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Screening for new ligands of the MB327-PAM-1 binding site of the nicotinic acetylcholine receptor

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ABSTRACT

Intoxications with organophosphorus compounds (OPCs) effect a severe impairment of cholinergic neurotransmission that, as a result of overstimulation may lead to desensitization of nicotinic acetylcholine receptors (nAChRs) and finally to death due to respiratory paralysis. So far, therapeutics, that are capable to address and revert desensitized neuromuscular nAChRs into their resting, i.e. functional state are still missing. Still, among a class of compounds termed bispyridinium salts, which are characterized by the presence of two pyridinium subunits, constituents have been identified, that can counteract organophosphate poisoning by resensitizing desensitized nAChRs. According to comprehensive modeling studies this effect is mediated by an allosteric binding site at the nAChR termed MB327-PAM-1 site. For MB327, the most prominent representative of the bispyridinium salts and all other analogues studied so far, the affinity for the aforementioned binding site and the intrinsic activity measured in *ex vivo* and in *in vivo* experiments are distinctly too low, to meet the criteria to be fulfilled for therapeutic use. Hence, in order to identify new compounds with higher affinities for the MB327-PAM-1 binding site, as a basic requirement for an enhanced potency, two compound libraries, the ChemDiv library with 60 constituents and the Tocriscreen Plus library with 1280 members have been screened for hit compounds addressing the MB327-PAM-1 binding site, utilizing the [$^2H_6$]MB327 MS Binding Assay recently developed by us. This led to the identification of a set of 10 chemically diverse compounds, all of which exhibit an IC_{50} value of $\leq$ 10 μM (in the [$^2H_6$]MB327 MS Binding Assay), which had been defined as selection criteria. The three most affine ligands, which besides a quinazoline scaffold share similarities with regard to the substitution pattern and the nature of the substituents, are UNC0638, UNC0642 and UNC0646. With binding affinities expressed as pK_i values of 6.01 ± 0.10, 5.97 ± 0.05 and 6.23 ± 0.02, respectively, these compounds exceed the binding affinity of MB327 by more than one log unit. This renders them promising starting points for the development of drugs for the treatment of organophosphorus poisoning by addressing the MB327-PAM-1 binding site of the nAChR.

1. Introduction

The use of sarin as a chemical warfare agent in a brutal civil war in Syria in recent years (OPCW 2020a) as well as the confirmed, repeated use of organophosphates in poisoning individuals as in case of Alexei Nawalny (OPCW 2020b) and of Sergei Skripal and his daughter (OPCW, 2018) show that organophosphorus compounds (OPCs) still pose a serious threat to humanity. This class of compounds exerts its detrimental pathophysiological effect via inactivation of the enzyme acetylcholinesterase (AChE). The AChE is a fundamental part of signal

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Fig. 1. Structure of MB327, [$^2H_6$]MB327 and PTM0022.

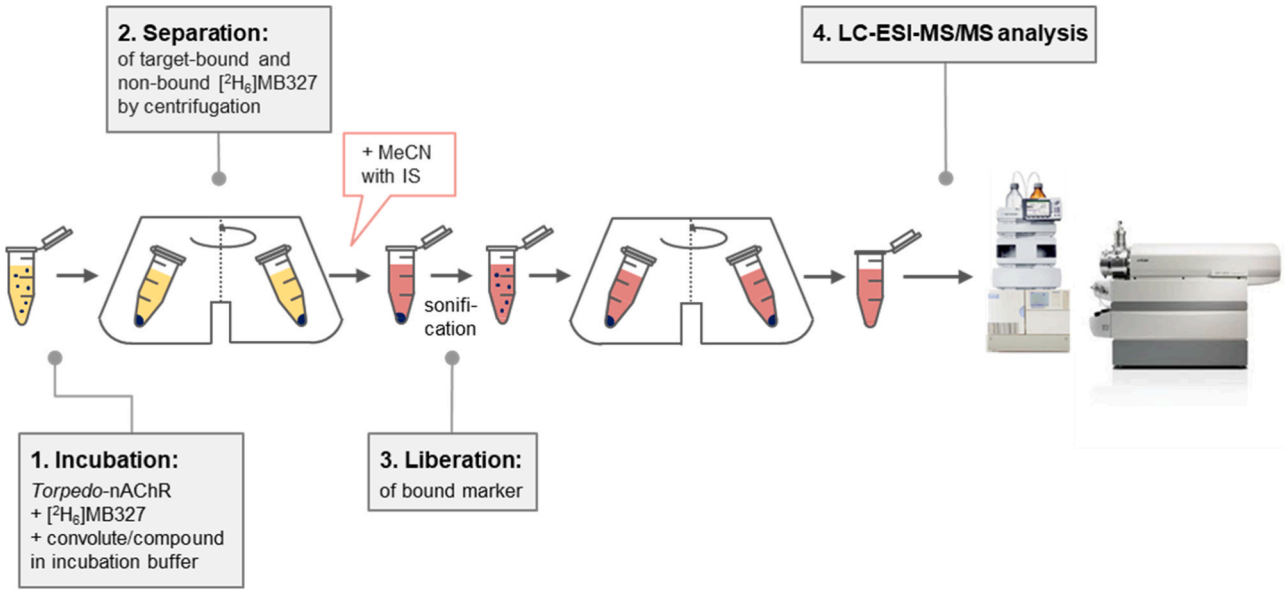


Fig. 2. MS Binding Assay workflow. The process of preparation and processing of binding samples was conducted as previously described comprising the following four steps: (1) Incubation of [$^2H_6$]MB327 as marker and aliquots of the Torpedo membrane preparation in incubation buffer, (2) Separation, (3) Liberation of bound marker and (4) LC-ESI-MS/MS analysis for quantification of bound marker. IS = internal standard {[$^2H_{18}$]MB327}.

transmission of the nervous system and responsible for the clearance of the released neurotransmitter acetylcholine from the synaptic cleft. Thus, inactivation of AChE leads to an accumulation of acetylcholine and as a consequence thereof to an overstimulation of muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptors. mAChR overstimulation can be antagonized by atropine, for instance, whereas no specific therapeutic compound is available to counteract the overstimulation of nAChR, which ultimately shifts into a non-conducting state of desensitization thereby disrupting cholinergic signaling (Papke, 2014). Desensitization of nicotinic acetylcholine receptors (nAChRs) at neuromuscular junctions leads finally to death due to respiratory paralysis.

