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NMDA-receptor-inhibitors“

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1 Introduction

1.1 The N-methyl-D-aspartate-receptor (NMDAR)

1.1.1 Occurrence

Most research has been on N-methyl-D-aspartate-receptors (NMDARs) in the central nervous system (CNS). NMDAR-ion-channels represent a subtype of ionotropic glutamate (=excitatory amino acid) receptors, and are involved in a wide number of excitatory neurotransmission processes in the brains of vertebrates [1].

NMDARs play a pivotal role in regulation of synaptic development and function and the generation of rhythms for breathing and locomotion. Moreover, NMDARs are important for refinement of synaptic connections with experience and synaptic plasticity, which are processes underlying learning and memory [1]. Though, NMDAR-overstimulation in neurons contributes to several progressive brain disorders as stroke, pain and numerous neurological diseases like for instance epilepsy, Parkinson, Alzheimer's and Huntington's disease [1,2-7].

Originally, there were a number of studies proving the presence of NMDAR-like receptors in invertebrates such as arthropodes, nematodes, molluscs, honeybees, crabs, drosophilae, etc. [1,8-12]. An initial study with rats suggested the appearance of postsynaptic NMDA-receptors in neurons distributed throughout the nervous system. This study suggested that NMDARs mainly

occur in neurons in brain structures, in particular in the hippocampus, the cerebellum, the hypothalamus, the brainstem and the spinal cord [13]. Another study showed the evidence of both pre- and postsynaptic NMDARs in the ventral and dorsal horn of the spinal cord [14]. Interestingly, the presence of NMDARs in neurons is not limited to synapses since there are also extrasynaptic receptors [15]. Beside neurons, one study has shown that the NMDA-receptor is present in primary human astrocytes [16]. Meanwhile, it is also established that NMDAR subunits are present in brain endothelial cells. Latest studies showed that the NMDAR in brain endothelial cells could be a promising target for the treatment of ischemic insults and epilepsy [17].

Further studies demonstrated NMDAR-occurrence apart from the brain. For instance the presence of NMDARs was identified in peripheral nerves in rats and mice, where they are responsible for encephalitis in certain circumstances and even in cells of the bone (osteoclasts, osteoblasts) of rats, where the receptor is important for normal osteoclast function [18-22]. Studies with NMDAR modulators suggested NMDAR-presence in the intestine or in chondrocytes of rats [23,24]. Thereby it was found that blockers of the NMDARs such as ketamine could inhibit the transmission of pain signals from the intestine and reduce visceromotor responses and motility reflexes in rats. In human chondrocytes for instance the NMDAR is linked to nNOS (= reactive oxygen species) -activity [25,26]. The occurrence of NMDA-receptors was also confirmed in lung regions and alveolar macrophages of rats, where over-activation of these channels, a process called excitotoxicity, could cause pulmonary edema [27,28].

1.1.2 Subunits

The major subunits contributing to the NMDAR are the GluN1 (NR1), GluN2 (NR2) and GluN3 (NR3). Each subunit consists of an extracellular N-terminus with a ligand-binding domain, an intracellular C-terminus and a membrane region, consisting of three transmembrane (TM) segments (cylinder shown labelled with 1, 3 and 4), a re-entrant pore loop (small cylinder labelled with 2) and an extracellular loop between TM3 and TM4 (see Figure 1.1) [29].

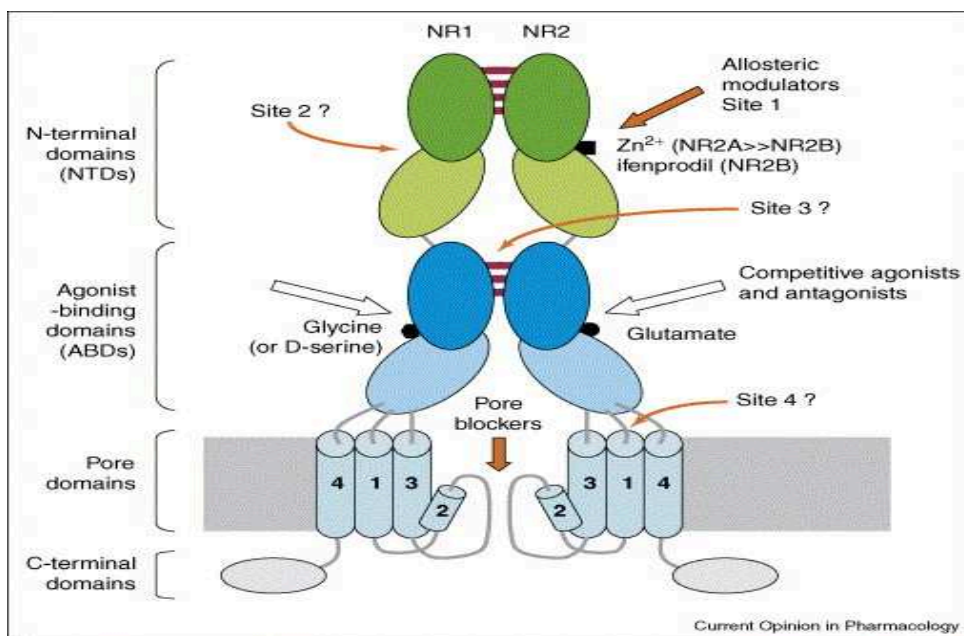


Figure 1.1 Structure and regulatory sites of the NMDAR (here one NR1/NR2 heterodimer is shown): Thick blank arrows indicate the binding sites of competitive agonists such as glycine and glutamate. Site 1: Thick filled out arrows mark the binding of non-competitive blockers such as zinc (more NR2A selective) or ifenprodil (NR2B selective) and the position, where channel (pore) blockers such as Mg^{2+} , MK-801, memantine and ketamine bind. Site 2, 3 and 4: Thin colored arrows mark presumed modulatory sites, on which either positive or negative modulators could bind. Graphic was adapted from [29].

Most often NMDA-receptor-isoforms appear to function as heteromeric assemblies consisting of two NR1-subunits, the glycine-binding domains, in combination with two types of NR2-subunits (NR2A and NR2B), where glutamate and allosteric modulators such as zinc bind. Whilst NR1 subunits undergo alternative splicing, NR2 subunits (NR2A, NR2B, NR2C, NR2D), as well as NR3 (NR3A and NR3B) subunits are encoded by different genes [29]. NR3A- and NR3B subunits, which can bind glycine as the NR1 subunit does, have an inhibitory impact on receptors activity [30,31]. It is accepted that these subunits could form ternary NR1/NR2/NR3 complexes [29]. In addition NMDARs have the ability to form NR1/NR3 heterodimers. NR1/NR3 dimers only require glycine for activation [32]. It was shown that glycine binding to the NR3 subunit of such a dimer led to ion channel opening while glycine binding to NR1 causes inhibition due to rapid desensitization [1,33]. However, if glycine binds to the NR1 subunit of the NR1/NR2 heterodimer the NMDAR is activated [1]. Therefore, the lower current through triheteromeric NR1/NR2/NR3 receptors may be due to glycine binding to the NR1 subunit in the NR1/NR3 dimer conducting inhibition of the NMDAR [1,31]. Hence NMDA-receptors exist in numerous different combinations of NR1, NR2 and NR3 subunits, resulting in receptors with different properties [1,34,35].

1.1.3 Activation and functionality

NMDA-receptors belong together with the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainat-receptors to the group of ionotropic glutamate receptors [36].

The activation of the NMDAR necessitates two coincident conditions. On the one hand, glutamate and either glycine or serine must be liberated per pre-synaptic membranes and then bind to the receptor (see Figure 1.1). On the other hand, the postsynaptic cell has to be depolarized [36]. At resting potentials Mg^{2+} -ions sit in the NMDAR-channels hindering ions in- and efflux [36]. At depolarized membrane potentials, cation influx into the neuron via AMPA receptors leading to a positive charge in the cell, by which positively charged Mg^{2+} -ions leave the NMDA-channel pore and consequently Ca^{2+} - and Na^{+} -currents into and K^{+} -currents out of the cell via NMDARs occur [1,37-39]. Nonhomologous asparagine residues on NMDAR-subunits (NR1, NR2) ensure Ca^{2+} - but not Mg^{2+} -ions to enter the cell [40]. Under physiological conditions the intracellular calcium-overload is prevented by calcium-dependent NMDAR-inhibition [36,41]. Both calcium as well as multiple other factors play an important role for NMDAR-mediated neuroprotection including the cAMP response element binding protein (CREB), nuclear factor kappa B, ERK1/2, Akt1, phosphatidylinositol 3-kinase, protein kinase C epsilon, and brain derived neurotrophic factor [42]. Under pathological conditions as they can be found in diseases such as stroke, Parkinson and epilepsy, however, this protection mechanism of

regulation is turned off, resulting in excessive calcium-influx through the NMDAR-channels. Thus, calcium-overload creates an irreversible death of neuronal cells in the brain [36,43,44].

The NMDA-receptor exhibits a relatively complex functionality. Physiologically NMDARs are important in terms of cellular mechanisms for learning and memory, so called synaptic refinement and plasticity [45]. There are two major forms of synaptic plasticity, namely long term depression (LTD) and long term potentiation (LTP), which are characterized by long-lasting enhancement and reduction of synaptic transmission between two adjacent neurons after repetitive stimulation, respectively [45]. NMDAR-dependent calcium influx induces both LTP and LTD. Therefore, the cell must have an opportunity to either potentiate or to depress a synaptic connection [46]. Nowadays it is understood that marginally activation of the NMDAR causing low increases in postsynaptic calcium leading to LTD, while stronger activation resulting in high increases in postsynaptic calcium and causes LTP [46,47]. Many proteins in the NMDAR complex are involved in these processes. One of them is Ca^{2+} /calmodulin-dependent protein kinase II, a serine/threonine protein kinase, of which large amounts can be found in the post-synaptic density region [48,49]. Another player in terms of LTP is D-serine. Once released from an astrocyte, D-serine regulates NMDAR-dependent plasticity in many thousands of excitatory synapses nearby [50].

In the pathological status the NMDA-receptor contributes to the so called excitotoxicity [51]. Excitotoxicity occurs for instance in case of stroke due to too much glutamate available, which leads to over-activation of the NMDAR and subsequently to increased calcium-influx which activates enzymes that will damage membranes and proteins being essential for cellular integrity [44]. Moreover, calcium-overload set off several downstream lethal reactions including nitrosative stress, oxidative stress and mitochondrial dysfunction [52,53,54]. NMDAR-related excitotoxicity has been implicated not only to acute insults such as stroke but also to adult-onset diseases such as Alzheimer, amyotrophic lateral sclerosis, etc. [52].

It should be mentioned that the functionality of the NMDA-receptor is dependent on its localization. Aforementioned, two types of NMDARs can be distinguished: synaptic situated receptors and extrasynaptic receptors. NMDARs located at the synapse provoke the cAMP response element binding protein (CREB)-mediated transcription and neuronal survival, while extrasynaptic receptors are linked to a CREB shut-off pathway, causing cell death. Due to regionalized availability D-serine is preferentially bound to synaptic receptors, whereas glycine binds to extrasynaptic ones. Thus, selective blocking of glycine binding to extrasynaptic NMDARs could lead to inhibited apoptosis without hindering neuronal survival through the synaptic NMDA-receptors [15,55].

1.2 NMDA-receptor-agonists and antagonists

Due to the complex structure of the NMDAR there are several binding sites for NMDAR-modulators to regulate the activity of this receptor (see Figure 1.1).

1.2.1 Agonists at the NR1-subunit

The binding of glycine (see Figure 1.2) to the S1S2 site on the NR1 subunit (see Figure 1.1) is required for the activation of NMDARs [1,56]. At first, it was assumed that endogenous levels of extracellular glycine were sufficient to saturate the glycine binding site. In later studies it was discovered that further positive modulators could definitely influence the NMDAR-activity [1,57]. For instance amino acids such as (D)-alanine and (D)-serine (see Figure 1.2) show high affinities for the glycine site. Thus, they act as full agonists [1,58].

Conformationally constrained analogues of glycine such as 1-aminocyclopropane-1-carboxylic acid (see Figure 1.2), a cyclopropyl analogue (=ACPC), and 1-aminocyclobutane-1-carboxylic acid, a cyclobutane analogue (=ACBC) behave as partial agonists showing different levels of potency [1,59,60].

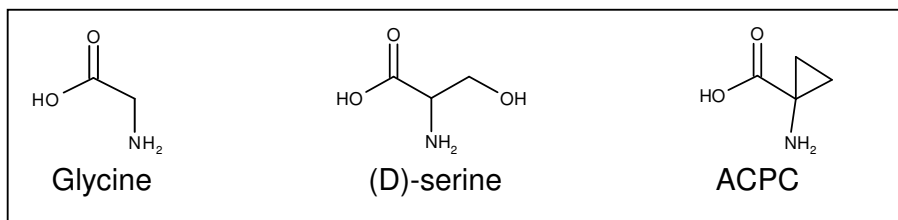


Figure 1.2 Chemical structures of glycine, (D)-serine and ACPC

1.2.2 Antagonists at the NR1-subunit

The first full antagonist discovered binding to the NR1-glycine-site was kynurenic acid (see Figure 1.3) [1,61]. By means of structural variation of the basal structure of kynurenic acid a number of potent glycine antagonists such as 5,7 dichlorokynurenic acid (5,7-DCKA), L-683,344, L-689,560, L-701,324 (see Figure 1.3), GV150526A, and GV196771A had been developed [1,62-66]. Glycine site antagonists enhanced therapeutic ratios by keeping anti-convulsant, neuroprotective, and analgesic properties while showing psychotomimetic effects [1,58]. Unfortunately, none of these compounds has achieved clinical use to treat stroke or epilepsy. One reason might be that these compounds exhibit high affinity plasma protein binding. Thus adequate bioavailability in the brain is uncertain [1,67]. However, a study in 2007 suggested antagonists based on the quinoline nucleus (see Figure 1.3, rightmost) dosed orally indicating good hydrous solubility and splendid bioavailability based on plasma concentration and activity in an in vivo model of neuropathic pain [1,68].

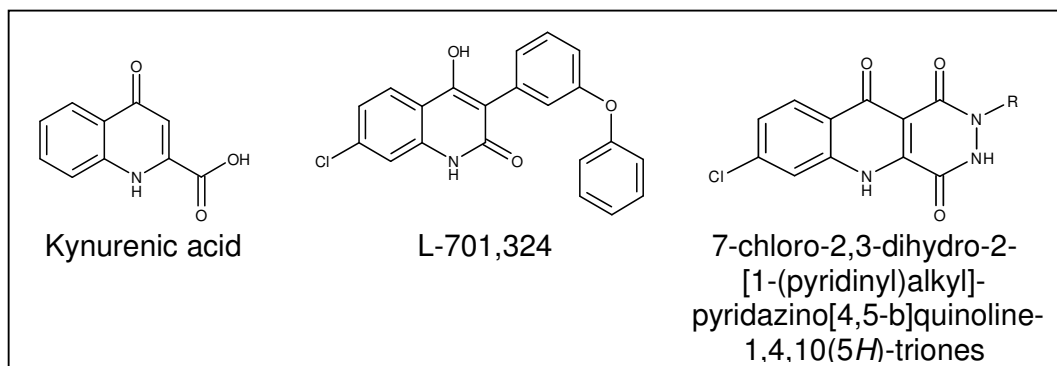


Figure 1.3 Chemical structures of kynurenic acid, L-701,324 and 7-chloro-2,3-dihydro-2-[1-(pyridinyl)-alkyl]-pyridazino[4,5-b]quinolin-1,4,10(5*H*)-triones

1.2.3 Non-competitive antagonists (channel blockers)

Channel blockers block the NMDAR only in the open state such as the dissociative anesthetics phencyclidine and ketamine (see Figure 1.4) [1,69-71]. For non-competitive channel blocking the asparagine residue (N598) deep within the pore lining M2 segment of an NMDAR is essential, which was identified with site-specific mutagenesis [1,72]. In an ischemic insult neurons degenerate by excessive Ca^{2+} -entry through NMDARs. It was thought that NMDAR channel blockers counteract with this mechanism and could therefore be used to treat ischemia. This led to the development of the high affinity channel blocker MK-801 [73]. However, MK-801 (see Figure 1.4) demonstrated adverse effects such as ataxia, memory and learning impairment, and neuronal vacuolization, maybe due to its slow kinetics of dissociation from the binding site in the NMDA-channel [1,74,75]. Memantine (see Figure 1.4) is the only channel blocker, which is currently now in clinical use under the trade names Ebixa®, Axura®, and Namenda®. It is indicated for cognitive deficits in moderate to severe Alzheimer's disease. In contrast to MK-801, memantine is a low affinity channel blocker showing fast on-and off-kinetics on NMDARs and therefore causing minor adverse reactions such as psychotomimetic effects [74].

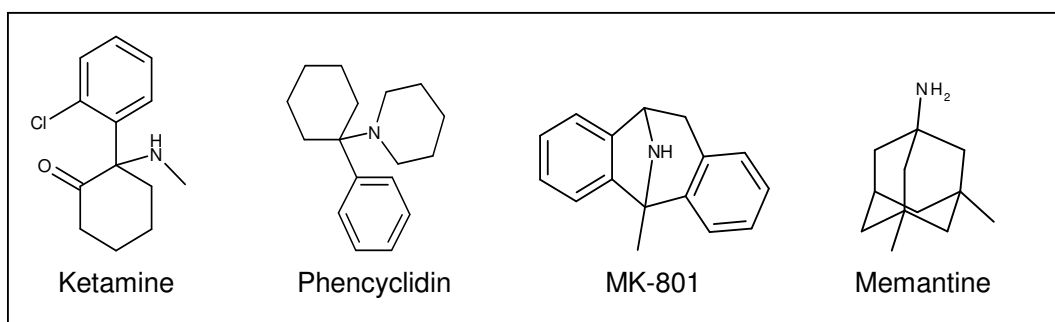


Figure 1.4 Chemical structures of ketamine, phencyclidin, MK-801 and memantine

1.3 Assays for determining binding/functionality of NMDARs in vitro

Most of the NMDAR binding experiments are done by radioligand binding assays with substances of which binding sites to the NMDAR are well understood such as [^3H]-glutamate, [^3H]-MK-801 or the competitive antagonist [^3H]-CGP-39653. For instance Buchstaller et al. (2006) used [^3H]-glycine binding assays to detect whether potential glycine antagonists have the ability to suppress [^3H]-glycine binding [76,77].

For the investigation of the functionality of NMDARs one possibility is to use an electrophysiological method, called the patch-clamp-technique. Therefore, NR1/NR2 expressing oocytes are voltage clamped to a holding potential of -70 mV. By application of NMDAR-agonists such as glutamate and glycine a deviation to the holding potential occurs due to ionic currents, which can be measured through microelectrodes connected to an amplifier. For example concomitant administration of a NMDAR antagonist resulted in lower ionic currents [78]. A further method to detect NMDAR-functionality utilizes a fluorimetric determination of ion-concentrations such as Ca^{2+} via fluorimetric imaging plate reader (FLIPR)-assays. In this regard, changes in intracellular Ca^{2+} induced by NMDAR-agonists or -antagonists were measured through calcium-sensitive fluorophores [78]. If NMDAR functionality is linked to cell viability or toxicity, a lot of different types of cell based assays can be used to determine the number of viable cells at the end of an experiment [79]. Three different assays are de-

scribed in more detail in the “material and methods-section” of this work: the XTT-assay and the Bioluminescence- and LDH-detection-test (see section 2.2.2). In addition to these three assays further cell viability tests could be used. One is the MTT-assay, which is the predecessor of the XTT assay. In contrast to XTT, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is positively charged and therefore able to penetrate eukaryotic viable cells. Thus formazan is created by reduction of MTT through mitochondrial dehydrogenase and subsequently spectrophotometrically measured. However, the resulting formazan product is insoluble. Before measurement formazan has to be solubilized. For instance acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent can be used for this solubilization step [79].

Secondly, the number of viable cells could be examined with the phosphatase-assay using their ability of hydrolyzing p-nitrophenyl phosphate through intracellular acid phosphatase. The produced p-nitrophenol is then measured spectrophotometrically [80].

Thirdly, the [^3H]-thymidine-assay is a cell proliferation assay, whereby [^3H]-labelled thymidine is incorporated into the cell nucleus of proliferating cells. Since this method uses radioligands there are increased risks to laboratory personnel and environment [80].

1.4 Different cells for NMDAR-testing

There are numerous ways to examine NMDAR-functionality by using a variety of different cells.

For instance oocytes expressing subunits of the NMDAR are often used. For that, the corresponding complementary DNA (cDNA) of the required NMDAR-subunits is simply injected into the nucleus of the oocyte [78]. Also PC12 cells which already express NMDAR-subunits can be purchased from different sources and are therefore a widespread model in order to investigate NMDAR-functionality. Though, it was shown that these cells do not form a normal complement of NMDA-receptor subunits [81]. Furthermore, it can be worked with rat cerebellar granule- or NMDAR-expressing mouse fibroblast cells for determining NMDAR-functionality [76,81-83]. As well there are several studies carried out using human embryonic kidney (HEK)-293- or baby hamster kidney (BHK)-21 cells which each could express NMDAR-subunits through different expression vectors [78,84-90]. Last mentioned option in examining NMDAR-functionality is the usage of NMDAR-expressing COS-7 cells, which are kidney fibroblast cells of the African green monkey [91,92].

However, it has to be pointed out that the cells mentioned above represent only a portion of cell types that are used for the investigation of NMDA-receptors.

1.5 Aim of the work

The aim of the present work was to establish a cell culture assay based on NMDAR functionality for testing potential NMDAR-inhibitors.

For the assay, HEK-cells over-expressing a functional NMDAR-Ca²⁺-channel consisting of NR1/NR2A subunits inducible by tetracycline should be used.

HEK-cells should undergo cell death after induction of NR1/NR2A expression.

The optimization of this assay should lead to a difference (=measurement window) of at least 50% cell viability or 2-fold cell toxicity between NR1/NR2A induced and non-induced HEK-cells, respectively. In order to achieve this difference, several parameters such as cell seeding density, time point of addition of tetracycline, tetracycline and serum concentration, procedure of medium exchange and the choice of the medium used should be varied.

Finally, to test the qualification of the assay, dose-response curves of reference compounds (MK-801, memantine and L-701,324), glycine antagonists, that were derived from the NMDAR-antagonist 3-phenyl-4-hydroxyquinolin-2(1H)-one, and other compounds suspicious interacting with NMDARs should be created. From these curves the IC₅₀ of mentioned substances should be determined and subsequently compared with results from the literature.

2 Material and Methods

2.1 Material

2.1.1 Chemicals and substances

Dimethylsulfoxide (DMSO), 41647, Sigma-Aldrich

Aqua purificata, 143AD-2029, Elisabeth Schubert GmbH

Ethanol, 1016AD-814, Elisabeth Schubert GmbH

Tetracycline-hydrochloride, T3383, Sigma-Aldrich

Memantine-hydrochloride, SLBB7723V, Sigma-Aldrich

(+)-MK-801 hydrogen maleate, 022M4616V, Sigma-Aldrich

Magnolol, by courtesy of the Department of Pharmacognosy, Vienna, Austria

Honokiol, by courtesy of the Department of Pharmacognosy, Vienna, Austria

L-701,324, 3B/88582, Tocris bioscience

Bu-081, Bu-082 , Bu-085, Bu-090, Bu-093, Bu-094, Bu-097, Bu-099, Bu-101,

Bu-105, Bu-106, Bu-108, Bu-112, Bu-113 (=Cs-294), Cs-182, Cs-183, Cs-191

(=Cs-241), Cs-199, Cs-224, Sie-20, 1F1, „in house-glycine-antagonists“, Vienna, Austria

HX531, 3912, Tocris bioscience

Wy-14,643, C7081, Sigma-Aldrich

2.1.2 Devices and software

Microplate-reader, Infinite M200Pro Tecan, Austria GmbH, Grödig, Austria

Light microscope, TMS, Nikon, Japan

Laminar Air Flow Biosafe 3, Ehret GmbH & CoKG, Tulln, Austria

Thoma-chamber, Paul Marienfeld GmbH & CoKG, Lauda-Königshofen, Germany

Centrifuge, 5810R, Eppendorf AG, Hamburg, Germany

Waterbath, 355100, Müller-Scherr GmbH & CoKG, Linz, Austria

Fridge, 4°C, Liebherr GmbH, Kirchdorf, Germany

Freezer, -20°C, Liebherr GmbH, Kirchdorf, Germany

CO₂-incubator, CO₂6000, NAPCO, Ehret GmbH & CoKG, Tulln, Austria

GraphpadPrism6™-software, free 30 days demo version, accessed on 20th of November, 2014 and downloaded from <http://www.graphpad.com/scientific-software/prism/>

ChemBioDraw® Ultra 14, two weeks free trial, accessed on 17th of February, 2015 and downloaded from <http://www.cambridgesoft.com>

Microsoft Excel 97™

2.1.3 Cell culture material

Phosphate buffered saline (PBS), sterile, 14190-094, Invitrogen life technologies (Gibco™, Carlsbad, CA, USA)

Trypsin/EDTA-solution, 1.5x, sterile, 15400-054, Invitrogen life technologies (Gibco™, Carlsbad, CA, USA)

Fetal calf serum (FCS), A15-151, 56°C heat-inactivated, # 10720-106, PAA laboratories GmbH, Pasching, Austria

Gentamycine, 10 mg/ml, G1272, Sigma-Aldrich

Glutamine, 200 mM, 25030-024, Invitrogen life technologies (Gibco™, Carlsbad, CA, USA)

Zeocine, 100 mg/ml, ant-zn-1, Invivogen

Blasticidine, 10 mg/ml, ant-bl-1, Invivogen

Poly-D-lysinyhydrobromide, P1024; Sigma-Aldrich

EMEM, MEM Eagle EBSS with phenol red and with glutamine, BE12-611F, BioWhitaker/Lonza

EMEM, MEM Eagle EBSS without phenol red and without glutamine, 2-fold, BE12-668F, BioWhitaker/Lonza

Cell culture flasks, surface 75 cm², 658175, Greiner Bio-One GmbH, Frickenhausen, Germany

96 well microplates, clear, Cellstar®, 655180, Greiner Bio-One GmbH, Frickenhausen, Germany

96 well microplates, white, Cellstar®, 655098, Greiner Bio-One GmbH, Frickenhausen, Germany

Syringe filters, Rotilabo® syringe filters, P666.1, PVDF, 0.22 µm, sterile, Carl Roth GmbH & CoKG, Karlsruhe, Germany

Needles, 100 Sterican®, 4665791, Braun Melsungen AG, Melsungen, Germany

Syringes, 10 ml, 20 ml, 30 ml, 57B-9140, BD Laagstraat, Temse, Belgium

Sterile pipettes, 5 ml, 10 ml, 25 ml, 86.1253.001, 86.1254.001, 86.1685.001, Sarstedt AG, Nürnberg, Germany

Vacuum driven disposable filtration system, PR02445, 1000 ml, Stericup, Millipore Corporation, Massachusetts, USA

Petri dishes, 101VR203V, 90 mm, Sterilin Ltd., Newport, UK

Multi channel pipettes, 100 µl, 300 µl, Eppendorf AG, Hamburg, Germany

Pipettes, 10 µl, 100 µl, 1000 µl, Eppendorf AG, Hamburg, Germany

Pipette tips, 0.1-10 µl, 1-200 µl, 5-300 µl, 100-1000 µl, 1-061-96-0, 1-110-96-1, 1-104-96-0, 1-200-96-2, AHN Biotechnologie GmbH, Nordhausen, Germany

Tubes, 15 ml, 50 ml, 188271, 227261, Cellstar®, Greiner Bio-One GmbH, Frickenhausen, Germany

Reagent-reservoirs, 89094-662, VWR, USA

Cell culture assays:

- EZ4U, BI-5000, Biomedica GmbH & CoKG, Vienna, Austria
- Luminescent viability assay, Celltiter Glo®, G7570, Promega Corporation, Madison, USA
- LDH-test, cytotoxicity detection kit, 11644793001, Roche diagnostics GmbH, Vienna, Austria

2.1.4 HEK293-NMDAR-NR1/NR2A-cells

The cells were a kind gift from Prof. Pifl from the Brain Research Institute of the Medical University in Vienna.

