PALACKÝ UNIVERSITY OLOMOUC

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The neuroprotective activity of 8-azapurine derivatives inspired by natural cytokinins performed on glutamate-induced model of oxidative damage

BACHELOR THESIS

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Abstrakt

Teoretická část bakalářské práce je soustředěna na stručný popis neurodegenerativních onemocnění a na jejich dosud dostupnou léčbu. Dále je v teoretické části popsaný význam cystin-glutamátového antiporteru, Nrf2-ARE dráhy, neurotransmiteru glutamátu v neurodegerativních onemocněních a potenciální způsoby, jak by mohli sloužit k vývoji účinné léčby těchto onemocnění. V teoretické části je taktéž popsaný použitý model, a to buněčná linie SH-SY5Y. V praktické části byla testována samotná cytotoxicita derivátů 8-azapurinů inspirovaných přírodními cytokininy a jejich neuroprotektivní vlastnosti na glutamátem indukovaném oxidativním poškození SH-SY5Y buněk. Bylo vyhodnoceno, které z testovaných látek mají nejlepší aktivitu a následně byly zhodnoceny vztahy mezi strukturami a aktivitami vybraných látek.

Klíčová slova	8-azapurinové deriváty, amyotrofická laterální skleróza, Alzeheimerova choroba, cytokininy, glutamátem indukované oxidativní poškození, Huntingtonova choroba, Parkinosonova choroba, SH-SY5Y
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Abstract

Theoretical part of this bachelor thesis is focused on defining neurodegenerative diseases and their available treatment. The function of cystine-glutamate antiporter, Nrf2-ARE pathway, the function of the neurotransmitter glutamate, their impact on neurodegenerative diseases, and their potential to be a target treatment for such diseases are also summarized in the theoretical part. Cell line SH-SY5Y that was used as *in vitro* model in the experimental part is also summarized in the theoretical part. In the experimental part, cytotoxicity of 8-azapurine derivatives inspired by natural cytokinins and their neuroprotective abilities were tested on glutamate-induced oxidative stress model on SH-SY5Y cell line. Derivatives with the most potent neuroprotective abilities and their chemical structures were concluded.

Keywords	8-azapurine derivatives, amyotrophic lateral sclerosis, Alzheimer's disease, cytokinins, glutamate-induced oxidative damage, Huntington's disease, Parkinson's disease, SH-SY5Y
Number of pages	47
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Language	English

I hereby declare that I elaborated this bachelor thesis with the supervision of Mgr. Gabriel Gonzalez, Ph.D. and with the information sources stated in the chapter References.

Olomouc, 31.7.2022 Karolína Ficeriová

Aknowledgment

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Contents

List of abbreviations

4-CPG – (S)-4-carboxyphenylglycine

Aβ – amyloid beta peptide

AD – Alzheimer's disease

ALS – amyotrophic lateral sclerosis

ARE – antioxidant respond elements

ATP – adenosine triphosphate

ATRA – *all-trans* retinoic acid

BBB – blood-brain barrier

COMT – catechol-*O*-methyltransferase

CYC – cystine

DHE – dihydroethidium

DMEM/F12 – Dulbecco's modified Eagle's Medium and Ham's F12 Nutrient Mixture

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

FBS – fetal bovine serum

FDA – Food and Drug Administration

GABA - gamma amino butyric acid

GSH – glutathione

HD – Huntington's disease

HTT – huntingtin gene

Keap 1 – Kelch-like erythroid cell-derived protein

LMSs – lower motor neurons

MAO – monoamine oxidase

mGlur – metabotropic glutamate receptors

mRNA – messenger ribonucleic acid

NAD(P)H - nicotinamide adenine dinucleotide phosphate

NDD – neurodegenerative disease

NMDA – *N*-methyl-*D*-aspartate

Nrf2 – nuclear factor erythroid 2-related factor 2

PBS – phosphate-buffered saline

PD – Parkinson's disease

PI – propidium iodide

R-LA – *R*-lipoic acid

RNA – ribonucleic acid

ROS – reactive oxygen species

SOD – superoxide dismutase

UDP – uridine diphosphate

UMNs – upper motor neurons

x_C^- – cystine-glutamate antiporter

1. Introduction and the aim of the thesis

1.1 Introduction

Neurodegenerative diseases (NDDs) are characterized by progressive loss of neurons in the central or peripheral nervous system. The most common NDDs are Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). It is estimated that NDDs globally affect more than 40 million people (Wood et al., 2015). The onset of NDDs is influenced by environmental, genetic, cellular, and molecular factors and typical symptoms include memory and locomotory impairment, changes in behaviour, and cognitive defects (Solanki et al., 2016).

On a molecular level NDDs are caused by elevated oxidative stress, mitochondrial dysfunction, protein aggregating and misfolding, dysregulated neuronal apoptosis, changes in metabolism, cell signalling, and gene expression can also be modified (Jellinger, 2001; Parihar et al., 2008; Solanki et al., 2016). In some NDDs, such as sporadic PD, the exact pathways of described mechanisms still remain undefined.

Nowadays only a few treatments approved by the Food and Drug Administration (FDA) of NDDs exist, their success rate is very low and they are focused on keeping the patient's health from deteriorating for a certain period of time (D. Kumar et al., 2020; Solanki et al., 2016). Specific drug treatments of AD, PD, HD, and ALS are described in this thesis. Ongoing studies are focused on defining the exact mechanism of each NDD, as well as on developing a treatment that would stop NDDs from progressing.

Besides describing the characteristics of AD, PD, HD, ALS, and their available treatment, this thesis describes the molecular mechanism Nrf2-ARE pathway, cystine-glutamate antiporter, and their importance in NDDs. The thesis is also focused on defining neuroprotective and antioxidative abilities of derivatives of plant hormones, cytokinins. Several studies have proven that these compounds have antioxidative, cytoprotective, and neuroprotective abilities when tested on various toxicity-induced models on different cell lines (Gonzalez et al., 2021; Voller et al., 2017).

1.2 Aim of the thesis

The aims of the thesis are following:

1. Literature research focused on neurodegenerative diseases, their current treatment, cystine-glutamate antiporter, Nrf2-ARE pathway, and glutamate-induced oxidative damage model on neuronal SH-SY-5Y cell line.
2. Screening of cytotoxicity of 8-azapurine derivatives inspired by natural cytokinins compared to the standards on glutamate-induced oxidative damage model on neuronal SH-SY-5Y cell line, measurement of the activity of the chemical compounds based on the production of superoxide radical
3. Comparison of the results of the performed experiments with available literature.
4. Evaluation of the chemical compounds, determination of the relation between the chemical structure and its activity.

2. Theoretical part

2.1 Characteristics and treatment of neurodegenerative diseases

As implied in the introduction, NDDs are defined by progressive loss of the structure and function of the neurons in specific regions of the nervous system, ultimately causing neuronal cell death (Przedborski et al., 2003). Degeneration or loss of the neurons results in the impairment of memory, movement, and cognitive abilities, as well as in difficulties with behaviour and emotions. AD, HD, PD, multiple sclerosis, and ALS are all classified as neurodegenerative disorders. NDDs can be induced by environmental aspects, however, the main causes of the degeneration are the alteration of mechanisms on cellular and molecular levels, such as mutation and modification of expression of genes, modification of cell signalling, mitochondrial dysfunction, protein aggregation, oxidative stress, production of free radicals, inflammatory response, and apoptosis of neurons (Solanki et al., 2016). Oxidative stress was found to be one of the main causes of the damage of the neurons. It is induced by increased generation of reactive oxygen and nitrogen species, such as hydrogen peroxide, hydroxyl and superoxide radicals and, nitric oxide as well as by the impairment of cellular antioxidant defence systems (Poddar et al., 2021). Treatment of such diseases is very limited, as there are only a few drugs that are able to affect them. Therapy is not focused on full recovery from the disease, the main purpose of the drugs applied to patients is to stop the progression of the symptoms (Solanki et al., 2016). All the available therapies are discussed in the following subchapters.

2.1.1 Alzheimer's disease

Progressive loss of cholinergic neurons in the temporal lobe of the brain resulting in forgetfulness is the first symptom of AD, followed by confusion, memory loss, and anxiety (Rowinska-Zyrek et al., 2015; Solanki et al., 2016). Post mortem examination of AD patient's brain showed neurofibrillary tangles within neuronal cells and amyloid plaques concentrated around the cells. The two mentioned structures are considered to be the main hallmarks of AD. Amyloid plaques contain amyloid beta peptide (Aβ), whose accumulation and deposition of toxic oligomers initiate the onset of AD. Accumulated oligomers produced by Aβ can block proteasome function and inhibit the activity of mitochondria, which makes them neurotoxic. Furthermore, Aβ directly influences signalling pathways of tubulin-associated unit, known as tau protein phosphorylation, leading to the protein becoming hyperphosphorylated.

The main function of tau-protein is to form the internal skeleton of nerve axons in the brain. However, once tau-protein is hyperphosphorylated, protein aggregation is initiated, resulting in the formation of neurofibrillary tangles.

Ionotropic glutamate receptors permeable to Na^+, K^+, and highly permeable to Ca^{2+} are called *N*-methyl-*D*-aspartate (NMDA) receptors. The main function of these receptors is a synaptic transmission of glutamate and synaptic plasticity, which determines learning abilities, quality of memory, as well as proper development and function of the central nervous system. Dysfunction of these receptors was found to have an impact on NDDs, especially on AD, PD, and HD (Chohan and Iqbal, 2006; Liu et al., 2019). It was proven that NMDA receptors are dysregulated by Aβ oligomers. Once dysregulated, glutamatergic synaptic transmission is disrupted, leading to the glutamate levels being extremely increased, which augments the activity outside NMDA receptor synapsis and induces excitotoxicity. Furthermore, Ca^{2+} uptake is excessively increased as well, resulting in the damage of mitochondria and endoplasmic reticulum, eventually causing cell death and initiation of early cognitive impairment in AD (Liu et al., 2019; Y. Zhang et al., 2016).