MB327 (see Fig. 1) has been described as a potential antidote in case of organophosphate poisoning and, interestingly, has been discussed as a resensitizer of the nAChR. Recent electrophysiological studies from Niessen et al. demonstrate that the bispyridinium compound MB327 can functionally recover ("resensitize") the nAChR from desensitization (Niessen et al., 2018, 2016). In addition to the agonist, i.e. orthosteric binding site, the nAChR exhibits allosteric binding sites, as well, which have been found to allow a modulation of the function of the receptor (Arias, 2011, 2010; Changeux and Edelstein, 2005; Chatzidaki and Millar, 2015). Only recently modeling studies with MB327 identified an allosteric binding site at the nAChR termed MB327-PAM-1, which is believed to mediate its resensitizing effect on the desensitized receptor (Kaiser et al., 2023). Prior to that, pharmacological studies had demonstrated, that MB327 exerts a beneficial pharmacological effect against OPC poisoning e.g. was found to increase survival in tabun-poisoned guinea pigs *in vivo* (Turner et al., 2011). Also, for soman-impaired neuromuscular transmission in human intercostal muscle and rat diaphragm preparations, MB327 could be shown to restore muscle force (Seeger et al., 2012).

However, the toxicity of MB327 limits its use as an antidote in OPC poisoning (Price et al., 2016). To induce recovery of poisoned muscle *in vitro*, MB327 must be applied in concentrations of at least 100–200 $\mu mol \bullet L^{-1}$ to exert a clear therapeutic effect (Seeger et al., 2012), whilst its administration is accompanied with toxic effects in a concentration only marginally higher. Accordingly, its therapeutic window for effective treatment of OPC poisoning is likely to be too narrow. In fact, MB327 has been discussed to be also an AChE inhibitor and ion channel blocker at micromolar concentrations (Niessen et al., 2011; Tattersall, 1993; Turner et al., 2011). This lack of potency and selectivity of MB327 is to a large extent attributed to its low affinity for the target. In consequence, for an increased therapeutic efficacy new compounds with enhanced affinity for the MB327 binding site are to be identified.

The aim of the present study was to identify new compounds targeting the MB327-PAM-1 binding site at the nAChR, with distinctly higher affinity as compared to MB327. To this end commercially available compound libraries should be screened utilizing the [$^2H_6$]MB327 MS Binding Assay developed by us.

In a previous publication, we introduced MS Binding Assays (mass spectrometry based binding assays) as test system for the characterization of ligands binding to the MB327-PAM-1 binding site of the nAChR (Sichler et al., 2018). MS Binding Assays are based upon the same setup as conventional radioligand binding assays but use a native marker, termed MS marker, instead of a radiolabeled ligand (Höfner and Wanner, 2015). While MS Binding Assays offer a simple working principle and provide reliable results, same as radioligand binding assays, all disadvantages and restrictions, that arise from the use and the handling of radioactivity when performing radioligand binding experiments, e.g. the necessity to comply with safety regulations, the need to safely

dispose radioactive waste, etc. can be circumvented (Grimm et al., 2015; Hess et al., 2011; Neiens et al., 2015). The general setup and performance of MS Binding Assay is illustrated in Fig. 2 and involves the four following distinct steps: (1) incubation of marker, also termed reporter ligand, with the target protein, (2) separation of target protein with bound marker from the incubation system e.g. by filtration or centrifugation, (3) liberation of bound marker from the target protein and (4) LC-MS/MS analysis for the quantification of formerly bound MS marker.

For the quantification of the marker, a deuterated analogue of MB327, [$^2H_6$]MB327 (see Fig. 1), that is used in our MS Binding Assays addressing the MB327-PAM-1 binding site, a reliable, sensitive, and robust LC-ESI-MS/MS method has been developed and validated according to the recommendation of the FDA guidance for bioanalytical method validation (Sichler et al., 2018).

As target *Torpedo*-nAChR (from *Torpedo californica* also termed *Tetronarce californica*), which is a well-established substitute of the human muscle-type nAChR has been used, as the latter, unfortunately, is not available in the amounts required for this type of binding experiments (Millar, 2003; Navedo et al., 2004). In our binding assays, we had found the binding affinity of MB327 to be in the micromolar range {saturation binding experiments using [$^2H_6$]MB327 as marker yielded a K_d of 15.5 ± 0.9 μmol•L^{-1}; autocompetition binding experiments using [$^2H_6$]MB327 as marker and MB327 as competitor yielded a $K_{i,MB327}$ of 18.3 ± 2.6 μmol•L^{-1} (Sichler et al., 2018)}, which is in line with the potencies found in functional assays before (Seeger et al., 2012; Niessen et al., 2016).

In most recent work, we synthesized two series of structurally different MB327 analogues and characterized these in terms of their binding affinities towards the MB327-PAM-1 binding site (Rappenglück et al., 2018a; Rappenglück et al., 2018b). One series consisted of a set of symmetric analogues of MB327 with lipophilic and hydrophilic substituents at the pyridinium subunits. Following evidence that the putative MB327 binding sites could be non-symmetrical with regard to their polarity (Wein et al., 2018), in addition, a second series of bispyridinium salts, this time exhibiting hydrophilic on the one and lipophilic residues on the other of the two pyridinium subunits, has been synthesized. When the constituents of these two series of compounds were characterized with regard to their binding affinity for the MB327-PAM-1 binding site, PTM0022, an at both pyridinium rings 3-phenyl substituted analogue of MB327, (see Fig. 1), was identified as the most affine ligand, its pK_i value amounting to 5.16 ± 0.07.

In the pursuit to identify further ligands at the MB327 binding site with higher affinities as compared to MB327, as a second, additional route, a screening approach, as a very common technique in biomedical research, should be followed. That way, on the one hand the suitability of the [$^2H_6$]MB327 MS Binding Assay established by us for compound library screening should be demonstrated and on the other, new compounds with an affinity towards the MB327 binding site distinctly surpassing that of MB327 be discovered.

