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Library Screening by Means of Mass Spectrometry (MS) Binding Assays—Exemplarily Demonstrated for a Pseudostatic Library Addressing γ-Aminobutyric Acid (GABA) Transporter 1 (GAT1)

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Dedicated to Prof. Herbert Mayr with warmest wishes on the occasion of his 65th birthday

In the present study, the application of mass spectrometry (MS) binding assays as a tool for library screening is reported. For library generation, dynamic combinatorial chemistry (DCC) was used. These libraries can be screened by means of MS binding assays when appropriate measures are taken to render the libraries pseudostatic. That way, the efficiency of MS binding assays to determine ligand binding in compound screening with the ease of library generation by DCC is combined. The feasibility of this approach is shown for γ-aminobutyric acid (GABA) transporter 1 (GAT1) as a target, representing the most important subtype of the GABA transporters. For the screening, hydrazone libraries were employed that were generated in the presence of the target by reacting various sets of aldehydes with a hydrazine derivative that is delineated from piperidine-3-carboxylic acid (nipecotic acid), a common fragment of known GAT1 inhibitors. To ensure that the library generated is pseudostatic, a large excess of the nipecotic acid derivative is employed. As the library is generated in a buffer system suitable for binding and the target is already present, the mixtures can be directly analyzed by MS binding assays—the process of library generation and screening thus becoming simple to perform. The binding affinities of the hits identified by deconvolution were confirmed in conventional competitive MS binding assays performed with single compounds obtained by separate synthesis. In this way, two nipecotic acid derivatives exhibiting a biaryl moiety, 1-{2-[2′-(1,1′-biphenyl-2-ylmethylidene)hydrazine]ethyl}piperidine-3-carboxylic acid and 1-(2-{2′-[1-(2-thiophenylphenyl)methylidene]hydrazine}ethyl)piperidine-3-carboxylic acid, were found to be potent GAT1 ligands exhibiting pK_i values of 6.186 ± 0.028 and 6.229 ± 0.039, respectively. This method enables screening of libraries, whether generated by conventional chemistry or DCC, and is applicable to all kinds of targets including membrane-bound targets such as G protein coupled receptors (GPCRs), ion channels and transporters. As such, this strategy displays high potential in the drug discovery process.

Introduction

Hit identification is one of the fundamental steps in the early stages of the drug discovery process. Often large compound collections that might contain molecules originating from different sources are searched for ligands that bind and modulate the activity of a biopolymer that has been selected as a therapeutic target for the cure of a disease of interest. For functionally active targets like enzymes or receptors, luminescence- and fluorescence-based methods are quite common, which is mainly due to the high sample throughput that can be achieved with such techniques. In the last ten years, mass spectrometry (MS) has also become an important tool in drug screening. Different concepts have evolved that utilize MS for this purpose. In the most common approach, compounds formerly bound to the target under equilibrium or near equilibrium conditions are liberated and subsequently identified by MS. These MS-based techniques, which are termed affinity selection mass spectrometry (AS-MS), are quite powerful and have found widespread application in drug screening; however, they require monitoring of all constituents of the library by MS.[1,2]

MS binding assays represent a novel technique to perform saturation, competition and kinetic experiments. As such, they are closely related to radioligand binding assays. But with no labeling of the ligands for quantitation by MS being required, they avoid all the drawbacks that result from radioactivity associated with radiometric assays.[3]

So far, competitive MS binding assays have been widely used to characterize single compounds in competition studies by establishing competition curves to create precise affinity data.[3,4] Being easy and simple to perform, competitive MS binding assays could also be considered as readouts for drug screening. For this purpose, competitive MS binding assays could be reduced to a single-point determination quantifying the amount of bound reporter ligand competing with com-

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pound library members for the binding sites at a given concentration. Thus, the analytical demand for this kind of assay is far less than for the above-mentioned AS-MS methods, which require the quantification of all library components.[3] Of course, radioligand binding assays could also be used for drug screening but are largely avoided for comprehensive drug screening campaigns because of the radioactively labeled compounds required for this technique. Thus, by avoiding these drawbacks, competitive MS binding assays should have high potential for application in drug screening.

In this study, we demonstrate that competitive MS binding assays are a useful tool for drug screening. To this end, MS binding assays are applied to compound libraries generated by utilizing simple and efficient reactions known from dynamic combinatorial chemistry (DCC),[5–7] which results in a highly efficient method for library generation and screening, as outlined below.

Dynamic combinatorial libraries (DCLs) are generated by combining building blocks of suitable reactivity allowing the formation of all possible ligands that interchange in a thermodynamic equilibrium. Classical, adaptive DCLs are generated in the presence of the protein target that serves as a template, shifting the equilibrium towards the best binders. Identification of amplified compounds is carried out by monitoring the shift of the composition of the library effected by the target or by analyzing formed target–ligand complexes by an MS screening technique, such as electrospray ionization (ESI)-MS/MS,[8] ESI-Fourier transform ion cyclotron resonance (FTICR)-MS/MS[9] and ESI-MS with quadrupole time-of-flight (QTOF).[10]

When designing a DCL, the concept of isoenergetic compounds has to be taken into account to ensure the formation of equal amounts of each library member in an untemplated DCL.[5] This is of fundamental importance to facilitate the detection of amplification effects and to prevent false negative results due to low amounts of energetically disfavored but potent binders. Furthermore, until today, to ensure the detectability of amplification effects, this concept has typically been applied to targets like enzymes or carbohydrates.[5,11] This is likely to be because of their good availability in pure form and their high solubility in aqueous systems, allowing their application at relatively high, that is, micromolar, concentrations.

Next to adaptive libraries, there are pre-equilibrated[6] and iterative[12] forms. Both kinds of DCLs are generated under thermodynamic conditions but are analyzed when static, accomplished by changing the temperature or pH. Of course, these libraries are amenable to screening by conventional methods.[13–15]

Clearly, drug screening by DCC represents an intriguing concept, however, it is important to note that the analysis of dynamic libraries for the identification of the best binders is a challenging and demanding task.[16] Moreover, the generation of DCLs is commonly based on the most fundamental and efficient chemical reactions of organic chemistry, such as imine and oxime formation[17] or aldol condensation,[6] and so, the process is generally very easy to perform.

We considered it worthwhile to use DCLs for our project in which—as already outlined above—demonstrating the usefulness of competitive MS binding assays as tools in drug screening was a major aim. Combining DCC reactions for library generation and competitive MS binding assays for screening can be realized in the following manner: The DCL should be generated in the presence of the target, not to enhance the concentrations of best binders by a template effect, but rather to improve the assay performance. In addition, this approach could possibly help to accelerate the formation of best binders by kinetic template effects, thus amplifying their concentrations in the compound mixture, in case libraries had not reached thermodynamic equilibrium because of insufficient reaction time or inappropriate reaction conditions.

For the analysis of DCLs, the libraries should favorably consist of equal amounts of individual constituents. As the composition of dynamic libraries depends on the thermodynamic stabilities of the individual members, this requires the components to be mostly isoenergetic; however, this is rarely achieved. To overcome this problem, only a one-dimensional library should be used, in which a set of structurally diverse building blocks is reacted with a single compound of complementary chemical reactivity. In order to force the reaction equilibrium towards the products, the single compound with fixed structure should be employed in a large excess as compared with the structurally diverse fragments. In this way, the library should become pseudostatic, and by starting from a mixture with equal amounts of the structurally diverse fragments, a library with all constituents being present in similar amounts should be obtained (Figure 1).

Finally, adaptive DCLs can usually only be applied to targets available in reasonable amounts, like enzymes, because monitoring the library composition is a highly demanding task, however, the combination of library generation by DCC and analysis by MS binding assays should be applicable to membrane-bound proteins like G protein coupled receptors (GPCRs), ligand-gated ion channels and neurotransmitter transporters. Usually, only extremely low concentrations, typically in the sub-nanomolar range, can be reached for these kinds of

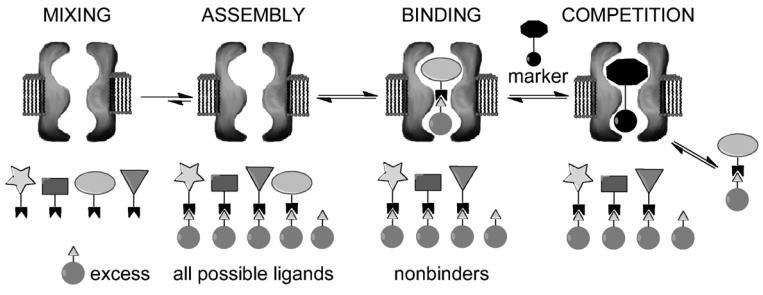


Figure 1. Graphical representation of the designed method comprising generation of pseudostatic libraries by DCC and analysis by competitive MS binding assays. Initially, a pseudostatic library is generated by combining a diverse set of building blocks in equal amounts with a large excess of one compound of complementary chemical reactivity. After equilibration of the mixture, a competitive MS binding assay is performed by adding a native marker and quantifying the amount of marker bound to the target to unravel the presence of hits.

targets. To demonstrate that the new method described here using MS binding assays as a readout can cope well under these conditions, a membrane-bound protein was employed in this study. In this context, it should also be added that MS binding assays can serve not only as readout for hit detection, but also for hit identification. The latter, however, will be the subject of further studies.