The originally human embryonic kidney (=HEK) -293-cell line was isolated from the kidney of an aborted human embryo and then transformed with sheared fragments of human adenovirus type 5 (Ad5) into chromosome 19 by Graham et al. (1977). This cell line is commercially available [84].

In order to obtain HEK-293-NMDAR-NR1/NR2A-cells from the parental HEK-293-cell line, cells were transformed with plasmids containing GRIN1 and GRIN2A genes. This led to the expression of the corresponding proteins, namely the subunits of the NMDA-receptor, which are the NR1-domain, the glycine-binding-site domain and the glutamate-binding subunit NR2A. The transformation was accomplished by subcloning the hNR1 subunit into the expression vector pcDNA4/TO (Invitrogen Life Science) and the cDNA of the NR2A subunit into the vector pcDNA1 (received from Dr. Antonio Ferrer-Montiel,

IBMC-UHC, Alicante) [85,86]. The selection of these expression vectors was done with the antibiotics zeocine and blasticidine. For adequate expression of the subunits NR1 and NR2A the tetracycline-regulated expression (T-Rex™) System was used [85,86].

It was already shown that these two domains (NR1 and NR2A) are sufficient for setting up a functional NMDA-receptor [87,88].

2.2 Methods

2.2.1 Cell culture of HEK293-NMDAR-NR1/NR2A-cells

2.2.1.1 Preparing HEK-cell culture media

Cultivation medium:

The ingredients of 500 ml MEM (minimum essential medium) Eagle EBSS (Earl's balanced salts) (= EMEM) are a number of vitamins, amino acids (glycine, glutamine, etc.), inorganic salts (CaCl_2 , KCl, MgSO_4 , etc.) glucose, etc. [93].

For the completion of the cell cultivation medium 50 ml (=10%) FCS and 2.75 ml (=50 mg/l) gentamycine were added to 500 ml EMEM with phenol red and glutamine.

Assay medium:

To receive a one-fold concentrated solution 250 ml 2-fold EMEM (without phenol red and without glutamine) were mixed with 250 ml sterile filtered aqua purificata. Then 5 ml of 200 mM glutamine (2 mM), 2.75 ml (=50 mg/l) gentamycine and FCS (in different concentrations, depending on the experiment accomplished) were added to 500 ml medium.

2.2.1.2 Freezing of HEK-cells

The freezing medium contained 10 ml MEM Eagle EBSS with (2 mM) glutamine, 2 ml FCS (20%), 0.05 ml gentamycine and 1 ml DMSO (dimethylsulfoxide sterile, 10%).

Cells were trypsinized in a T75-flask, taken up in 10 ml cultivation medium and centrifuged at 300g for 5 minutes. The supernatant was removed and the pellet was taken up in freezing medium and put in the isopropanol-cryobox over night at -80 °C. On the next day the cell pellet was transferred into liquid N₂.

2.2.1.3 Thawing HEK-cells

1 ml pre-warmed cultivation-medium was added to the cells in the cryovial. After that cells were re-suspended in 10 ml cultivation-medium and centrifuged at 300g for 5 minutes. Finally the supernatant was removed and the pellet was taken up in 15 ml medium and put in a poly-D-lysine-coated T75-flask.

2.2.1.4 Coating of T75-tissue culture flasks and 96-well plates

For better cell adherence culture flasks (or 96-well plates) were coated with poly-D-lysine-solution.

Therefore, 5 mg poly-D-lysine-hydrobromide were dissolved in 20 ml H₂O and subsequently sterile filtered (= 10x stock). This stock was stored at -20 °C in Eppendorf-tubes as 2 ml aliquots each.

For coating 5.4 ml sterile H₂O and 0.6 ml stock (45 µl H₂O and 5 µl stock in each well of a 96-well plate) were mixed and added in a T75-flask, incubated 2

hours in the laminar air flow, washed two times with 10 ml (100 µl per well) PBS and finally let dry.

2.2.1.5 Inducing NR1/NR2A-expression

For each induction, 5 mg tetracycline-hydrochloride were freshly dissolved in 1 ml H₂O and afterwards sterile filtered (=5 mg/ml stock). For instance, 1 µl of tetracycline-hydrochloride-solution-stock was supplemented to 50 ml complete medium (=0.1 µg/ml).

2.2.1.6 Addition of selecting antibiotics

The expression vectors pcDNA4/TO and pcDNA1 of the subunits NR1 and NR2A allow selection with the antibiotic zeocine in presence of blasticidine.

For instance, freshly thawed, 90 µl zeocine (from stock: 100 mg/ml, 100 µl aliquots were stored at -20°C) and 15 µl blasticidine (from stock: 10 mg/ml, 50 µl aliquots were stored at -20°C) were added to 30 ml medium.

2.2.1.7 Medium exchange

15 ml pre-warmed cultivation-medium was mixed with 45 µl from zeocine-stock and 7.5 µl from blasticidine-stock in a 50 ml tube. This medium-mixture was poured in at the side wall of the flask after removing the old medium.

2.2.1.8 Sub-cultivation of HEK-cells

Consumed medium was removed. Then, cells were washed two times with 10 ml PBS, treated with 3 ml trypsin/EDTA-solution (1.5-fold) per T75-flask and placed for 3 minutes at 37°C in the incubator. The detachment of the cells could only be achieved by heavy knocking of the T75-flask on the laboratory table. After the cells detached from the flask bottom they were resuspended in 10 ml pre-warmed cultivation medium and centrifuged at 300g for 5 minutes. The supernatant was discarded in order to remove the trypsin/EDTA. Then the cells were taken up in a corresponding amount of fresh pre-warmed cultivation medium (= cell suspension). For instance if splitting-ratio was 1:3 (to split at 90% confluence), 3 ml of a 9 ml re-suspended cell suspension were added in a new poly-D-lysine coated T75-flask and filled up to 15 ml with 12 ml cultivation medium. Finally, the flask was slightly panned, labelled with splitting ratio, passage number and initials and placed in the incubator at 37°C.

2.2.1.9 Cell seeding and counting

As described before in section 2.2.1.8 consumed medium was removed. Then, cells were washed two times with 10 ml PBS and treated with trypsin/EDTA-solution (1.5-fold). After the flask was placed in the incubator for 3 minutes cells were separated off the bottom of the flask by heavy knocking and subsequently resuspended in 10 ml pre-warmed cultivation medium. Eventually, trypsin/EDTA was removed via centrifugation and the cells were resuspended in 10 ml fresh pre-warmed medium.

Now, 20 μ l of the cell suspension was filled into each Thoma chamber and only cells in the chambers (squares) were counted. The actual number of cells per ml was calculated by multiplying the average of the number of cells counted in both chambers by 10.000 (due to a chamber volume of 0.1 μ l). Finally the suspension was diluted to the required cell concentration (cells/ml or cells/cm² in view of a well surface area of 0.34 cm² for 96-well plates) and subsequently 200 μ l (100 μ l) of this suspension were added in each well of a PDL-coated 96 well microtiter plate except those wells for blank-values.

2.2.2 Cell viability and cytotoxicity tests

A large number of different tests based on different methods for the determination of the amount of living cells exist [79].

In this work, three different detection methods were used: EZ4U (tetrazolium reduction), Bioluminescence- (ATP-detection) and LDH-test (LDH-activity).

2.2.2.1 EZ4U – a non-radioactive cell proliferation and cytotoxicity assay

This test is based on the mechanism that only viable cells with intact mitochondrial membrane and cell membrane are able to convert tetrazolium salts into a purple colored formazan product through mitochondrial dehydrogenase (see Figure 2.1). That means the formation of formazan requires viable cells and less number of viable cells results therefore in a reduced formazan production. Thus, the number of viable cells can be defined without harvesting by simply measuring of formed formazan spectrophotometrically [94,95].

The EZ4U-test includes XTT (=2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide), which is reduced to a soluble and stable non-toxic formazan-derivate (see Figure 2.1) [94,95]. A study from Berridge et al. (2005) suggested that phenazine methosulfate (PMS) is required for sufficient reduction of XTT to formazan since incubation of XTT in absence of PMS on human T-lymphoblastic Jurkat cells yielded only slight tetrazolium reduction [96]. According to Berridge et al. (2005) PMS ensures the reduction of the

tetrazolium salt (XTT) by taking up electrons at the cell surface or in an accessible position at the plasma membrane [94,96]. On the contrary, in accordance with the manufacturer's instruction, mitochondrial dehydrogenases are involved in reduction of XTT-reducing agents such as PMS [94]. Therefore, this mechanism is not clarified in detail yet.

Reaction scheme:

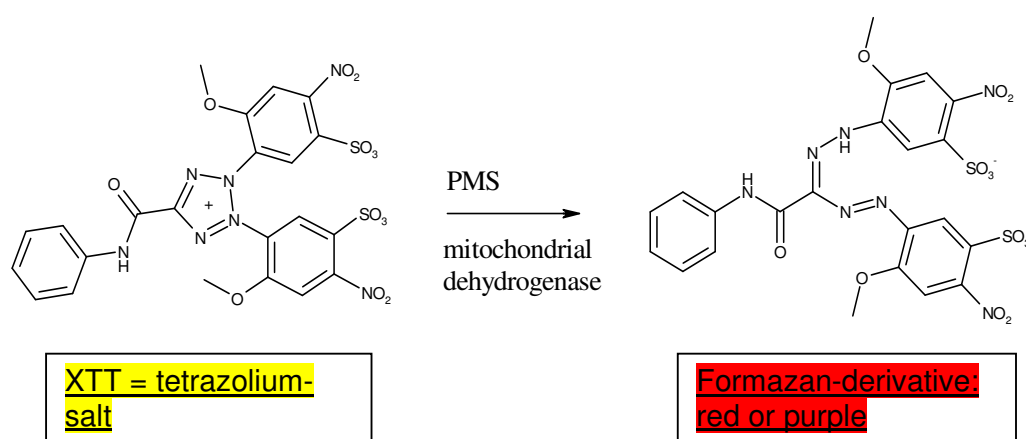


Figure 2.1 Reaction scheme: capability of viable cells to reduce XTT (yellow) into a formazan derivative (red) through mitochondrial dehydrogenase and phenazine methosulfate (=PMS) [94]

EZ4U-test-procedure:

EZ4U-test substrate (tetrazolium salt) was dissolved in 2.5 ml activator (electron coupling reagent=PMS). This yielded in a straw colored solution. 20 µl (10 µl) of this solution was added to 200 µl (100 µl) assay medium in each well of a 96-well-microplate. The absorbance was measured by a well-microplate-reader at different time points from 30 minutes to 4 hours. Wavelengths were set nearby the absorbance maximum of the emerging formazan-product (470nm) and at a wavelength, where this formazan-derivative shows no absorbance

(650nm) as a reference absorbance to eliminate nonspecific background values (caused by cell debris, fingerprints, or other potential interferences; see Figure 2.2) [95]. Between the measurements the plates were placed in the incubator at 37°C.

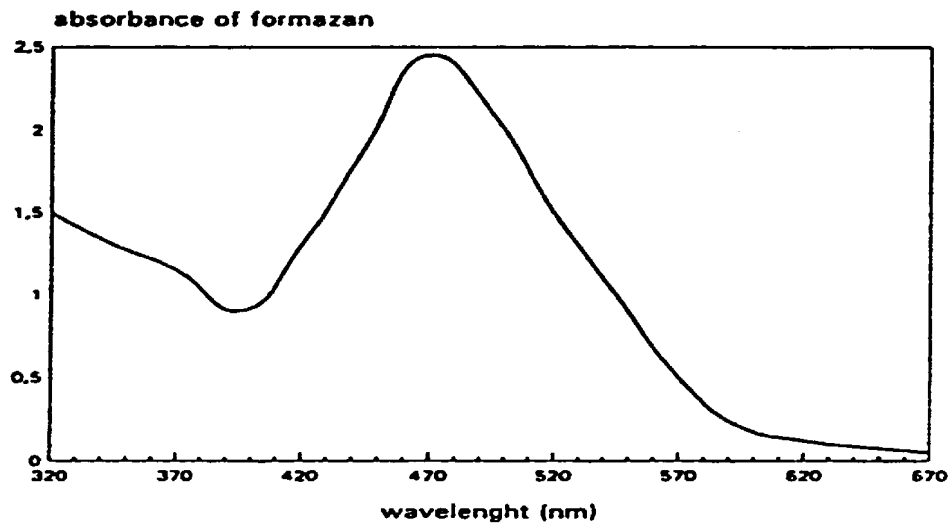


Figure 2.2 Absorbance spectrum of formazan [95]

2.2.2.2 Celltiter Glo® – a bioluminescence test

This kind of detection method is mostly used in high-throughput-screening (HTS) applications [79].

In this case, ATP serves as a valid marker of viable cells. When cells die, they lose membrane integrity and ATP is released. ATP-ase-inhibitors must be present so that ATP is not degraded by endogenous ATPases [79]. The Promega ATP detection reagent consists of beetle luciferin as a substrate and the stable form of recombinant luciferase purified from *Photuris pennsylvanica* (LucPpe2), to catalyze the reaction and to generate photons in presence of ATP, O₂ and Mg²⁺ (see Figure 2.3) [79,97].

Reaction scheme:

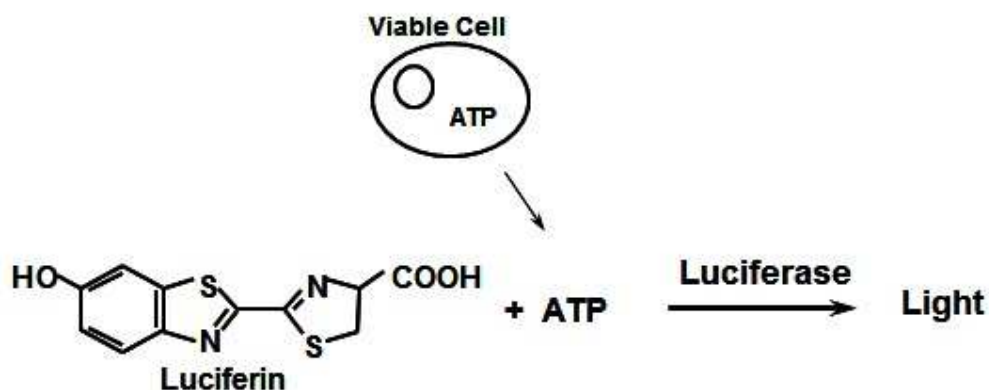


Figure 2.3 Reaction scheme: dying cells secrete ATP, which generates light in presence of luciferin through luciferase [79]

Bioluminescence-test-procedure:

Bioluminescence-test substrate (=beetle luciferin) and buffer were mixed together. After 30 minutes of plate equilibration at room temperature 100 µl of this mixture were added to 100 µl assay medium in each well of a white 96 well microplate. Then the plate was shaken for two minutes to induce cell lysis, which led to ATP-liberation. 10 minutes later the plate was measured with a well-microplate-reader at 250 to 1000 ms (milliseconds) integration time per well.

2.2.2.3 Cytotoxicity Detection Kit (LDH)

This detection method characterizes the number of viable cells by lactate dehydrogenase (LDH)-activity. Damaged or lysed cells release LDH [98]. The contents of the assay are a tetrazolium salt, INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) and the enzyme diaphorase as a catalyst. The detection of viable cells follows the conversion of lactate to pyruvate, subsequently transforming INT to a formazan-product through diaphorase-activity which can be detected spectrophotometrically (see Figure 2.4) [98].

Reaction scheme:

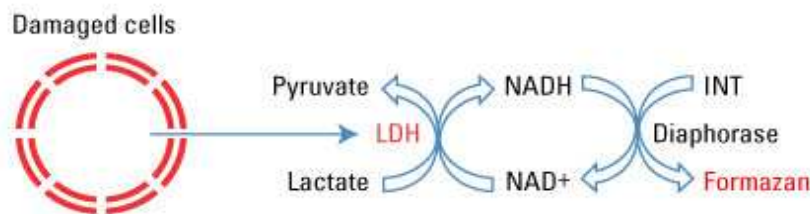


Figure 2.4 Reaction scheme: damaged cells secrete LDH, which convert existing lactate to pyruvate whereby NADH is emerged which in turn forms INT into formazan through diaphorase-activity [99]

LDH-test-procedure:

LDH-test dye solution (=INT) was mixed with catalyst (=diaphorase), which had been dissolved in 1 ml sterile filtered H₂O before. For instance 45 ml dye solution and 1 ml catalyst were mixed and 100 µl of this mixture were added to 100 µl assay medium in each well of a 96-well-microplate. After 15 minutes of incubation 50 µl stop solution was added per well to inactivate the enzymes. Finally,

the plates were measured with a well-microplate-reader at 492 nm and at 620 nm reference wavelength.

2.2.3 Optimization of the experimental assay setup

As mentioned above, the addition of tetracycline to the HEK-293-NR1/NR2A-cells causes an overexpression of the subunits NR1 and NR2A and thus a formation of functional NMDARs. This means that tetracycline (TC) treated cells were supposed to die due to over-activation of the receptor through a surplus of glutamate and glycine (contained in the HEK-293-cells medium) leading to increased intracellular Ca^{2+} inducing apoptosis.

For the optimization of the assay three cell viability test methods (EZ4U, LDH and Bioluminescence) were used. The aimed difference in measured values between tetracycline (TC)-treated and non-treated cells should amount to at least 50% cell viability (EZ4U, Bioluminescence) or 2-fold cell toxicity (LDH), respectively. In order to achieve this several experiments were accomplished by varying the following parameters:

For the experiments all in all cell seeding densities of 20.000, 40.000 and 80.000 cells/cm² were prepared. Tetracycline (TC) was added in concentrations of 0.1 or 1 µg/ml. Furthermore, three different time points of addition of tetracycline to the cells were attempted. Firstly, TC was added directly during cell seeding to the cell suspension, mixed and applied into the corresponding wells. Secondly, a 2-fold solution of TC in medium was added immediately after seeding a 2-fold cell suspension into the corresponding wells or thirdly a one-fold or a 2-fold solution of TC in medium depending on removing the whole or half of the consumed medium was added 2 hours or 24 hours after seeding of

the cells. In some experiments a washing step with 200 µl PBS per well was performed between the medium-exchange. The incubation time was either 24 hours or 48 hours. Moreover, in an experiment using EZ4U-test an additional medium-exchange was accomplished directly before measurement.

As cultivation medium EMEM with phenol red with 10% FCS or EMEM without phenol red with 3% FCS was used. As induction medium EMEM with phenol red with 10% FCS or EMEM without phenol red in serum concentrations of 10, 5, 3, 1, and 0% FCS were tested, respectively.

2.2.4 Calculation of the tetracycline-induction effect

The tetracycline induction effect between tetracycline (TC)-treated and TC-untreated cells was calculated as follows.

For instance, in one experiment using EZ4U-test the average of the readings obtained for pure cultivation-medium without cells resulted in 0.16 (= blank), the average of the readings obtained for TC-untreated cells was 1.18 (= TC-untreated) and the average of the measured values obtained for TC-treated cells was 1.04 (= TC-treated) (see Figure 2.5).

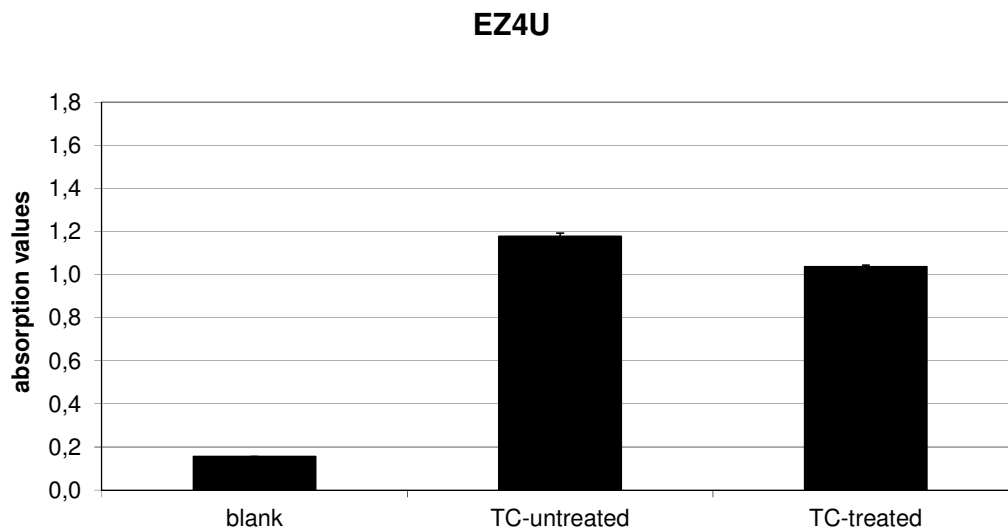


Figure 2.5 Average of the readings obtained for pure medium (=blank), TC-untreated- and TC-treated cells. Data are given as means $\pm$ SEM, n = 16 wells.

Now the values for TC-untreated- and TC-treated cells were subtracted by the “blank-value” (see Figure 2.6).

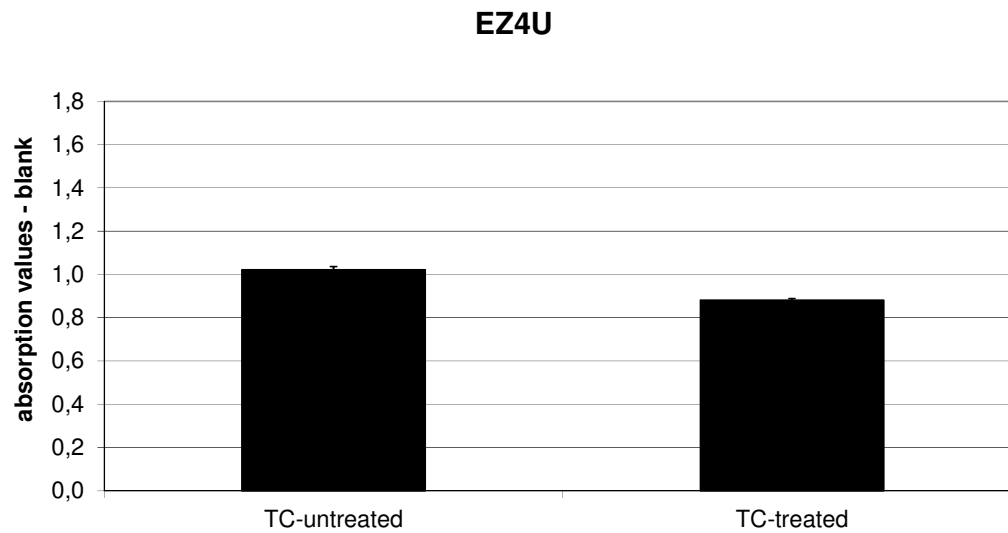


Figure 2.6 Average of the readings obtained for TC-untreated- and TC-treated cells subtracted by “blank-value” each. Data are given as means $\pm$ SEM, n = 16 wells.

Finally the “TC-untreated-value” minus “blank-value” was set as 100%, and the “TC-treated-value” minus “blank-value” was determined based on it (see Figure 2.7).

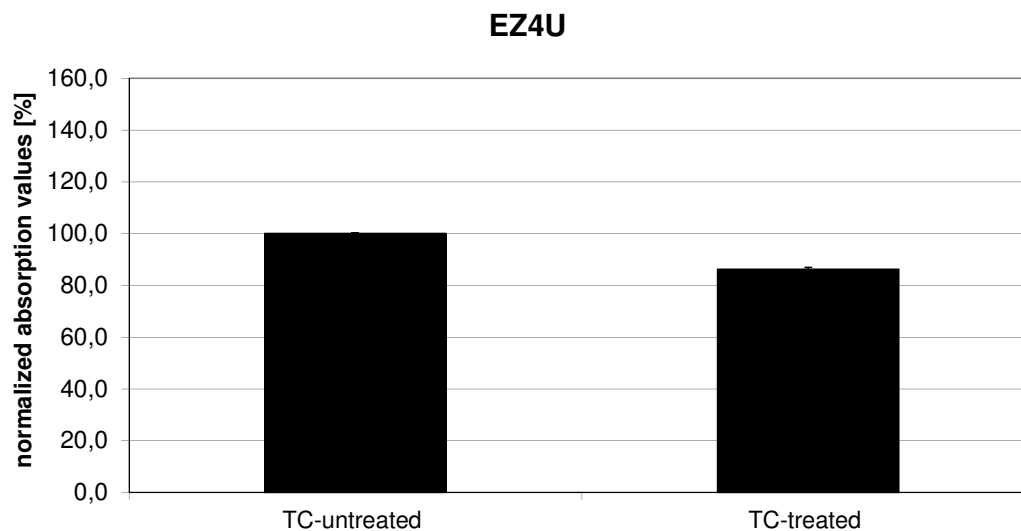


Figure 2.7 “TC-untreated-value” minus “blank-value” was set as 100% and “TC-treated-value” minus “blank-value” was calculated with regard to that. Data are given as means $\pm$ SEM, n = 16 wells.