The most used drugs for treating patients with AD are composed of cholinesterase inhibitors and NMDA antagonists approved by FDA. Cholinesterase inhibitors are used in order to improve the cognitive impairment caused by insufficient levels of acetylcholine in presynapse. They are able to increase acetylcholine levels by inhibiting its degradation, which should result in the improvement of AD patient's cognitive abilities (Rowinska-Zyrek et al., 2015). Only four inhibitors have been approved by FDA so far, but only three of them are currently being used for the treatment of AD – donepezil, galantamine, and rivastigmine. These inhibitors have proven to have a significant effect on maintaining the cognitive abilities of the patients and delaying the development of the symptoms by 6-12 months, however, they were effective only in 50% of the patients (Hong-Qi et al., 2012; Rowinska-Zyrek et al., 2015). Non-competitive NMDA receptor antagonist called memantime has been approved by FDA and is currently being used to treat patients with AD. Memantine is thought to reduce excessive excitatory signals by adjusting the activity of NDMA receptor, as well as to regulate abnormal neurotransmission of glutamate, meanwhile allowing the physiological transmission essential for normal cell functioning to occur (Hong-Qi et al., 2012). Memantine is able to restore the function of damaged nerve cells, to relieve the patients from the symptoms, and improve the quality of patients' lives. Moreover, patients being treated with donepezil and memantine have shown significant enhancement of cognitive and functional abilities (Hong-Qi et al., 2012; Liu et al., 2019).

However, no drug has been able to cure a patient completely or prevent the disease from further developing yet (Hong-Qi et al., 2012). Antioxidant vitamin E in high doses can be used as an alternative to the treatment of AD, as it moderates cognitive impairment (Solanki et al., 2016). In addition, stem cell-based therapy is thought to be one of the potential targets for the treatment of AD. Stem cells are able to be differentiated into neurons, therefore, they might be able to replace the progressive neuronal loss occurring in AD (Rowinska-Zyrek et al., 2015).

2.1.2 Huntington's disease

Symptoms of HD include chorea (involuntary, irregular, and unpredictable muscle movements), dystonia (involuntary muscle contractions resulting in twisting movements), bradykinesia (difficulty in movement), psychiatric disturbances, and cognitive impairment most likely caused by dysfunction of neurons and their cell death (Solanki et al., 2016). Unlike other neurodegenerative diseases, HD is a genetic autosomal disease caused by a mutation in the huntingtin gene (HTT). HTT contains an expansion of CAG trinucleotide repeat, resulting in encoding atypically elongated polyglutamine repeat in the expressed huntingtin protein. Therefore, misfolding of proteins occurs (Ross and Tabrizi, 2011). From the pathological point of view, selective degeneration of neurons located in the striatum is found in HD patients. Striatum is able to produce gamma amino butyric acid (GABA), however, when damaged, the production is significantly reduced. The lack of GABA causes atrophy of dorsal striatum, cerebral cortex, thalamus, and other brain regions, from which the atrophy of the striatum is considered to be the most severe one (Solanki et al., 2016). As already mentioned, the main reason for HD onset is the mutation of the HTT, encoding Htt protein. The protein is classified as cytoplasmic protein essential for vesicle transport, it can also be involved in the regulation of gene transcription. Once the mutation occurs, the length, as well as the conformation of the protein are changed which creates toxic *N*-terminal fragments (Ross and Tabrizi, 2011). These fragments are translocated into the nucleus, where they form toxic intranuclear aggregates of *N*-terminal Htt fragments, which are able to directly influence components involved in transcription, such as genomic DNA, histones, or several transcription factors. mRNA levels of several genes, such as the ones encoding neurotransmitter receptors or ligands were shown to be downregulated, moreover, neuronal cell death occurred only after mRNA downregulation of the particular genes (A. Kumar et al., 2014). Mutated HTT protein is also thought to affect mitochondria.

Once it is joined with the mitochondrial outer membrane, complexes II and III of the electron transport chain become impaired, intra-cellular levels of ATP decrease, and reactive oxygen species (ROS) levels are augmented (Labbadia and Morimoto, 2013).

There is no drug that would completely cure HD, nevertheless, tetrabenazine and its derivative (deutetrabenazine) are the only drugs approved by the FDA for the treatment of chorea so far (Mestre and Shannon, 2017). Although no cure specifically for HD is available yet, medications to diminish the symptoms connected with HD are widely used (Ross and Tabrizi, 2011). As for potential therapy targets, antisense oligonucleotides, which would be able to lower mutated HTT protein through RNA and DNA appears to be promising. Other potential targets include stem cell therapy or antibody therapy (Ferguson et al., 2022).

2.1.3 Parkinson's disease

Symptoms of PD include bradykinesia, impaired coordination, shuffling gait, trembling in upper extremities, inability to move spontaneously, micrographia, memory lapses, difficulty swallowing, as well as dementia, and depression (Rowinska-Zyrek et al., 2015). It is believed that PD is caused by the process of cumulative loss of dopamine neurons, which can be induced by endogenous toxins, excitotoxicity, and oxidative stress. The loss of dopaminergic neurons occurs in the basal ganglia of the midbrain called substantia nigra, more precisely in the front part of pars compacta. The structure plays a crucial role in proper movement and muscle tone of an individual. Further histopathological analysis of PD patient's brain has discovered inclusion composed of Lewy bodies with eosinophilic core, loss of dopamine in the cells located in the nigrostriatal pathway, as well as astrocytic cell death. The hallmark of PD is considered to be Lewy bodies, which are mostly composed of alpha-synuclein. Decreased ferritin level is another molecular reason behind the onset of PD. Lower ferritin levels result in high concentrations of free Fe^{3+} ions in the brain, which are processed in the oxidation cycle as well as other metal ions, resulting in forming ROS. Oxidative damage is also induced by excessive production of monoamine oxidase, which creates hydrogen peroxide molecules that are able to cause dopamine impairment.

The main function of the drugs used for treating PD is to augment the activity of dopamine receptor in order to increase dopamine-mediated synaptic inhibition. PD is treated with drugs containing L-DOPA (l-3,4-dihydroxyphenylalanine) which is able to cross the blood-brain barrier and is converted to dopamine in the brain. To prolong the effect of levodopa, catechol-*O*-methyltransferase (COMT) inhibitors, e.g. tolcapone, and monoamine oxidase (MAO) inhibitors, e.g *L*-deprenyl are used in order to prevent the breakdown of dopamine by the mentioned enzymes (Rowinska-Zyrek et al., 2015).

In addition, selective dopamine receptor agonists such as pramipexole, ropinirole, rotigotine, and apomorphine can be used as a treatment or to augment the effect of levodopa (Brooks, 2000).

2.1.4 Amyotrophic lateral sclerosis

ALS is a motor neuron disease, which causes degeneration of both lower population of the motor neurons (LMNs) situated in the spinal cord and brain stem, as well as the upper one (UMNs) situated in the specific region of the cerebral cortex called motor cortex (Hulisz D, 2018; Salameh et al., 2015). The symptoms include muscle paralysis, weakness, stiffness, impaired coordination of the limbs (UMNs), spontaneous muscle twitching, and atrophies caused by lost connection between the synapses connected to the muscles (LMNs) affecting the entire body (Hulisz D, 2018; Solanki et al., 2016). There are two types of ALS: familial (5-10% ALS patients) and the most occurring sporadic (idiopathic) ALS. Familial ALS is caused by genetic mutation of genes such as C9orf72, superoxide dismutase (SOD1), and genes encoding two RNA binding proteins (TDP4352, FUS) with onset in late teenage years or early adulthood. As for the sporadic type, the average age of the onset is around mid-fifties and the cause of the disease is unknown, however, it is thought to be caused by abnormalities in the immune system, mitochondrial dysfunction, and by increased glutamate levels. Glutamate becomes toxic as soon as it is not removed quickly enough from the synaptic area by astrocytes and excitatory amino acid transporter, causing repetitious and immoderate firing of action potentials. Dysregulation of glutamate results in mitochondrial and endoplasmic reticulum stress (Hulisz D, 2018).

The only drug that has been approved by FDA for treating ALS is called riluzole. The drug interferes with the neurotransmission of glutamate via complex mechanism of action, including the blockade of sodium channels and interactions with NMDA channels and metabotropic glutamate G-protein coupled receptors (mGlur). When riluzole interferes with the mentioned mechanisms, glutamate release is inhibited (Shaw and Ince, 1997). The ongoing development focused on treating or curing the disease includes antisense oligonucleotides and virus-delivered gene therapies, as well as stem cells therapy (Salameh et al., 2015).

2.2 Cystine/glutamate antiporter and its effect on neurodegenerative diseases

2.2.1 Definition and mechanism of cystine/glutamate antiporter

The cystine/glutamate antiporter, also known as the x_C^- system is defined as sodium-dependent system, which is able to transport anionic amino acids (Conrad and Sato, 2012; Had-Aissouni, 2012). The main role of the x_C^- system is to transport oxidized form of cysteine, cystine (CYC) into the cell in exchange for intracellular glutamate. The ratio of the exchange provided by the cystine/glutamate antiporter is 1:1 and the pathway is sodium independent and chloride-dependent in physiological conditions. Once CYC enters the intracellular space, it is reduced to cysteine, which is crucial for the biosynthesis of glutathione. Cysteine surplus is secreted back into the extracellular space via neutral amino acid transport systems. Glutathione is a tripeptide thiol composed of glutamic acid, cysteine, and glycine, furthermore, it is thought to be vital for protecting the cells from intracellular oxidative damage. Therefore oxygen, electrophilic agents, and bacterial lipopolysaccharide are supposed to trigger the expression of xCT which would result in increased biosynthesis of glutathione (GSH). However, over the past years, it has been proved that the x_C^- system is more likely to be a GSH-independent redox system, which preserves the redox cycle balance. By transporting extracellular cystine into the cell, the x_C^- system maintains reducing extracellular microenvironment by a constant flow of the surplus cysteine to the extracellular space, as presented in Figure 1.