To demonstrate the feasibility of the concept outlined above, mGAT1, one of the four transporters known to mediate transport of GABA across cellular membranes in the brain, was selected as the target in this study. For this transporter, a competitive MS binding assay, employing the native marker NO711 (**6**) has previously been established.[18,19]

GAT1 (species independent denomination of mGAT1 according to the human genome organization HUGO),[20] representing the most abundant GABA transporter in the brain, is primarily located in presynaptic cell membranes of GABAergic neurons and is thus the most important subtype for the reuptake of GABA and for the termination of GABAergic neurotransmission. Decreased GABAergic neurotransmission is assumed to be a major cause of neuronal diseases like epilepsy, Parkinson's disease and sleeping disorders. Accordingly, enhancing GABA concentration in the synaptic cleft by inhibition of GABA transporters, such as GAT1, to support restoration of the neurotransmitter balance in the central nervous system (CNS) represents a rational approach to the cure of these diseases. In fact, with the introduction of the GAT1 selective inhibitor tiagabine (**5**), this transporter subtype has already been demonstrated to be a valid drug target in the treatment of epilepsy.[21]

(*R*,*S*)-Nipecotic acid ((*R*,*S*)-**2**) and guvacine (**3**) were first described to be potent in vitro inhibitors of the neuronal and glial GABA transport in the 1970s.[22,23] However, due to their highly polar nature, these compounds are not able to readily cross the blood–brain barrier and are not orally active.[24] SK&F-8997-A (**4**), tiagabine (**5**) and NO711 (**6**) are lipophilic derivatives of amino acids **2** and **3**, and represent the first orally active GAT inhibitors described.[25] These compounds are characterized by the presence of a lipophilic aromatic domain, which is attached to the amino nitrogen of the cyclic amino acid with a spacer of a definite length. As a result of this modification, compounds **4**–**6** possess a distinctly improved ability to permeate the blood–brain barrier, they exhibit high subtype selectivity in favor of GAT1,[26–29] and they also act as anticonvulsants in animal models.[30] Of these compounds, tiagabine (Gabitril; **5**), having undergone successful clinical development, is in use as an add-on medication for the treatment of epilepsy.[21,22,31] In a study by Andersen et al. published in 2001, a number of series of GAT1 inhibitors were reported in which the lipophilic moiety had been modified.[32] Compounds **7**–**9**, which are among the most potent derivatives (IC_{50} = 55–111 nM), represent typical examples that nicely demonstrate the structural variations tolerated by the target like the insertion of an ether function or the different arrangement of the aryl rings.

Inspired by inhibitors **7**–**9** (Figure 2),[32] we aimed to develop combinatorial libraries of related structures that, as already stated, could be generated by DCC. The spacer linking the hydrophilic and lipophilic halves of the molecules was deemed to be the most suitable position for the functional group required for library generation. Hydrazone formation was considered a suitable reaction. However, under the conditions typically required for binding assays as such neutral pH, hydrazone libraries equilibrate only very slowly and thus do not fully represent dynamic libraries. That said, the assay conditions employed for the generation of dynamic and static libraries are more or less the same. With this in mind, using hydrazone libraries to validate the proposed drug screening concept still seemed justified. Moreover, the use of hydrazone chemistry could significantly ease the identification of best binders through their independent resynthesis.

Accordingly, libraries based on hydrazone chemistry and containing compounds that resemble GAT1 inhibitors **7**–**9** were chosen as model compounds for this study. In line with the conditions defined above, reaction of aldehyde libraries **11** with an excess of **10** in the presence of the target mGAT1 was

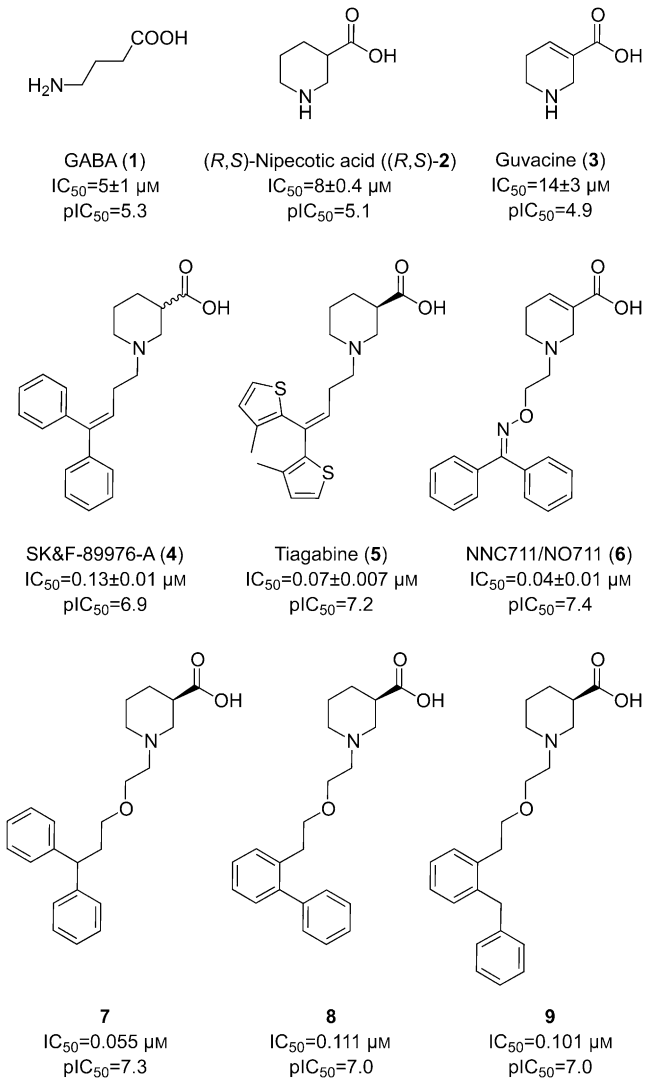
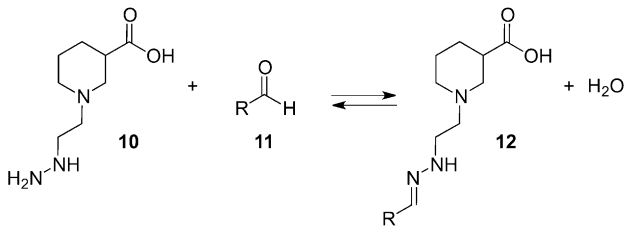


Figure 2. Structures of GABA and GAT-1 inhibitors. The pIC_{50} values quoted were calculated from the published IC_{50} values taken from Reference [30] (compounds **1**–**6**) and Reference [32] (compounds **7**–**9**).



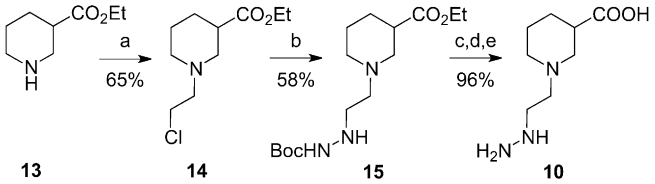
Scheme 1. Condensation of nipecotic-acid-derived hydrazine **10** with diverse aldehydes to give hydrazones with the general structure **12**.

suggested for the generation of the desired libraries (Scheme 1). Subsequent application of MS binding assays for mGAT1 to the resulting protein–ligand mixtures should identify active compounds.[18,19]

Results and Discussion

Chemistry

Synthesis of *N*-(2-hydrazinoethyl)nipecotic acid (**10**) was accomplished according to Scheme 2. Nipecotic acid ethyl ester (**13**) was transformed into **14** using a literature method.[33] Subsequent reaction with *N*-mono-Boc-protected hydrazine gave **15**. Removal of the *N*-Boc group and hydrolysis of the ester functionality under acidic conditions provided hydrazine **10**.



Scheme 2. *Reagents and conditions*: a) 1-bromo-2-chloroethane, acetone, K_2CO_3, RT, 20 h;[33] b) *tert*-butyl carbazate, EtOH, reflux, 1 h; c) TFA, CH_2Cl_2, RT, 30 min; d) 4 M HCl, 80 °C, 1 h; e) ion exchange chromatography.