The figure above (see Figure 2.7) shows a cell viability reduction through TC-treatment of about 14%. Concerning EZ4U-test the aimed difference of at least 50% was not obtained in this case.

The similar procedure for the LDH-assay is explained in section 2.2.6.

2.2.5 Application of the test substances – optimized procedure

After optimization of the assay several NMDAR-blockers and substances with unknown effects on NMDAR-functionality were tested. An overview of the test compounds is given below (see Table 2.1).

To characterize the potential of the NMDAR-inhibitors to block the tetracycline (TC) induced cell damage through NMDAR-over-activation, test compounds were applied to TC-treated cells in increasing concentrations up to 300 μ M. The same concentrations of the test substances were administered to TC-untreated cells to investigate sole cytotoxic effects unrelated to the receptor overexpression as a control.

Therefore, a cell suspension in a concentration of 136.000 cells/ml was prepared to achieve 40.000 cells/cm² cell seeding density. Hereupon 100 μ l of this cell suspension was applied into each well of a 96-well-microtiterplate, except those of the rows 1 and 12, in which pure medium was added. For this purpose medium with phenol red with a serum concentration of 10% FCS was used. After 24 hours of cultivation test substances were applied in the following manner: For instance in the case of memantine 215.8 g/l comply with a 1 M solution. Thus, 21.58 g/l or 21.58 mg/ml correspond to a 100 mM solution. Hence 10 mg memantine was weighed and dissolved in ca. 0.463 ml (= 463 μ l) sterile filtered H₂O to receive a 100 mM stock solution. 100 mM stock solutions of glycine antagonists were prepared in DMSO. These 100 mM stock-solutions were diluted to 30 and 10 mM. 30 mM-dilution was then diluted to 3 mM, which in

turn was diluted to 0.3 mM, whereas 10 mM dilution was then diluted to 1 mM, which in turn was diluted to 0.1 mM. Once all dilution steps were performed in the same solvent, the six dilutions prepared were applied to tetracycline- and non-tetracycline-containing medium without phenol red and a serum concentration of 5% FCS in a ratio of 1:100 to receive end-concentrations of 300, 100, 30, 10, 3 and 1 μ M. The exact application scheme is shown in the table below (see Table 2.2).

After 24 hours of incubation measurement was carried out via LDH-test and the measurement values obtained as described in section 2.2.6 indicating the effects of a test substance at the corresponding concentration were inserted into graphpad prism software for the generation of the dose-response-curves.

Exceptions to this method include the channel blocker MK-801, the glycine antagonist L-701,324, the novel glycine antagonists Bu-099, Cs-224 and Sie-20, and the compounds suspicious interacting with the NMDAR such as honokiol and HX531.

MK-801 dilutions in sterile filtered water were prepared up to a concentration of 1 nM due to its high efficacy on the NMDAR. L-701,324 was also used as 20 μ M and 50 μ M yielding a fine shaped dose-response curve. As well the novel glycine antagonists Bu-099 and Cs-224 were applied in additional concentrations to improve the shape of the obtained dose-response curves. To understand the exact course of the dose-response curve obtained for honokiol added to TC-untreated cells extra concentrations of 5 μ M and 20 μ M honokiol were also measured. HX531 was used in concentrations of 1, 3, 5, 10, 20, 30 and

200 μ M, while Sie-20 was applied in concentrations of 1, 3, 10, 20 and 30 μ M due to shortage of the substance.

2.2.5.1 Test compounds

Substance	Characteristics	Molecular weight (g/mol)
MK-801	Channel blocker	221.30
Memantine	Channel blocker	215.80
Bu-081	Glycine antagonist	409.89
Bu-082	Glycine antagonist	333.84
Bu-085	Glycine antagonist	271.33
Bu-090	Glycine antagonist	271.70
Bu-093	Glycine antagonist	261.27
Bu-094	Glycine antagonist	285.36
Bu-097	Glycine antagonist	291.75
Bu-099	Glycine antagonist	319.81
Bu-101	Glycine antagonist	271.33
Bu-104	Glycine antagonist	299.39
Bu-105	Glycine antagonist	237.25
Bu-106	Glycine antagonist	317.79
Bu-108	Glycine antagonist	305.78
Bu-109	Glycine antagonist	285.36
Bu-112	Glycine antagonist	277.73
Bu-113 (=Cs-294)	Glycine antagonist	425.93
Cs-182	Glycine antagonist	411.90
Cs-183	Glycine antagonist	453.98
Cs-191 (=Cs-241)	Glycine antagonist	411.90
Cs-199	Glycine antagonist	425.93
Cs-224	Glycine antagonist	413.87
1F1	Glycine antagonist	357.26
Sie-20	Glycine antagonist	397.88
L-701,324	Glycine antagonist	363,80
Wy-14,643	PPAR- α -agonist	323.80
Magnolol	Bioactive constituent of <i>Magnolia officinalis</i>	266.33
Honokiol	Bioactive constituent of <i>Magnolia officinalis</i>	266.33
HX531	Retinoid X receptor-antagonist	483.56

Table 2.1 Test substances, their characteristics and molecular weight

2.2.5.2 Application scheme

In grey “Blank-wells” and green “Control-wells” medium without phenol red with 5% FCS and 1% DMSO or sterile filtered water (memantine and MK-801) was applied.

Medium without phenol red with 5% FCS, 1% DMSO and 0.1 µg/ml tetracycline (TC) was added into the red “TC-wells”.

In the lime-green “x µM subst.-wells” medium without phenol red with 5% FCS and test substance in corresponding concentration was applied, whereas medium without phenol red with 5% FCS, test substance in corresponding concentration and TC (0.1 µg/ml) was added into the light red “x µM subst. + TC-wells” (see Table 2.2).

96-well-microtiterplate

○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○
○	○	○	○	○	○	○	○	○	○	○	○	○

Terms:	Blank-wells	pure medium (no cells)
	Control-wells	Tetracycline (TC)-untreated cells
	TC-wells	Tetracycline (TC)-treated cells
	x µM subst.-wells	Tetracycline (TC)-untreated cells + test substance in set concentration
	x µM subst. + TC-wells	Tetracycline (TC)-treated cells + test substance in set concentration

Table 2.2 Application scheme of the test substances on a 96-well-microtiterplate

2.2.6 Generation of dose-response curves

The generated dose response curve shows the capability of the test compounds in minimizing the TC-induced cell damage through NMDAR-over-expression received by adding those substances to TC-treated cells. The second curve demonstrates sole cytotoxic effects of the substance unrelated to the NMDAR by adding them to TC-untreated cells.

For example Bu-090 was applied in concentrations of 1, 3, 10, 30, 100 and 300 μM to TC-treated and TC-untreated cells in two independent 96-well-microtiterplates. Plates were measured and the average values of the obtained different readings were calculated. The average of the readings obtained for pure cultivation-medium without cells resulted in an absorption of 0.42 (= blank), the average of the readings obtained for TC-untreated cells was 0.63 (= TC-untreated) and the average of the readings obtained for TC-treated cells was 1.0 (= TC-treated). Further the average of the absorption readings for Bu-090 applied for instance in a concentration of 100 μM to tetracycline (TC)-treated cells was 0.79 (= TC-treated + 100 μM Bu-090) whereas the average of the readings for Bu-090 applied in a concentration of 100 μM to tetracycline (TC)-untreated cells was 0.57 (= TC-untreated + 100 μM Bu-090) (see Figure 2.8).

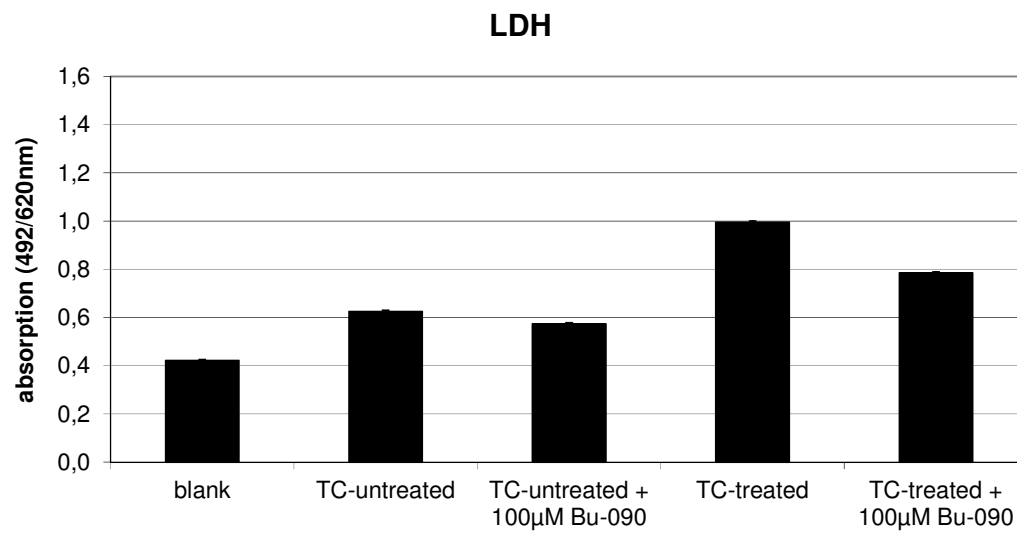


Figure 2.8 Average of the absorption readings obtained for blank, TC-untreated cells, TC-untreated cells + 100 µM Bu-090, TC-treated cells and TC-treated cells + 100 µM Bu-090. Data are given as means $\pm$ SEM, n = 8-32 wells from two independent plates.

Subsequently, the “blank-value” was subtracted from the other obtained values (see Figure 2.9).

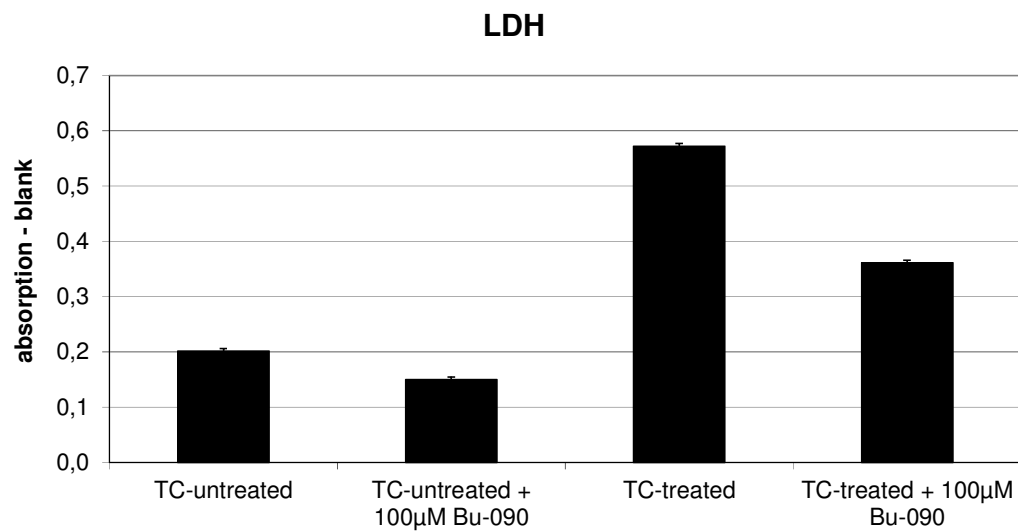


Figure 2.9 Average of the readings obtained for TC-untreated cells, TC-untreated cells + 100 µM Bu-090, TC-treated cells and TC-treated cells + 100 µM Bu-090 subtracted by “blank-value” each. Data are given as means $\pm$ SEM, $n = 8-32$ wells from two independent plates.

Then the “TC-untreated-value” minus “blank-value” was set as 100%, and the other values were determined based on it (see Figure 2.10).

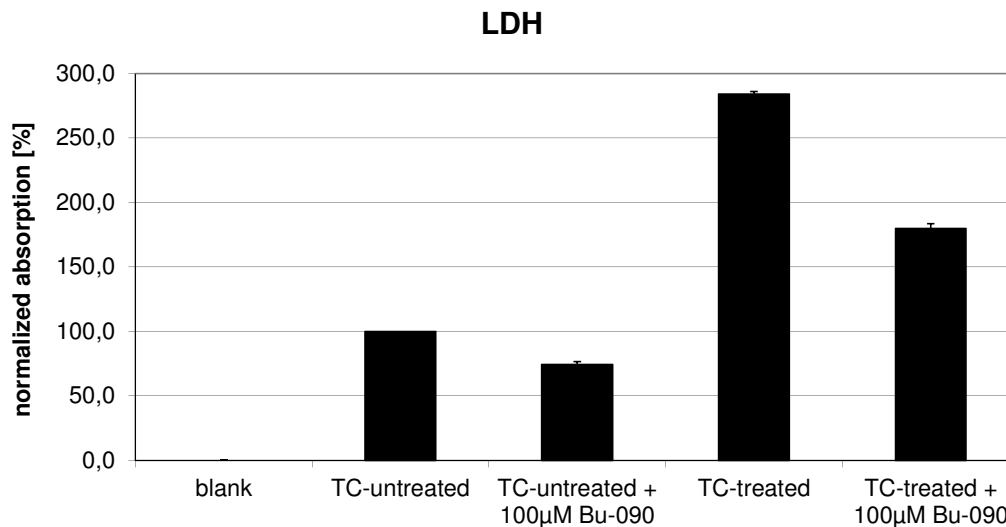


Figure 2.10 “Blank-value” was set as 0%, “TC-untreated-value” minus “blank-value” was set as 100%, and other values were calculated with regard to that. Data are given as means $\pm$ SEM, n = 8-32 wells from two independent plates.

In this example, the TC-treatment increased toxicity by more than 2-fold (100% vs. 280%) indicating that the data could be used for the calculation of a dose-response curve.

Finally, the difference of the TC-dependent measurement window between “TC-treated-value” minus “blank-value” (280%) and “TC-untreated-value” minus “blank-value” (100%) was set as 100%, in order to normalize the differences in the measurement windows between different experiments. Therefore, 100% of “TC-untreated-value” minus “blank-value” was set to 0% and 280% of “TC-treated-value” minus “blank-value” was set to 100% (see Figure 2.11).

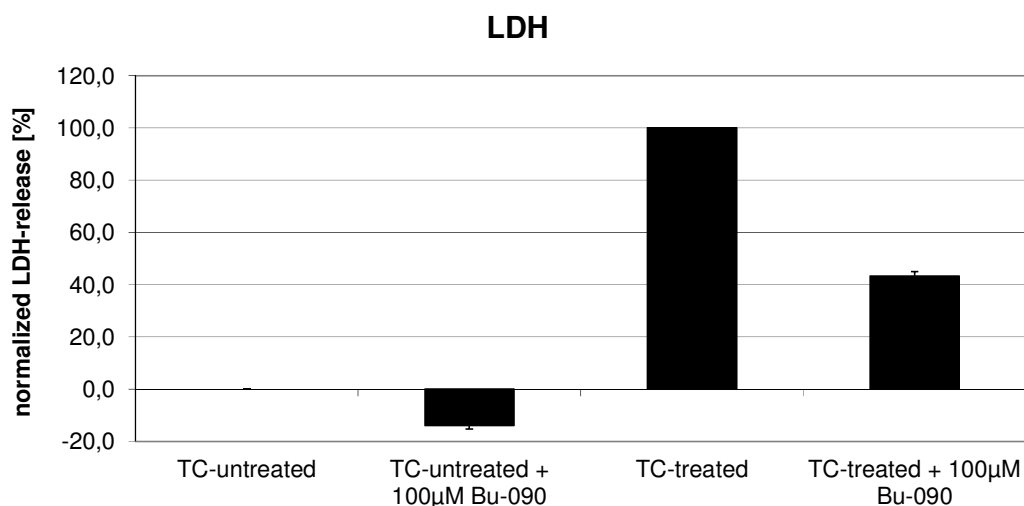


Figure 2.11 In this graph it is shown that Bu-090 at a concentration of 100 µM reduced the TC-induced cell damage, measured through LDH-release by about 57% and at this concentration Bu-090 indicated no toxic effects, due to “TC-untreated + 100 µM Bu-090-value” resulted in a value below 0%, regarding “TC-untreated-value” was set as 0%. Data are given as means ± SEM, n = 8 wells from two independent plates.

Now, for the generation of the dose –response curves graphpad prism software was used. Therefore, sample data “Dose-response – X is log (dose)” was selected. Then the applied molar concentrations of the corresponding substance were inserted as X values and the normalized differences in measurement windows obtained at these concentrations represented the Y values. After that X values were transformed to logX values and subsequently dose response curves were drawn using “nonlinear regression (curve fit)” and the equation “log (inhibitor) vs. normalized response - Variable slope”.

At higher concentrations (100, 300 µM) values of some test compounds were significantly above zero indicating strong sole cytotoxic effects at these concentrations. Thus, for proper calculation of the IC₅₀ value the dose-response curves

had to be adjusted. Therefore, data points at higher concentrations showing strong cytotoxic effects were equated to data points at which still no or slight cytotoxic consequences could be investigated and subsequently the curves were drawn.

In the case of test substances that could not considerably reduce the TC-induced cell damage through NMDAR-over-activation, no extra dose response curves were created.

2.3 Ranking by Spearman's rank-correlation-coefficient

This coefficient (r_s) is used to prove a statistical correlation between different ranking orders and is calculated as follows [100].

- $$r_s = 1 - \frac{6 \sum D^2}{n(n^2 - 1)}$$

Equation 2-1: r_s = spearman's rank-correlation-coefficient, D = differences of the rankpairs, n = number of the rankpairs [100]

For the correlation of the different outputs obtained in this work, they were listed, ranked and the square of the differences (D^2) of the n rankpairs were determined. Then the spearman's rank-correlation-coefficient (r_s) was calculated using Equation 2-1 and subsequently compared to the cutoff (r) at the probability levels (p) of 0.05 and 0.01 by using the table retrieved from the following website: <http://psychology.ucdavis.edu> (last accessed on 25th of February, 2015).

The null hypothesis, meaning that there is no correlation between the two independent distributions was rejected if $r_s > r$ and thus p value < 0.05 or < 0.01 , respectively (see results, Table 3.1 - Table 3.8).

3 Results

3.1 Optimization of the assay - results using EZ4U-test

Using EZ4U-detection-method there were a number of experiments carried out, in which the cell seeding density, the concentration of tetracycline (TC), the way of the addition of TC, and the incubation time were varied in order to obtain the aimed difference in measured values between TC-treated and TC-untreated cells. Also in one experiment a washing step with PBS was undertaken between the exchange of cultivation medium with incubation medium and in another test an additional medium exchange was accomplished shortly before measurement. Furthermore, EMEM with or without phenol red in several FCS-concentrations was used. For cultivation EMEM either with or without phenol red with 10% FCS each was applied, whereas EMEM without phenol red with 0, 1, 3, 5 or 10% FCS served as induction medium.

In the following, results of two different experiments are introduced.

At one experiment cells were prepared in a concentration of 136.000 cells/ml. 200 µl of this suspension were seeded in medium without phenol red with a serum concentration of 10% FCS. After 24 hours of cultivation half of the consumed medium (100µl) was removed and 100 µl of a 2-fold solution with TC (0.2 µg/ml) in medium without phenol red and 5% FCS (TC-treated cells) and 100 µl of a not TC-containing 2-fold medium without phenol red and 5% FCS (TC-untreated cells) were added into each appropriate well of a 96-well-microtiterplate.

After further 24 hours of incubation, measurement was accomplished via EZ4U-test and the difference in measured values obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. In this case TC-treatment resulted in a viability reduction of circa $14 \pm 1\%$ (see Figure 3.1).

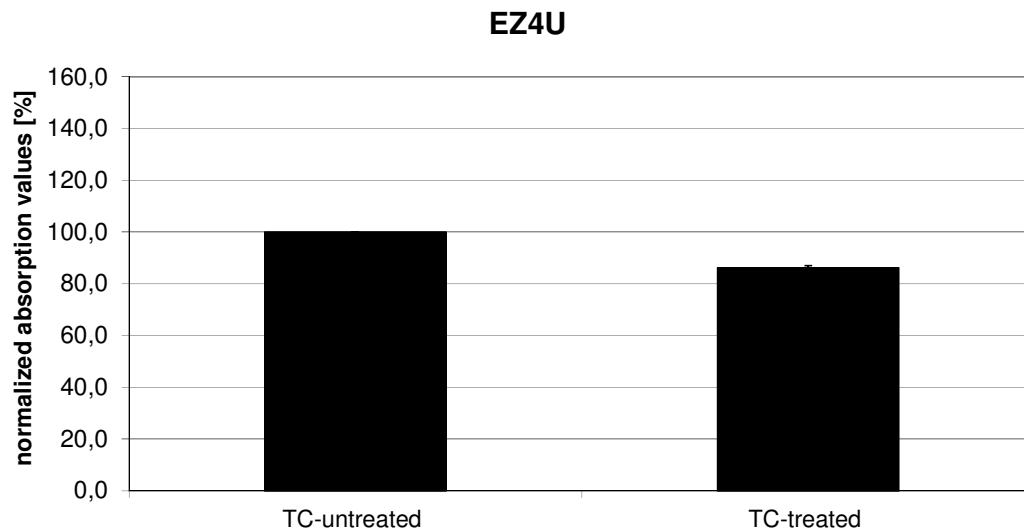


Figure 3.1 Obtained difference in measured values between tetracycline (TC)-untreated- and TC-treated cells using EZ4U-test. Data are given as means $\pm$ SEM, n = 16 wells.

For another experiment using EZ4U-test the following test setup was accomplished. Cells were seeded in a concentration of 136.000 cells/ml. 0.1 $\mu\text{g/ml}$ tetracycline was added directly to a part of the cell suspension and 200 μl of this mixture was applied into each appropriate well of a 96-well-microtiterplate (=TC-treated cells). The rest of the cell suspension was applied to 200 μl each into the appropriate wells (=TC-untreated cells). After 24 hours of incubation measurement was made via EZ4U-test and the difference in measured values

obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. In this case, addition of TC yielded in circa $43 \pm 2\%$ reduction of cell viability (see Figure 3.2).

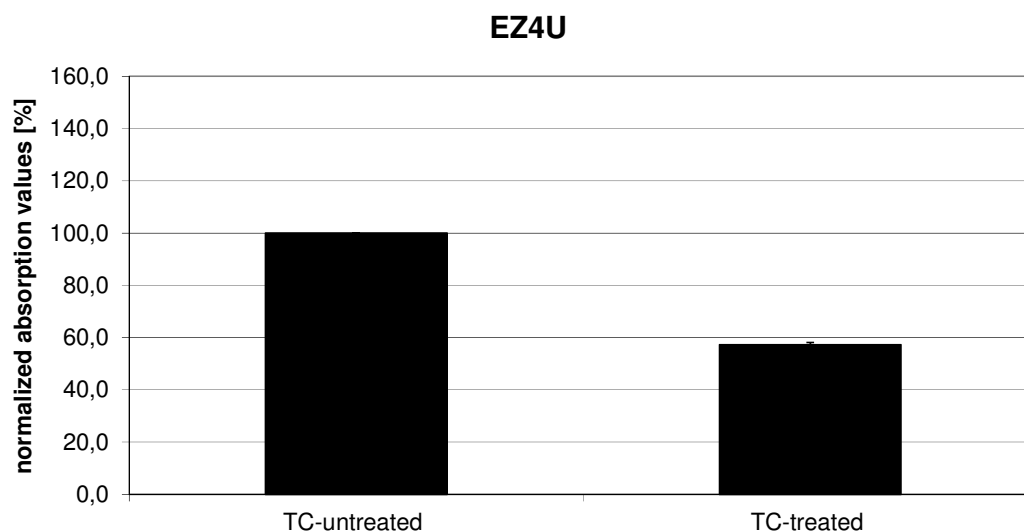


Figure 3.2 Highest obtained difference in measured values between tetracycline (TC)-untreated- and TC-treated cells using EZ4U-test, data are given as means $\pm$ SEM, n = 16 wells

By comparing the results shown in Figure 3.1 and Figure 3.2 it could be assumed that the time point and way of adding tetracycline (TC) to the cells may had a decisive impact on the difference in measured values between TC-treated and TC-untreated cells using EZ4U-test. However, by applying this test the maximal difference between TC-treated and TC-untreated cells resulted in circa 43% indicating that the aimed objective of at least 50% difference in measured values could not be achieved.

3.2 Optimization of the assay - results using Celltiter

Glo®-test

For example using the Bioluminescence-test, cells were seeded in a concentration of 68.000 cells/ml. 0.1 µg/ml tetracycline was added directly to a part of the cell suspension and 100 µl of this mixture was applied into each appropriate well of a white 96-well-microtiterplate (=TC-treated cells). The rest of the cell suspension was applied to 100 µl each to the appropriate wells (=TC-untreated cells). After 24 hours of incubation, measurement was performed via Bioluminescence-test and the difference in measured values obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. In this experiment the difference in measured values between tetracycline (TC)-treated and non-treated cells was not even 9%, which was far-off the desired objective of 50% (see Figure 3.3).