The activation of the system can be induced by electrophilic agents, oxygen, oxidative stress agents, tumour necrosis factor-alpha, or by Aβ (Conrad and Sato, 2012). Glutamate uptake by x_C^- antiporter can be inhibited by cystine, ibotenate, α-aminoadipate, and (S)-4-carboxyphenylglycine (4-CPG), which are presented in Figure 2 (Had-Aissouni, 2012).

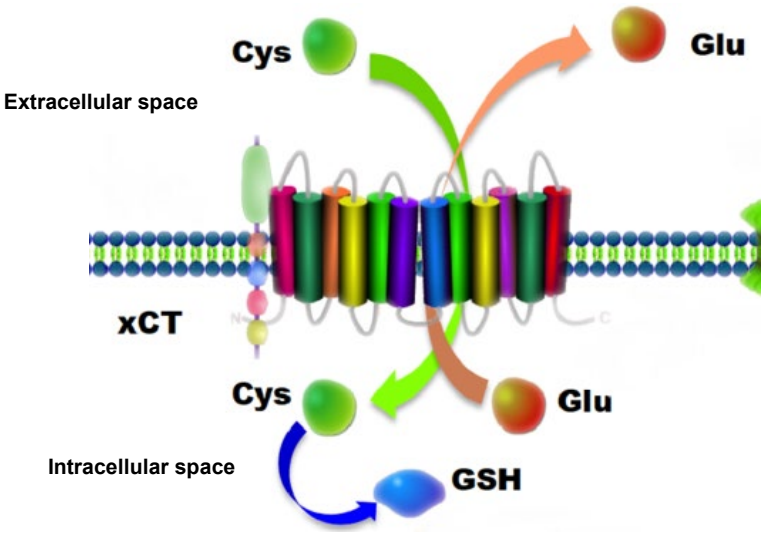

Figure 1 – The cystine/glutamate antiporter regulation scheme. Cysteine and glutamate are exchanged via x_c^- antiporter in 1:1 ratio. Cysteine taken into the cell is used for glutathione synthesis (edited and taken from Savaskan et al., 2015)

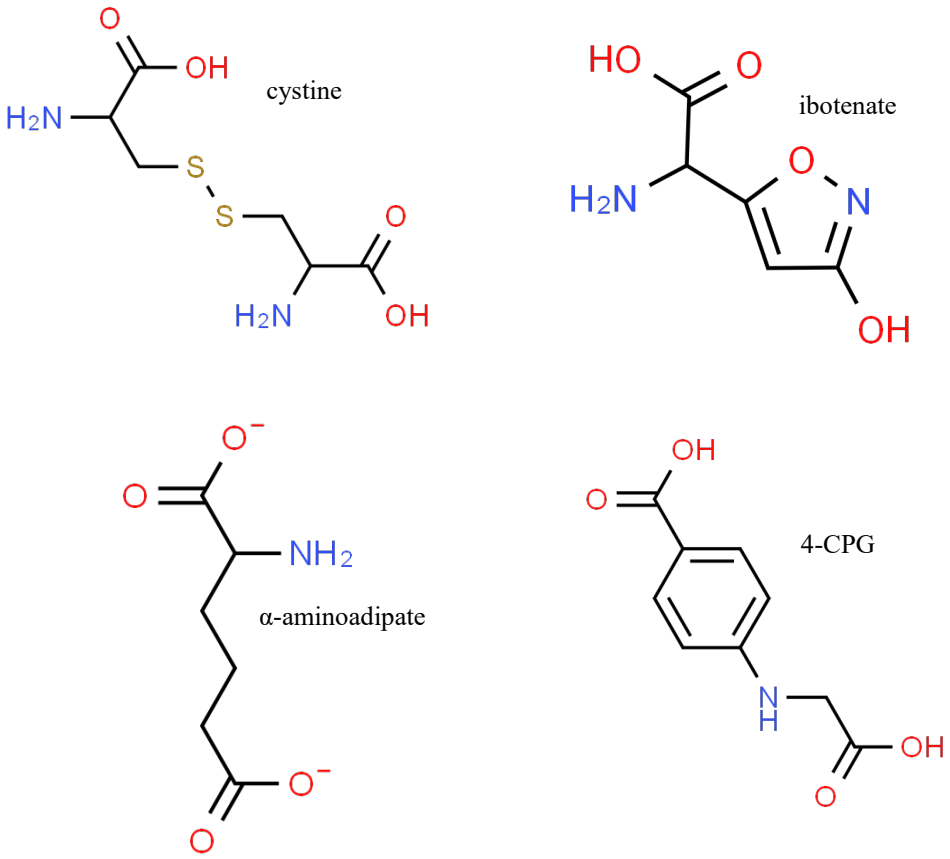

Figure 2 – Structures of x_C^- system inhibitors – cystine, ibotenate, α-aminoadipate, 4-CPG (downloaded from http://www.chemspider.com/)

2.2.2 Cystine/glutamate antiporter in neurodegenerative diseases

The x_C^- system located in glial and nerve cells is constitutively expressed in the brain, which is essential for the synthesis of glutathione. Neuronal cells are known to be sensitive to oxidative stress, therefore it is vital for the system to exchange cystine and glutamate in a 1:1 ratio. When extracellular levels of cysteine are increased, cell signalling and communication are reduced and do not have a toxic impact on the cells. On the contrary, augmented levels of extracellular glutamate may lead to neurodegeneration caused by glutamate-induced excitotoxicity by overstimulation of glutamate receptors (Had-Aissouni, 2012). Furthermore, increased levels of glutamate inhibit the synthesis of glutathione, which is caused by impaired uptake of cystine by x_C^- antiporter. Decreased levels of glutathione cause increased accumulation of ROS, elevated oxidative stress, and eventual neuronal cells (Conrad and Sato, 2012).

2.2.3 Glutamate toxicity in neurodegenerative diseases

The function of glutamate is directly connected to neurodegenerative diseases, including AD, PD, HD, and ALS (X. Zhang et al., 2020). Regarding such diseases, the excessive amount of glutamate is released into the synaptic cleft by nerve impulses. Most of the surplus glutamate is absorbed by microglia or astrocytes. Glutamate is able to regulate the excitation of neurons and synaptic transmission and can be classified as the most common excitatory neurotransmitter in the brain. The initial release of glutamate comes from cerebral cortical pyramidal neurons in the prefrontal cortex, from where it is carried into the striatum, thalamus, and brainstem. Its main function is to regulate the release of other neurotransmitters (e.g. gamma-aminobutyric acid) and innervate pyramidal neurons in the cerebral cortex. Once a nerve impulse reaches the cell, glutamate is released into the synaptic cleft, leading to the induction of a receptor-mediated signalling pathway.

The process of glutamate becoming toxic is called excitotoxicity and it is induced by prolonged activation of mGlur and ionotropic glutamate receptors, which triggers a neurotoxic cascade, ultimately resulting in cell death (Armada-Moreira et al., 2020). Overactivation of ionotropic NMDA receptors and voltage-dependent Ca^{2+} channels increase Na^+ and Ca^{2+} intracellular uptake through the plasma membrane. Overactivation of mGlu receptors results in the release of Ca^{2+} the from endoplasmic reticulum and mitochondria, which is a response to membrane being damaged by the disruption of ionic gradients (Mattson, 2019). Increased intracellular Ca^{2+} levels activate enzymes that degrade cytoskeletal proteins, lipids, membrane receptors, and metabolic enzymes.

Mitochondria play a crucial role in excitotoxicity-induced calcium influx, as they try to maintain low cytosol Ca^{2+} concentrations. As already mentioned, Ca^{2+} amounts are excessively increased and the uptake of these ions by mitochondria depolarizes the mitochondrial membrane and diminishes their anti-oxidant functions, resulting in high ROS production (e.g. hydrogen peroxide, superoxide anion radical), mitochondrial dysfunction, caspase activation and eventual cell death by apoptosis (Armada-Moreira et al., 2020). Cell death can be driven by necrosis or it can be caused by oxidative stress, as demonstrated in Figure 3.

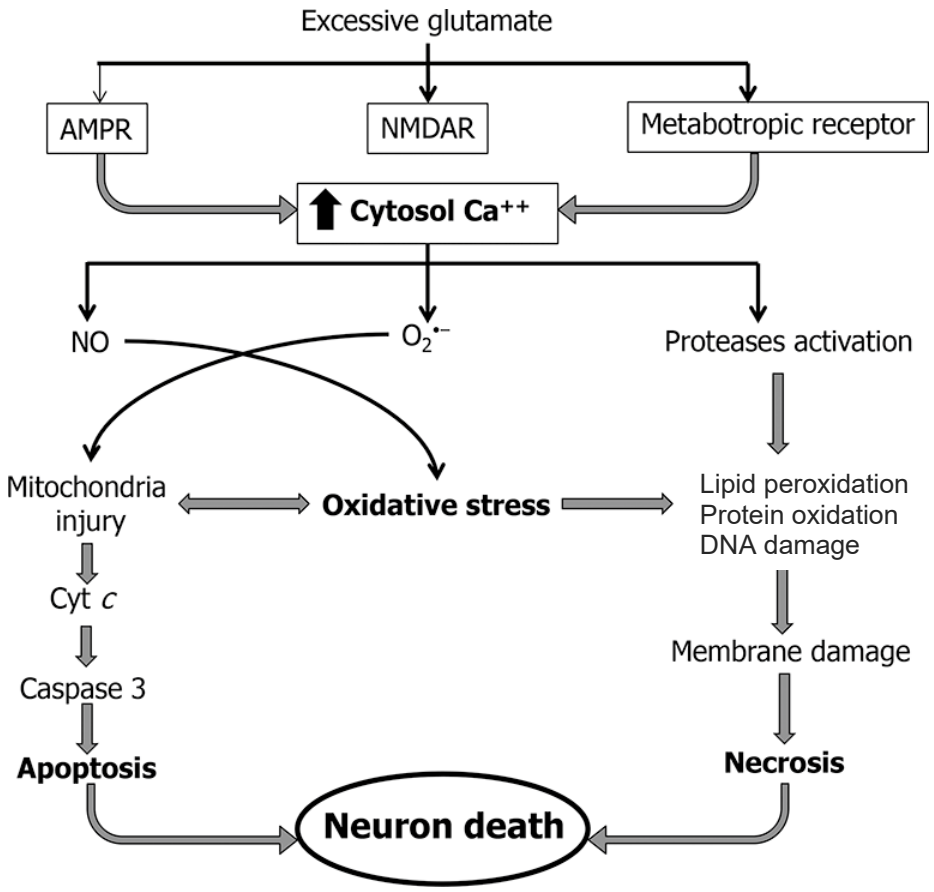

Figure 3 – The excitotoxicity induced by glutamate overactivates ionotropic (NMDA) and metabotropic receptors, increases intracellular Ca^{2+} uptake, ultimately resulting in depolarization of mitochondrial membrane, mitochondrial dysfunction and increased ROS production. Oxidative stress induced by increased ROS activates caspase leading to cell death by apoptosis or protein oxidation, DNA damage and lipid peroxidation, ultimately resulting in cell death by necrosis (taken from Rama and García, 2016)