Assay conditions

As already outlined above, for library screening, a competitive MS binding assay for mGAT1 based on NO711 (**6**) as a reporter ligand should be employed for hit detection.[18,19] To this end, a suitable buffer system had to be found that does not interfere with library formation, allows rapid library generation, and is compatible with the binding assay. For the original MS binding assay, a 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS; 50 mM) buffer containing sodium chloride (1 M) was used. As the buffer capacity of TRIS in the neutral pH range is rather low, and TRIS carries a primary amino functionality that could interfere with the hydrazone formation, this buffer was thought to be less suitable for the new assay.[18,19] Being nearly structurally inert and characterized by high buffer capacity, we decided to study a phosphate buffer (25 mM) containing sodium chloride (1 M) for improved binding of the reporter ligand[19] at varying near-neutral pH values.

To determine the implications of using a phosphate rather than a TRIS buffer, a series of NO711 saturation experiments at different pH values close to physiological pH were performed. The original binding assay carried out using TRIS buffer (pH 7.1) yielded a K_d value for NO711 of 23.4 ± 2.2 nM (n = 15, mean ± SEM).[18,19] The K_d values calculated for NO711 from the results of the saturation experiments performed with phosphate buffer are given in Table 1. The data shows that NO711 binding at pH 7.1 is unaffected by the buffer used (phosphate buffer: K_d = 26.5 ± 4.6 nM; TRIS buffer: K_d = 23.4 ± 2.2 nM). Furthermore, only slight changes in K_d values were observed when the pH was lowered to 6.8 (20.9 ± 3.2) or raised to 7.4 (24.7 ± 3.5), however, the K_d value varied more significantly when the pH of the binding assay was adjusted to 7.70 (42.9 ± 7.2 nM).

Table 1. Comparison of B_{max} and K_d values in phosphate-buffered solutions at varying pH levels.[a]

pH	6.80	7.10	7.40	7.70
B_{max}[b]	37.8 ± 0.5	39.1 ± 2.8	33.5 ± 0.7	40.6 ± 3.1
K_d[c]	20.9 ± 3.2	26.5 ± 4.6	24.7 ± 3.5	42.9 ± 7.2

[a] $NaH_2PO_4 \cdot H_2O$ (12.5 mM), $Na_2HPO_4 \cdot 2H_2O$ (12.5 mM), NaCl (1 M); [b] pmol mg^{-1} protein (n = 3, mean ± SEM); [c] nM (n = 3, mean ± SEM).

To study the influence of the phosphate buffer pH on the rate of hydrazone formation, a series of experiments at different pH levels were performed in which hydrazine **10** was reacted with 2-phenylbenzaldehyde (**24A**). Aldehyde **24A** was selected for two reasons: the structure is prototypic for the aldehydes intended for use, and the UV absorption of the resulting hydrazone (**24H**) differs from that of the free aldehyde to such an extent that the progress of the reaction can be directly monitored by following the increase in UV absorption of the reaction mixture without generating false results caused due to the decrease of aldehyde UV absorption at the chosen wavelength. The rate of hydrazone formation gradually increased as the pH of the phosphate buffer was lowered, with the fastest rate observed at pH 6.8, the lowest pH applied; however, the rate of hydrazone formation was still reasonably fast at pH 7.1 (Figure 3a). Therefore, this pH level was selected for the generation and screening of libraries as it represents a good compromise between the rate of hydrazone formation and the stability of the binding assay, the K_d value of NO711 varying only slightly when lowering (pH 6.8) or increasing (pH 7.4) the pH of the assay conditions.

For the current study, only small libraries containing four different hydrazones were used, with the concentration of the individual hydrazones amounting to 10 μM. This concentration was chosen as the minimum affinity—the highest IC_{50} value—a test compound should have to give a positive signal in the MS binding assay when no other active constituent is present in the library. In that case, the specific binding of the MS

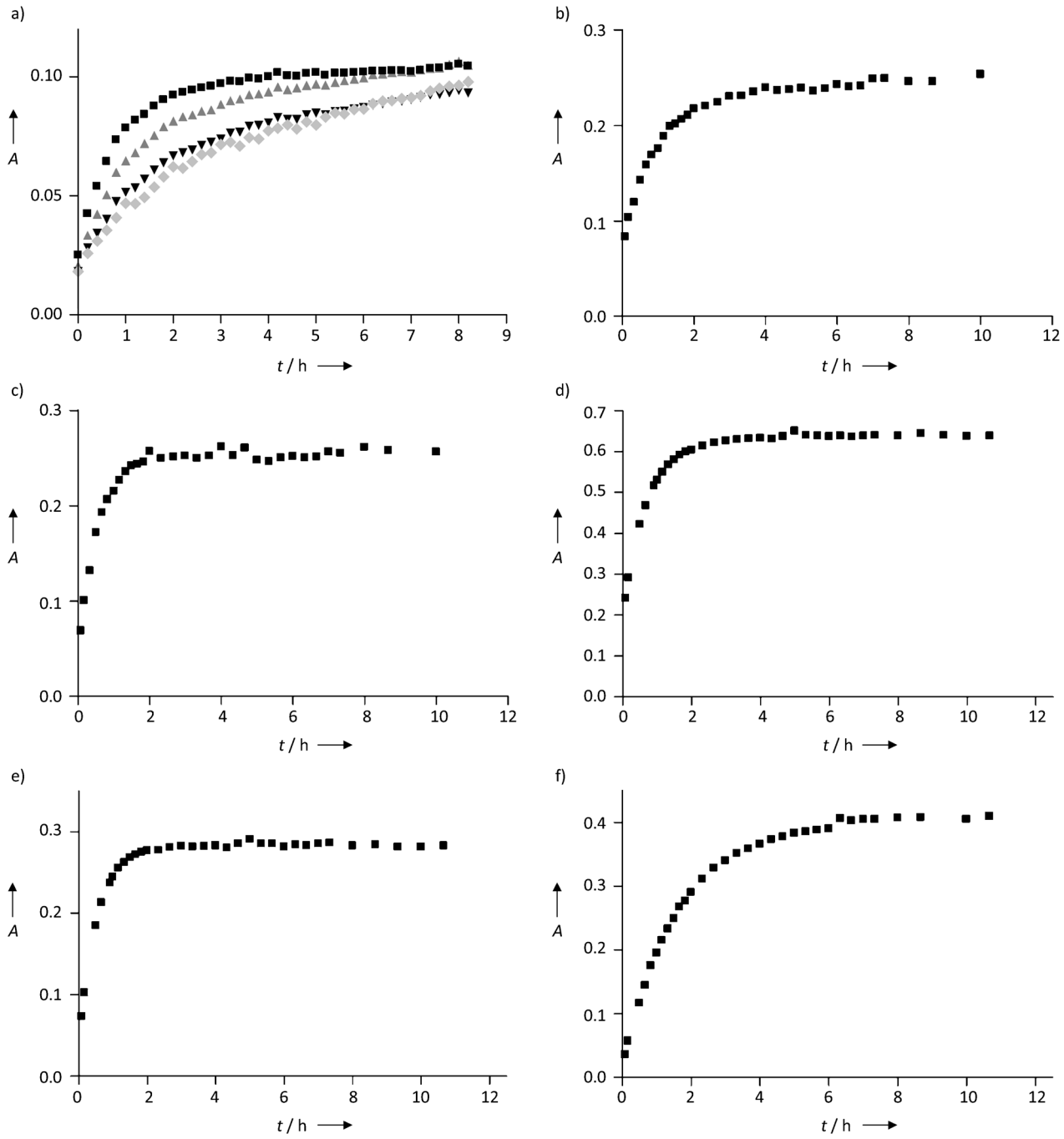


Figure 3. a) Absorbance (*A*) versus time (*t*) for the reaction of **24 A** (10 μM) with hydrazone **10** (100 μM) in phosphate buffer at different pH values: 6.8, ■; 7.1, ▲; 7.4, ▼; 7.7, ◆; ($n=12$). Reactions were monitored by UV: ε_{284nm} **10**: 180 L mol^{-1} cm^{-1}, ε_{284nm} **24 A**: 1700 L mol^{-1} cm^{-1}, ε_{284nm} **24 H**: 9720 L mol^{-1} cm^{-1}. Absorbance (*A*) versus time (*t*) for the reaction of b) **24 A**, c) **29 A**, d) **33 A**, e) **37 A** and f) **38 A** (40 μM) with hydrazine **10** (100 μM) in phosphate buffer at pH 7.1 ($n=3$).

marker NO711 (**6**) in the MS binding assay serving as a readout would be decreased to 50% or less. The concentration of nipecotic-acid-derived hydrazine **10** was set to 100 μM, which corresponds to a clear excess of the hydrazine over the aldehydes (ratio = 2.5:1), ensuring that in a potential state of equilibrium the products are favored. To apply hydrazine **10** in a still higher concentration (i.e., 1 mM) turned out to be less suitable, as in this case the hydrazine itself decreased marker binding to 62 ± 14% (mean ± SD of $n=10$ experiments with four replicates).