2. Material and methods

2.1. Material

MB327 and analogues [$^2H_6$]MB327 and [$^2H_{18}$]MB327 were synthesized in-house by S. Rappenglück and were of ≥ 95% purity measured by ^{1}H NMR spectroscopy (Rappenglück et al., 2018a). *Torpedo californica* (also termed *Tetronarce californica*) electroplaque tissue was obtained from Aquatic Research Consultants (San Pedro, CA, USA). Compound libraries Tocriscreen Plus and the ChemDiv ion channel ligand library were purchased from Tocris (Bristol, UK) and ChemDiv (San Diego, US), respectively. All test compounds were supplied 10 mmol•L^{-1} in DMSO. Water for incubation buffers and LC-MS-mobile phase was prepared in-house by distillation of demineralized water (prepared by reverse osmosis) and subsequent filtration using 0.45 μm filter material. For LC-MS, HPLC grade acetonitrile was obtained from VWR Prolabo (Darmstadt, Germany). Ammonium formate as additive for mobile phase buffer was purchased from Sigma-Aldrich (for mass spectrometry, ≥ 99%, Taufkirchen, Germany). All other chemicals were of analytical grade. All percentages and ratios given are specified as *v/v* ratios.

2.2. MS Binding Assays

MS Binding Assays were performed with [$^2H_6$]MB327 as marker and nAChR-enriched membranes, prepared from *Torpedo californica* electroplaque tissue, as previously described (Sichler et al., 2018; Rappenglück et al., 2018a). Membranes from frozen *Torpedo californica* electroplaque tissue were prepared as reported and stored in storage buffer (120 mmol•L^{-1} NaCl, 5 mmol•L^{-1} KCl, 8.05 mmol•L^{-1} Na_2HPO_4, 1.95 mmol•L^{-1} NaH_2PO_4, pH 7.4). Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as standard (Smith et al., 1985). All binding experiments were performed as described previously (Rappenglück et al., 2018a). For binding samples for convolute testing and deconvolution and samples for compound characterization i.e. competition experiments, aliquots of the membrane preparation (60–120 μg protein per sample) were incubated with [$^2H_6$]MB327 as marker at 10 μmol•L^{-1} in triplicates in storage buffer (= incubation buffer) in a total volume of 125 μL for 2 h at 25 °C. For each binding experiment, non-specific binding was determined by heat denaturation subjecting aliquots of the *Torpedo californica* membrane preparation to a temperature of 60 °C for 1 h in a shaking water bath prior to incubation with marker and test compounds. In competition experiments the concentrations of the competitors ranged from 1 nmol•L^{-1} - 100 μmol•L^{-1}. After incubation, binding samples were processed and analyzed exactly as previously described (Sichler et al., 2018), if not stated otherwise.

2.3. LC-MS-Instrumentation and marker quantification

LC-MS-analysis was performed on an API 3200 triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) with a Turbo-V source coupled to an Agilent 1200 HPLC system (vacuum degasser, quaternary pump, column oven; Agilent, Waldbronn, Germany) and a Shimadzu SIL-HTA autosampler (Shimadzu, Duisburg, Germany). For quantification of [$^2H_6$]MB327 as marker in MS Binding Assays the YMC Triart Diol-HILIC column (50 mm×2.0 mm, 3 μm; YMC Europe GmbH, Dinslaken, Germany) was used as stationary phase, protected by two in-line filters (0.5 and 0.2 μm, IDEX, Wertheim-Mondfeld, Germany) upstream to the column. For all experiments, the column temperature was set to 20 °C. A composition of acetonitrile and ammonium formate buffer (20 mmol•L^{-1}, pH 3.0) in a ratio of 80:20 was employed as mobile phase at a flow rate of 800 μL•min^{-1}. The samples, dissolved in acetonitrile and ammonium formate buffer (20 mmol•L^{-1}, pH 3.0) in a ratio of 90:10, were injected in a volume of 10 μL to the column. Within the API 3200 mass spectrometer, Q1 and Q3 were operated with unit resolution. [$^2H_6$]MB327 (marker) and [$^2H_{18}$]MB327 (internal standard) were monitored at the mass transitions of *m/z* 159.2/144.3 and *m/z* 165.2/147.2, respectively.

2.4. Library screening

For preparation of convolutes, we pooled 8 test compounds (10 mmol•L^{-1} in DMSO) of the respective library into one stock solution, by combining 2.5 μL of each compound solution and added incubation buffer to a final volume of 1 mL, resulting in a final concentration of 25 μmol•L^{-1} for each test compound. For testing of convolutes, we prepared and processed binding samples with [$^2H_6$]MB327 as marker at 10 μmol•L^{-1} and aliquots of the membrane preparation and the respective convolute comprising 8 test compounds at 10 μmol•L^{-1} (see MS Binding Assays for more detail). Active convolutes, i.e. convolutes that met the criteria set for selection were subjected to deconvolution experiments, where all 8 test compounds of one convolute were tested