2.3 Nrf2-ARE pathway

2.3.1 Characteristics, definition, and function of Nrf2-ARE

Nuclear factor erythroid 2-related factor 2, also known as Nrf2 is classified in the cap 'n' collar subfamily basic region leucine zipper transcription factors, which binds to the promoter DNA sequence known as antioxidant respond elements (ARE) (Ma, 2013). The activation of ARE is crucial for the initiation of drug-metabolizing enzymes, e.g. glutathione *S*-transferase, resulting in augmented detoxification and extermination of some exogenous and endogenous chemicals, especially oxidants and electrophiles.

2.3.2 Activation and mechanism of Nrf2-ARE pathway

Mechanism of Nrf2 affects the ROS and reactive nitrogen species homeostasis by x_C^- cystine/glutamate transport, induction of superoxide and peroxide catabolism via SOD 1, as well as by regeneration of oxidized cofactors and proteins. In normal conditions, Nrf2 is suppressed by ubiquitination and proteasomal degradation provided by Kelch-like erythroid cell-derived protein (Keap 1) (Ma, 2013). When activated by oxidants and electrophiles, critical cysteine thiols of Keap1 are modified. Nevertheless, Nrf2 can be regulated by Keap1-independent mechanism by being phosphorylated via glycogen synthase kinase-3, as well as the factor can be altered by the regulation of its transcription (Petri et al., 2012).

Nrf2-ARE pathway along with the Keap1 suppressor work as a cellular sensor for detecting the damage caused by the oxidants or electrophiles, therefore it can be activated by oxidative damage, inflammation, and environmental stressors. Once detected, Keap1-Nrf2 binding is decreased and Nrf2 is translocated into the nucleus by karyopherin-6. Heterodimers with small Maf proteins and other transcription factors are formed after the translocation (Petri et al., 2012). The created heterodimers bind to the ARE sequence and the transcription of more than 250 ARE genes is initiated, including the genes encoding phase II detoxification enzymes, anti-inflammatory response molecules, antioxidant cellular defence (antioxidant proteins), oxidant signalling, and drug metabolism, such as UDP-glucuronosyltransferase, NAD(P)H quinone oxidoreductase-1 or glutathione S-transferase. Therefore, the Nrf2-ARE pathway plays a pivotal role in cell survival (Lee et al., 2005; Petri et al., 2012). The described mechanisms are affected by dephosphorylation/phosphorylation and deacetylation/acetylation of Nrf2 (Ma, 2013). The activation mechanism is presented in Figure 4.

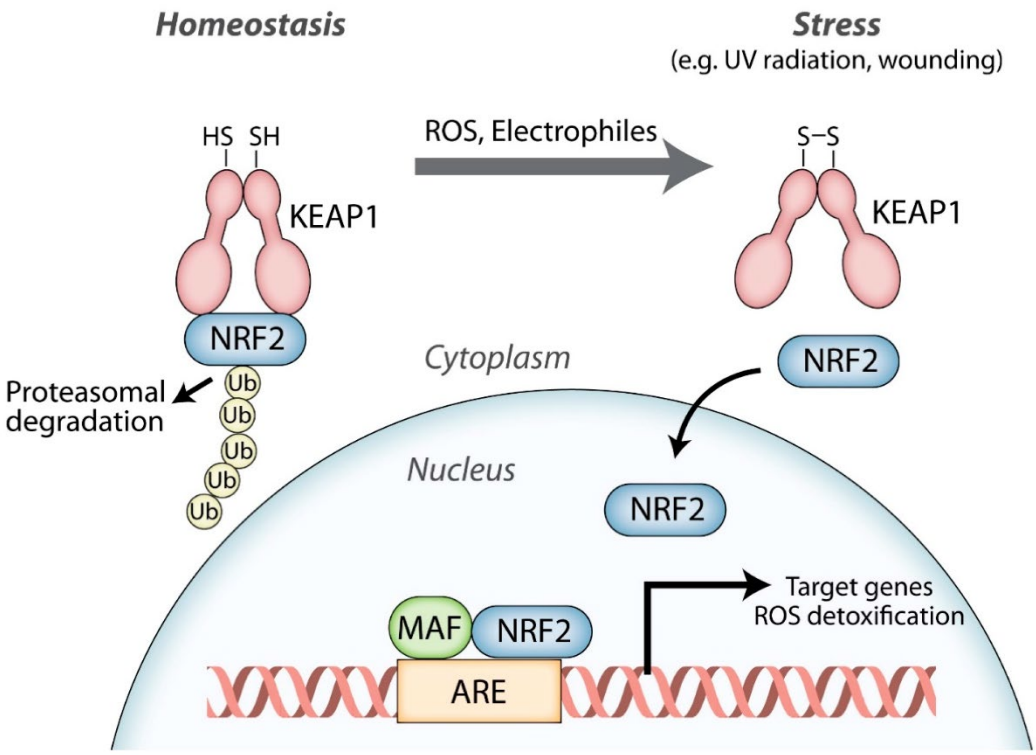

Figure 4 – Nrf2-ARE activation pathway. In normal conditions, Nrf2 is ubiquitinated through Keap1 and degraded by proteasome. Once Nrf2 is activated by ROS or other electrophiles, it becomes phosphorylated and is translocated from cytoplasm to nucleus, Keap1 is inactivated. Nrf2 with Maf protein heterodimers and other transcription factors bind to ARE sites in the nucleus and activate genes transcription of genes encoding phase II detoxification enzymes, anti-inflammatory response molecules etc. (taken from Hiebert & Werner, 2019)

2.3.3 Nrf2-ARE pathway in neurodegenerative diseases

Over the past years, many studies have demonstrated a direct connection between the dysfunction of the Nrf2-ARE pathway and neurodegenerative diseases, as one of the hallmarks of such diseases is the excess amount of free oxygen radicals (Ma, 2013). Once the Nrf2-ARE pathway is impaired, oxidative stress is increased and mitochondrial dysfunction occurs. According to the conclusions from the studies focused on neurodegenerative diseases performed by Petri et al., in 2012 dysfunction of the Nrf2 pathway in neurodegenerative diseases causes diminishment of Nrf2 nuclear translocation, which leads to decreased defence against oxidative stress.

2.3.4 Nrf2-ARE pathway as a potential target for the treatment of neurodegenerative diseases

Calkins et al. performed an observation in 2009, where they studied the protective abilities of the Nrf2 pathway in cell cultures of cortical neurons. Once the Nrf2-activating substances were added to the culture, the majority of Nrf2 activation took place in astrocytes. Another observation from Shih et al. performed on astrocyte cell cultures proved that the activation of astrocytic Nrf2 protein can protect neuronal cells from glutamate-induced oxidative damage (Calkins et al., 2009; Nguyen et al., 2009). In addition, other authors observed the protection from cell death caused by mitochondrial toxins, such as hydrogen peroxide, tert-butyl hydroperoxide, or 3-nitropropionic acid. Their studies are summarized in a review by Calkins et al., 2009.

Based on the studies mentioned above, Nrf2 activators became the main topic of many studies. Several Nrf2 activators have been discovered so far. A great number of the activators oxidize or alkylate cysteine residues in thiol-rich Keap1, forming covalent adducts (Li et al., 2020). Dimethyl fumarate, originally used for treating psoriasis, was used to treat multiple sclerosis with successful Nrf2 activation. The mechanism of activating Nrf2 by dimethyl fumarate is based on the covalent reaction of monomethyl fumarate (active dimethyl fumarate metabolite) with C151 of Keap1 in brain neurons and glial cells (Jayaram and Krishnamurthy, 2021). Sulforaphane is able to modify Cys171 residue of Keap, resulting in the protection of neurons against hydrogen peroxide or glutamate toxicity *in vivo* (Li et al., 2020).

The majority of potential Nrf2 activators are derived from natural products, such as curcumin and its derivates, which possess anti-inflammatory and antioxidative properties. Curcumin is able to react with cysteine groups in thiol-rich Keap1, resulting in the activation of Nrf2-ARE pathway, moreover, it is able to prevent ROS production and penetrate the blood-brain barrier (BBB). Other Nrf2 activators include polyphenols, such as caffeic acid phenethyl ester and ferulic acid ethyl, flavonoids such as eriodictyol-7-*O*-glucoside, quercetin, cardamomin, carnosic acid, carnosol, 3,4-dihydroxyphenylethanol, and resveratrol. All the mentioned substances were tested on different cell lines, such as rat pheochromocytoma PC12, neuronal SH-SY5Y, or human colorectal carcinoma HCT116 and presented the ability to activate Nrf2. Cardamomin and quercetin are even able to protect the cells from oxidative damage. Many potential Nrf2 activators are currently undergoing clinical trials. Structures of some mentioned activators are presented in Figure 5.