To get a rough estimate of the time necessary to accomplish an almost complete hydrazone formation under the conditions defined above for the generation of the library, five electronically diverse aromatic aldehydes (**24 A**, **29 A**, **33 A**, **37 A** and **38 A**) were reacted with hydrazine **10** (100 μM) in the absence of the target. To resemble the conditions of the library forma-

tion, the concentrations of individual aldehydes were set to the total concentration of aldehydes in the assay (40 μM; four aldehydes each at 10 μM). Like **24A**, aldehydes **29A**, **33A**, **37A** and **38A** were selected according to their UV characteristics so as to allow direct monitoring of the reaction by UV. In each case, almost complete equilibrium was reached within four hours (Figure 3b–f). Therefore, an incubation period of four hours for the generation of the libraries in the presence of the target, that is, in the final experiments, seemed sufficient.

In situ library generation and screening

With regard to the general considerations described above and the conclusions drawn for the assay conditions, the new method for the generation and screening of compound libraries was accomplished as follows.

1. *Generation of pseudostatic libraries by DCC*. For the generation of libraries, a set of four different aldehydes (each 10 μM) was incubated with hydrazine **10** (100 μM) and mGAT1 protein (10–20 μg per sample, total volume 250 μL) in phosphate buffer for 4 h at 37 °C in a 96-well microtiter plate.
2. *Hit detection by competitive MS binding assay*. For hit detection subsequent to library generation, NO711 was added to each sample (final concentration 20 nM) and allowed to equilibrate for 40 min at 37 °C. Experiments were terminated by filtration. Bound MS marker (NO711) was liberated by denaturing the isolated protein–ligand complexes (by drying at 50 °C for 1 h and elution with 300 μL MeOH) and quantified by LC-ESI-MS/MS. The amount of MS marker found was used to assess the potency of the library.

In the current study, 36 different aldehydes grouped in nine libraries each consisting of four members (Figure 4) were applied to test the feasibility of the concept outlined above. In each case, the generation and screening of the hydrazone libraries (generated by mixing the respective aldehyde library with hydrazine **10**, Figure 5a) was supplemented by a control experiment in which only the aldehyde library was applied to reveal the potency exerted by the aldehydes themselves. Similarly, hydrazine **10** was tested at the assay concentration (100 μM) for its inhibitory potency in the absence of aldehydes. Figure 5b shows the results of the control and library screening experiments. In control experiments, neither hydrazine **10** nor any of the aldehydes decreased marker binding to mGAT1 to a significant extent. For example, aldehyde library 1 (**16A**, **17A**, **18A**, and **19A**) had no considerable effect on NO711 binding, decreasing it slightly to 80±3%. For hydrazine **10**, the effect was even less pronounced (92±3%). However, hydrazone library 1 (generated from aldehyde library 1 and hydrazine **10**) led to a significant decrease in specific marker binding to just 26±6%, thus indicating that at least one potent hydrazone inhibitor is present in the mixture. With the hydrazine alone being almost inactive, the observed decrease must be due to hydrazone formation and cannot be the result of an additive effect of inhibition by aldehyde library and hydrazine **10**. For a purely additive effect, the decrease in NO711 binding would have to be less than the sum of the decreases observed for hydrazine **10** and the respective aldehyde library; however, this is never the case (Figure 5b).

To verify the validity of the result obtained for library 1, this library was also tested after it had been generated in an independent pre-equilibration experiment. For the formation of hydrazone library 1, hydrazine **10** was mixed with aldehyde library 1 at a 100-fold higher concentration for both components (1 mM of each aldehyde, 10 mM of hydrazine) in the absence of the target. According to ^{1}H NMR experiments, hydrazone formation was complete upon mixing (see Supporting Information). Subsequent dilution with incubation buffer by a factor of 100 gave the original concentration. This pre-equilibrated library was then evaluated in the MS binding assay, and the NO711 specific binding was found to be 31±3%. This result is in good agreement with that obtained for the four-hour experiments in which library generation takes place in the presence of the target (26±6%).

The same set of experiments with a four-hour incubation period for library generation and with a pre-equilibrated library was also carried out for aldehyde library 2. The results for these two experiments were very similar too; NO711 specific binding was found to be 29±2% after 4 h incubation (library generation in presence of target) and 25±2% when the pre-equilibrated library was used. As such, it was concluded that a four-hour incubation period in the presence of the target is sufficient to reach equilibrium and thus to obtain reliable results. Accordingly, the remaining hydrazone libraries were only generated and screened following the original procedure (4 h incubation in the presence of the target and screening by subsequent MS binding assay). Hydrazone libraries 3 and 5 turned out to be the most potent, decreasing marker binding to 7.5±3.5% and 13±1%, respectively. Though less potent, libraries 4, 8, and 9 also decreased marker binding to less than 50%, suggesting the presence of hydrazones with IC_{50} values below 10 μM. With NO711 specific binding around 50%, libraries 6 and 7 appear to be the least potent.

Deconvolution experiments

Of the nine hydrazone libraries, seven gave rise to a decrease in marker binding of at least 50%, indicating a high number of hydrazones with IC_{50} values below 10 μM. For this reason, criteria to detect an active library were tightened. Only compounds with an IC_{20} value of less than 10 μM, that is the concentration that gives rise to only 20% of the maximal activity, which corresponds to an IC_{50} value of 2.5 μM ($IC_{20} = 4 \times IC_{50}$), should be detected with this method. Consequently, the upper limit for defining a library as active was lowered to 20% remaining marker binding. This value was only achieved by the two most potent libraries, libraries 3 and 5.

For the identification of the most active compounds in libraries 3 and 5, the same protocol was followed as for library screening, except that single compounds were evaluated. Hydrazone formation was allowed to take place for four hours as for library screening (aldehyde: 10 μM; hydrazine **10**: 100 μM).

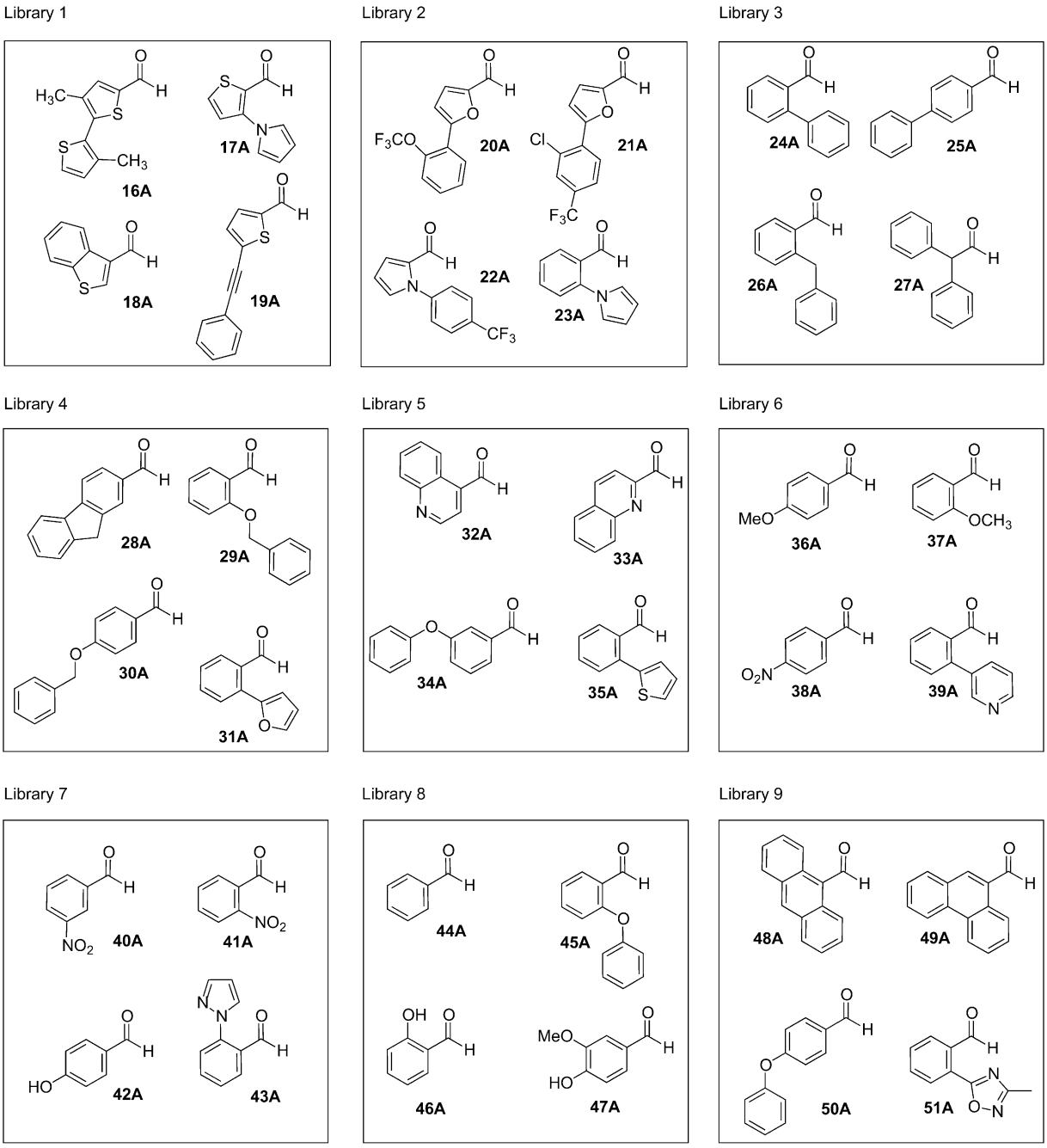


Figure 4. Aldehyde libraries 1–9 employed in drug screening.