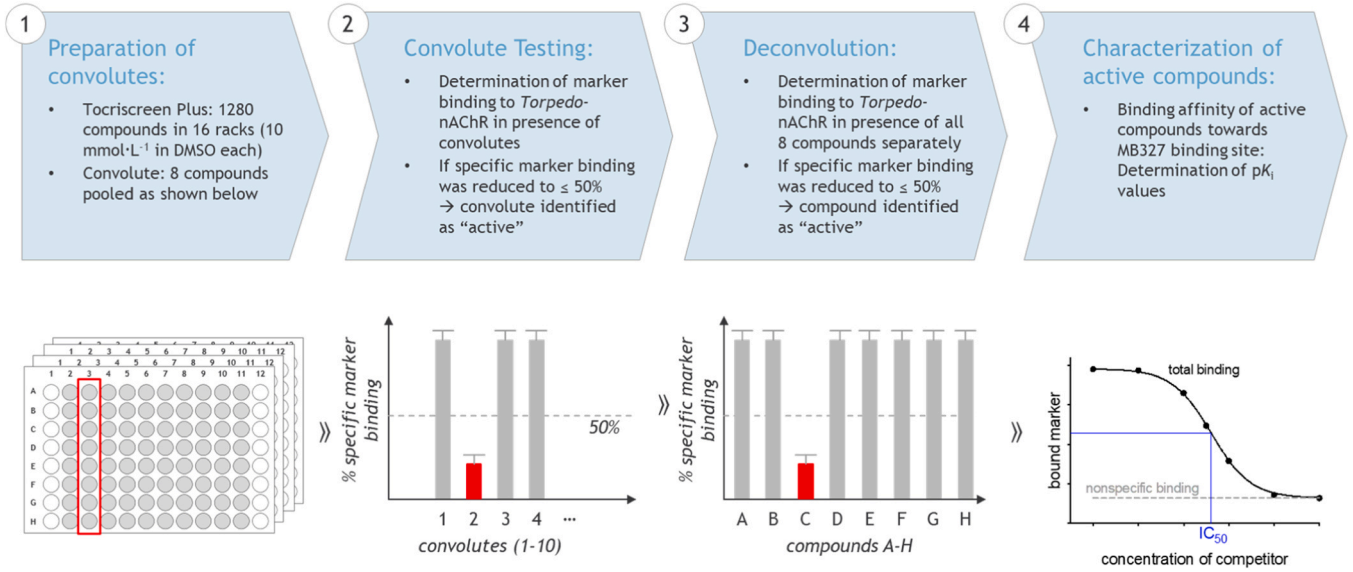


Fig. 3. Library Screening approach, illustrated with the Tocriscreen Plus library as example: For preparation of convolutes (step 1) 8 compounds in wells A-H per rack were pooled into a stock solution to be used in the binding assay. As 80 compounds were supplied per rack this makes a total of 10 convolutes per rack. Convolutes were tested in binding assays (at 10 μmol•L^{-1} for each compound) with [$^2H_6$]MB327 as reporter ligand and the *Torpedo*-nAChR as target. Convolutes were referred to as active, if their presence resulted in reduction of specific marker binding to a level ≤ 50% as compared to binding in absence of test compounds (shown in red for step 2). These active convolutes were subjected to deconvolution (step 3 in the screening process) i.e. single testing of each compound A-H (each at 10 μmol•L^{-1}) in the binding assay analogous to step 2. Analogous to convolute testing, a compound was considered as active, if its presence in binding samples resulted in reduction of specific marker binding to a level ≤ 50% as compared to specific binding in absence of a test compound. This is shown for compound C in the example. As final step, step 4, these compounds, identified as active, were characterized in terms of their binding affinity towards the MB327-PAM-1 binding site at the *Torpedo*-nAChR by determination of pK_i values in competition experiments.

separately at 10 μmol•L^{-1} in binding samples with [$^2H_6$]MB327 as marker and aliquots of the membrane preparation. Active compounds were further characterized in terms of their binding affinity towards the MB327 binding site. To this end, we conducted competition experiments with [$^2H_6$]MB327 as marker and active compounds as competitor in a concentration range from 1 nmol•L^{-1} to 100 μmol•L^{-1}. pK_i values were obtained by analysis of the respective competition curves with Prism v. 5.0 (GraphPad Software, La Jolla, CA, USA). Studies for the determination of the effect of AZ10417808 on [$^2H_6$]MB327 binding were conducted in analogy to competition experiments.

2.5. Data analysis

Based on the determined calibration function the concentration of bound marker in the binding samples was determined using Analyst v.1.6.1 (Sciex, Darmstadt, Germany). Specific binding was defined as the difference between total binding and non-specific binding. Competition curves were analyzed with the "One site - Fit Ki" regression tool (Prism v. 5.0, GraphPad Software, La Jolla, CA, USA) by means of nonlinear curve fitting, fixing top and bottom level of the sigmoidal competition curves to total binding (in absence of competitor, n = 3) and non-specific binding (determined by heat denaturation, n = 3). K_i values were calculated from the thus obtained IC_{50} values according to the Cheng-Prusoff equation by means of the aforementioned program {with K_d value for [$^2H_6$]MB327 from Rappenglück et al., 2018a}. If not stated otherwise, pK_i values are given as means ± SEM.

3. Results and Discussion

As already indicated, in this study compound libraries should be screened in order to identify new ligands of the MB327-PAM-1 binding site of the nAChR, that exhibit distinctly higher affinities compared to MB327. For this purpose, a compound library with a total of 60 ion channel ligands from ChemDiv Inc and a collection of 1280 compounds representing known bioactive compounds with diverse structures from Tocris, the Tocriscreen Plus library, should be studied. With the constituents of these libraries addressing ligand gated ion channels (library from ChemDiv Inc.) and dozens of pharmacological targets including GPCRs, ion channels, enzymes and transporters, to name only a few (Tocriscreen Plus library) the screening was thought to cover a broad set of different chemotypes, which might eventually lead to the identification of a reasonable number of novel binders of the MB327-PAM-1 binding site.

Fig. 3 illustrates the screening approach, we followed in this study for the screening of the Tocriscreen Plus library, which consists of the following four distinct steps: (1) Preparation of convolutes, (2) convolute testing, (3) deconvolution and (4) characterization of active compounds.

As an initial step in the screening approach all > 1300 compounds from both libraries to be tested were pooled into smaller sets of compounds, so-called convolutes. For this work, we chose to pool eight test compounds to make up one convolute, as a bigger convolute size is likely to lead to uncertain results and to a reduction of the efficiency of the screening process. In the case of the Tocriscreen Plus library, which was supplied in 16 racks with 80 compounds each, we combined all columns A to H into convolutes, i.e. 10 convolutes per rack, as illustrated in Fig. 3. As all test compounds from both libraries, the Tocriscreen Plus as well as the ChemDiv library, were supplied in a concentration of 10 mmol•L^{-1} in DMSO, dilution of one convolute i.e. eight test compounds to 10 μmol•L^{-1} in the incubation mix resulted in a final DMSO assay concentration of 0.8%. To ensure this would not adversely impact the binding experiment, we conducted autocompetition experiments, i.e. binding experiments with [$^2H_6$]MB327 as marker and MB327 as competitor, both in presence and absence of 0.8% DMSO in the assay, to compare affinity constants in both settings. As we did not observe any significant difference in competition curves nor corresponding pK_i values, we concluded that a concentration of 0.8% DMSO in the binding assay is well tolerated. As a next step, step 2, in the screening approach, we tested all convolutes in the binding assay with [$^2H_6$]MB327 as reporter ligand and the *Torpedo*-nAChR as target as previously described

Table 1
Results of convolute testing with [$^2H_6$]MB327 as reporter ligand and Torpedo-nAChR as target. Results are shown for active convolutes only.