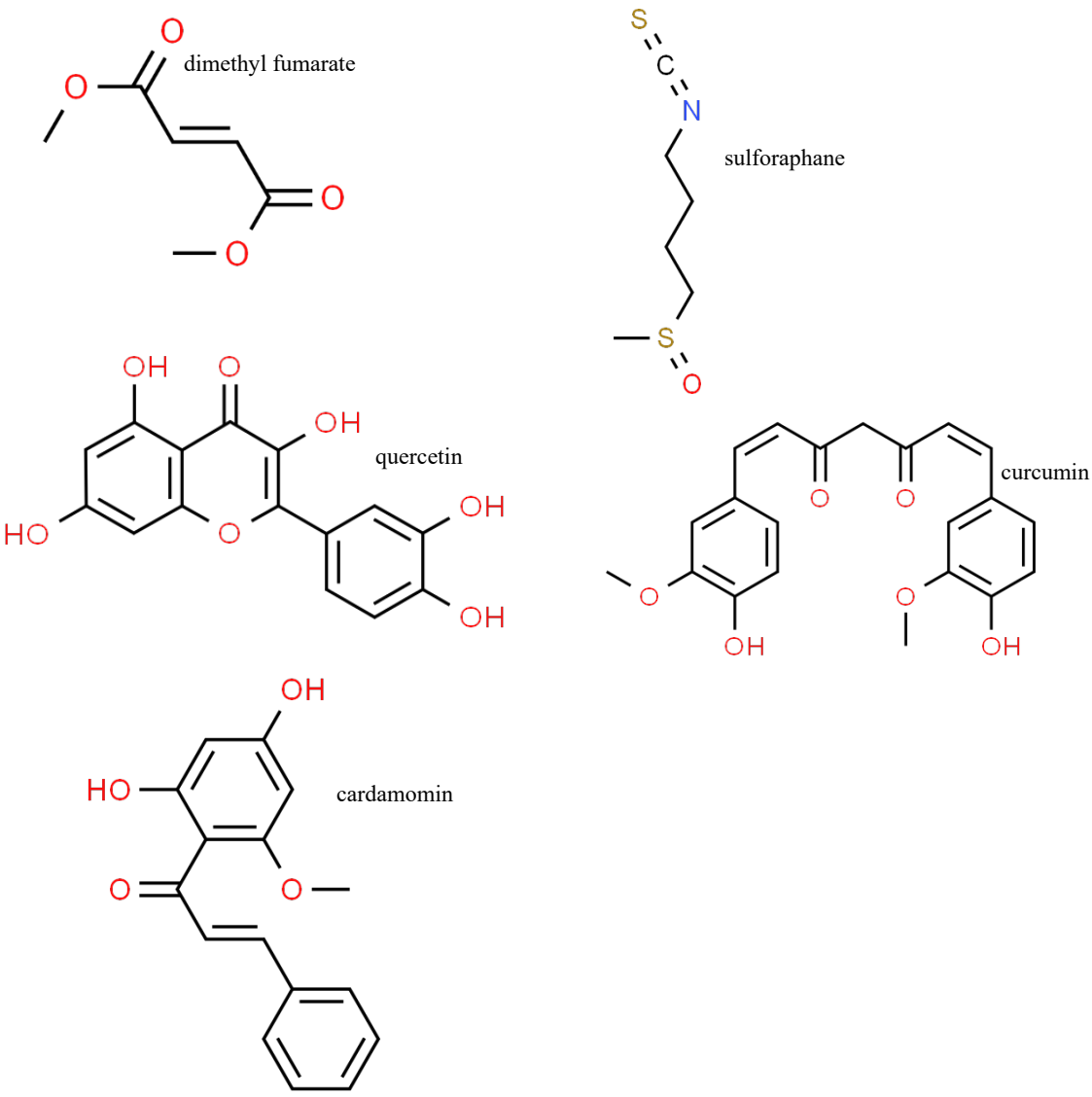

Figure 5 – Structures of Nrf2 activators – dimethyl fumarate, sulforaphane, quercetin, curcumin, cardamomin (downloaded from http://www.chemspider.com)

The main problem with Nrf2 activators is that they are electrophiles, which can bond not only to Keap1 cysteine group but also to other cysteine-rich proteins or other nucleophiles. This bond can trigger off-target effect and result in high toxicity (Li et al., 2020; Osama et al., 2020). A solution to this problem could be the use of competitive inhibitors on the Keap1-Nrf2 binding site, e.g ubiquitin-binding protein p62 which would augment the selectivity and minimize the side effects.

2.4. SH-SY5Y cell line as *in vitro* cell model in neurodegenerative diseases

SH-SY5Y is a homogenous neuroblast-like (N type) cell line derived SK-N-SH parental line, obtained from a metastatic bone marrow biopsy (Kovalevich and Langford, 2013). This cell line has become widely used *in vitro* cell model for studying neuronal differentiation, metabolism, neurodegenerative processes, neurotoxicity, as well as neuroprotection (Xie et al., 2010). The cells are capable of proliferating in culture for long periods of time without contamination, they can be differentiated to mature neuron-like phenotype, moreover, they express numerous neuronal markers including human-specific proteins and enzymes, which are described further in this subchapter (Kovalevich and Langford, 2013).

Differentiation is a crucial process in experiments with the SH-SY5Y cell line. After differentiation, the cells stop proliferating, form a stable population, and start to morphologically resemble human neurons located in the brain (Xie et al., 2010). Based on the used differentiating agent, they can be differentiated into cholinergic, adrenergic, and dopaminergic adult neuron phenotypes. Differentiation induces formation and extension of neuritic processes, augmentation of electrical excitability of the plasma membrane, initiation of neuron-specific enzymes, neurotransmitters neurotransmitter receptors, and some neurofilament proteins. More specifically, differentiated and high passage undifferentiated SH-SY5Y cell lines express neuron-specific enzymes tyrosine hydroxylase, which catalyzes dopamine, and dopamine-β-hydroxylase, which catalyses the formation of noradrenaline from dopamine. Furthermore, they express dopamine transporter and dopamine receptor subtypes 2 and 3, which are characteristic for dopaminergic neurons, as well as norepinephrine transporter and vesicular monoamine transporter, which are hallmarks of adrenergic neurons (Kovalevich and Langford, 2013).

One of the most commonly used differentiating agents is vitamin A derivative, *all-trans* retinoic acid (ATRA), which is added to the cell culture media (Kovalevich and Langford, 2013). It has been proved that when SH-SY5Y cells are differentiated by ATRA, the expression of choline acetyltransferase and vesicular monoamine transporters are increased, resulting in forming cholinergic phenotype. Dopaminergic phenotype can be induced by ATRA with the use of additional differentiating agents, such as phorbol ester. Differentiated cells show increased levels of tyrosine hydroxylase, dopamine receptors 2 and 3, and dopamine transporters. Furthermore, differentiated cells expressed NR1 subunit of ionotropic NMDA glutamate receptor. The expression of human neuron-specific features makes the differentiated SH-SY5Y cell line an ideal *in vitro* cell model for studying mechanisms and treatment of neurodegenerative diseases.

3. Experimental part

3.1 Chemicals, solutions and biological material

Human neuroblastoma cell line SH-SY5Y (European Collection of Authenticated Cell Culture), *all-trans* retinoic acid (ATRA), dihydroethidium (DHE), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's Medium and Ham's F12 Nutrient Mixture (DMEM:F12, 1:1), fetal bovine serum (FBS), monosodium L-glutamate, 8-azapurine derivatives, penicillin-streptomycin, 1x PBS, propidium iodide (PI), selective inhibitor of necrosis IM-54, Triton X-100, Trypsin-EDTA solution – all the chemicals were provided by Merck, 8-azapurine derivatives were synthesized by Mgr. Gonzalez, Ph.D (Gonzalez, 2016)

3.2 Laboratory equipment and instruments

Analytical balance scales (Sartorius), automatic pipettes and automatic pipette tips (Eppendorf, Finpipette), centrifuge BR4 (Jouan), incubator MCO-18AIC (Sanyo), laminar flow cabinet (MSC ADvantage), microscope CKX41 (Olympus), pipette controller (Swiftpipet Pro), spectrophotometer infinite M200 Pro (Tecan), vortex Minishaker MS2 (IKA), 0,6 ml and 1,5 ml micro test tubes (Eppendorf), Petri dish, 15 ml, 50 ml test tubes and 96-well microtiter microplates (Techno Plastic Products).

3.3 Experimental methods

3.3.1 Cell culture

The cells were prepared in a total of three steps: cultivation, preparation for differentiation and differentiation. SH-SY5Y cell line was cultivated in DMEM/F12 media with 10% fetal bovine serum and 1% penicillin and streptomycin (cultivation media). The cell line within the media was placed into 50 cm^2 Petri dishes. Conditions for the cultivation were following: 37°C, 5% CO_2, cells were cultivated in the incubator. When the confluence of the cells in the Petri dishes reached 70-90%, the cells were rinsed by 1x PBS and passaged by trypsin. After PBS was aspirated, 1 ml of trypsin was added to detach the adherent cells for 5 minutes at laboratory temperature. Trypsin was then inhibited by 4 ml of cultivation media. From 5 ml of cell suspension, 2,5 ml was added to a new Petri dish containing 10 ml of fresh cultivation media.

3.3.2 Preparation of the cell for the experiments

After the cultivation, the media was removed and its residue was washed off by 1 ml of 1x PBS, followed by 5 min incubation of the cells with 1 ml of trypsin to make the cells nonadhesive. 4 ml of the cultivation media was added to the Petri dish containing cells, the cell suspension was transferred to 13ml centrifuge tube. The cell suspension was centrifuged for 5 min with 140g relative centrifugal force. After the centrifugation, the supernatant was removed, pellet containing the cells was added to 10 ml cultivation media, and 10 µl of the created cell suspension was pipetted to Bürker counting chamber. After establishing the final number of the cells contained in the suspension, the cells were diluted in order to reach the final number of the cells – 20 000 in 100 µl per well. The mentioned volume with stated cell number suspension was pipetted to 96-multiwell plates and left in the incubator for 24 hours.

After 24 hours, the cells were differentiated. 100 µl/well of DMEM/F12 media with 1% fetal bovine serum and 10µM ATRA were added to the microplate. The cells were left to be differentiated for 48 hours in the incubator.