The data for the control samples, consisting either only of hydrazine derivative **10** or aldehyde, were generated in the same way. According to the data obtained in the deconvolution experiments, the most potent inhibitors were based on 2-phenylbenzaldehyde (**24 A**) (library 3; Figure 6b) and 2-thiophen-2-ylbenzaldehyde (**35 A**) (library 5; Figure 6d). The corresponding hydrazones decreased the specific binding of NO711 to $\leq 5\%$ (**24 H**) and to $8.2 \pm 0.4\%$ (**35 H**). In addition, library 4 was evaluated as an example of a medium potency library ($30 \pm 3\%$). Aldehyde **31 A** was found to give rise to the best binding hydrazone derivative in this library. However, the inhibitory potency of **31 H** was limited, decreasing specific NO711 binding to only ~$37 \pm 1\%$. To further verify the validity of the new library generation and screening assay, the individual components of libraries 1 and 8 were also analyzed to determine the best binders. In line with the results obtained for libraries 1 and 8, none of the library members exhibited significant inhibitory potency (Figure 6a and e).

Confirmation of deconvolution results

To verify the results of the deconvolution experiments, hit compounds **24 H** and **35 H**, both decreasing marker binding to less than 20%, and medium potency hydrazones **26 H**, **31 H**

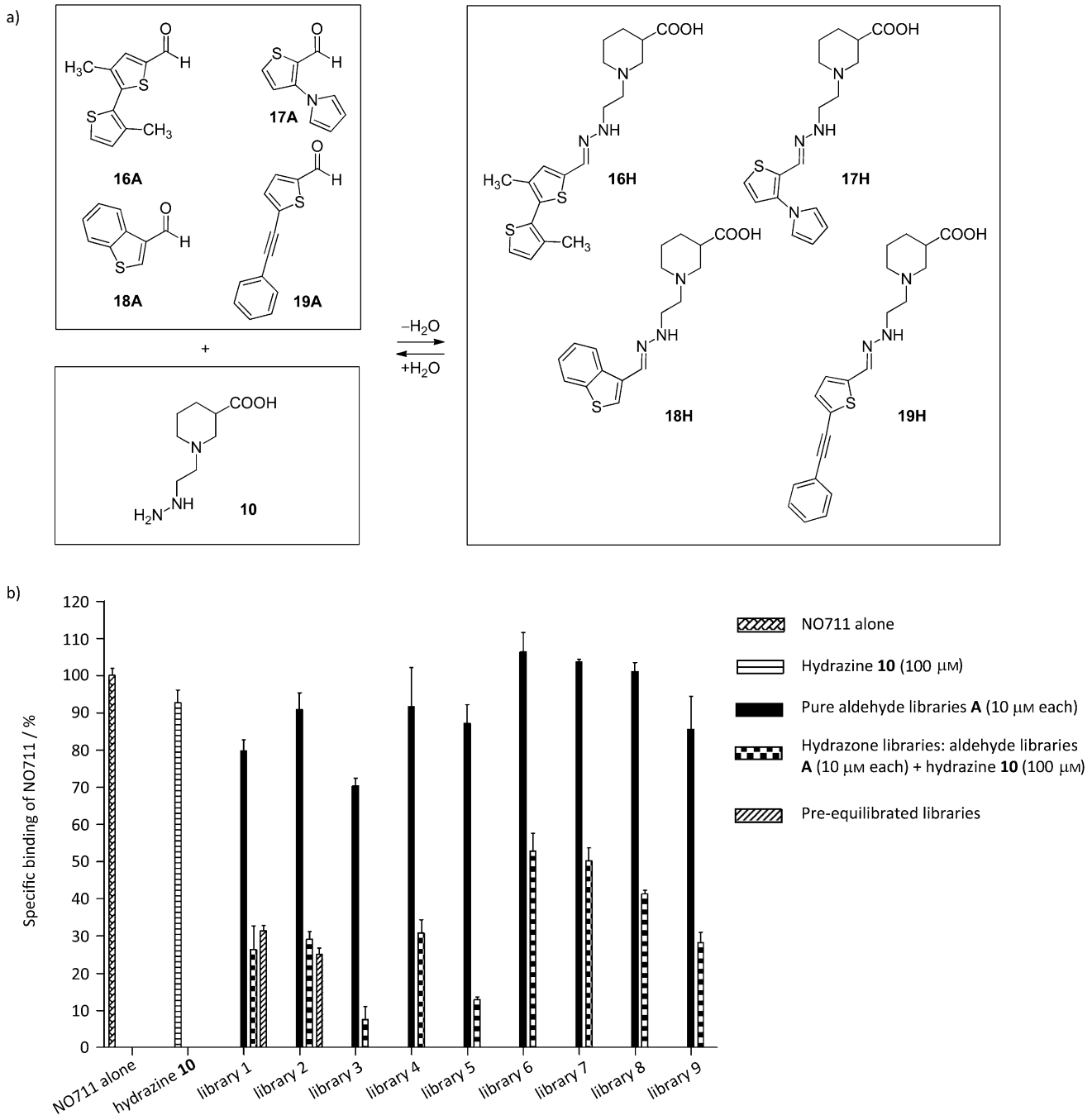


Figure 5. a) Library formation exemplified by the reaction of aldehyde library 1 with hydrazine **10**. b) Specific binding of NO711 in the presence of hydrazine **10**, pure aldehyde libraries 1–9 (for library compounds, see Figure 4), and the corresponding hydrazone libraries after an incubation time of 4 h for library generation and 40 min for marker binding. Data for pre-equilibrated hydrazone libraries 1 and 2 is also shown. Data represents the mean ± SD of four replicates.

and **45H**, each decreasing marker binding to slightly greater than 20%, were synthesized and characterized in competitive MS binding experiments to determine the binding affinities (pK_i) for mGAT1. Interestingly, like **24H** and **35H**, hydrazones **26H**, **31H** and **45H** are derived from *ortho*-substituted benzaldehydes too. Hydrazone **51H** was also evaluated as it contains an *ortho*-biaryl moiety that, considering the results obtained for compounds **24H** and **35H**, could be favorable for binding. For this compound, a member of the weakly active library 9, a binding experiment identical to the deconvolution experiment was performed (10 μM **51A** and 100 μM hydrazine **10**). However, with a remaining NO711 binding of 65 ± 8%, hydrazone **51H** exhibited only low affinity (data not shown).

Hydrolysis of the hydrazone products is possible in the absence of excess hydrazine **10**, and this could lead to false results from the binding assays. To exclude this possibility, the