Library	Convolute ID	Specific marker binding[a]
Tocriscreen Plus	02–05	33.1 ± 4.2
	03–02	12.1 ± 7.7
	03–04	**285.2 ± 27.8**
	05–01	48.6 ± 4.6
	07–03	41.3 ± 14.3
	09–07	28.0 ± 4.7
	11–05	27.4 ± 5.5
	12–05	17.6 ± 6.6
	12–07	15.5 ± 12.5
	14–04	4.8 ± 3.8
	14–05	5.7 ± 1.9
	14–09	12.6 ± 4.4
	14–10	19.8 ± 4.5
	15–03	32.5 ± 2.1
	15–08	40.4 ± 5.2

[a] % specific binding (mean ± SD, n = 3) of 10 μmol•L^{-1} [$^2H_6$]MB327 in presence of 8 test compounds of a convolute, each at a concentration of 10 μmol•L^{-1} as compared to specific marker binding in absence of test compounds.

(Rappenglück et al., 2018a). Accordingly, for each convolute comprising eight test compounds at a final concentration of 10 μmol•L^{-1} one binding sample was prepared, in which, in addition, the reporter ligand at the same concentration (10 μmol•L^{-1}) and aliquots of the *Torpedo* membrane preparation in incubation buffer was contained. Further, binding samples were prepared in absence of test compounds with [$^2H_6$]MB327 at 10 μmol•L^{-1} to determine total as well as non-specific binding (using heat shock as previously described) as a control. Comparing specific marker binding in presence of the convolute, i.e. the eight test compounds, with marker binding towards the *Torpedo*-nAChR in absence indicates if one or more test compounds have an effect on [$^2H_6$]MB327 binding. As we were looking for hit compounds with an IC_{50} of ≤ 10 μmol•L^{-1}, we considered a convolute as active, if its presence in binding samples resulted in a reduction of specific marker binding, defined as the difference between total and non-specific binding, to a level of ≤ 50% (specific marker binding in absence of any competitor was defined as 100%). Under these conditions, with all test compounds being present at a concentration of 10 μmol•L^{-1} in the binding assay, it is to be expected that one compound within the convolute targets the MB327 binding site with an IC_{50} value of ≤ 10 μmol•L^{-1}, provided no additive or allosteric effects are present. The phenomenon of additive effects will be discussed later in this chapter. The third step in the screening approach, called deconvolution, aims at the identification of the hit or possibly hit compounds within the active convolute responsible for the reduction of specific marker binding to ≤ 50%. As the sample size was quite small, we decided to test all compounds of the respective active convolute separately in the same setting as in convolute testing. With the focus on the identification of the hit compounds with an IC_{50} of ≤ 10 μmol•L^{-1} {in the [$^2H_6$]MB327 MS Binding Assay} the threshold for a compound to be referred to as active, remained, of course, unchanged, i.e. it had to reduce specific marker binding to a level of ≤ 50%. The final step of the screening approach aims at the characterization of the binding affinity (pK_i values) towards the

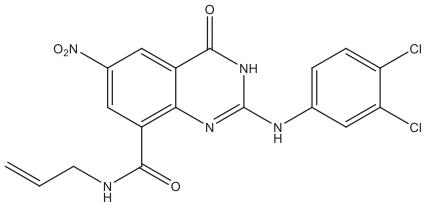


Fig. 5. AZ10417808.

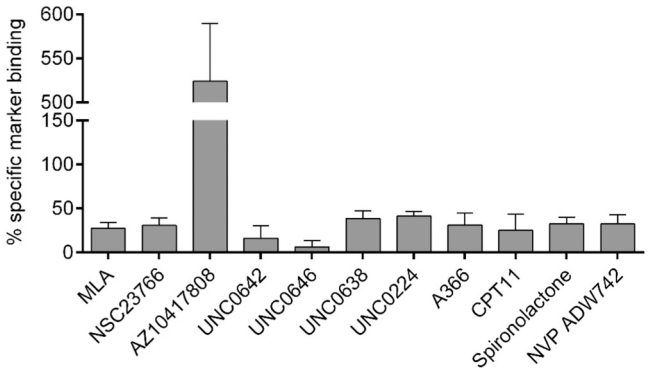


Fig. 6. Results from deconvolution experiments. Only test compounds are shown, in whose (at 10 μmol•L^{-1}) presence specific binding of [$^2H_6$]MB327 (10 μmol•L^{-1}) towards the Torpedo-nAChR was reduced below 50%, with the exception of AZ10417808, where a potentiation of marker binding was observed instead. Specific marker binding (mean ± SD, n = 3) is given as percentage compared to specific marker binding in absence of test compounds.

