of 10 mM GABA). The concentration of a competing test compound that inhibited 50% of specific binding (IC_{50}) was calculated with nonlinear regression (Prism 4.00, GraphPad Software, San Diego, CA, USA) for sigmoidal dose-response curves obtained in competitive binding experiments. The top and bottom of the sigmoidal curve were constrained to values obtained for the controls without competitor (total binding, top) or with GABA (10 mM, bottom). K_i values were determined according to Cheng and Prusoff.[37] Marker depletion was negligible in all experiments ($<10\%$). Values for the equilibrium dissociation constant (K_d) and the maximum density of binding sites (B_{max}) were gained from saturation binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 4.00). Nonlinear curve fitting in kinetic MS-binding experiments yielded the dissociation rate constant k_{-1} (one-phase exponential decay) and the observed rate constant k_{obs} (one-phase exponential association) from which the association rate constant k_{+1} was calculated according to $k_{+1}=(k_{obs}-k_{-1})/[L]$, in which [L] = concentration of free ligand NO 711.

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[1] C. P. Pert, S. H. Snyder, *Science* **1973**, *179*, 1011 – 1013.
[2] P. M. Sweetnam, C. H. Price, J. W. Ferkany, *Burger's Medicinal Chemistry and Drug Discovery, Vol.* 1, 5th ed. (Ed.: M. E. Wolff), Wiley, Hoboken, NJ, **1995**, pp. 698 – 730.
[3] R. P. Ekins, *Clin. Chem.* **1998**, *44*, 2015 – 2030.