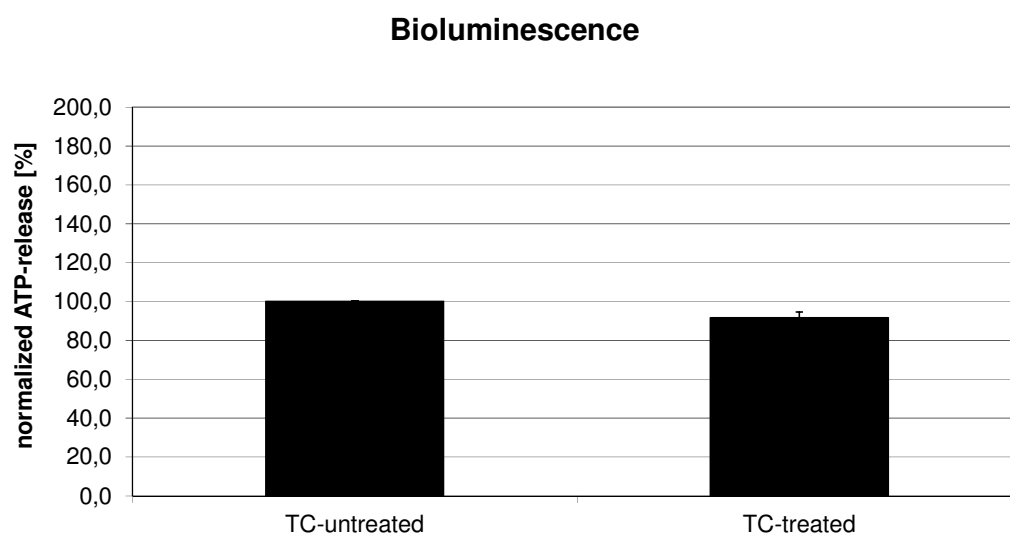


Figure 3.3 Obtained difference in measured values between tetracycline (TC)-untreated- and TC-treated cells using Bioluminescence-test. Data are given as means $\pm$ SEM, n = 24 wells.

3.3 Optimization of the assay - results using LDH-test-method

Using LDH-detection-method numerous experiments were implemented varying the way of the addition of tetracycline (TC) and using EMEM with or without phenol red in different FCS concentrations such as in EZ4U-test.

At one experiment using LDH-test the following test setup was applied. Cells were seeded in a concentration of 272.000 cells/ml. 50 µl of this suspension were seeded in medium without phenol red with a serum concentration of 3% FCS. Equal then 50 µl of a 2-fold solution of TC (0.2 µg/ml) in medium without phenol red and 3% FCS (TC-treated cells) and 50 µl of a 2-fold not TC-containing medium without phenol red and 3% FCS (TC-untreated cells) were added into each appropriate well of a 96-well-microtiterplate. After 24 hours of incubation measurement was made via LDH-test and the difference in measured values obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. In this case, TC increased the amount of cell toxicity to circa $19 \pm 2\%$ (see Figure 3.4).

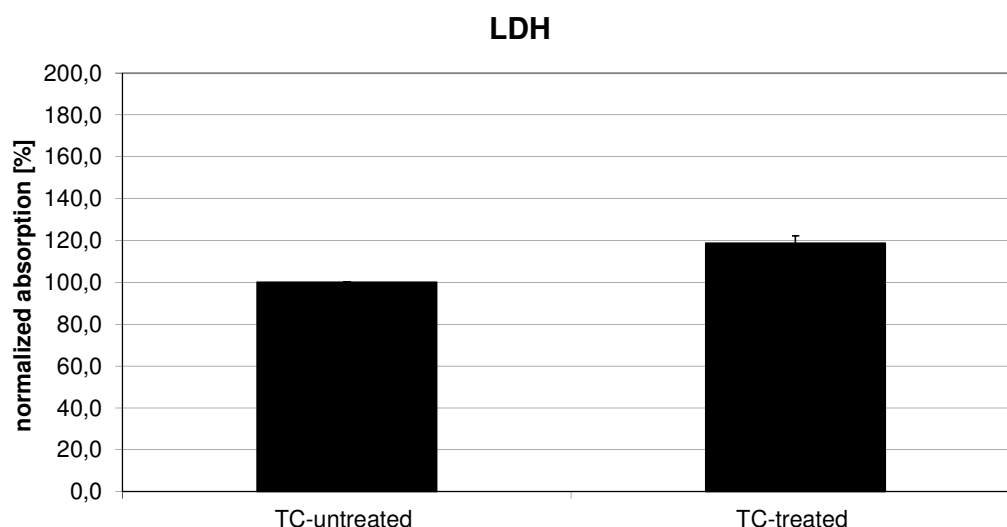


Figure 3.4 Obtained difference in measured values between tetracycline (TC)-untreated- and TC-treated cells using LDH-test. Data are given as means $\pm$ SEM, n = 16 wells.

For another experiment using LDH-test the following test setup was used. Cells were seeded in a concentration of 136.000 cells/ml. 100 μ l of the suspension (136.000 cells/ml) were seeded in medium without phenol red with a serum concentration of 10% FCS. After 24 hours of cultivation complete consumed medium was removed and 100 μ l of a solution of TC (0.1 μ g/ml) in medium without phenol red and 5% FCS (TC-treated cells) and 100 μ l of a solution of not TC-containing medium without phenol red and 5% FCS (TC-untreated cells) were added into each appropriate well of a 96-well-microtiterplate. After further 24 hours of incubation measurement was conducted via LDH-test and the difference in measured values obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. In this case, TC treatment resulted in $50 \pm 4\%$ increased cell toxicity (see Figure 3.5).

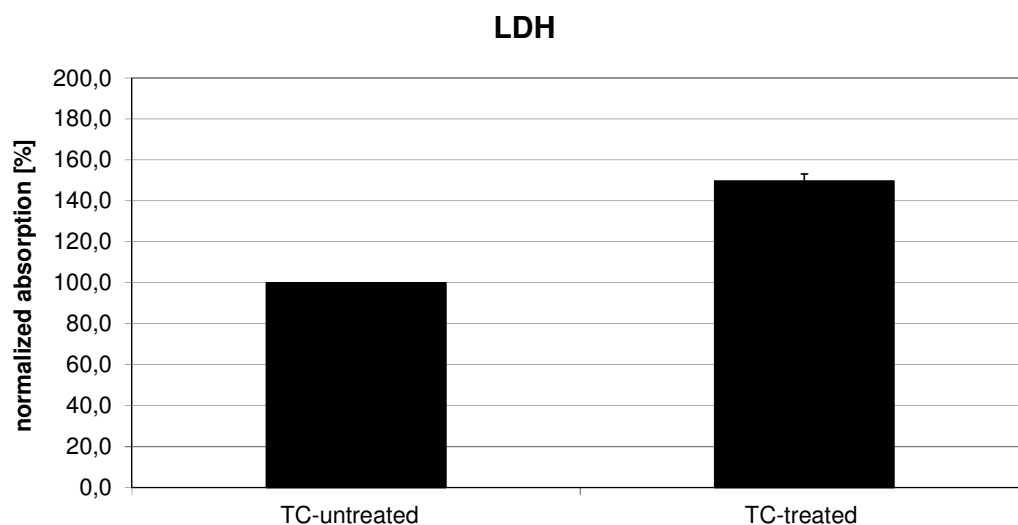


Figure 3.5 Obtained difference in measured values between tetracycline (TC)-untreated and TC-treated cells using LDH-test. Data are given as means $\pm$ SEM, n = 16 wells.

Comparing results shown in Figure 3.4 and Figure 3.5 using LDH-test - unlike EZ4U-test - a higher difference in measured values between TC-treated and TC-untreated cells could be obtained. Addition of tetracycline to the cells 24 hours after cell seeding towards previously addition of tetracycline immediately after cell seeding seemed to be advantageous for the LDH-assay set-up.

Using LDH-test the highest difference in measured values between TC-untreated- and TC-treated cells was obtained by applying the following test setup. Cells were seeded in a concentration of 136.000 cells/ml. 100 µl of this suspension were seeded in medium with phenol red with a serum concentration of 10% FCS. After 24 hours of cultivation consumed medium was removed and 100 µl of a solution of TC (0.1 µg/ml) in medium without phenol red and 5% FCS (TC-treated cells) and 100 µl of a solution of not TC-containing medium without phenol red and 5% FCS (TC-untreated cells) were added into each appropriate well of a 96-well-microtiterplate. After further 24 hours of incubation measurement was made via LDH-test and the difference in measured values obtained for TC-untreated- and TC-treated cells was determined as described under section 2.2.4. Applying this setup an over 2-fold (239% - 380%) TC-induced cell toxicity was achieved over several passages (PN17 - PN29) (see Figure 3.6).

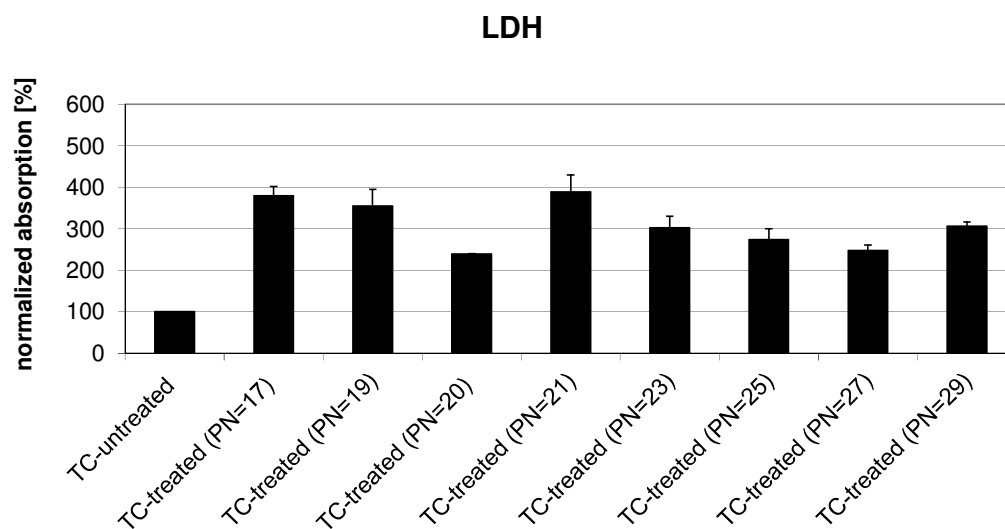


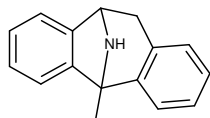
Figure 3.6 Obtained differences in measured values between TC-treated and TC-untreated cells depending on the cells passage number (PN) using LDH-test. Data are given as means $\pm$ SEM, n = 4-18 from at least 4 independent plates.

Thus, the LDH-test with the optimized procedure was chosen to be used for generating the dose-response curves of the test substances.

3.4 Dose-response curves of the test substances

3.4.1 Channel (pore) blockers

3.4.1.1 MK-801



= [5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

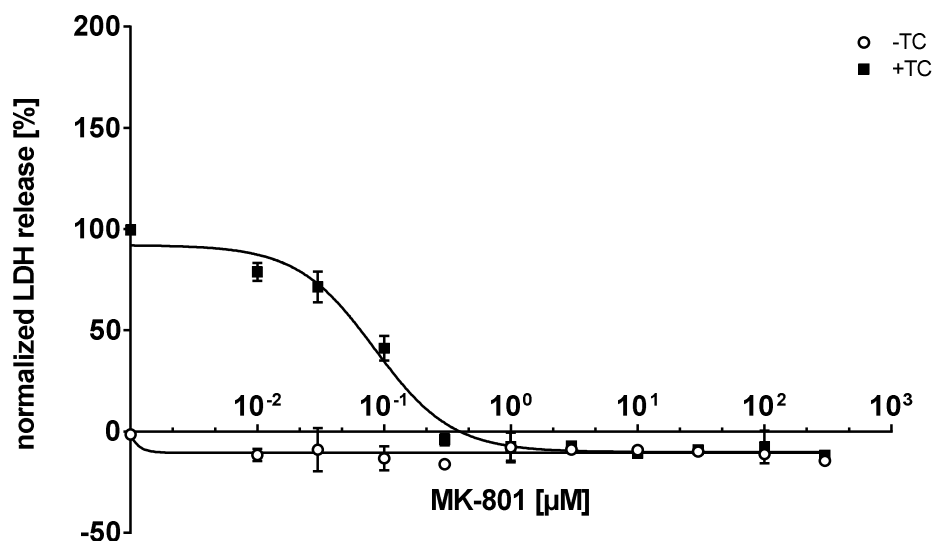
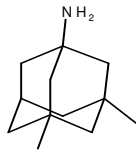


Figure 3.7 Fitted sigmoidal dose-response curves for MK-801 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (0.001-300 μM) – 16 (1 μM) wells from at least two independent plates.

MK-801 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of 84 nM and as indicated by the single data points, MK-801 exhibited no NMDAR-unrelated cytotoxic effects up to a concentration of 300 μM .

3.4.1.2 Memantine



= 3,5-dimethyltricyclo[3.3.1.1^{3,7}]decan-1-amine

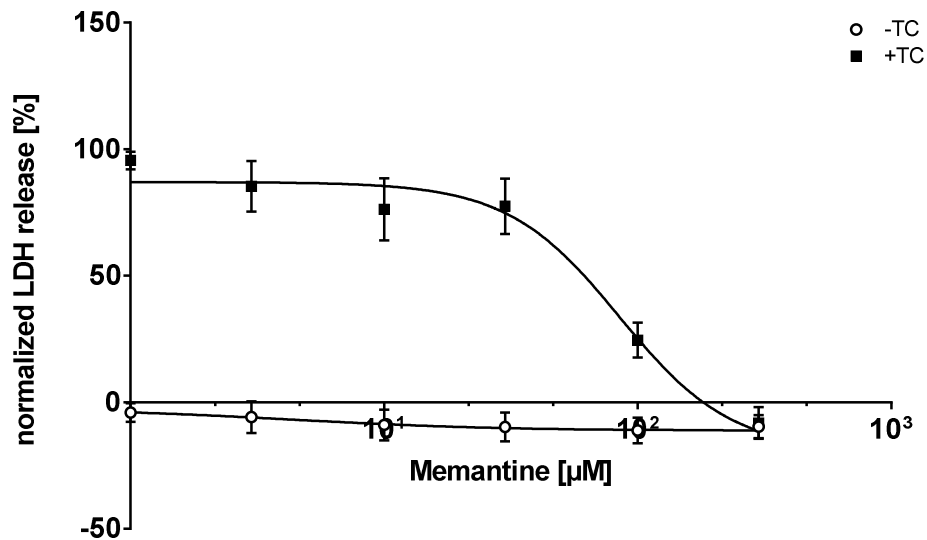
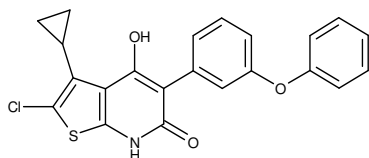


Figure 3.8 Fitted sigmoidal dose-response curves for memantine and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Memantine was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of 84 μM and as indicated by the single data points, memantine exhibited no NMDAR-unrelated cytotoxic effects up to a concentration of 300 μM .

3.4.2 Glycine antagonists

3.4.2.1 Bu-081



= 2-chloro-4-hydroxy-3-isopropyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

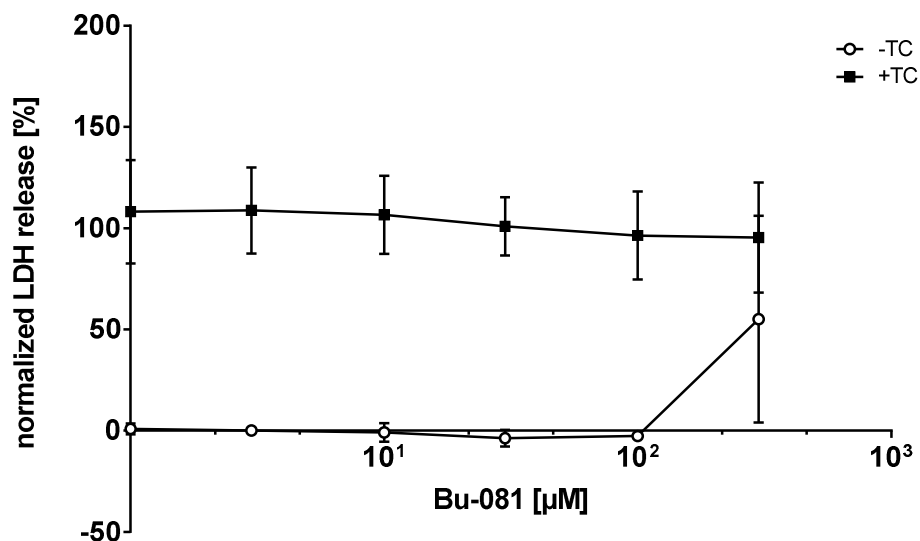
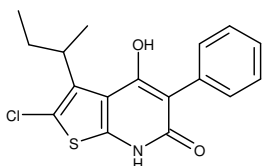


Figure 3.9 Connecting line between the calculated data points for Bu-081 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=12$ (1-300 μM) wells from three independent plates.

It was demonstrated that Bu-081 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μM . At a concentration of 300 μM Bu-081 exhibited strong NMDAR-unrelated cytotoxic effects.

3.4.2.2 Bu-082



= 2-chloro-4-hydroxy-3-isobutyl-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

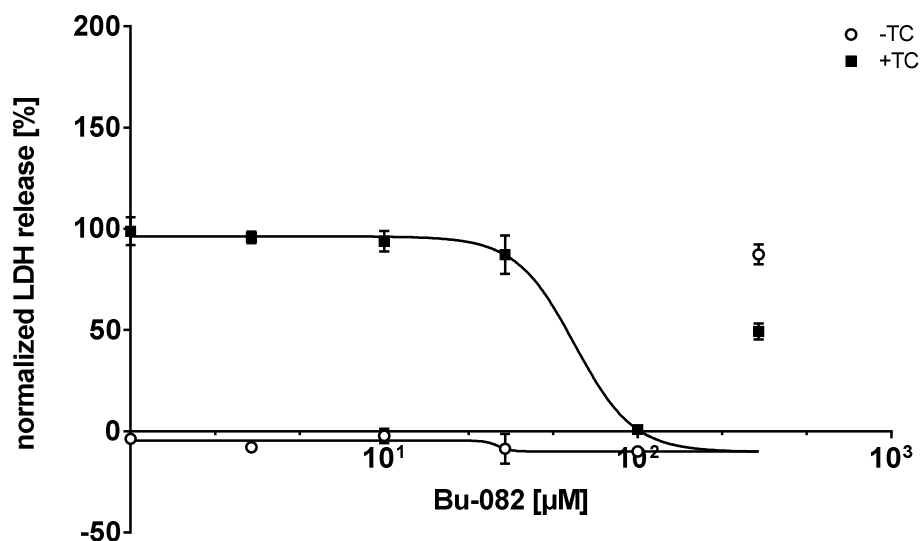
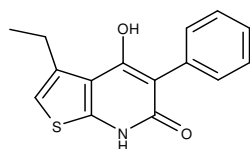


Figure 3.10 Fitted sigmoidal dose-response curves for Bu-082 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

Bu-082 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 56 μ M ($IC_{50}=56.38 \mu$ M) and as indicated by the single data points at a concentration of 300 μ M Bu-082 showed strong NMDAR-unrelated cytotoxic effects.

3.4.2.3 Bu-085



= 3-ethyl-4-hydroxy-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

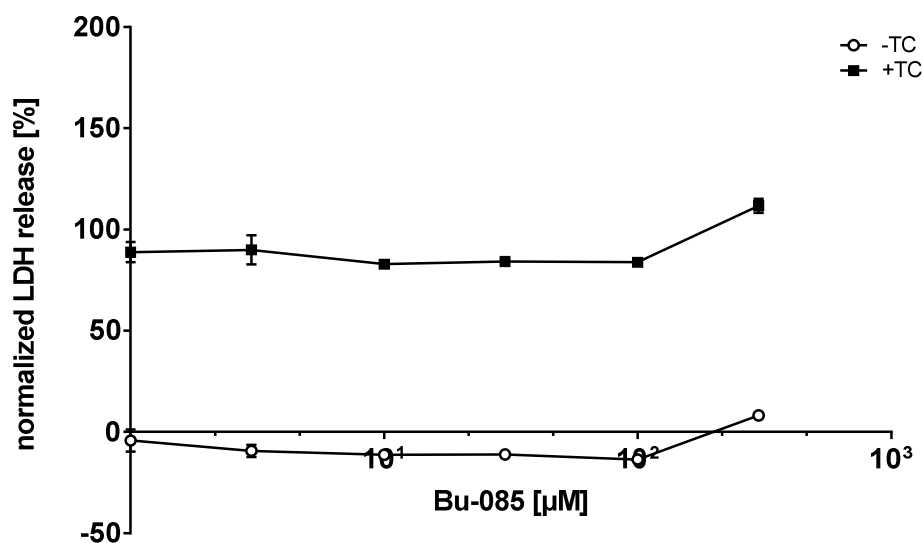
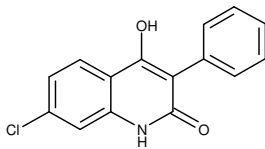


Figure 3.11 Connecting line between the calculated data points for Bu-085 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-085 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. At a concentration of 300 μ M Bu-085 revealed slight NMDAR-unrelated cytotoxic effects.

3.4.2.4 Bu-090



= 7-chloro-4-hydroxy-3-phenyl-2(1H)-quinolin-2-one

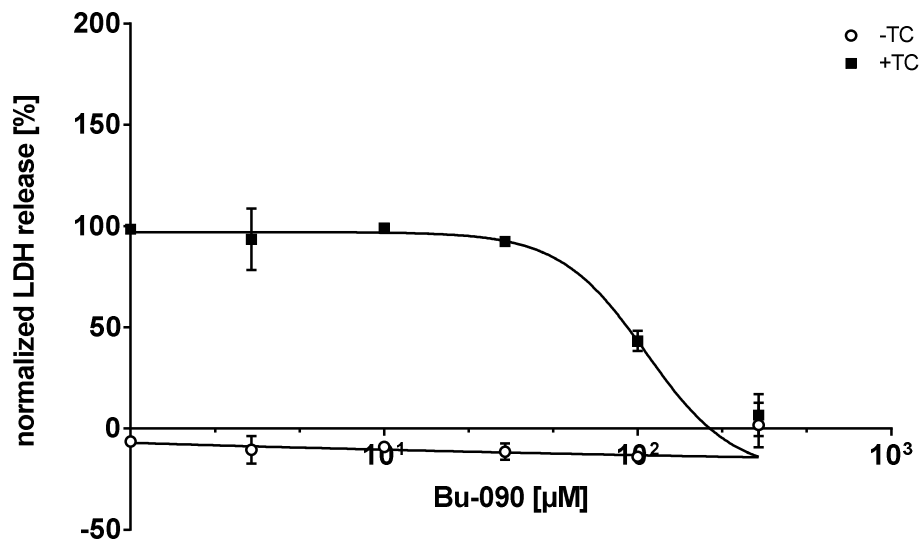
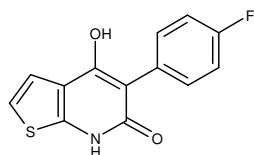


Figure 3.12 Fitted sigmoidal dose-response curves for Bu-090 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Bu-090 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 89 μM ($\text{IC}_{50}=89.65 \mu\text{M}$) and as indicated by the single data points at a concentration of 300 μM Bu-090 showed slight NMDAR-unrelated cytotoxic effects.

3.4.2.5 Bu-093



= 5-(4-fluorophenyl)-4-hydroxy-thieno[2,3-b]pyridin-6(7H)-one

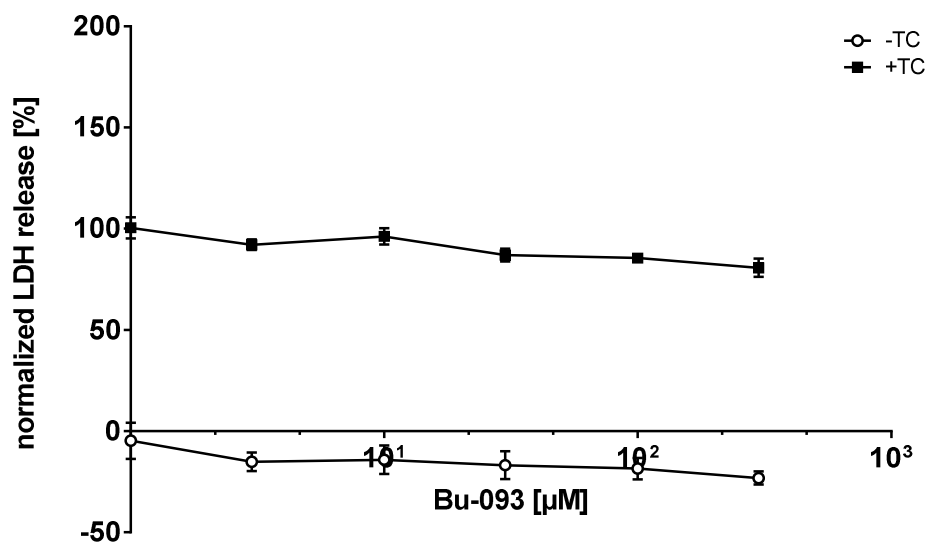
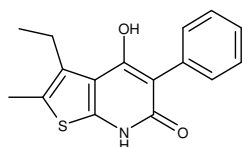


Figure 3.13 Connecting line between the calculated data points for Bu-093 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-093 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. Up to a concentration of 300 μ M Bu-093 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.6 Bu-094



= 3-ethyl-4-hydroxy-2-methyl-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

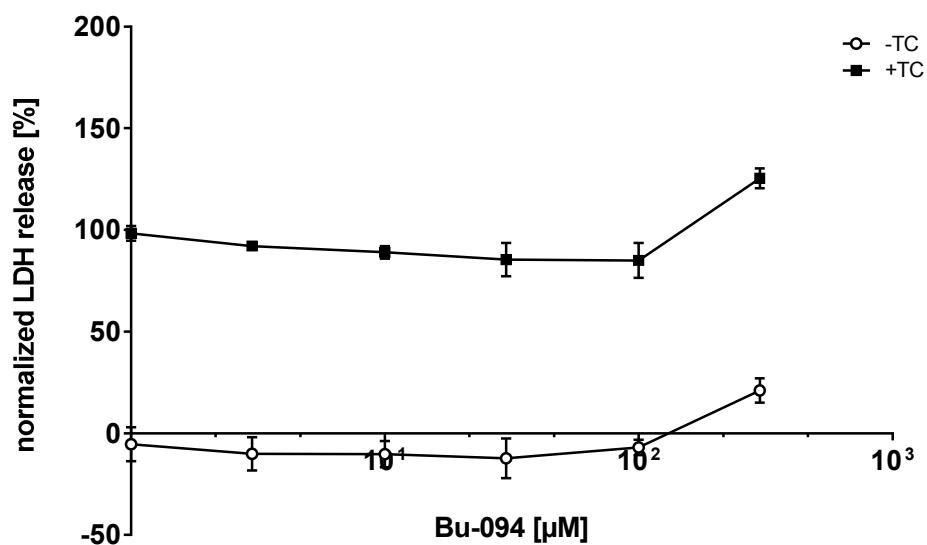
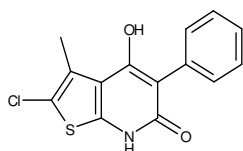


Figure 3.14 Connecting line between the calculated data points for Bu-094 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-094 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. At a concentration of 300 μ M Bu-094 revealed NMDAR-unrelated cytotoxic effects.