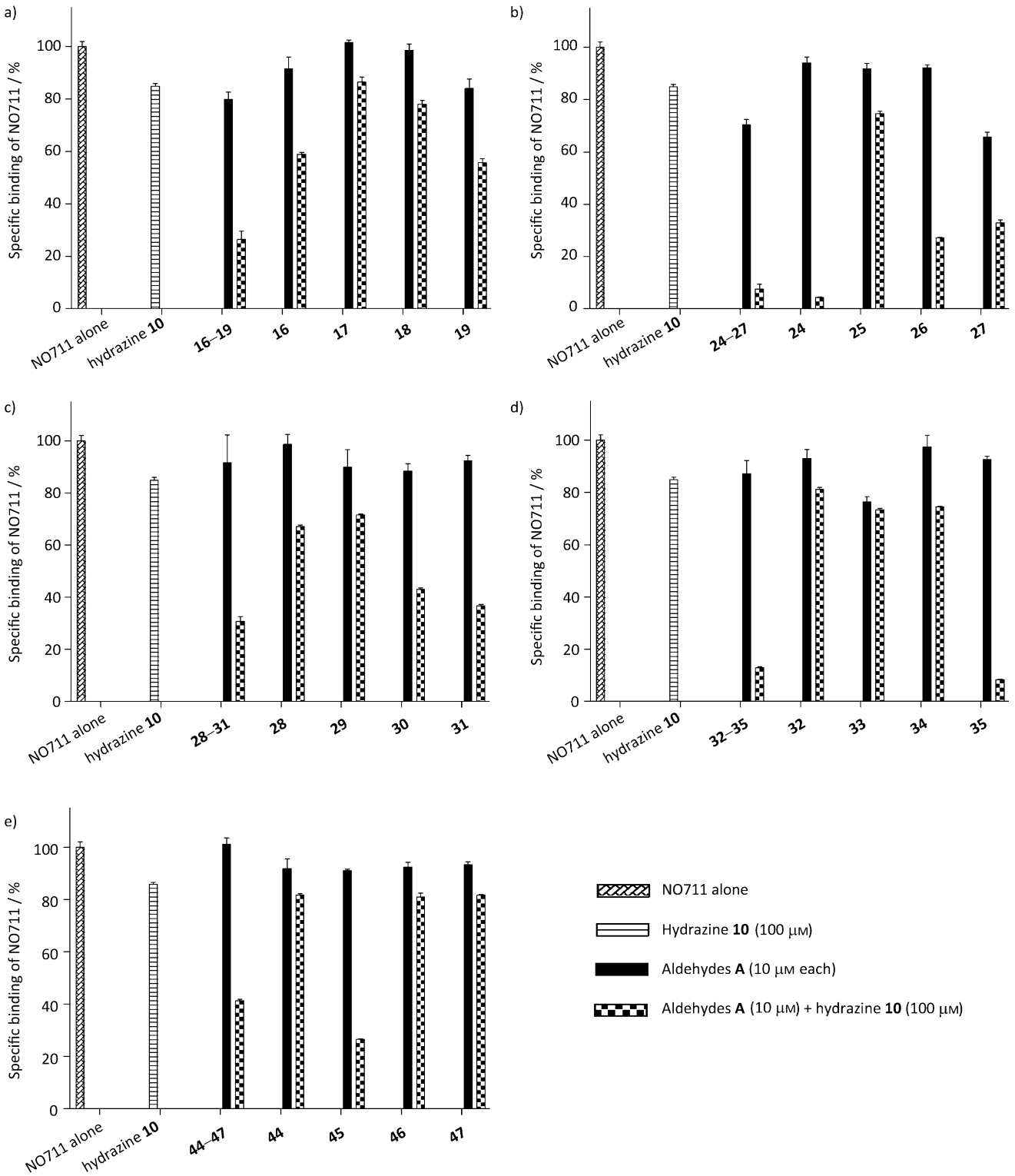


Figure 6. Deconvolution experiments of hydrazone libraries a) 1, b) 3, c) 4, d) 5, and e) 8 including control experiments (aldehyde libraries **A**, hydrazone libraries **H**, and hydrazine **10**).

stability of three representative but structurally diverse compounds (**35H**, **36H** and **38H**) was monitored at pH 7.1 by ^{1}H NMR spectroscopy (see Supporting Information). According to the results of these experiments, the tested hydrazones are sufficiently stable to be characterized in conventional competitive MS binding assays, which take two hours to perform and provide full competition curves and corresponding binding affinities. Only hydrazone **36H** decomposed slightly within the two-hour evaluation period. However, with the release of the corresponding aldehyde being only 5% of the total com-

pound, evaluation of the hydrazone should still deliver a reliable K_i value. For the sake of completeness, it should be added that the equilibrium was reached after eight hours, the ratio of hydrazone **36H** to aldehyde **36A** amounting to 84:16. For hydrazones **35H** and **38H**, no decomposition products could be seen by ^{1}H NMR after two hours. However, the dynamic character of hydrazones **35H** and **38H** became evident, too, upon extended monitoring of the equilibration. After seven hours (**35H**) and one week (**38H**), equilibrium had been reached with the hydrazones still clearly predominating, and the ratios of hydrazone to aldehyde amounting to 96:4 (**35H**)and 98:2 (**38H**).

For the determination of the binding affinities of the abovementioned hydrazone derivatives, the MS binding assay for mGAT1 as described by Zepperitz et al.[18,19] was used, except that the original TRIS buffer was replaced by the phosphate buffer employed for screening. Among the compounds tested, hit compounds **24H** and **35H** identified in the deconvolution experiments of libraries 3 and 5 again exhibited the highest potencies, with pK_i values of 6.186 ± 0.028 and 6.229 ± 0.039 (mean ± SEM, $n = 3$), respectively (Figure 7). For hydrazones **26H**, **31H**, **45H** and **51H**, which exhibited specific NO711

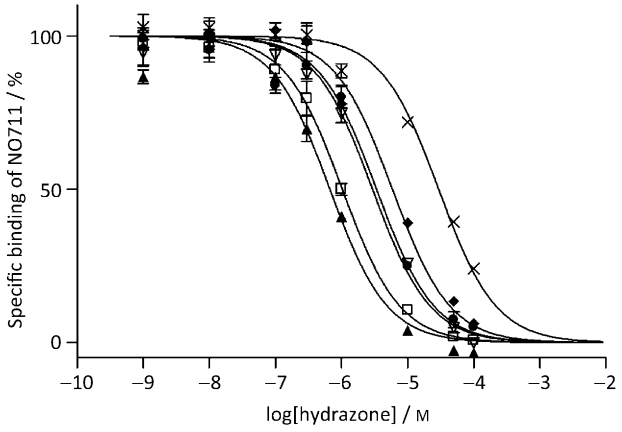
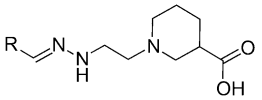
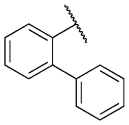
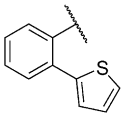
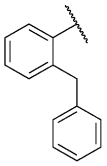
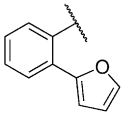
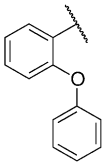
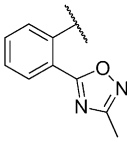


Figure 7. Representative competition curves for hydrazones **24H** (▲), **35H** (□), **26H** (●), **31H** (▽), **45H** (◆) and **51H** (×) obtained from competitive MS binding assays. Data points represent specific binding of NO711 (mean ± SD from triplicate values) in the presence of different concentrations of test compounds. Binding curves were generated by nonlinear regression.

Table 2. Comparison of NO711 binding data determined in deconvolution experiments (pK_i) and functional GAT1 activity (pIC_{50}).



Compd	R	SB[a] [%]	pK_i[b]	pIC_{50}[c]
24H		≤ 5	6.186 ± 0.028	5.308 ± 0.096
35H		8.2 ± 0.4	6.229 ± 0.039	5.542 ± 0.107
26H		27 ± 1	5.542 ± 0.042	5.186 ± 0.084
31H		37 ± 1	5.577 ± 0.037	4.895 ± 0.152
45H		24 ± 1	5.445 ± 0.075	4.879[d]
51H		65 ± 8[e]	4.479 ± 0.064	4.022[d]

[a] Specific binding (SB) of NO711 determined in deconvolution experiments under standard assay conditions: aldehyde (10 μM), **10** (100 μM), 4 h equilibration period. Specific binding < 5% indicates NO711 binding was below the lower limit of quantification (50 pM). [b] pK_i values were determined by competitive MS binding assay with NO711 performed in an mGAT1-expressing HEK293 cell membrane preparation. Data represent the mean ± SEM of three independent experiments. [c] pIC_{50} values were determined by [^{3}H]-GABA uptake assay performed in mGAT1-expressing HEK293 cells. Data represent the mean ± SEM of three independent experiments. [d] Value is the result of a single experiment. [e] Deconvolution graph not shown in Figure 6.

binding values of 27 ± 1%, 37 ± 1%, 24 ± 1%, and 65 ± 8%, respectively, in the deconvolution experiments, pK_i values of 5.542 ± 0.042, 5.577 ± 0.037, 5.445 ± 0.075, and 4.479 ± 0.064 were found (mean ± SEM, $n \geq 3$). These results not only confirm the high potency of the best hits **24H** and **35H** identified in the deconvolution experiments, but also indicate that the percentage values obtained in the deconvolution experiments provide a reasonable estimate of the pK_i values of the respective compounds, with the rank orders in both series being comparable.

In addition, hydrazone derivatives **24H**, **35H**, **26H**, **31H**, **45H** and **51H** were characterized with respect to their functional GAT1 activity. The results of these studies, accomplished by employing a [^{3}H]-GABA uptake assay for mGAT1 as described in the literature,[34] are summarized in Table 2. As can be seen from the data, the pIC_{50} values for [^{3}H]-GABA uptake by mGAT1 in first approximation run parallel to the pK_i values for binding affinity for this transporter. That the pK_i values are slightly higher than the pIC_{50} values is a common phenomenon for these assays. Furthermore, the potencies of the best hydrazone inhibitors **24H** and **35H** at mGAT1 are close to those of the model compounds **7**–**9**, which served as prototypes for the design of the hydrazone libraries. According to these results, hydrazone libraries are well suited to exploring the structural motifs required for efficient binding to a target, and hits

identified as best binders can be considered suitable templates for the development of stable analogues.