3.3.2 Glutamate-induced oxidative damage and primary screening of 8-azapurine derivatives

The effect of glutamate and its pathway in neuronal cells is described in detail in various subchapters of the theoretical part of the thesis. The cell line used in the following experiments is summarized in the subchapter 2.4.

To induce the oxidative damage, a calculated amount of monosodium L-glutamate was dissolved in DMEM/F12 1% FBS media and filtered using 0.2 µm filter in order to reach concentration of 160 mM or 100 mM. Each 8-azapurine derivative was dissolved in DMSO to stock the concentration of 10 mM. The solutions of the compounds and IM-54 were added to L-glutamate media so the final concentration of the substances in the media was 10 µM. The solutions were pipetted into the microplate and were diluted to 10 µM, 1 µM and 0,1 µM. 100 µl/well of the solutions were applied on differentiated cells after the medium containing ATRA was aspirated from the microplate. Each compound in each concentration was applied in triplicates. In order to analyze their effect, the same volume of L-glutamate medium as well as DMEM/F12% 1% FBS media containing DMSO (0,1% v/v) were added to 9 wells each per microplate, from which 3 wells were used as a blank.

Cell death was assessed by PI, which is a membrane impermeant fluorescent nucleic acid dye, which binds to DNA by intercalating between base pairs. The dye was used to distinguish viable cells from the non-viable ones.

PI (1 mg/ml) was diluted in 1x PBS and 100 µl/well of the solution was added to the microplate, so the final concentration of PI solution was 1 µm/ml. The microplate was then incubated at laboratory temperature in the dark for 15 minutes. The microplates were centrifuged for 1 minute at 500x g, then they were measured by spectrophotometer set for excitation/emission 535/617 nm. The measured signals of the compounds and IM-54 diluted in 160 mM or 100 mM L-glutamate media were compared to the glutamate control signal.

3.3.3 Measurement of oxidative stress induced by glutamate

The technique of co-treating the cells with 160mM glutamate and solutions of 8-azapurine derivatives was the same as in the previous subchapter. Oxidative stress was assessed by DHE. DHE is a fluorescent probe used for detecting ROS, especially intracellular superoxide and hydrogen peroxide (Kritis et al., 2015). Once DHE enters the cell, it is oxidized by superoxide, forming 2-hydroxyethidium with excitation/emission maximum at 500-530/590-620 nm. Moreover, it can be oxidized by different ROS, forming ethidium with excitation/emission maximum at 480/576 nm, which is intercalated into nuclear DNA.

After 4 hours of the co-treatment of the cells, the microplate was centrifuged at 500x g for 5 minutes and 30 seconds. The media containing 160 mM L-glutamate and the compounds was aspirated from the microplate and replaced with 100 µl/well of 10 µM DHE solution diluted in 1x PB. The microplate containing DHE solution was incubated at laboratory temperature in the dark for 30 minutes. The microplate was measured by spectrophotometer set for excitation/emission 500/580 nm. The measured signals of the compounds and IM-54 standard diluted in 160 mM L-glutamate media were compared to the glutamate control signal.

3.3.4 Cytotoxicity of 8-azapurine derivatives

Cytotoxicity is defined as a quality of chemicals or substances to cause cell death by apoptosis or necrosis. The technique of treating the cells is the same as in subchapter 3.3.2, however, no L-glutamate was added to the media, the compounds were diluted only in DMEM/F12 1% FBS media.

As for the control, three wells contained 50 µl of the medium, three wells below these contained 100 µl medium with DMSO (control DMSO). After 24 hours, 50 µl of 2% Triton X-100 was added to the wells containing 50 µl of the media to make the cell membranes permeable. Cytotoxicity was assessed by PI. The measured signals of the compounds diluted in DMEM/F12 1% FBS media were compared to the signal of medium containing 2% Triton X-100.

3.3.5 Data

All data were expressed as means ± standard error of the mean (SEM) calculated using Prism 8.4.3 (GraphPad software, La Jolla, CA, USA). Results of PI and DHE assays were normalized to the effect of glutamate considered as 100% of toxic effect or superoxide radicals formation, so that protective effect was observed as reduction of PI and DHE signals.

4. Results

4.1 Cytotoxicity of 8-azapurine derivatives

The definition and the method of the experiment are described in the previous subchapters and the result is presented in table 1. The results were normalized and the cell death induced by 2% Triton X-100 was set for 100 %. A substance is classified as toxic when the difference between the substance's signal and the signal of control DMSO is higher than 10 %. Results in Table 1 show that none of the tested substances is cytotoxic. The chemical structures of all the tested 8-azapurine derivatives are presented in Appendices.

Table 1 – The cytotoxicity of 8-azapurine derivatives tested on differentiated SH-SY5Y cell line assesed by PI after 24 hours. Cytotoxicity data are presented as percentages of cell death mean ± standard error of mean (SEM) of triplicates from at least 3 different days.

	Mean (%)	±SEM				
Control (DMSO)	5,464	0,316				
	0,1 µM	± SEM	1 µM	± SEM	10 µM	± SEM
MARK 11	5,12	0,63	5,41	0,46	5,09	0,41
MARK 13	5,23	0,63	5,86	0,72	5,49	0,67
MARK 14	6,19	0,61	6,23	0,67	6,12	0,59
MARK 15	4,49	0,76	5,47	0,38	4,28	0,48
MARK 17	4,48	0,67	6,78	0,61	5,22	0,58
MARK 21	3,35	0,45	3,98	0,34	3,71	0,45
MARK 22	6,26	0,39	4,50	0,39	3,94	0,53
MARK 23	4,95	0,54	5,28	0,41	5,14	0,54
MARK 24-o-BAP	5,54	0,25	4,41	0,55	4,15	0,64
MARK 25	5,40	0,45	5,69	0,50	4,71	0,40
MARK 26	4,36	0,28	5,86	0,69	3,78	0,42
MARK 27	5,45	0,60	5,98	0,39	5,20	0,65
MARK 28	7,11	1,99	7,34	1,79	6,06	2,02
MARK 29	3,70	0,63	4,67	0,56	3,93	0,62
MARK OEt	5,82	0,76	6,08	0,70	5,66	0,96
IM-54	4,72	0,32	3,25	0,36	5,24	0,36

4.2 Primary screening of 8-azapurine derivatives

4.2.1 Primary screening of 8-azapurine derivatives on 160mM glutamate-induced oxidative damage model

The experiment was performed as described in the subchapter 3.3.2 and the result of the experiment is presented in table 2. Cells treated with 160mM glutamate exhibit just above 4-times increased cell death compared to healthy control (control DMSO). The results were normalized and the cell death induced by glutamate was set for 100 %. The aim of the experiment was to select the compounds, whose effect would decrease cell death induced by glutamate by at least 10 %. The effect of the tested compounds was compared to the standard used in this experiment – a cell-permeable selective necrosis inhibitor IM-54. IM-54 is able to inhibit caspase-independent necrosis caused by oxidative stress, however, it is not able to inhibit caspase-dependent apoptosis (Sodeoka and Riken, 2010). The standard was able to decrease cell death by 20-25% at 0,1 μM (80,26 ± 1,75 %) and 1 μM (74,03 ± 1,68 %) after 24 hours of the co-treatment.

The compounds that decreased cell death by approximately 10 % are MARK 11 at 0,1 μM (92,60 ± 2,40 %), MARK 15 at 10 μM (93,57 ± 2,25 %), MARK 23 at 0,1 μM (90,15 ± 2,85 %) and 1 μM (92,28 ± 3,08 %), MARK 26 and MARK OEt at all three concentrations with little to no difference of decreased cell death when the concentrations were changed, MARK 28 at 0,1 μM (92,98 ± 1,38 %), and MARK 29 at 10 μM (92,78 ± 1,71 %). MARK 11, 15, 26, and 29 were evaluated as the most effective of the tested substances and were selected for further testing on co-treatment with 100 mM glutamate.

Table 2 – Primary screening of the neuroprotective activity of 8-azapurine derivatives and standard IM-54 on 160 mM glutamate-induced oxidative damage model performed on differentiated SH-SY5Y cell line assessed by PI after 24 hours. Data are presented as percentage of cell death mean ± standard error of mean (SEM) of triplicates from at least 3 different days.

	Mean (%)	± SEM				
Control (DMSO)	23,85	0,6151				
Glutamate 160 mM	100	0,6561				
	0,1 µM	± SEM	**1 µM**	± SEM	**10 µM**	± SEM
MARK 11	92,60	2,42	96,09	2,09	101,60	3,11
MARK 13	94,08	2,58	97,88	3,24	98,26	2,21
MARK 14	102,90	1,99	96,76	2,89	96,26	3,43
MARK 15	95,64	2,04	94,88	2,07	93,57	2,25
MARK 17	99,88	1,75	101,40	1,61	98,85	1,61
MARK 21	97,86	1,64	100,20	1,50	97,38	1,60
MARK 22	94,24	2,06	99,50	1,81	105,60	1,98
MARK 23	90,15	2,85	92,28	3,08	94,02	1,64
MARK 24-o-BAP	101,50	2,04	102,50	2,68	101,10	2,36
MARK 25	96,94	2,31	99,56	2,39	97,81	1,68
MARK 26	90,85	1,64	91,17	1,72	90,63	1,68
MARK 27	97,47	2,26	93,39	1,52	98,17	1,64
MARK 28	92,98	1,38	96,59	1,82	96,50	2,53
MARK 29	94,62	1,59	94,71	1,63	92,78	1,71
MARK OEt	93,42	2,29	91,95	2,90	92,22	2,68
IM-54	80,26	1,75	74,03	1,68	89,50	2,01

4.2.2 Primary screening of 8-azapurine derivatives on 100mM glutamate-induced oxidative damage model

The method used in this experiment is described in the subchapter 3.3.2 and the result of the experiment is presented in Figure 6. Cells treated with 100mM glutamate exhibit 1,4-times increased cell death compared to healthy control (control DMSO). The results were normalized and the cell death induced by glutamate was set for 100 %. The aim of the experiment was to select the most effective compounds from 160mM glutamate screening and demonstrate whether their effect was the same when applied in less toxic conditions.