3.4.2.7 Bu-097



= 2-chloro-4-hydroxy-3-methyl-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

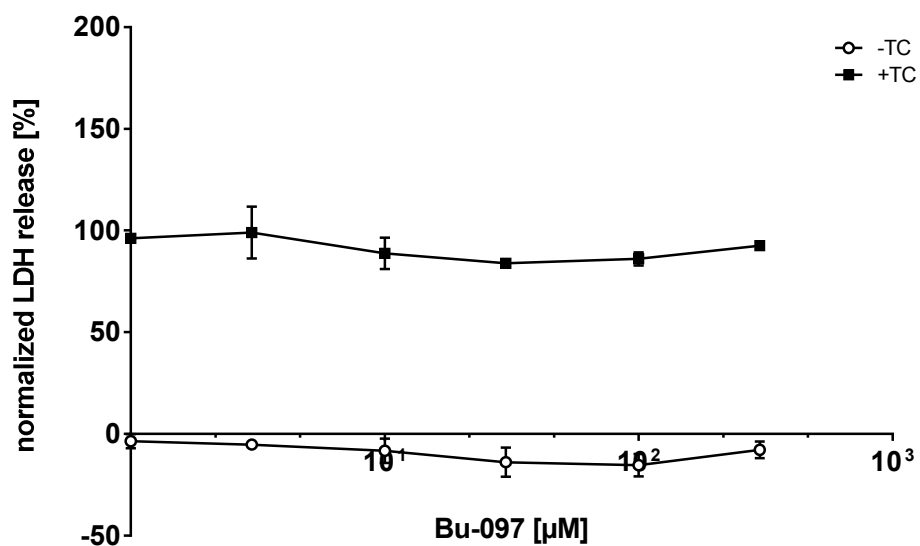
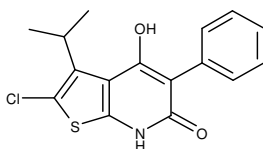


Figure 3.15 Connecting line between the calculated data points for Bu-097 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

It was demonstrated that Bu-097 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μM . Up to a concentration of 300 μM Bu-097 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.8 Bu-099



= 2-chloro-4-hydroxy-3-isopropyl-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

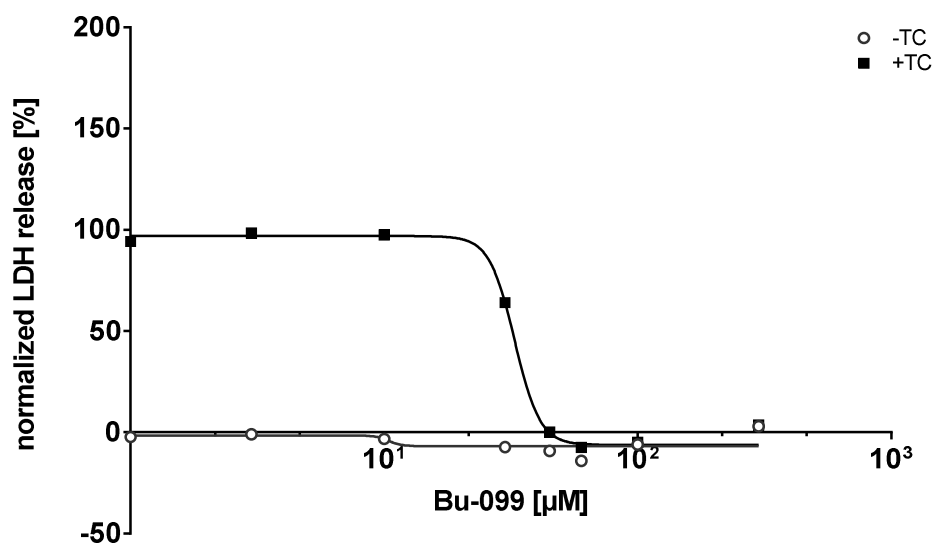
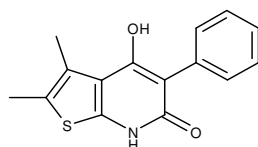


Figure 3.16 Fitted sigmoidal dose-response curves for Bu-099 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μ M) – 16 (10, 30 μ M) wells from at least two independent plates.

Bu-099 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 32 μ M ($IC_{50}=32.74 \mu$ M) and as indicated by the single data points at a concentration of 300 μ M Bu-099 showed slight NMDAR-unrelated cytotoxic effects.

3.4.2.9 Bu-101



= 2,3-dimethyl-4-hydroxy-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

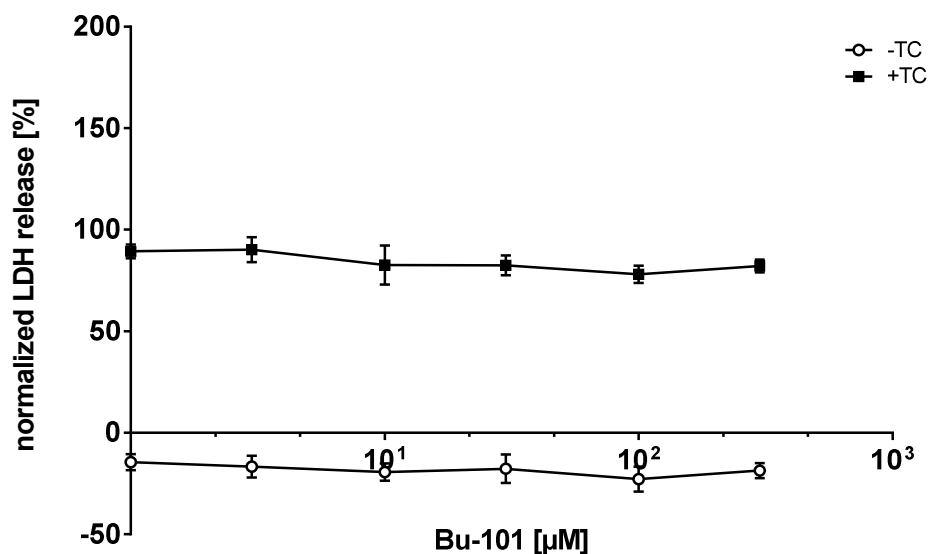
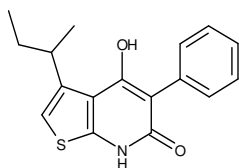


Figure 3.17 Connecting line between the calculated data points for Bu-101 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=16$ (1-300 μ M) wells from four independent plates.

It was demonstrated that Bu-101 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. Up to a concentration of 300 μ M Bu-101 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.10 Bu-104



= 3-isobutyl-4-hydroxy-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

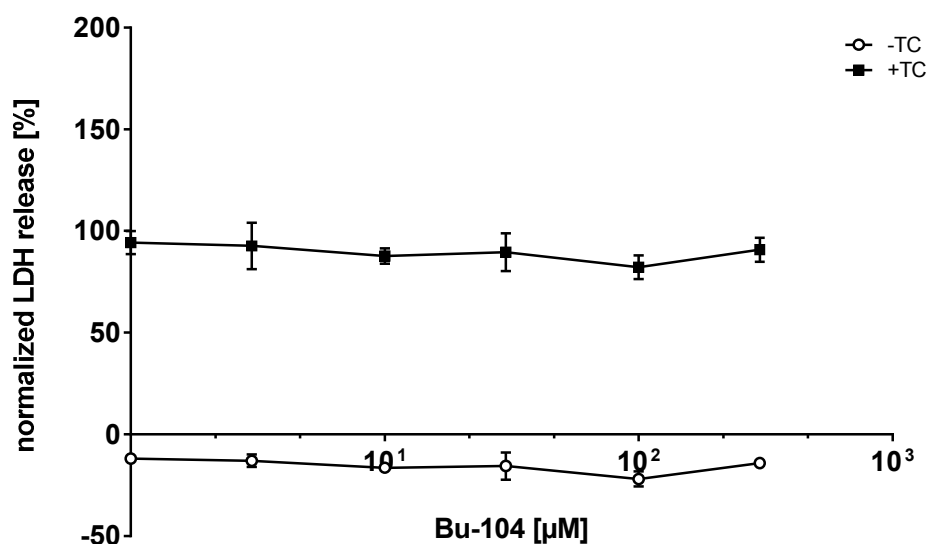
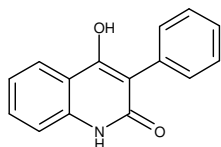


Figure 3.18 Connecting line between the calculated data points for Bu-104 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-104 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. Up to a concentration of 300 μ M Bu-104 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.11 Bu-105



= 4-hydroxy-3-phenyl-2(1H)-quinolin-2-one

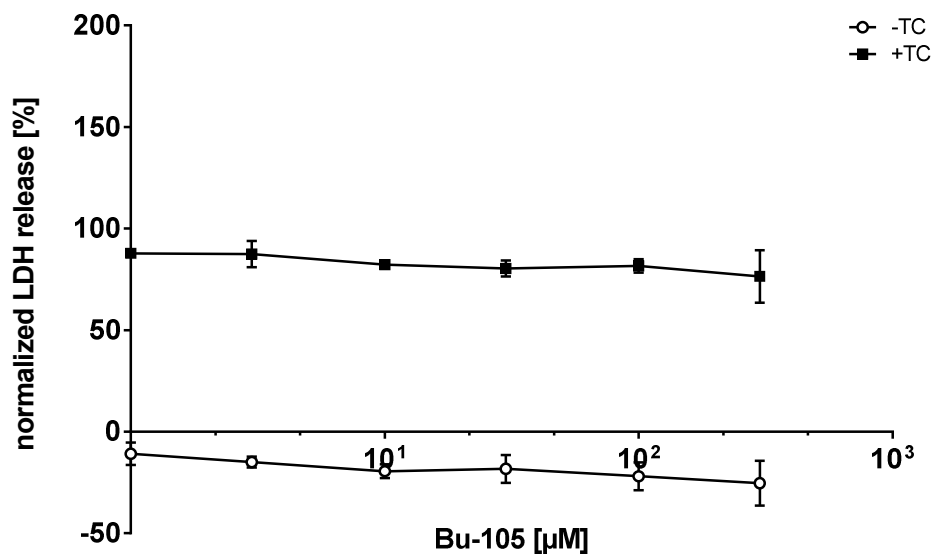
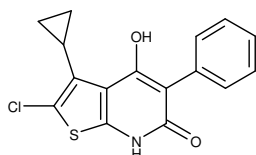


Figure 3.19 Connecting line between the calculated data points for Bu-105 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-105 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. Up to a concentration of 300 μ M Bu-105 exhibited no NMDAR-unrelated cytotoxic effects.

3.4.2.12 Bu-106



= 2-chloro-4-hydroxy-3-isopropyl-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

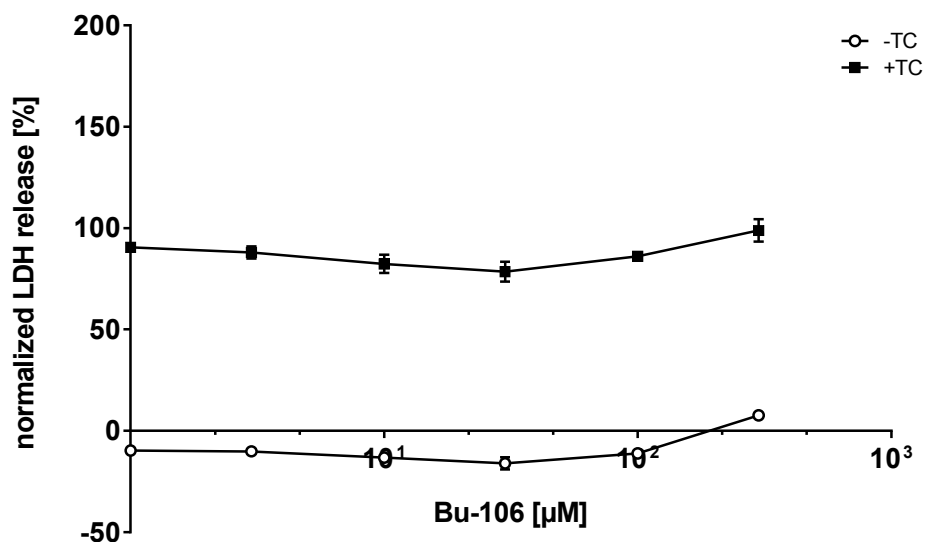
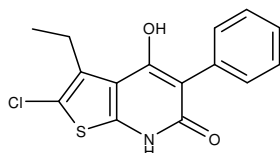


Figure 3.20 Connecting line between the calculated data points for Bu-106 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-106 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. At a concentration of 300 μ M Bu-106 revealed slight NMDAR-unrelated cytotoxic effects.

3.4.2.13 Bu-108



= 2-chloro-3-ethyl-4-hydroxy-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

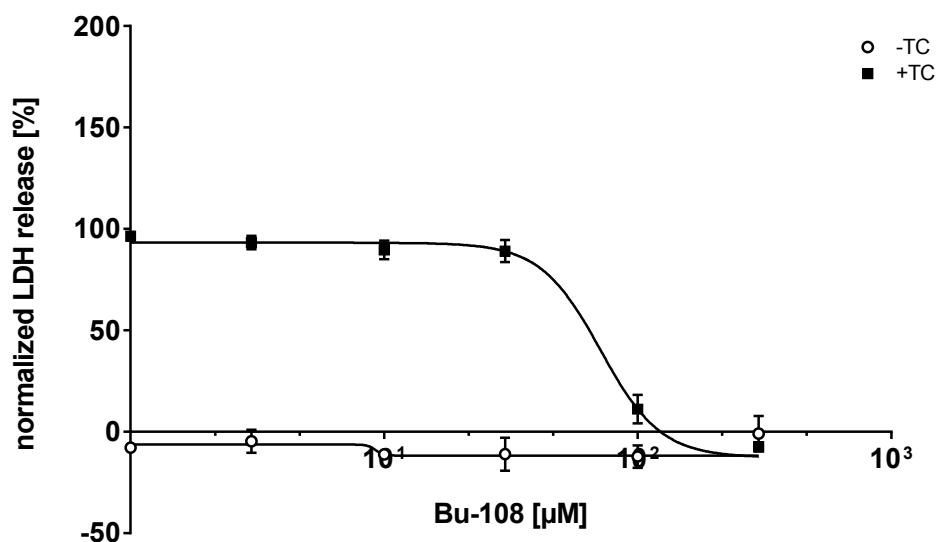
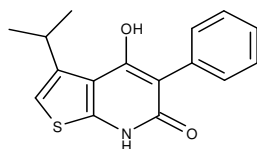


Figure 3.21 Fitted sigmoidal dose-response curves for Bu-108 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Bu-108 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 71 μM ($\text{IC}_{50}=71.37 \mu\text{M}$) and as indicated by the single data points, Bu-108 exhibited no NMDAR-unrelated cytotoxic effects up to a concentration of 300 μM .

3.4.2.14 Bu-109



= 3-isopropyl-4-hydroxy-5-phenyl-thieno[2,3-b]pyridin-6(7H)-one

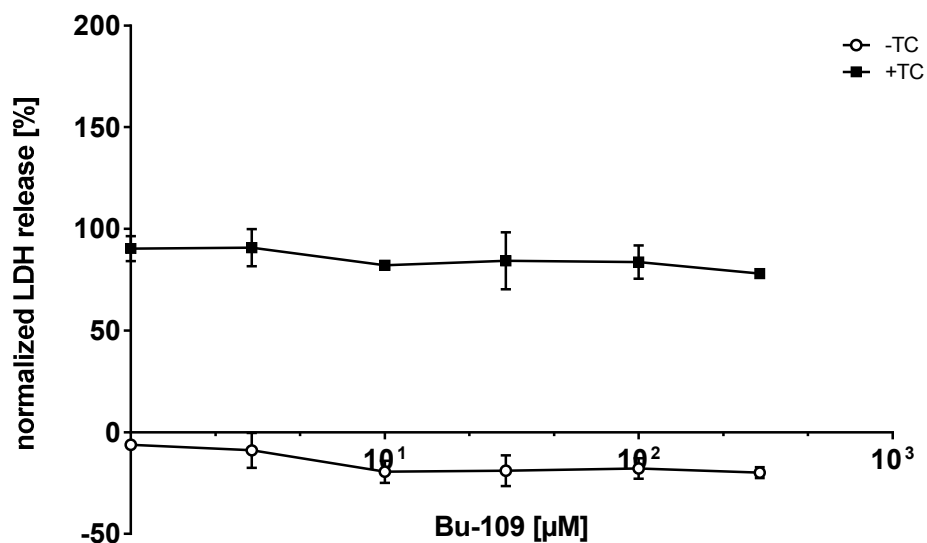
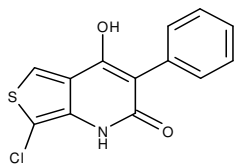


Figure 3.22 Connecting line between the calculated data points for Bu-109 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Bu-109 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. Up to a concentration of 300 μ M Bu-109 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.15 Bu-112



= 7-chloro-3-hydroxy-4-phenyl-thieno[3,4-b]pyridin-6(7H)-one

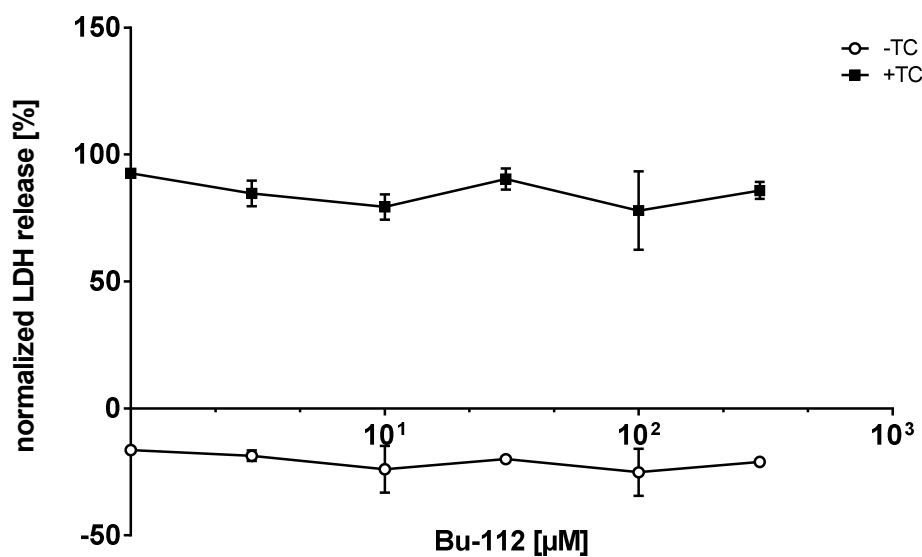
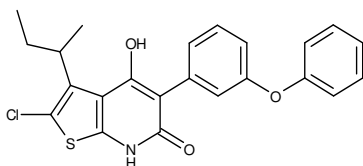


Figure 3.23 Connecting line between the calculated data points for Bu-112 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

It was demonstrated that Bu-112 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μM . Up to a concentration of 300 μM Bu-112 revealed no NMDAR-unrelated cytotoxic effects.

3.4.2.16 Bu-113



= 2-chloro-4-hydroxy-3-isobutyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

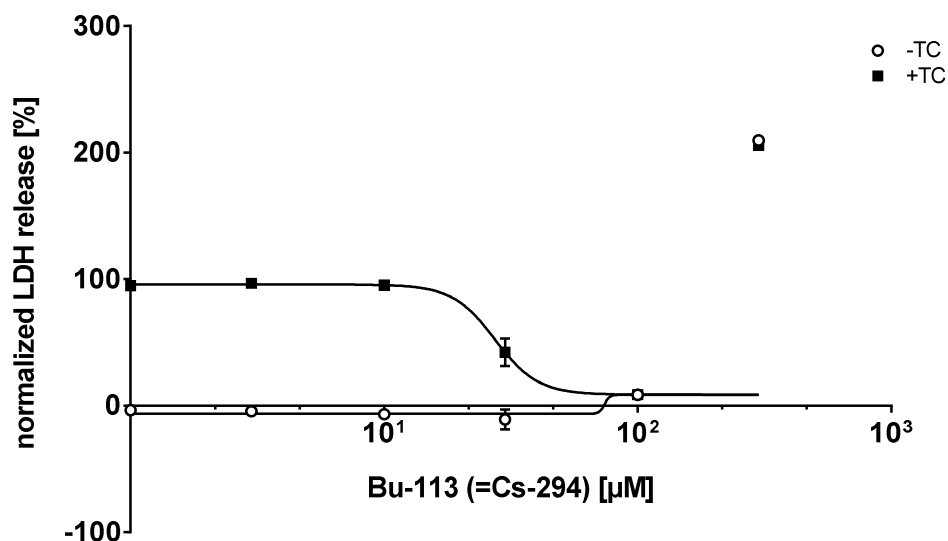
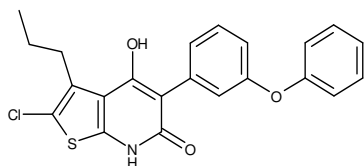


Figure 3.24 Fitted sigmoidal dose-response curves for Bu-113 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Bu-113 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 27 μM ($\text{IC}_{50}=27.41 \mu\text{M}$) and as indicated by the single data points at a concentration of 100 μM Bu-113 started to reveal slight NMDAR-unrelated cytotoxic effects.

3.4.2.17 Cs-182



= 2-chloro-4-hydroxy-3-propyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

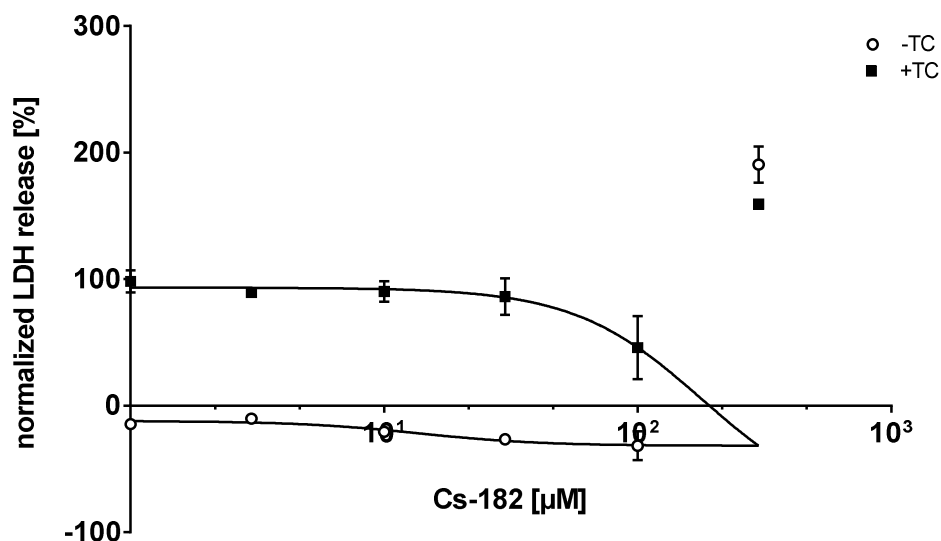
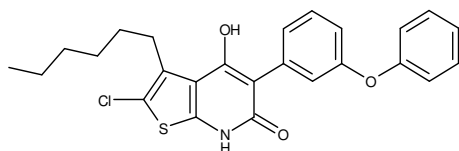


Figure 3.25 Fitted sigmoidal dose-response curves for Cs-182 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Cs-182 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 179 μM ($\text{IC}_{50}=179.8 \mu\text{M}$) and as indicated by the single data points at a concentration of 300 μM Cs-182 showed strong NMDAR-unrelated cytotoxic effects.

3.4.2.18 Cs-183



= 2-chloro-3-hexyl-4-hydroxy-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

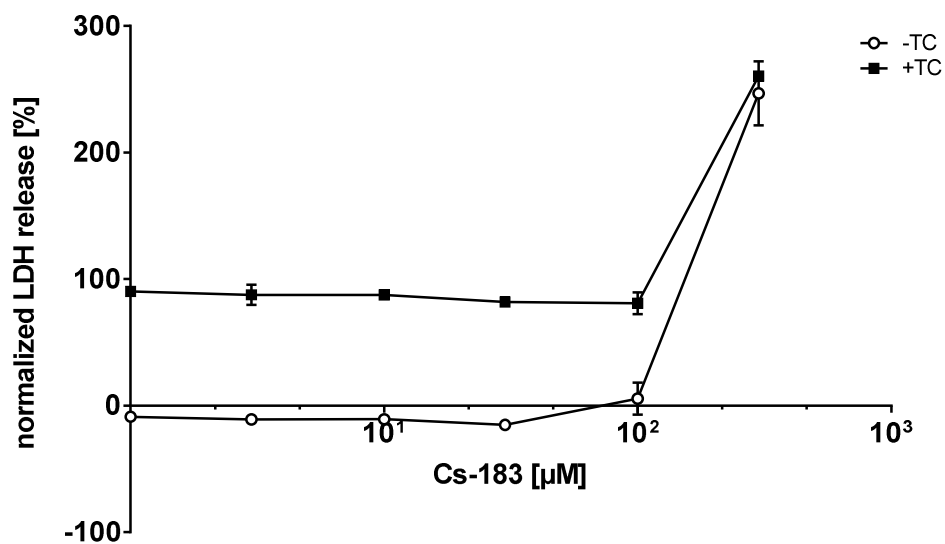
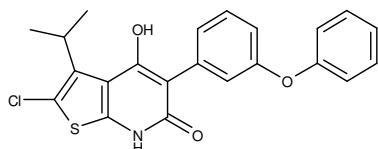


Figure 3.26 Connecting line between the calculated data points for Cs-183 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=8 (1-300 μ M) wells from two independent plates.