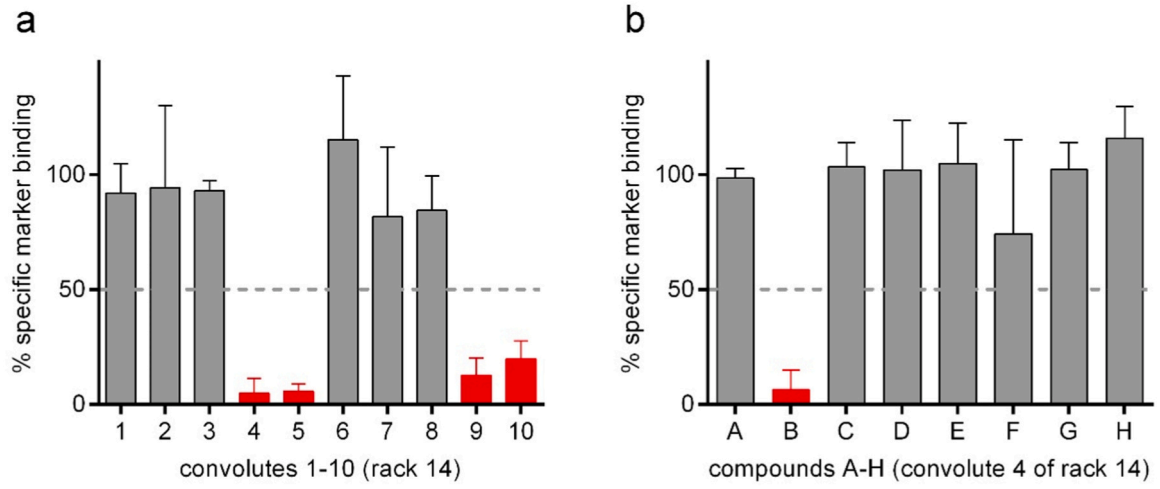
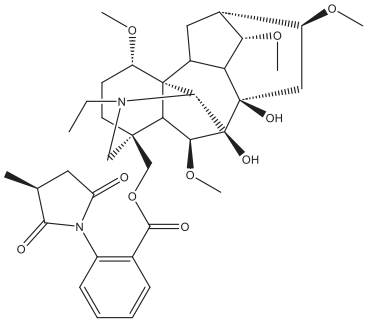
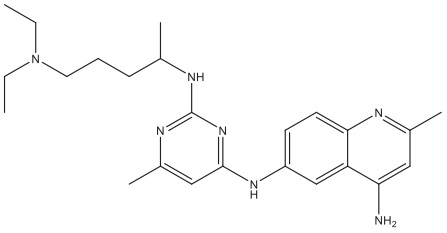
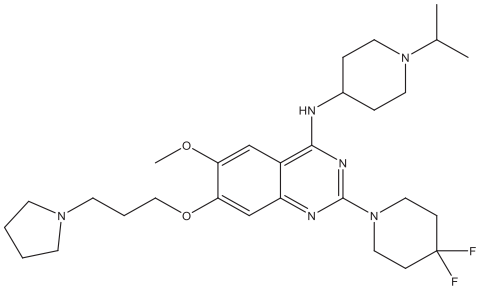
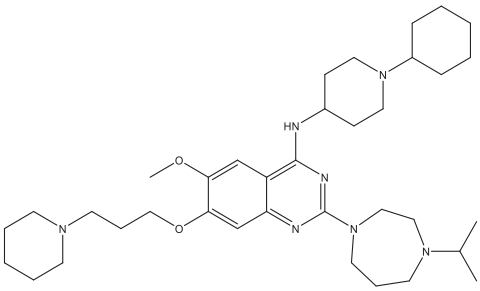
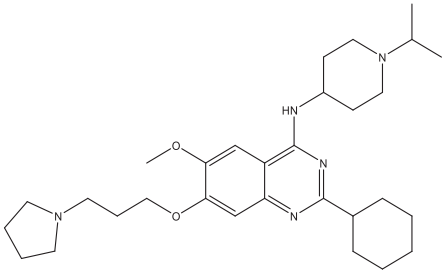
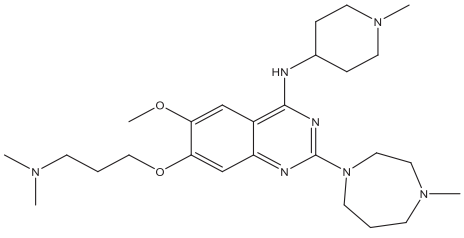


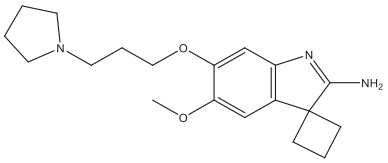
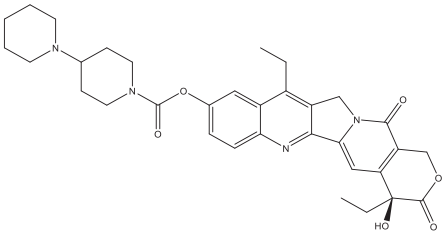
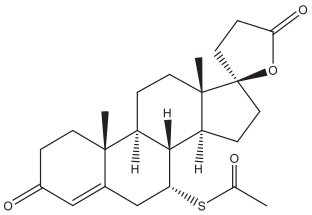
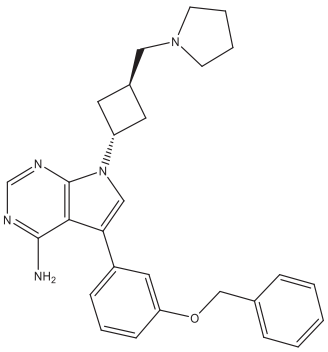
Fig. 4. Exemplary results of testing of convolutes 14–01 to 14–10 of the Tocriscreen Plus library, rack 14. Specific binding (mean ± SD, n = 3) of 10 μmol•L^{-1} [$^2H_6$]MB327 in presence of (a) 8 test compounds of a convolute or (b) one compound of the respective convolute, each at a concentration of 10 μmol•L^{-1}. Specific marker binding is given as percentage compared to specific marker binding in absence of test compounds.

Table 2
Results from competition binding experiments to determine pK_i values for active compounds. Binding experiments were conducted with 10 μmol•L^{-1} [$^2H_6$]MB327 as marker and *Torpedo*-nAChR as target.

Entry	Compound, Biological Activity[a]	Chemical Formula	pK_i[b]
1	**Methyllycaconitine** *α7 neuronal nicotinic receptor antagonist*		5.45 ± 0.18
2	**NSC23766** *Antioncogenic, Selective inhibitor of Rac1-GEF interaction*		5.49 ± 0.16
3	**UNC0642** *Potent and selective G9a and GLP histone lysine methyltransferase inhibitor*		5.97 ± 0.05
4	**UNC0646** *Potent and selective G9a and GLP histone lysine methyltransferase inhibitor*		6.23 ± 0.02
5	**UNC0638** *Potent and selective G9a and GLP histone lysine methyltransferase inhibitor*		6.01 ± 0.10
6	**UNC0224** *Potent and selective G9a and GLP histone lysine methyltransferase inhibitor*		5.23 ± 0.15

(*continued on next page*)

Table 2 (*continued*)

Entry	Compound, Biological Activity[a]	Chemical Formula	pK_i[b]
7	**A366** *Potent and selective G9a and GLP histone lysine methyltransferase inhibitor*		5.52 ± 0.15
8	**CPT11** *Antioncogenic, DNA topoisomerase I inhibitor*		5.15 ± 0.05
9	**Spironolactone** *Mineralocorticoid receptor antagonist*		5.07 ± 0.09
10	**NVP ADW742** *ATP-competitive inhibitor of IGF1R*		5.64 ± 0.08

[a] Data from Tocriscreen Plus.
[b] Data are given as mean ± SEM of three independent experiments.