Conclusions

In this study, competitive MS binding assays that have so far only been used for the characterization of the binding affinity of single compounds have been demonstrated to be applicable also as a readout in compound screening campaigns. By employing libraries generated by using dynamic combinatorial chemistry (DCC), a new kind of library generation and screening assay has been developed. In this assay, the dynamic libraries are generated in a way that renders them pseudostatic, with the library composition thus being well defined. Furthermore, the reaction is performed in a medium suitable for the binding assay and with the target already present. This allows the resulting mixtures to be directly used for competitive MS binding assays to determine the activity of the generated libraries. MS binding assays are highly sensitive, and as such, this method could be applied to targets like membrane-bound proteins for which typically only low concentrations can be reached.

The feasibility of this method was demonstrated for the membrane-associated protein GAT1, the most abundant γ-aminobutyric acid (GABA) transporter in the central nervous system (CNS), using pseudostatic hydrazone libraries. By combining equal amounts of four different aldehydes and one hydrazine derivative (**10**) in excess in the presence of the target, pseudostatic hydrazone libraries comprising equal amounts of the desired hydrazones were generated and subsequently screened by competitive MS binding assays employing NO711 (**6**) as a native mGAT1 marker. The binding potencies of the libraries were determined from the amount of bound marker found by LC-ESI-MS/MS as part of the competitive MS binding assay. In this way, two hit libraries were identified that decrease marker binding to less than 20%. Subsequent deconvolution experiments led to the identification of hydrazones **24H** and **35H** as compounds with the highest target affinity. After individual synthesis, full competitive MS binding assays confirmed the identified hits as potent binders of mGAT1, with pK_i values of 6.186 ± 0.028 and 6.229 ± 0.039, respectively.

Having been successfully applied to screening of compound libraries generated by DCC—a rather challenging task, competitive MS binding assays can be considered as powerful tools for the identification of active compounds, suitable for most types of libraries and targets, and therefore, they could find widespread application in drug discovery.

Experimental Section

Chemistry

Solvents used were of analytical grade and freshly distilled before use except for DMSO. Ethyl nipecotate was purchased from Sigma–Aldrich and freshly distilled before use. Other purchased reagents and reactants were used without further purification. Thin-layer chromatography (TLC) was carried out on precoated silica gel F_{254} glass plates (Merck). Flash chromatography (CC) was performed using Merck silica gel 60 (mesh: 0.040–0.063 mm). ^{1}H NMR spectra were recorded at room temperature (or 25 °C for samples solved in [D_6]DMSO) on a JNMR-GX (JEOL) at 400 or 500 MHz. ^{1}H NMR chemical shifts were internally referenced to tetramethylsilane (TMS) or 1,4-dioxane for samples dissolved in D_2O. The spectra were processed using MestReNova (version 5.1.1–3092 2007; Mestrelab Research, Santiago de Compostela, Spain). Broadened signals are designated by br (s_{br}, d_{br}, t_{br}). Infrared (IR) spectroscopy was performed with an FTIR Spectrometer 410 (Jasco). Samples were measured either as KBr pellets or as films on NaCl plates. A Hewlett Packard 5989 A with 59.980 B particle beam LC–MS interface was used for mass spectrometry (ionization: chemical (CH_5^+) or electron impact (70 eV)). High-resolution mass spectrometry (HRMS) was carried out using an LTQ FT (ThermoFinnigan) or a JMS GCmate II (Jeol).

1-(2-Chloroethyl)piperidine-3-carboxylic acid ethyl ester (**14**)[33] and 2-benzylbenzaldehyde (**26A**)[35] were prepared as described in the literature.

1-[2-(2-*tert*-Butoxycarbonylhydrazino)ethyl]piperidine-3-carboxylic acid ethyl ester (15): A stirred solution of **14** (158 mg, 0.717 mmol, 1.0 equiv) in abs EtOH (3.0 mL) was treated with Et_3N (73.0 mg, 0.721 mmol, 100.0 μL, 1.0 equiv) and *tert*-butyl carbazate (304 mg, 2.30 mmol, 3.2 equiv). The mixture was heated at reflux for at least 1 h (monitored by TLC). After cooling to RT, the solvent was completely evaporated in vacuo. The crude was purified by CC (step gradient; *n*-pentane/EtOAc/Et_3N, mixture 1) 1:1:0, mixture 2) 5:5:1) providing **15** as colorless oil (131 mg, 58%): $R_f = 0.60$ (*n*-pentane/EtOAc/Et_3N, 5:5:1); ^{1}H NMR (500 MHz, CD_2Cl_2): $\delta = 1.23$ (t, $J = 7.1$ Hz, 3H), 1.43 (s, 9H), 1.37–1.50 (m, 1H), 1.50–1.61 (m, 1H), 1.65–1.75 (m, 1H), 1.83–1.93 (m, 1H), 1.95–2.05 (m, 1H), 2.17 (t_{br}, $J = 9.8$ Hz, 1H), 2.41–2.50 (m, 2H), 2.53 (tt, $J = 10.3$, 3.8 Hz, 1H), 2.70–2.80 (m, 1H), 2.90 (td, $J = 6.0$, 2.6 Hz, 2H), 2.92–3.00 (m, 1H), 4.10 (q, $J = 7.1$ Hz, 2H), 4.04–4.26 (s_{br}, 1H), 6.30–6.64 ppm (s_{br}, 1H); ^{13}C NMR (101 MHz, CD_2Cl_2): $\delta = 14.4$, 25.1, 27.4, 28.5, 42.3, 48.6, 54.0, 56.0, 57.6, 60.6, 80.1, 157.0, 174.4 ppm; IR (film): $\tilde{\nu} = 3319$, 2977, 2940, 2811, 1730, 1720, 1454, 1367, 1281, 1252, 1155, 1031 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 315 (65) $[M]^+$, 270 (13), 242 (100), 215 (67), 214 (41), 138 (33); MS (CI, CH_5^+): *m/z* (%): 316 (100) $[M+H]^+$, 260 (77), 216 (26), 170 (77); HRMS-EI (70 eV): *m/z* $[M]^+$ calcd for $C_{15}H_{29}N_3O_4$: 315.2158, found: 315.2162.

1-(2-Hydrazinoethyl)piperidine-3-carboxylic acid (10): A stirred solution of **15** (353 mg, 1.12 mmol) in CH_2Cl_2 (2.0 mL) was treated with trifluoroacetic acid (12.6 g, 110 mmol, 8.50 mL, 100 equiv) at RT. After 1 h, the solvent was evaporated in vacuo. The resulting residue was dissolved in 5 M aq HCl (10 mmol, 2 mL, 9 equiv), and the solution was heated at 80 °C for 2 h. After evaporation to dryness, the resulting HCl salt[36] was purified by ion exchange chromatography (Amberlite IRA-120; solvent: 20% aq NH_3 solution) providing **10** as a colorless, amorphous solid (195.4 mg, 93%): ^{1}H NMR (500 MHz, D_2O/NaOD): $\delta = 1.29$ (qd, $J = 12.9$, 4.2 Hz, 1H), 1.50 (qt, $J = 13.1$, 3.8 Hz, 1H), 1.68–1.78 (m, 1H), 1.87–1.95 (m, 1H), 1.99 (dt, $J = 12.2$, 2.8 Hz, 1H), 2.04 (t, $J = 11.4$ Hz, 1H), 2.34 (tt, $J = 11.8$, 3.6 Hz, 1H), 2.46–2.54 (m, 2H), 2.82–2.93 (m, 3H), 3.01 ppm (d_{br}, $J = 11.3$ Hz, 1H); ^{13}C NMR (126 MHz, D_2O/NaOD): $\delta = 24.6$, 28.2, 45.4, 50.6, 53.8, 55.7, 56.7, 183.9 ppm; IR (KBr): $\tilde{\nu} = 3700$–2300, 2946, 1581, 1451, 1396 cm^{-1}; HRMS-ESI (ESI+): *m/z* $[M+CH_3CN]^+$ calcd for $C_{10}H_{20}N_4O_2$: 228.1568, found 228.1705.

Preparation of hydrazones: Experimental protocols and characterization data for the hydrazones described here are given in the Supporting Information.