IM-54 has shown to be very toxic in higher concentrations, but it decreased cell death at 0,1 µM (85,16 ± 6,66 %) and at 1 µM (79,02 ± 4,32 %).

Sufficient decreased cell death (by at least 10 %) was exhibited by MARK 11 at 10 µM (83,43 ± 11,58 %), MARK 15 at 10 µM (88,96 ± 6,74 %), MARK at 1 µM (79,21 ± 5,92 %) and at 10 µM (68,65 ± 74 %), and by MARK 29 at 10 µM (77,41 ± 5,31 %). These compounds were further tested on 160mM glutamate model in order to assess their antioxidative properties.

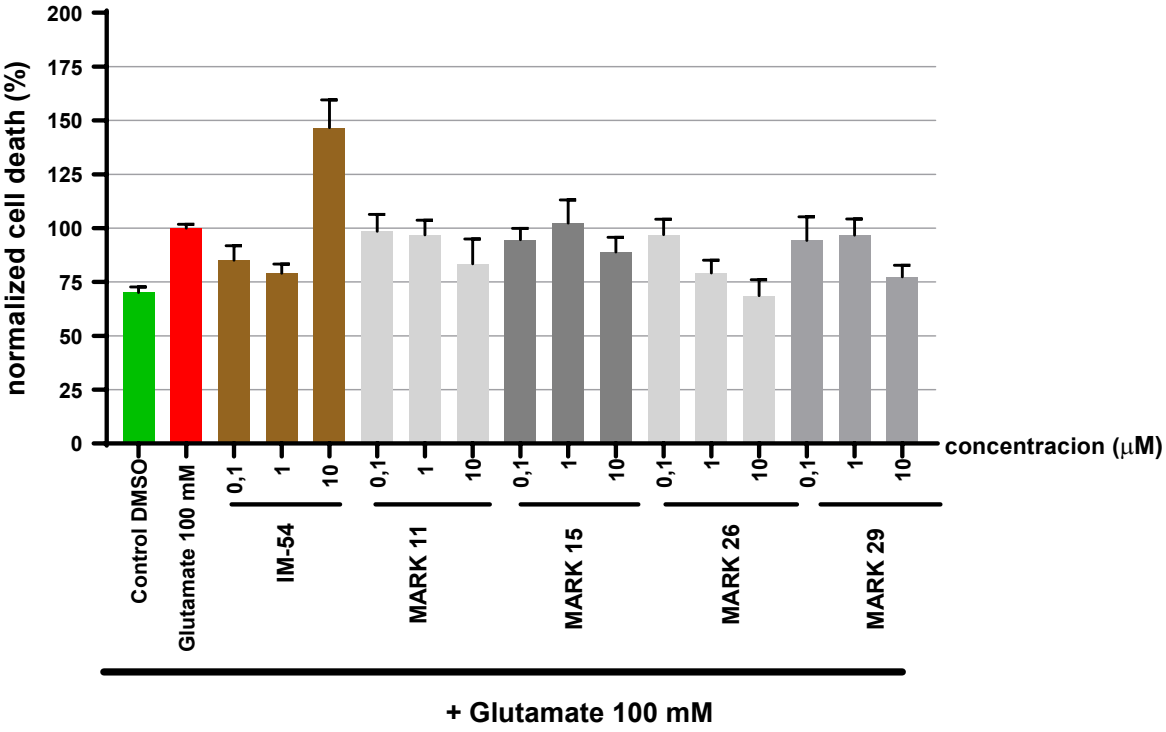

Figure 6 – Screening of the neuroprotective activity of 8-azapurine derivatives MARK 11, 15, 26, 29 (shades of gray) and standard IM-54 (brown) at 3 different concentrations on 100 mM glutamate-induced oxidative damage model performed on differentiated SH-SY5Y cell line assessed by PI after 24 hours. The data are compared to cells treated with 100 mM of glutamate (red) and to cells without toxin (control DMSO, green) Data are presented as percentage of cell death mean ± standard error of mean (SEM) of triplicates from at least different 3 days.

4.3 Oxidative stress assay

4.3.1 Oxidative stress assessed on 160mM glutamate-induced oxidative damage model

The method used in this experiment is described in the subchapter 4.3.3 and the result is presented in Figure 8. Cells treated with 100mM glutamate exhibit 2-times increased cell death compared to healthy control (control DMSO). The results were normalized and the superoxide radical formation induced by glutamate was set for 100 %.

Glutamate is able to induce oxidative stress in cells by various mechanisms. Since it is not certain, whether SH-SY5Y cell line expresses NMDA receptor, glutamate induces oxidative stress in this cell line by blocking the x_C^- antiporter, resulting in the GSH depletion and negative regulation of antioxidant enzyme, SOD. Oxidative stress can also be induced by ROS forming enzyme Rac-NADPH-oxidase (Maková et al., 2021).

To reduce the superoxide radical formation, *R*-lipoic acid (R-LA) was used as a positive control for its antioxidative properties not only in its original form but also after it is reduced in the cells to dihydrolipoate. R-LA is able to raise levels of intracellular glutathione within the cells, which protects the cells from oxidative damage (Packer et al., 1997). The results in Table 8 show that R-LA decreased superoxide radical formation by nearly 20 % at 50 µM (81,45 ± 6,06 %).

Sufficient decrease in superoxide radical formation was exhibited only by MARK 15 at 10 µM (79,50 ± 9,10 %) and MARK 26 at 1 µM (86,29 ± 7,90 %) and at 10 µM (87,50 ± 3,43 %).

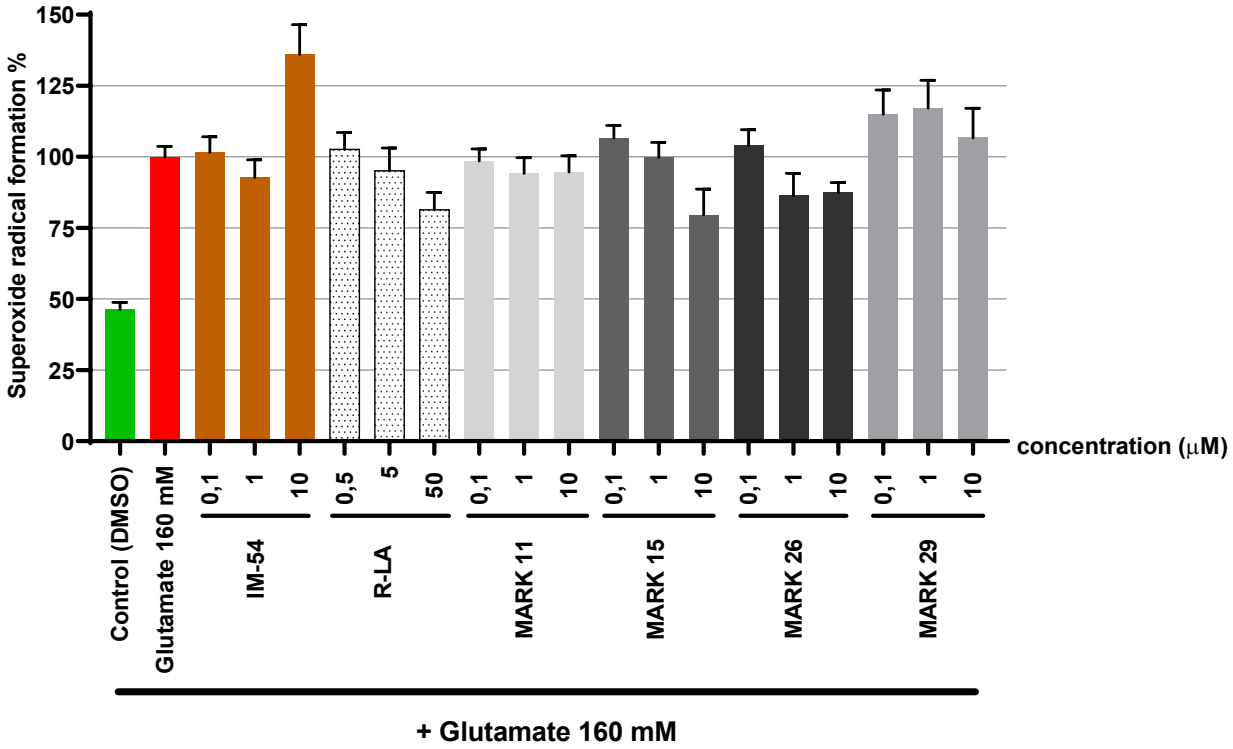

Figure 7 - Screening of the antioxidative activity of 8-azapurine derivatives MARK 11,15,26,29 (shades of gray), positive control R-LA (white with pattern) and standard IM-54 (brown) at 3 concentrations on 160mM glutamate-induced oxidative damage model performed on differentiated SH-SY5Y cell line assessed by DHE after 4 hours. The data are compared to superoxide radical formation induced by glutamate (red) and to cells without toxin (control DMSO, green). Data are presented as percentage of superoxide radical formation mean ± standard error of mean (SEM) of triplicates from at least 3 days. MARK 11 and R-LA were tested in triplicates in two different days.

5. Discussion

5.1 IM-54 on glutamate-induced oxidative damage model

IM-54 was developed by Sodeoka and Riken in 2010 in a study, where they focused on synthesising new selective inhibitors of necrosis. Their research showed, that IM-54 is able to inhibit necrosis induced by oxidative stress, especially by hydrogen peroxide. They proved that IM-54 is able to inhibit oxidative stress-induced necrosis, but it is unable to inhibit caspase-dependent apoptosis. These experiments were performed on cancer-derived HL60 cell line. It was concluded, that IM-54 is a selective inhibitor of caspase-independent necrosis induced by oxidative stress. Study by Alshangiti et al. in 2019 studied cytotoxic effect of a flavonoid isoliquiritigenin on human neuroblastoma cell line SK-N-BE. The study showed that IM-54 is able to block cell death induced by the flavonoid.