It was demonstrated that Cs-183 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. At a concentration of 300 μ M Cs-183 showed strong NMDAR-unrelated cytotoxic effects.

3.4.2.19 Cs-191



= 2-chloro-4-hydroxy-3-isopropyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

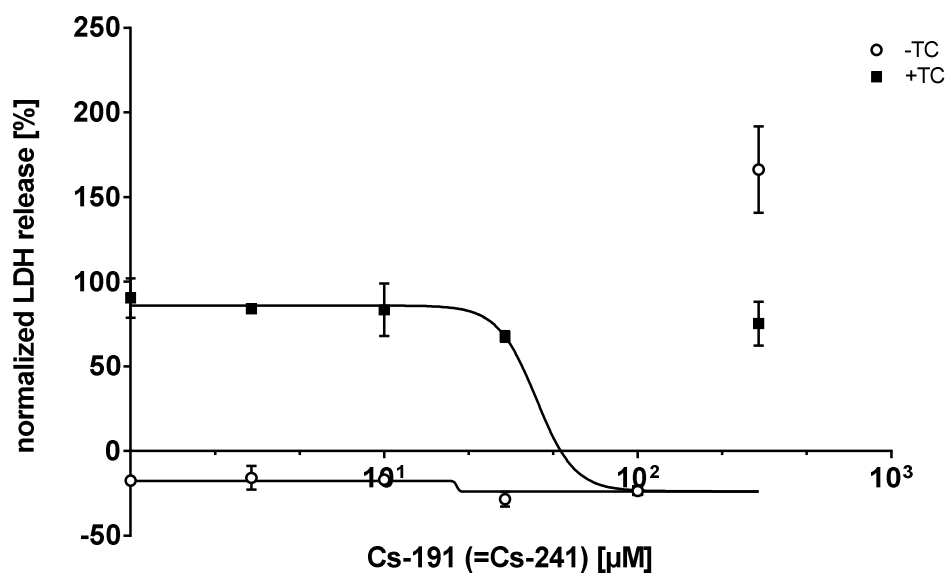
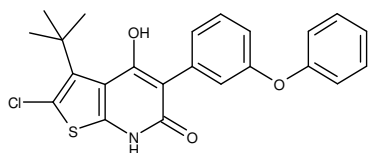


Figure 3.27 Fitted sigmoidal dose-response curves for Cs-191 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from two independent plates.

Cs-191 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 39 μM ($\text{IC}_{50}=39.76 \mu\text{M}$) and as indicated by the single data points at a concentration of 300 μM Cs-191 showed strong NMDAR-unrelated cytotoxic effects.

3.4.2.20 Cs-199



= 2-chloro-4-hydroxy-3-isobutyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

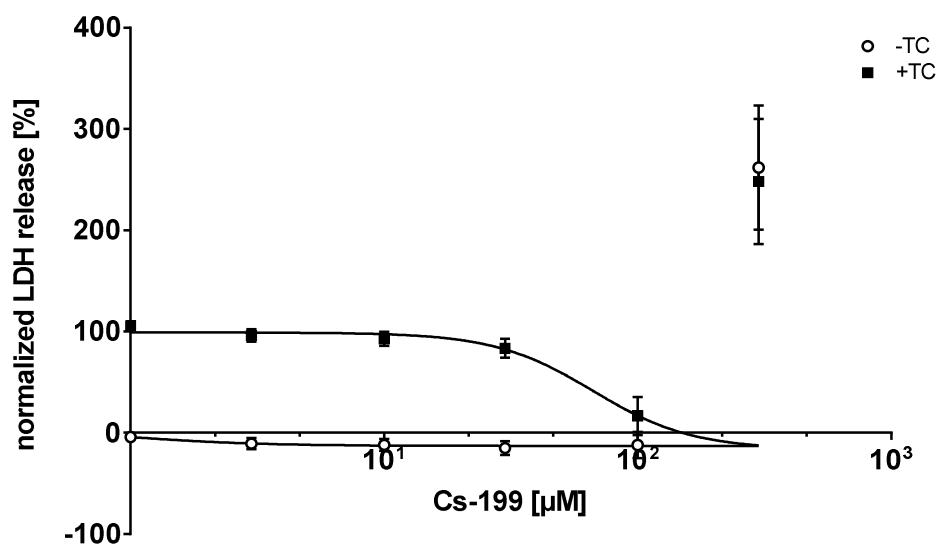
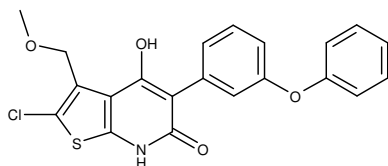


Figure 3.28 Fitted sigmoidal dose-response curves for Cs-199 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=16$ (1-300 μM) wells from four independent plates.

Cs-199 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 66 μM ($\text{IC}_{50}=66.70 \mu\text{M}$) and as indicated by the single data points at a concentration of 300 μM Cs-199 showed strong NMDAR-unrelated cytotoxic effects.

3.4.2.21 Cs-224



= 2-chloro-4-hydroxy-3-methoxymethyl-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

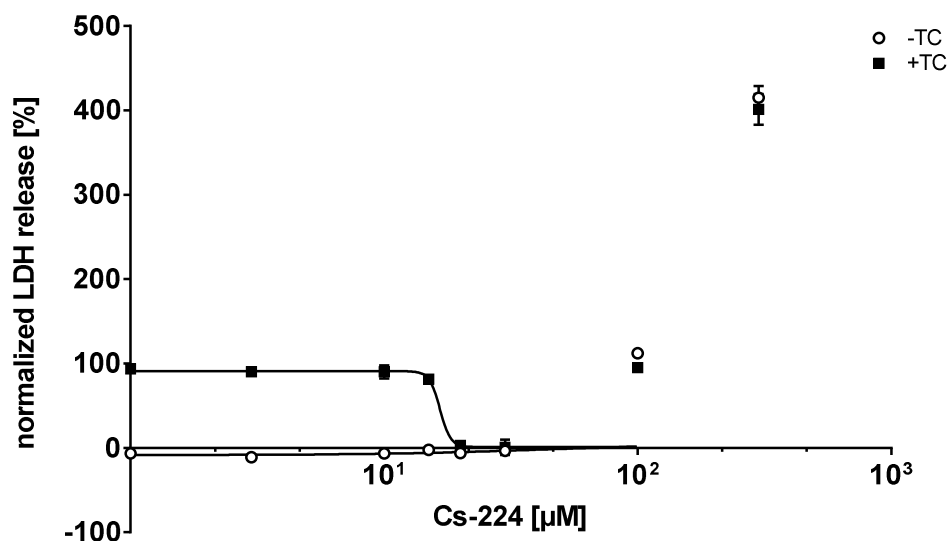
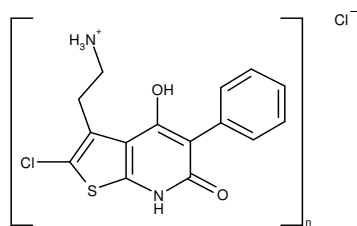


Figure 3.29 Fitted sigmoidal dose-response curves for Cs-224 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=4$ (15 μ M) – 8 (1-300 μ M) – 16 (30 μ M) – 20 (10 μ M) wells from at least one plate.

Cs-224 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 16 μ M ($IC_{50}=16.63 \mu$ M) and as indicated by the single data points Cs-224 showed NMDAR-unrelated cytotoxic effects already at a concentration of 100 μ M.

3.4.2.22 1F1



= 3-(2-amino-ethyl)-2-chloro-4-hydroxy-5-phenyl-thieno[2,3-b]pyridine-6(7H)-on-hydrochloride

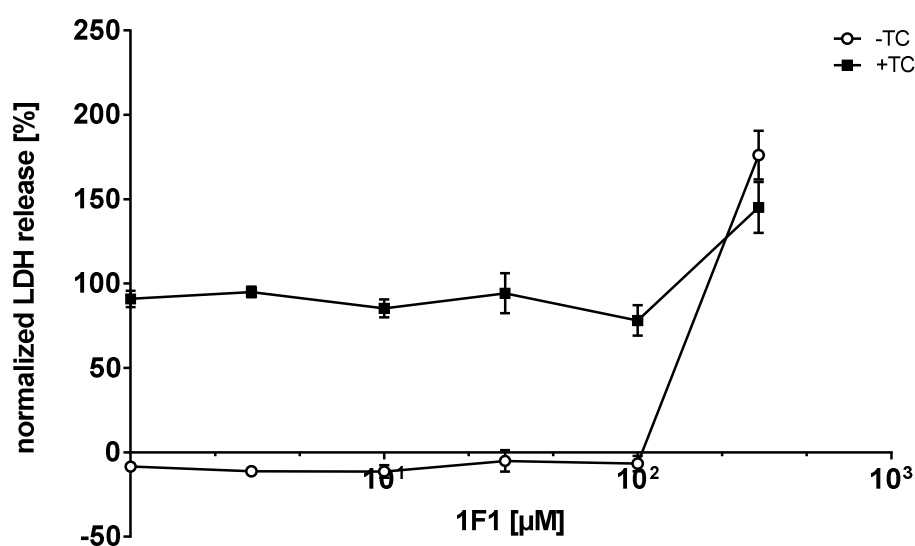
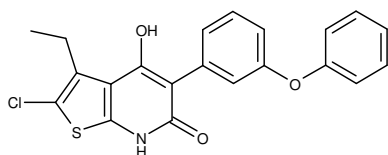


Figure 3.30 Connecting line between the calculated data points for 1F1 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=4 (1-300 μ M) wells from one plate.

It was demonstrated that 1F1 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μ M. At a concentration of 300 μ M 1F1 revealed strong NMDAR-unrelated cytotoxic effects.

3.4.2.23 Sie-20



= 2-chloro-3-ethyl-4-hydroxy-5-(3-phenoxyphenyl)-thieno[2,3-b]pyridin-6(7H)-one

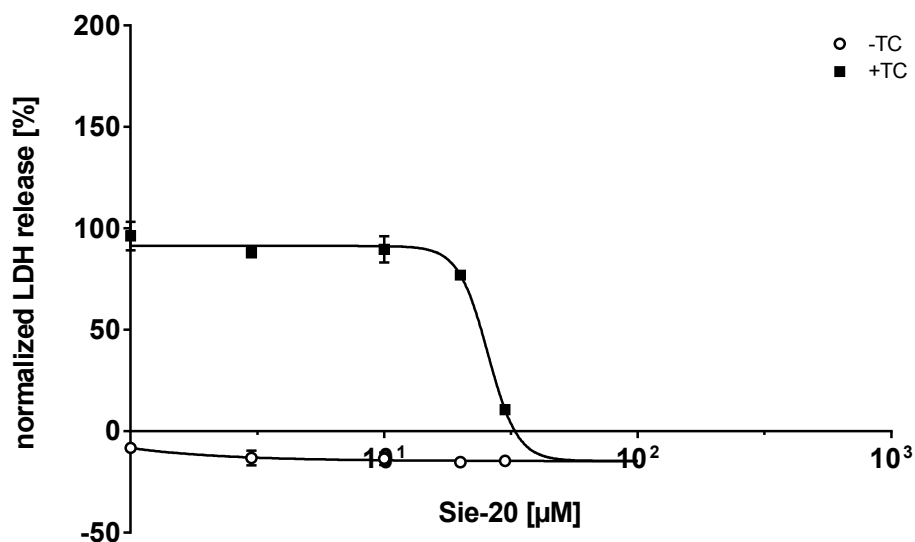
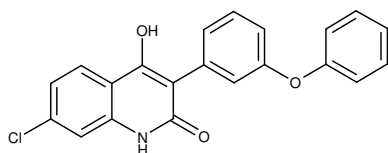


Figure 3.31 Fitted sigmoidal dose-response curves for Sie-20 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-30 μM) wells from two independent plates.

Sie-20 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 25 μM ($\text{IC}_{50}=25.66 \mu\text{M}$) and as indicated by the single data points, Sie-20 revealed no NMDAR-unrelated cytotoxic effects up to a concentration of 30 μM .

3.4.2.24 L-701,324



= 7-chloro-4-hydroxy-3-(3-phenoxyphenyl)-2(1H)-quinolin-2-one

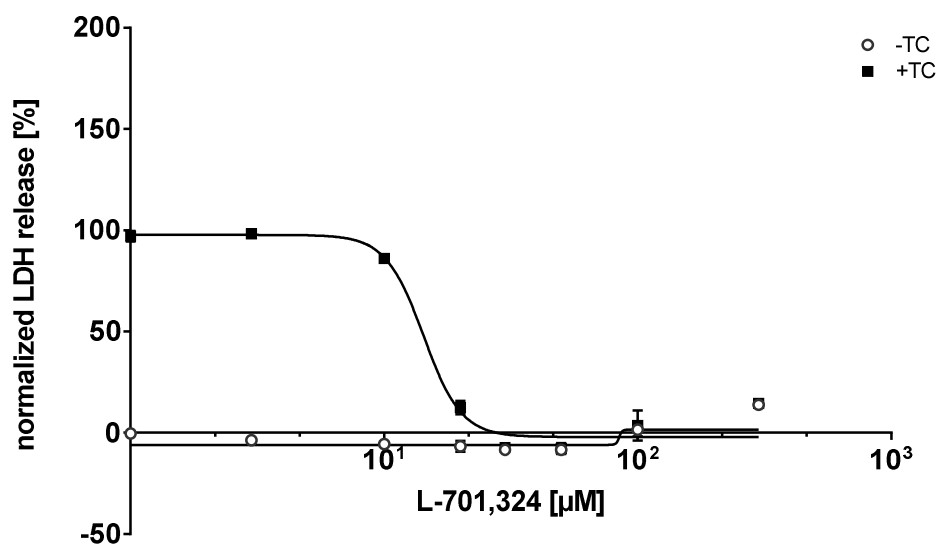
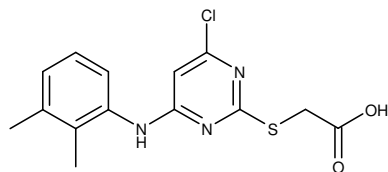


Figure 3.32 Fitted sigmoidal dose-response curves for L-701,324 and its effects on LDH release in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=24$ (3-100 μM) – 8 (1, 300 μM) – 16 (20, 50 μM) wells from at least two independent plates.

L-701,324 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 14 μM ($\text{IC}_{50}=14.22 \mu\text{M}$) and as indicated by the single data points L-701,324 started to reveal slight NMDAR-unrelated cytotoxic effects at a concentration of 100 μM .

3.4.3 Potentially novel NMDAR-modulators

3.4.3.1 Wy-14,643



= 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid

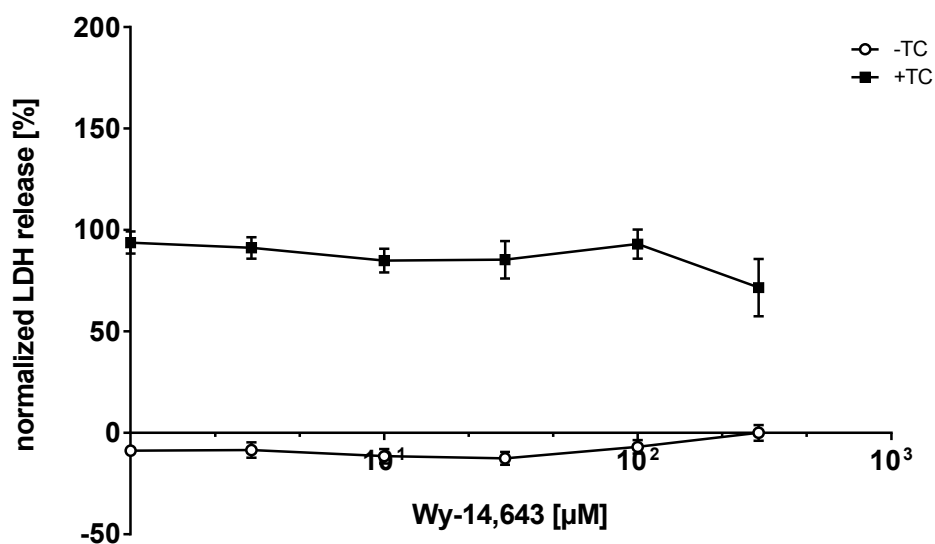
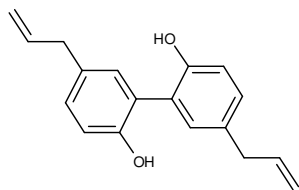


Figure 3.33 Connecting line between the calculated data points for Wy-14,643 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=16$ (1-300 μM) wells from four independent plates.

It was demonstrated that Wy-14,643 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 300 μM . Up to a concentration of 300 μM Wy-14,643 revealed no NMDAR-unrelated cytotoxic effects.

3.4.3.2 Magnolol



= 4-Allyl-2-(5-allyl-2-hydroxy-phenyl)phenol

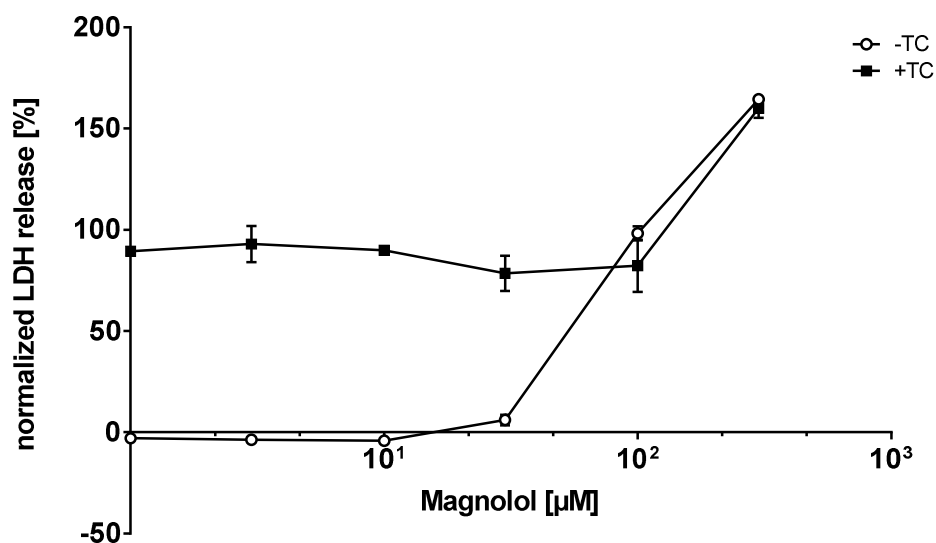
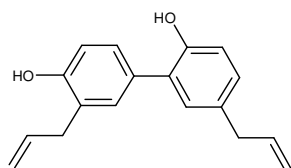


Figure 3.34 Connecting line between the calculated data points for magnolol at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=8$ (1-300 μM) wells from four independent plates.

It was demonstrated that magnolol did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation up to a concentration of 30 μM . At a concentration of 30 μM magnolol started to reveal slight NMDAR-unrelated cytotoxic effects.

3.4.3.3 Honokiol



= 2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol

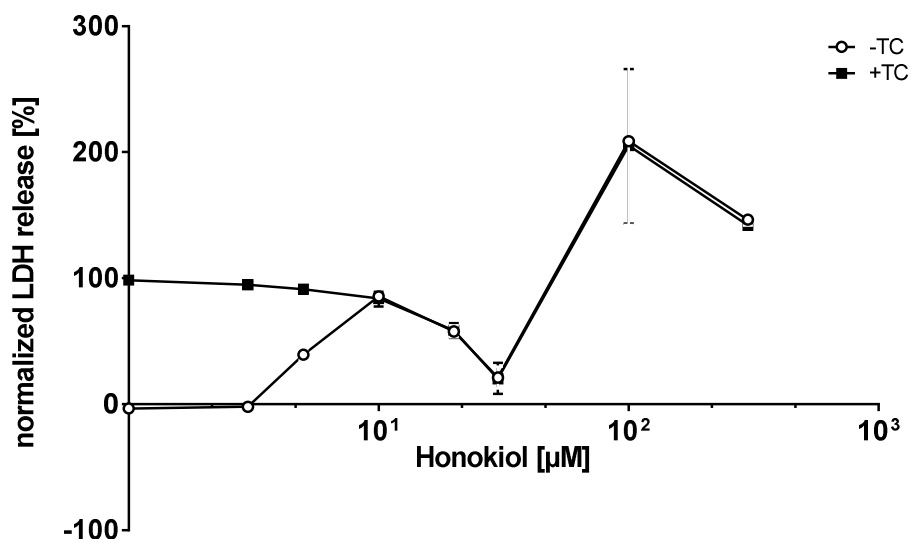


Figure 3.35 Connecting line between the calculated data points for honokiol at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=4$ (300 μ M) – 16 (1-100 μ M) – 24 (3, 10, 30 μ M) wells from at least two independent plates.

Honokiol was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 20 μ M ($IC_{50} \sim 20.08 \mu$ M). The fitted sigmoidal dose-response-curves for honokiol are not demonstrated in this figure to clearly show the incomprehensible NMDAR-unrelated cytotoxic effects of honokiol. At a concentration of 5 μ M honokiol started to reveal NMDAR-unrelated cytotoxic effects. Interestingly, honokiol concentrations of 20 and 30

μM caused significantly fewer NMDAR-unrelated cytotoxic effects than a concentration of $10\ \mu\text{M}$.

3.4.3.4 Honokiol with HX531

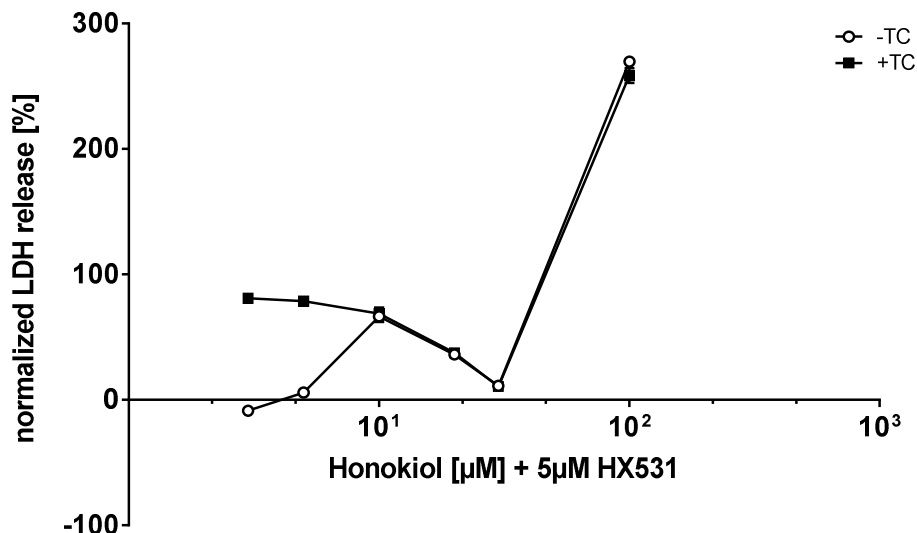
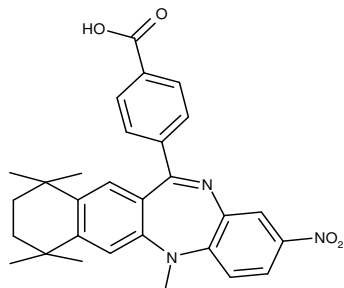


Figure 3.36 Connecting line between the calculated data points for honokiol + 5 μM HX531 at each applied concentration of honokiol in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means ± SD, n=8 (3-100 μM) wells from two independent plates.

It was found in another study that honokiol can activate the retinoic X receptor (RXR) as well [101]. Hence, it was assumed in this work that the unexpected NMDAR-unrelated cytotoxic effects of honokiol possibly were connected to an interaction with the RXR. Thus, by applying honokiol with the RXR-antagonist HX531 [102] these effects should be blocked. The fitted sigmoidal dose-response-curves for honokiol with HX531 are not shown in this figure to clearly demonstrate the incomprehensible NMDAR-unrelated cytotoxic effects of honokiol. Honokiol with 5 μM HX531 was able to inhibit the TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC_{50} value of about 17 μM ($IC_{50}=17.39$ μM). At a concentration of 5 μM honokiol started to reveal

NMDAR-unrelated cytotoxic effects and again concentrations of 20 μ M and 30 μ M of honokiol caused deciding fewer NMDAR-unrelated cytotoxic effects than a concentration of 10 μ M.

3.4.3.5 HX531



= 4-(7,8,9,10-Tetrahydro-5,7,7,10,10-pentamethyl-2-nitro-5H-benzo[b]naphtho[2,3-e][1,4]-diazepin-12-yl)-benzoic acid

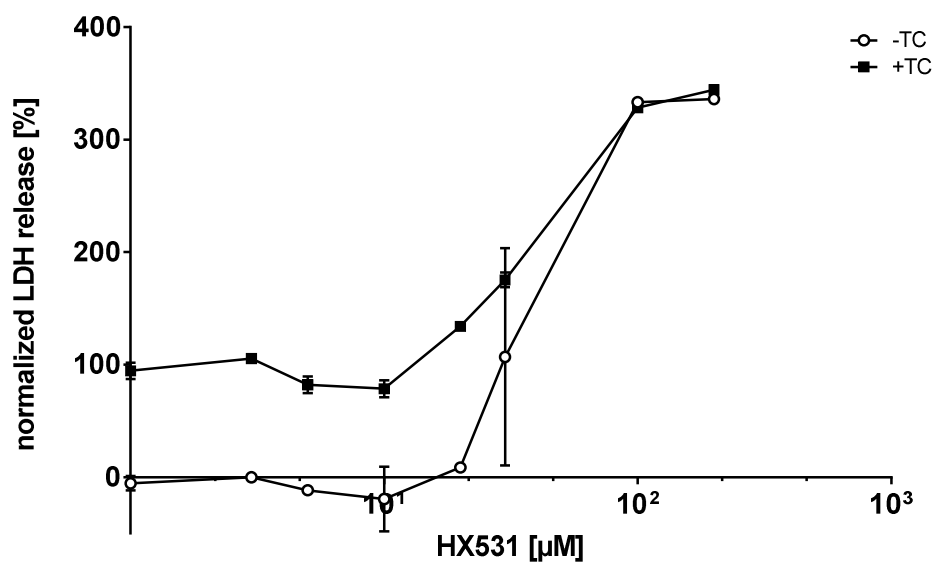


Figure 3.37 Connecting line between the calculated data points for HX531 at each applied concentration in TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, n=4 (3-300 μ M) – 8 (5, 30 μ M) – 12 (1 μ M) – 24 (10 μ M) wells from at least one plate.