[4] D. G. Haylett in *Receptor Pharmacology*, 2nd ed. (Eds.: J. C. Foreman, T. Johansen), CRC, Boca Raton FL, **2003**, pp. 153 – 180.
[5] S. Lazareno, *J. Recept. Signal Transduction Res.* **2001**, *21*, 136 – 165.
[6] R. Hovius, P. Vallotton, T. Wohland, H. Vogel, *Trends Pharmacol. Sci.* **2000**, *21*, 266 – 273.
[7] J. Liu, A. Zacco, T. M. Piser, C. W. Scott, *Anal. Biochem.* **2002**, *308*, 127 – 133.
[8] K. F. Geoghegan, M. A. Kelly, *Mass Spectrom. Rev.* **2005**, *24*, 347 – 366.
[9] K. Breuker, *Angew. Chem.* **2004**, *116*, 22 – 25; *Angew. Chem. Int. Ed.* **2004**, *43*, 22 – 25.
[10] M. M. Siegel, *Front. Med. Chem.* **2004**, *1*, 273 – 292.
[11] G. Hopfgartner, E. Varesio, *TrAC Trends Anal. Chem.* **2005**, *25*, 583 – 589.
[12] I. Muckenschnabel, R. Falchetto, L. M. Mayr, I. Filipuzzi, *Anal. Biochem.* **2004**, *324*, 241 – 249.
[13] G. Höfner, K. T. Wanner, *Angew. Chem.* **2003**, *115*, 5393 – 5395; *Angew. Chem. Int. Ed.* **2003**, *42*, 5235 – 5237.
[14] K. Niessen, G. Höfner, K. T. Wanner, *ChemBioChem* **2005**, *6*, 1769 – 1775.
[15] M. L. Nedved, S. Habibi-Goudarzi, B. Ganem, J. D. Henion, *Anal. Chem.* **1996**, *68*, 4228 – 4236.
[16] J. P. Bennet, H. I. Yamamura in *Neurotransmitter Receptor Binding* (Ed.: H. I. Yamamura, S. J. Enna, M. J. Kuhar), Raven Press, New York, **1985**, pp. 69 – 73.
[17] J. Guastrella, N. Nelson, H. Nelson, L. Czyzyk, S. Keynan, M. C. Miedel, N. Davidson, H. A. Lester, B. I. Kanner, *Science* **1990**, *249*, 1303 – 1306.
[18] N. O. Dalby, *Eur. J. Pharmacol.* **2003**, *479*, 127 – 137, and references therein.
[19] L. Iversen, *Biochem. Pharmacol.* **2004**, *68*, 1537 – 1540, and references therein.
[20] A. Schousboe, Al. Sarup, O. M. Larsson, H. S. White, *Biochem. Pharmacol.* **2004**, *68*, 1557 – 1563.
[21] B. S. Meldrum, A. G. Chapman, *Epilepsia* **1999**, *40*, S2 – S6.
[22] C. Braestrup, E. B. Nielsen, U. Sonnewald, L. J. S. Knutsen, K. E. Andersen, J. S. Jansen, K. Frederiksen, P. H. Andersen, A. Mortensen, P. D. Suzdak, *J. Neurochem.* **1990**, *54*, 639 – 647.
[23] I. Sundman-Eriksson, P. Allard, J. Marcusson, *Brain Res.* **1999**, *851*, 183 – 188.
[24] I. Sundman-Eriksson, P. Allard, *J. Affective Disord.* **2002**, *71*, 29 – 33.
[25] P. D. Suzdak, K. Frederiksen, K. E. Andersen, P. O. Sørensen, L. J. Knutsen, E. B. Nielsen, *Eur. J. Pharmacol.* **1992**, *224*, 189 – 198.
[26] J. S. Knutsen, K. E. Andersen, J. Lau, B. F. Lundt, R. F. Henry, E. E. Morton, L. Nærum, H. Petersen, H. Stephensen, P. D. Suzdak, M. D. B. Swedberg, C. Thomsen, P. O. Sørensen, *J. Med. Chem.* **1999**, *42*, 3447 – 3462.
[27] A. Lachmann, *J. Am. Chem. Soc.* **1924**, *46*, 1477 – 1483.
[28] S. B. Christensen, P. Krogsgaard-Larsen, *J. Labelled Compd. Radiopharm.* **1980**, *17*, 191 – 202.
[29] T. Tsukamoto, T. Komori, *Yakugaku Zasshi* **1953**, *73*, 779 – 780.
[30] Hartmann Analytic GmbH, Mascheroder Weg 1b, Braunschweig, Germany.
[31] A. Pingoud, C. Urban, J. Hoggett, A. Jeltsch, *Biochemical Methods*, Wiley-VCH, Weinheim, **2002**, pp. 56.
[32] G. Hopfgartner, K. Bean, J. Henion, *J. Chromatogr.* **1993**, *647*, 51 – 61.
[33] M. Bradford, *Anal. Biochem.* **1976**, *72*, 248 – 259.
[34] L. A. Borden, T. G. M. Dhar, K. E. Smith, R. L. Weinshank, T. A. Branchek, C. Gluchowski, *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **1994**, *269*, 219 – 224.
[35] K. Wanner, G. Fülep, G. Höfner, WO 0014064, **2000**. [*Chem. Abstr.* **2000**, *132*, 194656.]
[36] X. Zhao, C. E. Hoesl, G. C. Hoefner, K. T. Wanner, *Eur. J. Med. Chem.* **2005**, *40*, 231 – 247.
[37] Y.-C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099 – 3108.
[38] L. Nielsen, L. Brehm, P. Krogsgaard-Larsen, *J. Med. Chem.* **1990**, *33*, 71 – 77.
[39] E. Falch, A. Hedegaard, L. Nielsen, B. R. Jensen, H. Hjeds, P. Krogsgaard-Larsen, *J. Neurochem.* **1986**, *47*, 898 – 903.
[40] D. A. Brown, M. Galvan, *Brit. J. Pharmacol.* **1977**, *59*, 373 – 378.
[41] L. Brehm, P. Krogsgaard-Larsen, *Acta Chem. Scand. Ser. B* **1979**, *33*, 52 – 56.
[42] T. G. M. Dhar, L. A. Borden, S. Tyagarajan, K. E. Smith, T. A. Branchek, R. L. Weinshank, C. Gluchowski, *J. Med. Chem.* **1994**, *37*, 2334 – 2342.

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