Torpedo-nAChR of the respective hit compounds, to which end full scale competitive [$^2H_6$]MB327 MS Binding Assays should be performed.

In case of the ChemDiv compound library, convolute testing showed no distinct reduction of [$^2H_6$]MB327 binding in presence of any of the tested convolutes (see supplementary information). In contrast, testing of Tocriscreen Plus convolutes showed significant reduction of [$^2H_6$]MB327 binding in presence of convolutes (comprising eight test compounds) in several cases. In the screening 14 out of 160 convolutes were found to be active. These are listed in Table 1 (for the complete set of results of convolute testing see supplementary information). There, in Table 1, as well as in the following text, for an easier reference, convolutes originating from the Tocriscreen Plus library will be characterized by the ID XX-YY, where XX is the rack number (1−16) and YY (1−10) represents the convolute number of the corresponding rack. Interestingly, in one case, i.e. in presence of convolute 03–04, highlighted in bold in Table 1, a distinct increase of specific marker binding up to 285.2 ± 27.8% was observed.

Of the 14 convolutes identified as active four were found on rack 14. The results of the screening of the 10 convolutes (convolutes 1–10) contained in rack 14 of the Tocriscreen Plus library are exemplarily given in Fig. 4a.

Of these four active convolutes, convolute 14–04 reduced specific [$^2H_6$]MB327 binding down to a value as low as 4.8 ± 6.6% (mean ± SD, n = 3). A subsequently performed deconvolution of convolute 14–04 revealed, that the activity of this convolute arises from compound B, identified as compound UNC0646, which reduced specific marker binding to 6.4 ± 8.5% (mean ± SD, n = 3), whereas all other compounds (A and C-H) are inactive (see Fig. 4b) according to the set selection criteria {reduction of [$^2H_6$]MB327 binding to ≤ 50%}.

In case of convolute 14–10, during deconvolution even two active compounds, that reduced specific [$^2H_6$]MB327 binding to 41.4 ± 3.6% and 31.2 ± 9.5% (mean ± SD, n = 3) in the binding assay, respectively, have been identified. In contrast, though convolutes 05–01, 07–03, 09–07, 11–05 and 12–05 had fulfilled the activity criteria, deconvolution experiments did not lead to any hit compound {reduction of [$^2H_6$]MB327 binding to ≤ 50%}. This is certainly to be attributed to a so-called additive effect, where several compounds within the convolute have only minor effects on marker binding, which, however, together are large enough to reduce marker binding to ≤ 50%, wherefrom convolutes are considered as active.

Interestingly, convolute 03–04 was found to exhibit a distinct potentiation of reporter ligand binding. In subsequent deconvolution experiments, AZ10417808 was identified as the compound of this convolute, to be responsible for this effect. When tested separately in a binding sample (at 10 μmol•L^{-1}) AZ10417808, the structure of which is given in Fig. 5, caused an increase of [$^2H_6$]MB327 binding up to 523.9. ± 79.9% (mean ± SD), or in other words by a factor of about 5.

Overall, in deconvolution experiments in total 10 compounds have

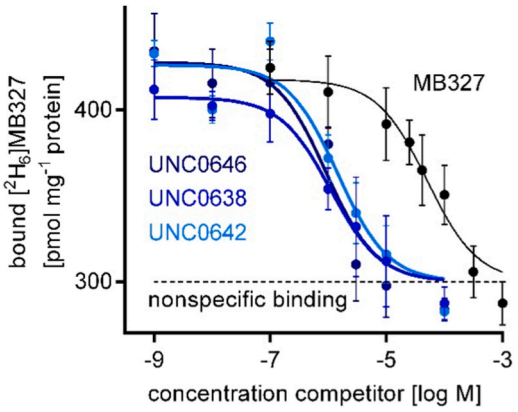


Fig. 7. Representative competition experiments conducted with 10 μmol•L^{-1} [$^2H_6$]MB327 as marker and *Torpedo*-nAChR as target employing UNC0646, UNC0638, UNC0642 and MB327 as competitor. Total binding (mean ± SD) of marker in presence of the respective competitor in a concentration range of 1 nmol•L^{-1} to 1 mmol•L^{-1}. Non-specific binding of marker was determined in triplicates by heat denaturation subjecting aliquots of the Torpedo membrane preparation to a temperature of 60 °C for 1 h in a shaking water bath prior to incubation with marker and test compounds. Nonlinear regression analysis yielded pK_i values of 6.27, 6.19, and 6.07 for UNC0646, UNC0638, UNC0642, respectively, in the particular experiments.

been identified as active. The results of the deconvolution experiments, i.e. for the compounds which due to the reduction of marker binding to ≤ 50% are considered to target the MB327 binding site with an $IC_{50} \leq 10$ μmol•L^{-1}, and for AZ10417808 enhancing MB327 binding are summarized in Fig. 6.

As final step in the screening approach, each of the hit compounds has been characterized with regard to its binding affinity constant (pK_i value) for the MB327 binding site in full scale competitive [$^2H_6$]MB327 MS Binding Assays. The results, the pK_i values for active compounds determined in 3 independent binding experiments are listed in Table 2. In Table 2 also the structures of the hit compounds as well as the biological activity, they are known for in literature, is contained.