It was demonstrated that HX531 did not inhibit the TC-induced cell membrane damage in a concentration dependent manner through NMDAR-over-activation

up to a concentration of 300 μM . HX531 started to reveal slight NMDAR-unrelated cytotoxic effects at a concentration of 20 μM .

3.4.3.6 Honokiol without cells

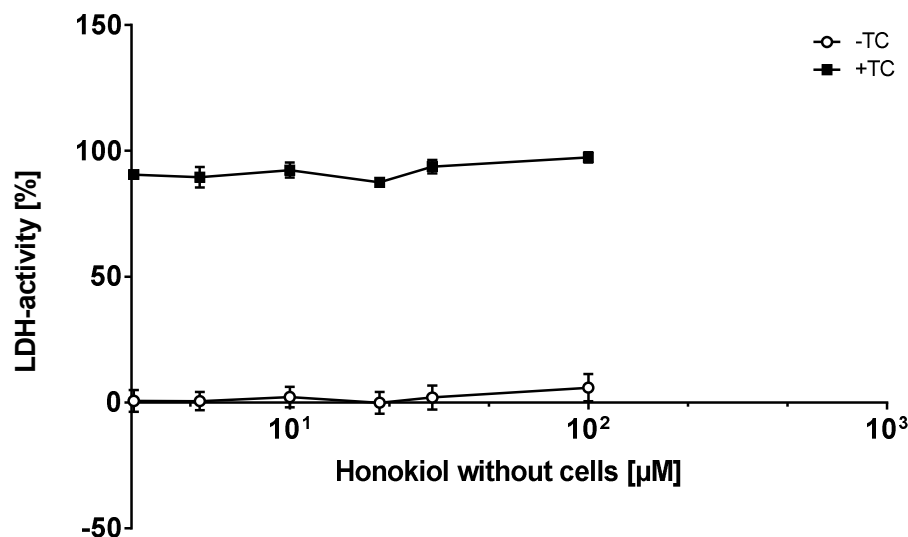


Figure 3.38 Connecting line between the calculated data points for honokiol at each applied concentration in pure medium of previously TC-treated- (+TC, filled square), and TC-untreated cells (-TC, white circle). Data are given as means $\pm$ SD, $n=4$ (3-100 μM) wells from one plate.

In order to determine, whether the incomprehensible NMDAR-unrelated cytotoxic effects of honokiol (see Figure 3.35) might be due to an interaction of honokiol with the LDH-test-solutions, honokiol was applied to pure LDH-containing medium.

However, only minor, not significant deviations between the data points were detected. These data confirmed that honokiol did not interact with the LDH-test.

3.5 Correlations of IC₅₀- and literature Ki-values with literature physico-chemical parameters

3.5.1 Correlation of IC₅₀- with MlogP-values

Test compound	IC ₅₀ [μM] (this work)	MlogP (Kainz J.)	Rank IC ₅₀ (this work)	Rank MlogP (Kainz J.)	D ²
Bu-081	>300	2.680	12	2	100
Bu-082	56.38	2.598	4	3	1
Bu-090	89.65	2.040	6	11	25
Bu-093	>300	1.481	12	17	25
Bu-094	>300	2.118	12	9	9
Bu-097	>300	1.868	12	12.5	0.25
Bu-099	32.74	2.361	3	6	9
Bu-101	>300	1.868	12	12.5	0.25
Bu-104	>300	2.361	12	6	36
Bu-105	>300	1.791	12	14	4
Bu-106	>300	2.361	12	6	36
Bu-108	71.37	2.118	5	9	16
Bu-109	>300	2.118	12	9	9
Bu-112	>300	1.612	12	16	16
Cs-183	>300	3.303	12	1	121
Cs-224	16.63	1.677	1	15	196
Sie-20	25.66	2.465	2	4	4
r_s (df=15) = 0.2555, $p > 0.01$, $p > 0.05$				sum	607.5

Table 3.1 Correlation of IC₅₀-values from the assay of this work with MlogP-values (determined through an equation with 13 parameters obtained from counting atoms, bonds, and fragments or functional groups using regression analysis) from the work of Kainz J. (2004) using Spearman's rank correlation coefficient (r_s).

In this case, it was assumed that the lower the IC₅₀-value, the higher the MlogP-value was. MlogP-values of some in the assay of this work tested glycine antagonists (Bu-085, Bu-113, Cs-182, Cs-191, Cs-199, 1F1, L-701,324) were not available. Pairs were ranked to show a positive correlation. However

$r_s < r$ (=cutoff) at the two probability levels indicated that there was no correlation between the obtained IC₅₀-values of this work and the MlogP-values.

3.5.2 Correlation of IC₅₀- with logPTsar-values

Test compound	IC ₅₀ [μM] (this work)	logPTsar (Kainz J.)	Rank IC ₅₀ (this work)	Rank logPTsar (Kainz J.)	D ²
Bu-081	>300	3.362	12	3	81
Bu-082	56.38	2.833	4	4	0
Bu-090	89.65	2.158	6	9	9
Bu-093	>300	1.250	12	17	25
Bu-094	>300	1.988	12	11	1
Bu-097	>300	1.710	12	13	1
Bu-099	32.74	2.437	3	6	9
Bu-101	>300	1.592	12	15	9
Bu-104	>300	2.700	12	5	49
Bu-105	>300	1.640	12	14	4
Bu-106	>300	1.933	12	12	0
Bu-108	71.37	2.106	5	10	25
Bu-109	>300	2.304	12	8	16
Bu-112	>300	1.300	12	16	16
Cs-183	>300	5.121	12	1	121
Cs-224	16.63	2.415	1	7	36
Sie-20	25.66	3.535	2	2	0
r_s (df=15) = 0.5074, $p > 0.01$, $p < 0.05$				sum	402

Table 3.2 Correlation of IC₅₀-values from the assay of this work with logPTsar-values (a sum of atomic partial logP-values) from the work of Kainz J. (2004) using Spearman's rank correlation coefficient (r_s).

The assumption was the lower the IC₅₀-value, the higher the logPTsar-value. logPTsar-values of some in the assay of this work tested glycine antagonists (Bu-085, Bu-113, Cs-182, Cs-191, Cs-199, 1F1, L-701,324) were not available. Pairs were ranked to show a positive correlation. In contrast to the correlation

to MlogP-values, ranking orders between the obtained IC₅₀-values from this work and the logPTsar-values correlated with a probability of 95%.

3.5.3 Correlation of IC₅₀- with ClogP-values

Test compound	IC ₅₀ [μM] (this work)	ClogP	Rank IC ₅₀ (this work)	Rank ClogP	D ²
Bu-081	>300	6.109	18	8	100
Bu-082	56.38	5.024	7	10	9
Bu-085	>300	3.364	18	19	1
Bu-090	89.65	3.308	10	20	100
Bu-093	>300	2.49	18	23	25
Bu-094	>300	3.813	18	16	4
Bu-097	>300	3.567	18	18	0
Bu-099	32.74	4.495	5	11	36
Bu-101	>300	3.284	18	21	9
Bu-104	>300	4.292	18	13	25
Bu-105	>300	2.473	18	12	36
Bu-106	>300	4.011	18	15	9
Bu-108	71.37	4.096	9	14	25
Bu-109	>300	3.763	18	17	1
Bu-112	>300	3.068	18	22	16
Bu-113	27.41	7.122	4	2	4
Cs-182	179.8	6.723	11	5	36
Cs-183	>300	8.31	18	1	289
Cs-191	39.76	6.593	6	6	0
Cs-199	66.70	6.992	8	3	25
Cs-224	16.63	4.964	2	4	4
1F1	>300	-1.316	18	24	36
Sie-20	25.66	6.194	3	7	16
L-701,324	14.22	5.406	1	9	64
r _s (df=22) = 0.6217, p < 0.01, p < 0.05				sum	870

Table 3.3 Correlation of IC₅₀-values from the assay of this work with ClogP (logarithmic octanol/water partition coefficient) –values calculated by ChemBioDraw® Ultra 14 using Spearman's rank correlation coefficient (r_s).

The assumption was the lower the IC₅₀-value, the higher the ClogP-value. Pairs were ranked to show a positive correlation. As for the logPTsar-values there was a correlation between the obtained IC₅₀-values from this work and the ClogP-values with a probability of 99%.

3.5.4 Correlation of literature Ki- with MlogP-values

Test compound	Ki [μM] (Buchstaller et al.)	MlogP (Kainz J.)	Rank Ki (Buchstaller et al.)	Rank MlogP (Kainz J.)	D ²
Bu-081	0.00950	2.680	6	2	16
Bu-082	0.0058	2.598	3	3	0
Bu-084	9.5	1.612	19	20.5	2.25
Bu-087	3.1	1.868	15	15	0
Bu-088	23.1	1.948	21	13	64
Bu-090	0.151	2.040	9	12	9
Bu-092	46.0	1.156	23	24	1
Bu-095	8.3	1.612	18	20.5	6.25
Bu-096	4.75	2.118	17	10	49
Bu-097	0.267	1.868	10	15	25
Bu-099	0.0066	2.361	4	6.5	6.25
Bu-101	1.27	1.868	12	15	9
Bu-104	0.94	2.361	11	6.5	20.25
Bu-105	12.2	1.791	20	17	9
Bu-106	0.058	2.361	7	6.5	0.25
Bu-107	1.77	1.741	14	18	16
Bu-108	0.0089	2.118	5	10	25
Bu-109	1.53	2.118	13	10	9
Bu-110	97.5	1.348	24	22.5	2.25
Bu-111	4.3	2.361	16	6.5	90.25
Bu-117	26.0	1.348	22	22.5	0.25
Cs-183	0.080	3.303	8	1	49
Cs-224	0.00160	1.677	2	19	289
Sie-20	0.00111	2.465	1	4	9
r_s (df=22) = 0.6926, $p < 0.05$, $p < 0.01$				sum	707

Table 3.4 Correlation of Ki-values from the study of Buchstaller et al. (2006) with MlogP-values (determined through an equation with 13 parameters obtained from counting atoms, bonds, and fragments or functional groups using regression analysis) from the work of Kainz J. (2004) using Spearman's rank correlation coefficient (r_s).

The presumption was the lower the Ki-value, the higher the MlogP-value. Pairs were ranked to show a positive correlation. The correlation coefficient r_s was higher than r (=cutoff) at the two probability levels indicating that there was a

significant correlation between the Ki-values from the work of Buchstaller et al. (2006) and the MlogP-values with a probability of 99%.

3.5.5 Correlation of literature Ki- with logPTsar-values

Test compound	Ki [μ M] (Buchstaller et al.)	logPTsar (Kainz J.)	Rank Ki (Buchstaller et al.)	Rank logPTsar (Kainz J.)	D ²
Bu-081	0.00950	3.362	6	3	9
Bu-082	0.0058	2.833	3	4	1
Bu-084	9.5	1.125	19	22	9
Bu-087	3.1	1.593	15	16	1
Bu-088	23.1	1.244	21	20	1
Bu-090	0.151	2.158	9	9	0
Bu-092	46.0	0.869	23	24	1
Bu-095	8.3	1.577	18	18	0
Bu-096	4.75	1.800	17	13	16
Bu-097	0.267	1.710	10	14	16
Bu-099	0.0066	2.437	4	6	4
Bu-101	1.27	1.592	12	17	25
Bu-104	0.94	2.700	11	5	36
Bu-105	12.2	1.640	20	15	25
Bu-106	0.058	1.933	7	12	25
Bu-107	1.77	1.543	14	19	25
Bu-108	0.0089	2.106	5	10	25
Bu-109	1.53	2.304	13	8	25
Bu-110	97.5	0.932	24	23	1
Bu-111	4.3	1.953	16	11	25
Bu-117	26.0	1.167	22	21	1
Cs-183	0.080	5.121	8	1	49
Cs-224	0.00160	2.415	2	7	25
Sie-20	0.00111	3.535	1	2	1
r_s (df=22) = 0.8496, $p < 0.05$, $p < 0.01$				sum	346

Table 3.5 Correlation of Ki-values from the study of Buchstaller et al. (2006) with logPTsar-values (a sum of atomic partial logP values) from the work of Kainz J. (2004) using Spearman's rank correlation coefficient (r_s)

The assumption was the lower the Ki-value, the higher the logPTsar-value. Pairs were ranked to show a positive correlation. As well here $r_s > r$ (=cutoff) at the two probability levels indicated that there was a correlation between the Ki-values from the work of Buchstaller et al. (2006) and the logPTsar-values with a probability of 99%.

3.5.6 Correlation of literature Ki- with ClogP-values

Test compound	Ki [μ M] (Buchstaller et al.)	ClogP	Rank Ki (Buchstaller et al.)	Rank ClogP	D ²
Bu-081	0.00950	6.109	11	7	16
Bu-082	0.0058	5.024	8	9	1
Bu-084	9.5	2.835	25	25.5	0.25
Bu-085	0.53	3.364	16	19.5	12.25
Bu-087	3.1	3.364	21	19.5	2.25
Bu-088	23.1	3.794	27	16	121
Bu-090	0.151	3.308	14	21	49
Bu-092	46.0	2.721	29	27	4
Bu-095	8.3	2.835	24	25.5	2.25
Bu-096	4.75	3.279	23	23	0
Bu-097	0.267	3.567	15	18	9
Bu-099	0.0066	4.495	9	11	4
Bu-101	1.27	3.284	18	22	16
Bu-104	0.94	4.292	17	12	25
Bu-105	12.2	2.473	26	29	9
Bu-106	0.058	4.011	12	14	4
Bu-107	1.77	3.218	20	24	16
Bu-108	0.0089	4.096	10	13	9
Bu-109	1.53	3.763	19	17	4
Bu-110	97.5	2.546	30	28	4
Bu-111	4.3	3.908	22	15	49
Bu-113	0.0020	7.122	5	2	9
Bu-117	26.0	2.336	28	30	4
Cs-182	0.00412	6.723	7	4	9
Cs-183	0.080	8.31	13	1	144
Cs-191	0.00146	6.593	3	5	4
Cs-199	0.0033	6.992	6	3	9
Cs-224	0.00160	4.964	4	10	36
Sie-20	0.00111	6.194	1	6	25
L-701,324	0.0014	5.406	2	8	36
r_s (df=28) = 0.8592, $p < 0.05$, $p < 0.01$				sum	633

Table 3.6 Correlation of Ki-values from the study of Buchstaller et al. (2006) with ClogP (logarithmic octanol/water partition coefficient) –values calculated by ChemBioDraw® Ultra 14 using Spearman's rank correlation coefficient (r_s).

The assumption was the lower the Ki-value, the higher the ClogP-value. Pairs were ranked to show a positive correlation. The correlation coefficient r_s was higher than r (=cutoff) at the two probability levels indicating that there was a correlation between the Ki-values from the work of Buchstaller et al. (2006) and the ClogP-values with a 99% probability.

3.6 Correlations of IC₅₀- with literature Ki- and literature IC₅₀-values

3.6.1 Correlation of IC₅₀- with literature Ki-values

Test compound	IC ₅₀ [μM] (this work)	Ki [μM] (Buchstaller et al.)	Rank IC ₅₀ (this work)	Rank Ki (Buchstaller et al.)	D ²
L-701,324	14.22	0.0014	20	19	1
Bu-081	>300	0.00950	5	10	25
Bu-082	56.38	0.0058	14	13	1
Bu-085	>300	0.53	5	5	0
Bu-090	89.65	0.15	11	7	16
Bu-097	>300	0.267	5	6	1
Bu-099	32.74	0.0066	16	12	16
Bu-101	>300	1.27	5	3	4
Bu-104	>300	0.94	5	4	1
Bu-105	>300	12.2	5	1	16
Bu-106	>300	0.058	5	9	16
Bu-108	71.37	0.0089	12	11	1
Bu-109	>300	1.53	5	2	9
Bu-113 (=Cs-294)	27.41	0.0020	17	16	1
Cs-182	179.8	0.00412	10	14	16
Cs-183	>300	0.080	5	8	9
Cs-191 (=Cs-241)	39.76	0.00146	15	18	9
Cs-199	66.70	0.0033	13	15	4
Cs-224	16.63	0.00160	19	17	4
Sie-20	25.66	0.00111	18	20	4
r_s (df=18) = 0.8842, $p < 0.05$, $p < 0.01$				sum	154

Table 3.7 Correlation of IC₅₀-values from this work with Ki-values from Buchstaller et al. (2006) using Spearman's rank correlation coefficient (r_s).

Here the assumption was, the lower the IC₅₀-value, the lower the Ki-value should be, wherein the pairs were ranked to show a positive correlation. Ki-values of some glycine antagonists tested in this work (Bu-093, Bu-94, Bu-112,

1F1, L-701,324) were not available from Buchstaller et al. (2006). The correlation coefficient (r_s) was higher than r (=cutoff) at the two probability levels (95 and 99%) indicating that there was a correlation between the IC_{50} -values obtained with the assay of this work and the K_i -values from the work of Buchstaller et al. (2006) with a probability of 99%.

3.6.2 Correlation of IC_{50} - with literature IC_{50} -values

Test compound	IC_{50} [μ M] (this work)	IC_{50} [μ M] (Buchstaller et al.)	Rank IC_{50} (this work)	Rank IC_{50} (Buchstaller et al.)	D ²
L-701,324	14.22	3.79	7	7	0
Bu-113 (=Cs-294)	27.41	7.9	5	5	0
Cs-182	179.8	15.9	2	2	0
Cs-183	>300	>50	1	1	0
Cs-191 (=Cs-241)	39.76	5.2	4	6	4
Cs-199	66.70	15.5	3	4	1
Cs-224	16.63	15.7	6	3	9
r_s (df=5) = 0.75, $p < 0.05$, $p > 0.01$				sum	14

Table 3.8 Correlation of IC_{50} -values from the assay of this work with IC_{50} -values from Buchstaller et al. (2006) using Spearman's rank correlation coefficient (r_s).

The presumption was the lower the IC_{50} -value obtained from the assay of this work, the lower the IC_{50} -value from the work of Buchstaller et al. (2006). Buchstaller et al. (2006) did not report IC_{50} -values of several in the assay of this work tested glycine antagonists (Bu-081, Bu-082, Bu-085, Bu-090, Bu-093, Bu-094, Bu-097, Bu-099, Bu-101, Bu-104, Bu-105, Bu-106, Bu-108, Bu-109, Bu-112, 1F1, Sie-20).

The pairs were ranked to show a positive correlation. In this case the correlation coefficient (r_s) with a number of pairs = 7 was between the probability levels of 0.05 and 0.01. This indicated with a probability of 95% that there was a correlation between the IC_{50} -values from this work and the IC_{50} -values from Buchstaller et al. (2006).

4 Discussion

4.1 Assay optimization

The first aim of the diploma thesis was to find optimal conditions to provide a 2-fold difference between the measurement values obtained for TC-treated and TC-untreated cells.

The principle of the assay was that HEK293 cells overexpressing functional NR1/NR2A after induction with tetracycline will die due to activation of the NMDAR by glutamate and increased Ca^{2+} -influx. Addition of NMDAR-inhibitors should decrease the percentage of dead cells detectable by a cell viability/toxicity assay. In the present work three different assays were tested, namely the EZ4U- (XTT based), the Celltiter Glo®- and the LDH-cytotoxicity-kit. After variation of seeding cell number, assay medium composition, time point of tetracycline addition (immediately with cell seeding, 2 h or 24 hours after cell seeding), serum concentration, time point of measurement, etc. following optimized protocol using the LDH-kit was established.

For the assay a cell suspension was prepared in a concentration of 136.000 cells/ml. 100 µl of this cell suspension were then applied into each well of a 96-well-microtiter plate (- except those of the rows 1 and 12, which contained pure medium -) leading to 40.000 cells/cm². For that, medium with phenol red with a serum concentration of 10% FCS was used. After 24 hours of cultivation, tetracycline in a concentration of 0.1 µg/ml and test substances in corresponding concentrations in medium without phenol red and 5% FCS were applied into

the corresponding wells (see section 2.2.5.2). After another 24 hours measurements were accomplished using LDH-activity-detection-method. This method enabled an over 2-fold difference between the values obtained for TC-induced and non-induced cells whereupon the IC_{50} -values of NMDAR blockers were determined.

In this regard, it was interesting to note that these conditions were quite similar to a recently published method by Buchstaller et al. (2006) in which they used mouse fibroblast cells overexpressing NMDAR-subunits NR1/NR2A induced by dexamethasone [76].

4.2 Qualification of the assay

4.2.1 Reference substances

In the developed assay of this work the channel blockers MK-801 and memantine, and the glycine antagonist L-701,324 were tested as reference substances.

For the qualification of the assay of the present work obtained results for these substances were compared with results in the literature using test assays with similar conditions in comparison to the assay of this work (see section 4.1).

One study showed through patch-clamp experiments with retinal ganglion cells of rats that memantine inhibited 200 μ M NMDA-evoked responses leading to cell death at a holding potential of -60mV resulting in an IC_{50} value of about 1 μ M - circa 80-fold lower than in the assay of this work [104]. According to Bre-

sink et al. (1996) memantine blocked L-glutamate-induced responses causing cell damage on HEK-cells expressing NR1/NR2A in a concentration dependent manner yielding in an IC_{50} value of $0.93 \pm 0.15 \mu M$, similar to the one obtained by Chen et al. (1997) [104,105]. Another study observed an IC_{50} value ($2.23 \pm 0.3 \mu M$) for MK-801 on NMDA-elicited currents using NR1/NR2A expressing HEK-cells, which was not very different from memantine referred to both aforementioned studies. Interestingly, they also showed an IC_{50} range ($105 \pm 54 nM$) for MK-801 on NMDA-induced currents in high affinity hippocampal neurons of rats [106], in which the IC_{50} value for MK-801 obtained by the assay of this work (84 nM) was encountered.

Gilling et al. (2009) measured changes in intracellular Ca^{2+} levels on HEK-cells expressing NRs by using fluorimetric imaging plate reader (FLIPR) assay. They declared lower IC_{50} -values for memantine ($IC_{50}=9.23 \pm 1.03 \mu M$) and as well for MK-801 ($IC_{50}=25 nM$) than in the assay of this work (see Figure 3.7 and Figure 3.8). However according to this study although the absolute numbers were lower in contrast to results obtained with the assay of this work (MK-801=84 nM, memantine=84 μM) it confirmed that MK-801 possesses a significantly lower IC_{50} than memantine [78].

Lopes et al. (2013) using rat cortical neuron cells reported an IC_{50} for memantine on NMDA-evoked neuronal cell damage between 0.75 and 1.31 μM determined by LDH-detection, whereas they also suggested an IC_{50} value for MK-801 (=70nM), which was approximately in the region of what the assay of this work revealed (~84nM) [107].

By comparing the results for memantine in the assay of this work with the results of Sommerauer et al. (2012), who implemented a phosphatase-activity assay using HEK cells expressing NRs by tetracycline induction, similar results for memantine were found despite the use of different cell viability tests. In the assay of this work memantine was able in blocking the tetracycline (TC)-induced cytotoxicity at concentrations of 3 μM and above (see Figure 3.8), whereas according to Sommerauer et al. (2012) memantine blocked TC-induced cell damage at a concentration of 10 μM and above. Moreover, memantine showed no NMDAR-unrelated cell cytotoxicity up to the tested concentrations in the assay of this work (300 μM) as well as in the work of Sommerauer et al. (2012) (100 μM) [86].

One study reported that L-701,324 was able to block NMDA-induced depolarizations on rat cortical slices with an IC_{50} of just 28 nM and provided an ED_{50} of 0.9 mg/kg for L-701,324 in protecting DBA/2 mice from audiogenic seizure, regardless of intraperitoneal or oral administration [108].

Also Buchstaller et al. (2006) tested the effects of the glycine antagonist L-701,324. On the one hand they used a glycine-competition-binding-assay and on the other hand they accomplished cell toxicity assays using LDH-cell toxicity tests on mouse fibroblast cells expressing NRs by dexamethason induction. They observed a 4-fold lower IC_{50} -value ($=3.79 \mu\text{M}$) for L-701,324 in comparison to the IC_{50} -value ($=14.22 \mu\text{M}$) obtained in the assay of this work. Possibly, this difference could be explained by the usage of different cell types [76].

In summary, it can be claimed that the IC_{50} value for memantine actually might be lower than it was in the assay of the present work, while the IC_{50} values for both L-701,324 and MK-801 were in a reliable concentration range compared to the literature, indicating the validity of this assay to a certain extent.

4.2.2 Glycine antagonists

The tested glycine antagonists in this work were mainly thieno-pyridone, which originally had been developed by Gottschlich et al. [82]. Exceptions were the 4-hydroxyquinolone-compounds Bu-090, Bu-105 and the reference compound L-701,324 (see Table 4.1).

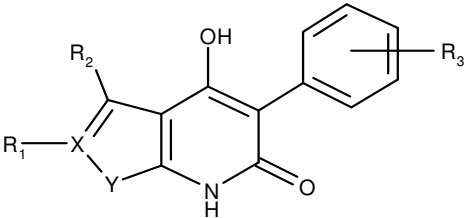
Basic structure						
						
Test compound	X	Y	R ₁	R ₂	R ₃	IC ₅₀ [μM]
Bu-081	C	S	Cl	c-C ₃ H ₅	m-phenoxy	>300
Bu-082	C	S	Cl	sec-C ₄ H ₉	H	56.38
Bu-085	C	S	H	C ₂ H ₅	H	>300
Bu-090	benzene-analogue		Cl	H	H	89.65
Bu-093	C	S	H	H	p-F	>300
Bu-094	C	S	CH ₃	C ₂ H ₅	H	>300
Bu-097	C	S	Cl	CH ₃	H	>300
Bu-099	C	S	Cl	i-C ₃ H ₇	H	32.74
Bu-101	C	S	CH ₃	CH ₃	H	>300
Bu-104	C	S	H	sec-C ₄ H ₉	H	>300
Bu-105	benzene-analogue		H	H	H	>300
Bu-106	C	S	Cl	c-C ₃ H ₅	H	>300
Bu-108	C	S	Cl	C ₂ H ₅	H	71.37
Bu-109	C	S	H	i-C ₃ H ₇	H	>300
Bu-112	S	C-Cl	H	H	H	>300
Bu-113 (=Cs-294)	C	S	Cl	sec-C ₄ H ₉	m-phenoxy	27.41
Cs-182	C	S	Cl	(CH ₂) ₂ CH ₃	m-phenoxy	179.8
Cs-183	C	S	Cl	n-C ₆ H ₁₃	m-phenoxy	>300
Cs-191 (=Cs-241)	C	S	Cl	i-C ₃ H ₇	m-phenoxy	39.76
Cs-199	C	S	Cl	tert-C ₄ H ₉	m-phenoxy	66.70
Cs-224	C	S	Cl	CH ₂ OCH ₃	m-phenoxy	16.63
Sie-20	C	S	Cl	C ₂ H ₅	m-phenoxy	25.66
1F1	C	S	Cl	(CH ₂) ₂ NH ₃ ⁺ Cl ⁻	H	>300
L-701,324	benzene-analogue		Cl	H	m-phenoxy	14.22

Table 4.1 Chemical structures of the tested glycine antagonists and their effects in the developed assay of the present work (IC₅₀).