MS Binding experiments

mGAT1 Membrane preparation: Membrane preparations of HEK293 cells stably expressing mGAT1[34] were prepared as described previously and stored at −80 °C.[19] On the day of the assay, an aliquot was rapidly thawed and diluted in a 20-fold volume of cold aq 0.9% NaCl (*m/v*). After centrifugation at 15000 rpm and 4 °C for 20 min (CP56GII, P70AT, Hitachi Ltd., Tokyo, Japan), the pellet was resuspended in ice-cold assay buffer (see below) to a protein concentration of approximately 0.1 mg mL^{-1} (determined as described previously).[19]

MS Binding assay: Saturation experiments (at various pH levels) and competition experiments for isolated hydrazones were performed as described recently,[18,19] but substituting the original incubation buffer for phosphate buffer (12.5 mM $Na_2HPO_4{\cdot}2H_2O$, 12.5 mM $NaH_2PO_4{\cdot}H_2O$, 1 M NaCl adjusted to the respective pH value with 2 M NaOH). Unless otherwise indicated, phosphate buffer at pH 7.1 was used in binding assays.

Library screening: Library screening experiments were performed with quadruplicate samples in a total volume of 250 μL in 1.2 mL polystyrene deep-well plates (Sarstedt, Nümbrecht, Germany). A solution of four individual aldehydes (100 μM each) in 10% DMSO/phosphate buffer (*v/v*, pH 7.1; 25 μL) and a solution of **10** (1 mM) in phosphate buffer (pH 7.1, 25 μL) were added to additional phosphate buffer (pH 7.1, 125 μL). The first incubation period (library equilibration) was initiated by addition of the mGAT1 membrane preparation (50 μL) immediately after combining hydrazine and aldehydes (delay <1 min). After 4 h at 37 °C (shaking water bath), NO711 (25 μL, 200 nM) was added to start the second incubation period (binding experiment). The binding experiment was stopped after 40 min at 37 °C (shaking water bath) by transferring an aliquot (200 μL) of each sample onto a 96-well filter plate (Acroprep, glass fiber, 1.0 μm, 350 μL; Pall Corp, Dreieich, Germany) with a 12-channel pipette and subsequent vacuum filtration. The filter plate was washed ice-cold 1 M NaCl solution (5×150 μL per well) using a 12-channel pipette, dried (60 min, 50 °C) and cooled to room temperature. Afterwards, liberation of the marker was achieved by elution of the filter plate with MeOH (3×100 μL per well) into a 1.2 mL polypropylene deep-well plate (Sarstedt, Nümbrecht, Germany). Finally, each sample was supplemented with 1 nM [$^2H_{10}$]NO711 in MeOH (200 μL) as an internal standard and then dried for 12–16 h (50 °C). For quantification by LC-ESI-MS/MS, samples were reconstituted in 10 mM NH_4HCO_2 buffer (pH 7.0)/MeOH (95:5, *v/v*, 200 μL).

Each library screening experiment included samples characterizing the pure aldehyde libraries (phosphate buffer (pH 7.1, 150 μL), a solution of four individual aldehydes (25 μL; 100 μM each), and 10% DMSO/phosphate buffer (pH 7.1, *v/v*), mGAT1 membrane preparation (50 μL) and 200 nM NO711 (25 μL)) and pure hydrazine **10** (phosphate buffer (pH 7.1, 125 μL), 10% DMSO/phosphate buffer (pH 7.1, *v/v*, 25 μL), hydrazine **10** (1 mM) in phosphate buffer (pH 7.1, 25 μL), mGAT1 membrane preparation (50 μL) and 200 nM NO711 (25 μL)). Total binding and nonspecific binding of NO711 was determined in analogously constituted samples lacking any inhibitor or in the presence of 100 mM GABA, respectively. Additionally, matrix blanks, zero samples and matrix standards were obtained in the same way, performing the binding experiment without NO711 and inhibitors. After filtration and elution, samples were supplemented with MeOH (200 μL), 1 nM [$^2H_{10}$]NO711 in MeOH (200 μL) or methanolic calibration standards containing 1 nM [$^2H_{10}$]NO711 and 50 pM, 100 pM, 500 pM or 1 nM NO711, respectively. Calibration curves for marker quantitation were generated using these standards.

Deconvolution experiments: The deconvolution experiments were carried out in the same way as described for library screening, except that a solution of single aldehyde in 10% DMSO/phosphate buffer was used (pH 7.1, *v/v*, 100 μM, 25 μL).

Pre-equilibrated libraries: A solution of four individual aldehydes (1 mM each) in 10% DMSO/phosphate buffer (pH 7.1, *v/v*, 10 μL) and a solution of **10** (10 mM) in phosphate buffer (pH 7.1, 10 μL) were added to phosphate buffer (pH 7.1, 80 μL). The mixtures were incubated for 1 h at 37 °C (shaking water bath) to guarantee full conversion to the corresponding hydrazones. After addition of phosphate buffer (pH 7.1, 900 μL), aliquots of the resulting solutions (25 μL) were supplemented with phosphate buffer (pH 7.1, 150 μL), mGAT1 membrane preparation (50 μL), and 200 nM NO711 (25 μL). Subsequently, the samples were processed as described for library screening.

Analysis

LC-ESI-MS/MS: Quantitation by LC-ESI-MS/MS was performed as described previously using a API 3200 triple-quadrupole mass spectrometer and by drying and reconstituting the methanolic eluates.[18,19]

Analysis of binding experiments: Marker depletion was negligible (<10%) in all binding experiments. Equilibrium dissociation constant (K_d) and density of binding sites (B_{max}) were calculated from one-site saturation isotherms of specific binding by means of the nonlinear curve-fitting program Prism 4.02 (GraphPad Software, San Diego, CA, USA). Specific binding was defined as the difference between total and nonspecific binding. Nonspecific binding below 50 pM could not be determined experimentally, but was extrapolated by linear regression for nonspecific NO711 binding concentrations ≥50 pM. The concentration of a competitor that inhibits 50% of specific binding (IC_{50}) was calculated from competition curves plotting NO711 specific binding concentrations against the log of the competitor concentration (eight different concentrations per competitor) with Prism 4.02 using the equation for one-site competition and nonlinear curve fitting. Specific binding determined for control samples in the absence of any competitor was set to 100%, whereas the bottom level was set to 0%. K_i values were calculated according to Cheng and Prusoff[37] and are expressed as pK_i values. Unless stated otherwise, all results are expressed as the mean ± SEM. pK_i values were determined in at least three separate experiments.

UV-monitoring of hydrazone formation: UV monitoring was carried out in a 96-well quartz glass UV plate (Hellma) with a SpetraMax M2e (Molecular Devices) plate reader and analyzed with SoftMax Pro 5.4 software. The incubation buffer described for the MS binding assay was used at a pH 7.1 except for comparing various pH values (pH 6.8, 7.1, 7.4, 7.7). The total volume of the samples was 300 μL. Each experiment was supplemented with blank samples that were composed of 270 μL phosphate buffer and 30 μL of 10% DMSO (*v/v*) in phosphate buffer (pH 7.1).

Comparison of different pH values: The experiment was performed with twelve identically constituted replicates. Phosphate buffer (240 μL) was supplemented with a solution of **24A** (30 μL, 100 μM) in 10% DMSO/phosphate buffer (*v/v*). To start the reaction, hydrazine **10** (1 mM, 30 μL) was added. Absorption values at 284 nm were determined immediately after placing the plate into

the instrument. Further absorption values were recorded at 12 min intervals for 8 h.

Monitoring of reaction progress: The experiment was performed with identically constituted triplicates. Phosphate buffer (240 μL) was supplemented with the desired aldehyde (400 μM, 30 μL) and 10% DMSO/phosphate buffer (*v*/*v*). To start the reaction, hydrazine **10** (1 mM, 30 μL) was added. Absorption values at a wavelength of 284 nm for conversion of **24A**, **29A**, **33A** and **37A** and 356 nm for conversion of **38A** were determined immediately after placing the plate in the instrument and then at intervals of 10 min or 20 min for 11 h.

GABA uptake assays: [^{3}H]GABA uptake assays were performed as previously described.[34]

Keywords: binding assays · dynamic combinatorial chemistry · hydrazones · mass spectrometry · membrane proteins

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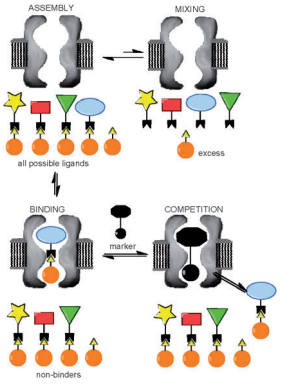
Library Screening by Means of Mass Spectrometry (MS) Binding Assays—Exemplarily Demonstrated for a Pseudostatic Library Addressing γ-Aminobutyric Acid (GABA) Transporter 1 (GAT1)



Mix and measure: The generation of compound libraries by dynamic combinatorial chemistry in the presence of a target and subsequent library screening by competitive mass spectrometry (MS) binding assays represents a new and highly efficient approach to drug discovery. This method, which requires the compound libraries to be rendered pseudostatic, has been successfully applied to mGAT1, the most abundant GABA transporter in the brain, leading to potent hits for this target.