As mentioned above, AZ10417808, which is described as selective non-peptide caspase-3 inhibitor in literature, did not reduce but increase [$^2H_6$]MB327 binding. To shed some light on the effect of AZ10417808, additional [$^2H_6$]MB327 binding experiments were performed, in which the concentration of AZ10417808 has been gradually increased from 10 nmol•L^{-1} to 100 μmol•L^{-1}. This led to a continuous rise without any sign of plateauing of [$^2H_6$]MB327 binding, with a value of 9128 ± 1796% (mean ± SEM, n = 3) reached at 100 μmol•L^{-1} of AZ10417808, the highest concentration applied. With the amount of [$^2H_6$]MB327 binding {approx. 9000 pmol•[mg protein]$^{-1}$ in presence of 100 μmol•L^{-1} AZ10417808} being way above the nAChR concentration B_{max} {approx. 300 pmol•[mg protein]$^{-1}$,Sichler et al., 2018; Rappenglück et al., 2018a} in the binding experiments, it appears unlikely that the observed phenomenon may arise from any kind of specific binding. Hence, this topic was not pursued any further.

Each of the 10 hit compounds, shown in Table 2, targets the MB327 binding site at the *Torpedo*-nAChR with an affinity that is higher, than that exhibited by MB327 (pK_i = 4.73 ± 0.03, Rappenglück et al., 2018a). The pK_i values of three of these compounds, UNC0224 (Table 2, Entry 6), CPT11 (Table 2, Entry 8) and Spironolacton (Table 2, Entry 9) towards the MB327 binding site amounting to 5.23 ± 0.15, 5.15 ± 0.05 and 5.07 ± 0.09, respectively, are in the same range as the value of PTM0022 (Fig. 1, pK_i = 5.16 ± 0.07, Rappenglück et al., 2018a), which represents the compound with the highest affinity towards the MB327 binding site described so far. In comparison to MB327 (pK_i = 4.73 ± 0.03, Rappenglück et al., 2018a) the binding affinity (pK_i) for the MB327 binding site of *Torpedo*-nAChR of Methyllycaconitincitrat (MLA, pK_i = 5.45 ± 0.18, Table 2, Entry 1), which has been described as a potent antagonist of α7 nAChR, is increased by 0.70 log units. For NSC23766 (Table 2, Entry 2), A366 (Table 2, Entry 7) and NVP ADW742 (Table 2, Entry 10) the increase in binding affinity is even somewhat higher, the differences of the pK_i values amounting to 0.74, 0.77 and 0.89, respectively. The compounds with the highest affinities, UNC0638 (Table 2, Entry 5), UNC0642 (Table 2, Entry 3) and UNC0646 (Table 2, Entry 4), all of which belong to the same class of compounds, show an increase in binding affinities towards the *Torpedo*-nAChR of even more than one log unit with pK_i values of 6.01 ± 0.10, 5.97 ± 0.05 and 6.23 ± 0.02, respectively. Representative competition curves obtained for these three compounds, UNC0638, UNC0642 and UNC0646, are displayed in Fig. 7 visualizing the distinct shift and increased binding affinity of these compounds as compared to MB327, of which UNC0646 represents the first submicromolar binder. Previously, these compounds have been described as selective inhibitors of the G9a and GLP histone lysine methyltransferase. Here, we demonstrate for the first time, that they are also potent ligands of the MB327-PAM-1 binding site of the *Torpedo*-nAChR. Interestingly, the pK_i value of UNC0224 is distinctly lower than those of the other three quinazoline derivatives, UNC0638, UNC0642 and UNC0646, despite the fact, that these compounds show a high degree of structural similarities. Detailed studies are required to clarify the effect of the individual structural characteristics such as the substitution pattern and the nature of substituents and of the heterocyclic scaffold on the binding affinity to finally establish structure activity relationships for this class of compounds. Such studies as well as studies aiming at the determination of the intrinsic activity of these compounds, in particular, in *ex vivo* assays with rat diaphragm preparations impaired by OPC treatment – showing first positive results for UNC0638 and UNC0642 (Nitsche et. al., 2024) – and in animal models of organophosphate intoxication are underway.

4. Conclusion

In this study, more than 1300 compounds were tested regarding their affinity to the MB327-PAM-1 binding site of the *Torpedo californica*-nAChR within a screening approach applying the recently developed [$^2H_6$]MB327 MS Binding Assay. A set of 10 compounds was identified as active, i.e. showed a reduction of specific [$^2H_6$]MB327 binding to a level of ≤ 50%, when applied in a concentration of 10 μmol•L^{-1}, which equals an IC_{50} value of ≤ 10 μmol•L^{-1}.

These 10 active compounds were characterized in terms of their binding affinities to the MB327 binding site at the *Torpedo californica*-nAChR by means of the recently developed [$^2H_6$]MB327 MS Binding Assay. As compared to the bispyridinium salt PTM0022, a derivative of the prototypic ligand MB327 addressing the MB327-PAM-1 binding site, with the highest affinity known so far for the aforementioned binding site, the binding affinities of some of these new ligands are at least similar to that of this bispyridinium salt. For some compounds, the binding affinity even exceeds that of PTM0022 by up to one log unit (pK_i). The ligand with the highest affinity overall was identified as UNC0646 with a binding affinity towards the MB327 binding site in the submicromolar range (pK_i = 6.23 ± 0.02).

The identification of UNC0638, UNC0642 and UNC0646 representing the most affine ligands of the MB327-PAM-1 binding site known so far also nicely underlines the potency and efficiency of the [$^2H_6$]MB327 MS Binding Assay as a tool in library screening as well as for affinity characterization of identified hit compounds.

So far it is still unknown, to what extent the hit compounds identified in this study are capable to counteract organophosphate poisoning in *in vivo* models, which to clarify will have to be the subject of future experiments. Most of all UNC0638, UNC0642 and UNC0646 as the compounds with the highest affinity for the MB327-PAM-1 binding site identified so far appear as the most promising starting points for the development of antidots against organophosphate poisoning. This is, in particular, true for UNC0638 and UNC0642, which have already been found to restore muscle function of soman-poisoned muscle tissue in *ex*

vivo experiments (Nitsche et al., 2024).

CRediT authorship contribution statement

Klaus T. Wanner: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Sonja Sichler:** Investigation, Validation, Writing – original draft. **Thomas Seeger:** Investigation, Writing – review & editing. **Franz Worek:** Writing – review & editing. **Franz F. Paintner:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Georg Höfner:** Conceptualization, Methodology, Validation. **Valentin Nitsche:** Investigation, Validation, Writing – original draft. **Karin V. Niessen:** Writing – review & editing.

Declaration of Competing Interest

Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2024.02.004.

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