4.2.2.1 Molecular analysis of the glycine antagonists based on the results obtained with the assay of this work

Aforementioned, in present work beside L-701,324 two glycine antagonists with quinolone basic structure, namely Bu-090 and Bu-105 were tested. These two compounds differ only by the chlorine residue in position 7. It could be suggested here that the chlorine residue in this position seemed to play a critical role in terms of in vitro effectiveness on NMDARs since Bu-090 could clearly inhibit TC-induced cell damage ($IC_{50} \sim 90 \mu M$) in contrast to Bu-105 (see Table 4.1). Interestingly, Buchstaller et al. (2006) showed a significant difference for these two compounds in terms of NMDAR binding as well. It is also noteworthy that Bu-090 was the only compound tested in the assay of this work which could diminish the TC-induced cell damage at a concentration lower than $300 \mu M$ by showing a K_i -value higher than $0.1 \mu M$ according to Buchstaller et al. (2006) [76].

The tested thieno-pyridone glycine antagonists can roughly be divided into compounds with or without a phenoxy group in 3'-position on the phenyl-cycle, respectively.

In the group of substances without the phenoxy group in 3' position a "chlorine-effect" could be investigated as well. For instance Bu-094 with a methyl-residue in 2-position showed no reasonable effects as opposed to Bu-108 substituted with chlorine in this position. Moreover, it could be assumed that not only chlorine per se seemed to be important for an effect on the NMDAR, but also its

position. One reason for this adoption might be that Bu-112 with a chlorine residue on another position was not able to block the NMDAR significantly. However, it should be mentioned that Bu-112 came up with a [3,4-b]-annealing of the thiophene cycle over a [2,3-b]-fused one as it was with all the other thieno-pyridone antagonists tested and this compound (Bu-112) had no other residues on the thiophene ring.

It could be found in this group of thieno-pyridone compounds without the phenoxy-group on 3'-position that small changes in the residue on 3-position could lead to a loss of activity on the NMDAR when comparing the results for Bu-097 with a methyl group and Bu-108 with an ethyl group in this position (see Table 4.1).

In this group of antagonists only Bu-082, Bu-099 and Bu-108 managed to halt TC-induced cell damage of which Bu-099 was the most effective in the assay of this work. Thus, the results for Bu-099 suggesting the isopropyl group in 3-position very beneficial for NMDAR-blocking.

The thieno-pyridone antagonists Bu-106 and 1F1 without the m-phenoxy residue on the phenyl ring, which both had a chlorine residue in 2-position and residues such as a cyclopropyl group and an ammonioethyl residue in 3-position respectively demonstrated no significant effects in inhibiting the TC-induced cell damage via NMDAR overactivation. Hence, these kinds of residues could interfere with the NMDAR blockade. Even in the group of 3'-phenoxy-thienopyridone glycine antagonists, Bu-081 with a cyclopropyl group

in 3-position as well as in Bu-106 did not considerably block the NMDAR (see Table 4.1).

In the group of 3'-phenoxy-thienopyridone antagonists all had a chlorine residue in 2-position, but different residues in 3-position. The reason why most of these compounds showed an effect in the assay of this work was probably due to the fact that their basic chemical structure was similar to that of L-701,324 with the difference of an [2,3-b]-annealed thiophene-cycle instead of the quinoline nucleus. Also their strong effects in the assay of this work could be explained by the NMDAR-binding-studies from Buchstaller et al. (2006) [76]. In any case, it might be assumed that the 3-phenoxy group in 3'-position on the phenyl cycle is advantageous for reducing cell damage through over-activated NMDARs.

Buchstaller et al. (2006) using mouse fibroblast cells with a NR1/NR2A dexamethasone induction system reported IC_{50} -values for all of the tested thienopyridones with a phenoxy residue in 3'-position on the phenyl ring, except for Bu-081 and Sie-20. Each of these IC_{50} -values was much lower in comparison to the corresponding one obtained by the assay of this work [76]. For instance Cs-182 with a propyl residue in 3-position showed an IC_{50} of 180 μ M in the assay of this work compared to an IC_{50} of about 16 μ M according to Buchstaller et al. (2006). Furthermore Buchstaller et al. (2006) published IC_{50} -values for Cs-182, Cs-199 and Cs-224 almost equal around 15 μ M each, whereas there consisted a greater difference among the results for these substances obtained with the assay of this work (see Table 4.1). As well Bu-113 (= Cs-294) showed

stronger effects than Cs-191 (= Cs-241) in the assay of the present work, while it was the other way around according to Buchstaller et al. (2006). However, both assays revealed that Cs-183 with a hexyl residue in 3-position was not capable to block NMDAR-induced cell damage, suggesting that this bulky residue was inconvenient for inhibiting NMDAR functionality [76].

Interestingly, as mentioned before under the group of thienopyridone antagonists without a phenoxy group in 3'-position, Bu-099 with an isopropyl-residue in 3-position such as in Cs-191 (=Cs-241) exhibited a stronger effect in blocking the NMDAR as compared to substances with an isobutyl- (Bu-082) and an ethyl-residue (Bu-108) in this position. However, in the assay of this work Cs-191 (=Cs-241) was weaker than Bu-113 (=Cs-294) with an isobutyl-residue and Sie-20 with an ethyl-residue each in 3-position.

Among all of the tested 3'-phenoxy-thienopyridone derivatives tested in the assay of this work Cs-224 was the strongest with a methoxymethyl residue in 3-position showing an IC_{50} of 16.63 μ M, though in the work of Buchstaller it was Cs-191 (=Cs-241) with an isopropyl residue in 3-position reaching an IC_{50} -value of 5.2 μ M.

Due to the differences between the IC_{50} values of the test substances Bu-113 (= Cs-294), Cs-191 (= Cs-241), Cs-199 and Cs-224 obtained with the assay of this work and the ones Buchstaller et al. (2006) reported, it is not clear which residue in 3-position was better than the other in terms of blocking the NMDAR. In addition one compound with a residue on the phenyl ring, which was not a phenoxy group, was tested in the assay of this work. This substance (Bu-093)

had a fluorine residue in 4'-position, but no chlorine residue in 2-position. This type of substitution showed no effects on NMDAR-blockade in the assay of this work (see Table 4.1).

Among of all the tested glycine antagonists, L-701,324 showed the strongest effects in the assay of the present work indicating that it should be clarified, whether the annealing of the thiophene cycle would bring an advantage over the quinoline nucleus of L-701,324 in terms of efficacy at all.

4.2.2.2 Existing literature about the novel glycine antagonists

Leeson et al. (1993) accomplished [^3H]-L-689,560-competition binding assays using 4-hydroxyquinoline-derivatives. They found that Bu-090 with a phenyl-group in 3-position was more active on the glycine NMDAR binding site in comparison to 4-hydroxyquinoline-compounds with residues such as hydrogen, cyclohexyl, methyloxycarbonyl- and 3-phenylcarbonyl in this position. On the other hand, they reported 4-hydroxy-3-phenyl-quinoline-substances with residues such as chlorine or ethyl in 5-position more potent than Bu-090. Furthermore, Leeson et al. (1993) showed an IC_{50} of Bu-090 in inhibiting NMDA-evoked depolarizations in rat cortical slices of $0.88\ \mu\text{M}$, which was about 100-fold lower than it was in the assay of this work [109].

Kulagowski et al. (1994) determined the inhibition of Bu-090 and Bu-105 regarding glycine binding on NMDARs together with some other 4-hydroxyquinoline derivatives through [^3H]-L-689,560-competition binding assays on rat brain membranes and their protection from audiogenic seizure in mice. Among the tested compounds, they suggested 3'-(aryloxy)-3-phenyl-4-hydroxyquinolin-2(1H)-ones as the most potent glycine antagonists, both in vitro and in vivo. Interesting to note that in this work and the work of Buchstaller et al. (2006) 3'-(aryloxy)-3-phenyl-4-hydroxy-thieno-pyridone-antagonists showed better results compared to 3-phenyl-4-hydroxy-thieno-pyridone-compounds confirming the importance of the 3'-aryloxy-group [108].

Another study from Carling et al. (1997) tested acidic and non-acidic 4-substituted-3-phenylquinoline-glycine antagonists in [3H]-L-689,560-competition binding assays and their in vivo activity against audiogenic seizure in mice just as in the previously described study. They showed that anionic functionality of these glycine antagonists was not essential for strong binding to the glycine site on the NMDAR. For the binding of 4-substituted-3-phenylquinoline substances to the glycine site on the NMDAR they also examined a ligand/receptor hydrogen bond interaction between the acceptor attached to the 4-position of the ligand and a hydrogen bond applied to the receptor. This binding mode concerns both acidic and nonacidic antagonists. However, they obtained a clear distinction between the in vitro results for anions and neutral compounds, suggesting a subtle difference regarding the binding mode. In this study the only compound showing significant anticonvulsant activity was 7-chloro-3'-(phenoxy)-3-phenyl-quinolin-2(1H)-one with an alkoxy-acetonitrile-substituent in 4-position [110].

Fuchigami et al. (2008) tested iodine and ethyl-C-5-substituted analogues of 4-hydroxy-3-(3-[¹¹C]methoxyphenyl)-2(1H)-quinolone ([¹¹C]4HQ) for their in vivo brain uptake. They showed that the blood radioactivity level after intravenous application of the ¹¹C-labelled 5-substituted analogue was nearly twice of that of the ¹¹C-labelled un-substituted compound. However, no differences in brain radioactivity levels were observed. Furthermore, in this study it was found that warfarin altered the brain uptake of 5-substituted (5Et-) and un-substituted [¹¹C]4HQ less than that of [¹¹C]L-703,717. This might be due to the additional

phenyl-group on the 3-phenyl-ring contributed to the binding to albumin in blood. Though, as mentioned before this phenyl moiety can be crucial in terms of potency according to Kulagowski et al. (1994) [108]. Fuchigami et al. (2008) also reported higher cerebellar concentration than in other brain areas at 1 minute after injection of 5Et-[¹¹C]4HQ. Administration of the nonradioactive ligand at 30 minutes before treatment with the same radioactive compounds weakly blocked 5Et-[¹¹C]4HQ binding, but did not hinder 5I-[¹¹C]4HQ binding. Moreover, they noted that 25% of radioactivity of the brain tissue was not linked to actual compounds after 40 minutes of injection, suggesting that metabolites in the brain might influence the specific binding of 5Et-[¹¹C]4HQ and 5I-[¹¹C]4HQ after intravenous injection [111].

Furthermore, with regard to brain uptake one study tested Bu-090 and Bu-105 as well as several other 4-hydroxyquinolone glycine antagonists for their plasma protein binding and consequently their ability in crossing the blood brain barrier (BBB). It was shown that 4-hydroxyquinolone glycine antagonists could readily cross the BBB. However, this penetration is limited by plasma protein binding of the compounds. Furthermore, they found out that the binding to plasma protein increased with their logP and suggested that low plasma protein binding, is good for high in vitro/in vivo ratios [83].

Another study investigated tightness properties of the cell lines PBMEC/C1-2 (=porcine brain microvessel endothelial cells) and ECV304 in order to establish a blood brain barrier mimicking cell culture model. The authors suggested that both cell lines were useful for BBB drug transport studies. Additionally they de-

terminated the permeability of diazepam and the glycine antagonist Bu-101 observing no correlation between permeability of diazepam and Bu-101 and TEER (=transendothelial electrical resistance), which is used for measuring tightness of biological membranes. It was found out that Bu-101 migrated significantly slower through both BBB in vitro models than diazepam [112].

In this context, a study from Mayrhofer T. was accomplished, in which permeation of Bu-090 and Bu-105 as well as of some in the present work tested thieno-pyridone-glycine antagonists (Bu-112, Bu-093, Bu-101, and Bu-081, see Table 4.1) across PBMEC/C1-2 layers cultured in astrocyte conditioned medium was investigated. This study suggested that it was likely that these glycine antagonists could not cross the blood-brain barrier, because they were all weak acids and hence at physiological state dissociated to a large extent. Although these compounds would therefore probably show less CNS-side effects, it remained unclear whether they would lead to a desired effect in vivo. However, it was also indicated in this study that some of these compounds may be liable to active transport processes. Bu-090 was the only substance tested by Mayrhofer T. which was able to block the TC-induced cell membrane damage through NMDAR-over-activation in a concentration-dependent manner in the assay of this work. According to Mayrhofer T. Bu-090 showed lower penetrability across porcine brain microvessel endothelial cells than diazepam serving as a transcellular permeation marker [113].

As in the previously mentioned study from Buchstaller et al. (2006) in addition to L-701,324 two thieno-pyridone glycine antagonists (Sie-20, Cs-191) were

tested in a mouse seizure model and beside L-701,324 the blood and brain concentrations of one of these two substances (Cs-191) were measured in rats [76]. In protection of maximal e-shock in the mouse seizure model the thienopyridone-glycine antagonists showed markedly less effects than L-701,324 and the blood and brain concentration-levels at 6 minutes as well as one hour after administration of Cs-191 were significantly lower than those of L-701,324. Despite these results thienopyridone-antagonists tested in the assay of this work could possess increased plasma stability and optimal BBB permeability properties.

One study using different cells (transfected HEK-cells, H9c2 cardiac myocytes) determined an inhibitory effect of in the present work tested derivatives of 4-hydroxy-3-phenylquinolin-2(1H)-one glycine antagonists on TRPM7 receptor, which is a calcium permeating ion channel such as the NMDAR [115]. They proved that blocking TRPM7 protein decreased cell damage induced by oxygen and glucose deprivation as in the case of ischemia [115].

Besides, it was found that derivatives of the glycine antagonists Bu-090 and Bu-105 with 4-hydroxyquinoline basic structure could inhibit fatty acid synthase (fas). This kind of synthase is highly expressed in cancer cells, which synthesize endogenous sources of fatty acids leading to cancer progression. Thus, these glycine antagonists could have the potential for treating cancer [114]. Moreover, according to Hallakou-Bozec et al. (2009) some of the tested thienopyridone-glycine antagonists (Bu-081, Bu-082, Bu-096, Bu-099, Bu-101, Bu-104, Bu-109) in this work could act as AMPK (=AMP-activated protein kinase)-

activators as well. Due to this finding these glycine antagonists might be used for development of drugs against diabetes, metabolic syndrome and obesity [116].

4.2.2.3 Correlations of IC₅₀-, Ki-values and physicochemical parameters and literature data

Since it is commonly claimed that glycine antagonists could be useful for neuroprotection against brain damage occurring during cerebral ischemia, hypoxia or traumatic brain injury, it would be interesting to investigate blood-brain barrier (BBB) permeability and plasma stability for the novel glycine antagonists of this work. Thus, it should be clarified whether these competitive NMDAR-inhibitors, especially thieno-pyridone antagonists can cross the BBB and will show sufficient in vivo effects albeit their plasma protein binding [1,67].

According to Rowley et al. (1997) BBB permeability of 4-hydroxyquinolone glycine antagonists is limited to plasma protein binding [83]. This may also apply to thieno-pyridone-glycine antagonists. A common method to predict the extent of a compound in binding to plasma protein is to determine its lipophilicity. There are a lot of calculation processes to obtain parameters describing the lipophilicity of a substance.

In the present work three different calculated lipophilicity parameters (MlogP, logPTsar, ClogP) of Bu-090, Bu-105 and of several thieno-pyridone-antagonists were used to investigate the correlation between the NMDAR blocking properties of tested compounds and their lipophilicity. Therefore, these lipophilicity parameters were correlated with the effects of the glycine antagonists (IC₅₀) using the Spearman's rank-correlation-coefficient (see results, Table 3.1, Table 3.2 and Table 3.3) [100]. It was shown with 95% probability that there was a

correlation between the lipophilicity parameters such as the logPTsar- and ClogP values and the activity of the test substances in the assay of this work (IC_{50}). However, no correlation was observed between IC_{50} - and MlogP values. Moreover, the lipophilicity parameters were correlated with the K_i -values reported by Buchstaller et al. (2006) obtained by glycine-competition-binding assays using the radioligand [^3H]-glycine and rat brain membranes [76]. There was a significant correlation between all of the three lipophilicity parameters (MlogP-, logPTsar-, ClogP-values) and the K_i -values from Buchstaller et al. (2006) (see results, Table 3.4, Table 3.5 and Table 3.6). Thus, lipophilicity of the tested glycine antagonists indeed seemed to influence the extent of their binding to the NR1 subunit of the NMDAR as well as their potential to inhibit cell damage via NMDAR-overactivation in vitro. However, the question remains open how important the parameter lipophilicity would be for effects in in-vivo models.

Then, for a more specific qualification of the assay in this work, both K_i - and IC_{50} values from the aforementioned work of Buchstaller et al. (2006) were ranked against the IC_{50} -values received by the assay of this work using again the Spearman's rank-correlation-coefficient [100, 76]. The ranking of K_i -values from Buchstaller et al. (2006) correlated significantly with the IC_{50} -values from this work with 99% probability. Also a correlation between both IC_{50} -value rankings was confirmed with a probability of 95% (see results, Table 3.7 and Table 3.8). These obtained results underline the validity of the assay established in this work.

4.2.3 Potentially novel NMDAR-modulators

4.2.3.1 Wy-14,643

This PPAR α agonist was suggested for the treatment of stroke due to its effects against oxidative stress [117]. Since overactivation of NMDARs among other factors is responsible for the clinical picture of stroke it should be investigated in this work whether Wy-14,643 has an effect on the NMDAR as well.

Wy-14,643 did not inhibit the TC-induced cell membrane damage via NMDAR overactivation up to a concentration of 300 μ M. However, this compound showed the tendency of halting the TC-induced cell damage at higher concentrations, but it cannot be excluded that Wy-14,643 would also show NMDAR-unrelated cytotoxic effects at concentrations above 300 μ M.

4.2.3.2 Honokiol, Magnolol, HX531 and Honokiol with HX531

In the assay of this work honokiol was able to inhibit TC-sensitive cell toxicity in a concentration dependent manner yielding in an IC₅₀ value of about 20 μ M (IC₅₀=20.08 μ M), whereas magnolol was not up to a concentration of 300 μ M. In contrast to this, Lin et al. (2005, 2006) reported similar effects of honokiol and magnolol using rat cerebellar granule cells. They showed by using a MTT assay (see section 1.3) that honokiol and magnolol could both reverse glutamate and NMDA effects on mitochondrial activity in a concentration dependent manner [81, 118].

Additionally, Lin et al. (2005, 2006) using rat cerebellar granule cells observed the effects of honokiol and magnolol on NMDA, KCl and glutamate evoked Na^+ - and Ca^{2+} -influx by fluorimetric determination. They suggested that both honokiol and magnolol blocked glutamate and KCl evoked Ca^{2+} influx, but not the KCl evoked Na^+ influx. However, in concordance to the results obtained by the assay of the present work they found out that honokiol blocked NMDA-induced Ca^{2+} -influx stronger than magnolol [81,118].

When comparing the conditions of the work from Lin et al. (2005, 2006) and those in the assay of this work several differences could be noticed. The main difference was probably that Lin et al. (2005, 2006) used rat cerebellar granule cells instead of NR1/NR2A-expressing HEK-cells. The work of Lin et al. (2005, 2006) focused on NMDAR-activation, achieved by NMDA-receptor agonists NMDA and glutamate, whereas in the assay of this work tetracycline-supplementation and the occurrence of glutamate and glycine in the medium led to NMDAR overactivation. Finally, Lin et al. (2005, 2006) used a MTT assay and fluorimetry as detection methods while in this work LDH-tests were accomplished [81,118].

Interestingly, in this work honokiol showed the same changes of LDH-activity regardless the cells were treated with TC or not (see Figure 3.35). It was assumed that these effects resulted by the fact that according to Kotani et al. (2010) honokiol is interacting with the retinoic X receptor (RXR) as well [101]. Hence, it was attempted to confirm that NMDAR-unrelated effects of honokiol was RXR-dependent by adding 5 μM retinoid X receptor antagonist HX531

[102] to each applied concentration of honokiol. There was only a minor difference occurring between the results of honokiol alone (see Figure 3.35) and the results of honokiol plus HX531 (see Figure 3.36). Moreover, HX531 alone showed no effects on the NMDAR-functionality up to a concentration of 200 μ M, indicating that RXR played no role in NMDAR induced toxicity in the assay of this work (see Figure 3.37). Finally, different honokiol-concentrations were applied to the pure medium-supernatant of TC-treated and TC-untreated cells to investigate if honokiol alone has an influence on LDH-activity itself. It was found that honokiol did not affect the LDH-activity (see Figure 3.38). Thus, it has to be emphasized that it demands further studies to find out how exactly honokiol interacts with NMDAR to comprehend its curious NMDAR-unrelated cytotoxic effects. It could be speculated that honokiol might act as NMDAR activator or inhibitor in a concentration dependent manner and dependent on the amount of NMDAR and its resultant total functionality status.

4.3 Summary

The aim of this work was to establish a cell culture assay for measuring the activity of NMDAR-inhibitors. For adequate measurement the first objective was to obtain a difference in measured values between tetracycline (TC)-treated and non-treated cells of at least 50% cell viability (EZ4U, Bioluminescence) or 2-fold cell toxicity (LDH) respectively. This aim was impressively achieved by using a LDH-test.

The next objective was to qualify the assay by testing the effects of several NMDAR-antagonists and potential inhibitors, and subsequently comparing the results with already published studies. Results for reference compounds MK-801 and L-701,324 were in a similar range according to the literature. In addition, ranking the results for other potential inhibitors correlated with results of another study using Spearman's rank correlation coefficient. The high correlation between the two methods emphasized the quality of the assay of this work. The lipophilicity is possibly crucial for the in vivo effects of the substances, regarding plasma protein binding and penetrability of biological barriers. Thus, three different calculated lipophilicity parameters (MlogP, logPTsar, ClogP) of the antagonists were correlated with the K_i -values from the literature as well as with the obtained IC_{50} -values of the present work. Results showed that lipophilicity seemed to be a major parameter for NMDAR binding and inhibition within the tested substance group.

Also further potential NMDAR modulators were tested in this work. For instance, honokiol was able to inhibit cell damage induced via NMDAR overactivation.

In conclusion, one receptor functionality based assay to determine IC₅₀-values of NMDAR inhibitors was successfully established, which could be applied for preclinical research.

5 References

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7 Appendix

7.1 Zusammenfassung

Die Aufgabenstellung dieser Arbeit war es, einen Zellkulturtest zu etablieren, womit ein rasches Feststellen der Wirkung einer Substanz auf den N-Methyl-D-Aspartat-Rezeptor (NMDAR) ermöglicht werden sollte. Für die Messung wurden HEK293 Zellen verwendet, die nach Zugabe von Tetracyclin einen funktionellen NMDAR exprimieren, was ohne Zusatz von NMDAR-Inhibitoren unweigerlich zum Zelltod führt.

Unter Verwendung eines LDH-Tests wurde letztendlich ein ausreichend großes Messfenster erreicht, um die konzentrationsabhängige Aufhebung des Zellschadens durch die verabreichten Testsubstanzen nachweisen zu können.

Für die Qualifizierung des Assays wurden als Referenzsubstanzen zwei NMDAR-Kanalblocker (MK-801 und Memantin) und ein Glycinantagonist (L-701,324) getestet. Zusätzlich wurden zahlreiche neuartige Antagonisten und einige Substanzen, bei denen der Verdacht besteht, dass sie eine Wirkung auf den NMDA-Rezeptor haben, getestet. Die Ergebnisse der Referenzsubstanzen und der neuartigen Antagonisten wurden mit Ergebnissen aus der Literatur verglichen. Zusätzlich wurde mit der Korrelation der K_i - und IC_{50} -Werte der Antagonisten aus der Literatur mit den IC_{50} -Werten der Antagonisten dieser Arbeit eine weitere Qualifizierung des Tests vorgenommen. Die Ergebnisse und die Korrelationen mit Literaturdaten bestätigten die Qualität des Testsystems, welches in dieser Arbeit etabliert wurde.

Darüber hinaus wurde überprüft, ob die Bindung einer Substanz an den NMDAR beziehungsweise dessen Wirkung auf eine Tetrazyklin-induzierte NMDAR-Überaktivierung in direktem Zusammenhang mit ihrer Lipophilie steht. Somit wurden Lipophilie-Parameter (ClogP, MlogP, logPTsar) mit Ki-Werten aus der Literatur beziehungsweise mit IC₅₀-Werten aus dieser Arbeit korreliert. Die Ergebnisse zeigten, dass die Lipophilie tatsächlich ein ausschlaggebender Faktor, sowohl was die NMDAR-Bindung, als auch die NMDAR-Blockade betrifft, ist.

Schließlich wurden in dieser Arbeit noch die Ergebnisse der Substanzen, bei denen der Verdacht einer Wirkung auf den NMDAR bestand, mit Resultaten anderer Studien verglichen. Unter jenen Substanzen gelang es lediglich Honokiol im etablierten Testsystem dieser Arbeit den TC-induzierten Zellschaden signifikant zu minimieren.

Zusammenfassend wurde ein auf NMDAR-Funktionalität basierender Test zur Überprüfung der Wirkung von potentiellen NMDAR-Inhibitoren etabliert, der für zukünftige präklinische Untersuchungen eingesetzt werden kann.

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