In this thesis, IM-54 was used on glutamate-induced oxidative damage model for the first time and the results correlate with the results from the available literature. However, structurally similar compound, necroptosis inhibitor necrostatin 1 was tested on glutamate-induced oxidative stress model on immortalized mouse hippocampal cell line HT-22 by Xu et al. in 2007 and the study proved that necrostatin 1 is able to protect the cells from glutamate induced oxytosis. Protective activity of necrostatin 1 was also tested in the study by Gonzalez et al. in 2021 on glutamate-induced oxidative stress model on SH-SY5Y cell line. The study showed that necrostatin 1 is able to significantly reduce cell death, as well as superoxide radical formation in the glutamate model. These results prove that not only cell death by apoptosis occurs in the glutamate model, but also cell death by caspase-independent necrosis. The structures are compared in Figure 8.

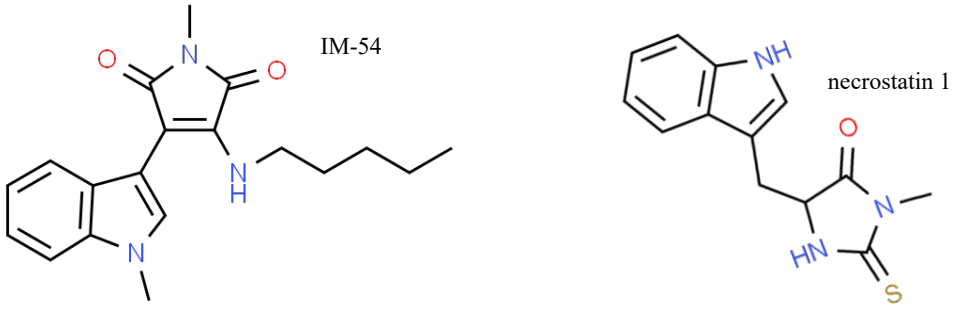

Figure 8 – Structures of IM-54 and necrostatin 1 (downloaded from http://www.chemspider.com/)

5.2 Cytoprotective activity of cytokinins

Cytokinins *para*-topolin, kinetin, isopententyladenine, and their derivates have demonstrated cytoprotective and neuroprotective abilities. Kinetin riboside-5′-triphosphate tested on SH-SY5Y cell line showed that this particular cytokinin increases kinase activity of PTEN-induced putative kinase 1 (PINK 1) and protected the cells from oxidative stress induced by mutated form of PINK 1, which is found in familial forms of recessive PD. *Trans*-zeatin was found to inhibit rat's acetylcholinesterase, as well as it is able to protect PC12 cells from toxicity induced by Aβ, which is the hallmark of AD. Kinetin riboside and *trans*-zeatin ribosides exhibited significant cytoprotective abilities when tested on HD model of PC12 cells. N^6-benzyladenosine and N^6-isopentenyladenosine induced augmented expression of ARE genes, as well as upregulation of Nrf2 transcription in several cancer cell lines (Voller et al., 2017). Structures of significant cytokinins are presented in Figure 9.

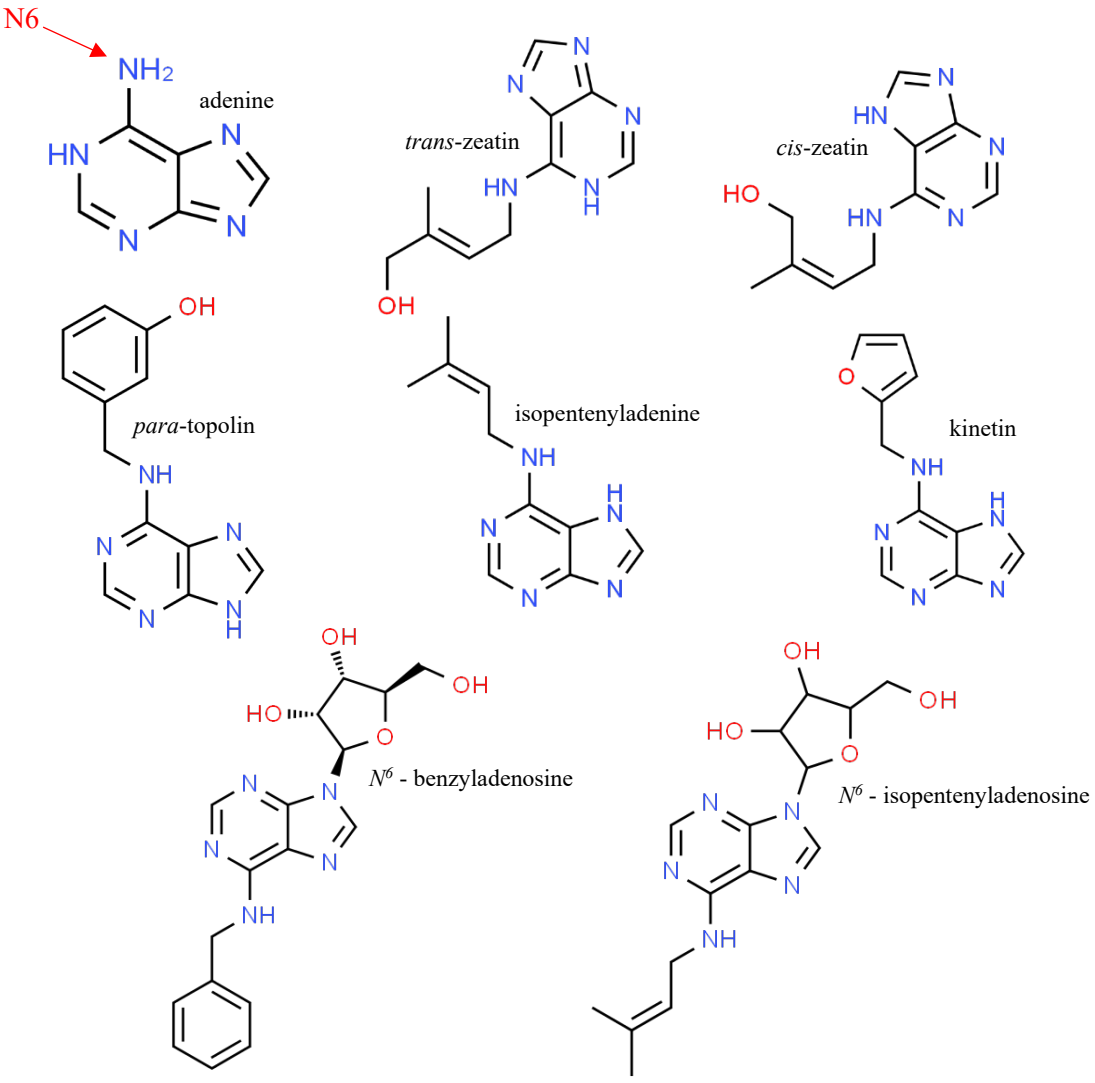

Figure 9 – adenine and cytokinins – *trans*-zeatin, *cis*-zeatin, *para*-topolin, isopentenyladenine, kinetin, N^6-benzyladenosine, N^6-isopentenyladenosine (downloaded from http://www.chemspider.com/)

Primary screening of 8-azapurine derivatives on 160mM glutamate-induced oxidative damage model on SH-SY5Y cell line showed that MARK 11,15,26 and 29 were able to decrease cell death induced by glutamate by approximately 10 % (Table 1). The structures of these compounds are presented in Figure 10. However, the neuroprotective activity of these compounds was not as effective as the activity of IM-54, as IM-54 decreased cell death by approximately 20 – 25 %. The concentration of glutamate was lowered to 100 mM and the same compounds were tested. The activity of these compounds at 10 μM corresponded with the activity of IM-54 at 1 μM. MARK 26 exhibited the most potent activity, as it was able to decrease cell death the most from all the tested compounds, including IM-54. MARK 29 at 10 μM decreased cell death similarly to IM-54 at 1 μM.

Structurally, MARK 26 is an analogue of *para*-topolin, MARK 29 and *trans*-zeatin have the same C^6 substituents. These cytokinins were proven to be potent cytoprotective and antioxidative agents by several studies mentioned in this subchapter. Hence, the result of the primary screening of 8-azapurine derivatives corresponds with available literature.

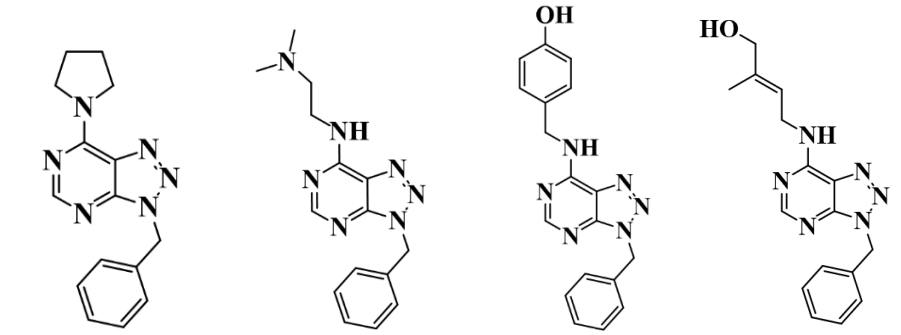

Figure 10 – structures of tested 8-azapurine derivatives MARK 11,15,26 and 29 with the most potent neuroprotective activity (synthetized Gonzalez, 2016)

5.3 Cytokinins as antioxidants

Brizzolari et al. studied antioxidative properties of kinetin, *para*-topolin and isopentenyladenine by fluorometric and spectrophotometric assays in 2016. The study proved that these cytokinins have potent oxygen radical absorbance capacity at 2,5 μM and 5 μM, hence they are classified as antioxidants. They are able to react with hydroxyl radicals, moreover, kinetin has been reported to partially reverse a diminishment of the activity of glutathione peroxidase and superoxide dismutase in cultured rat astrocytes co-treated with D-galactose (Voller et al., 2017), which induces ageing in animal models and is widely used to study age-related degenerative diseases (Long et al., 2007).

Study performed by Gonzalez et al. in 2021 tested neuroprotective abilities of cytokinin derivatives on salsolinol-induced PD model and glutamate-induced PD model on SH-SY5Y cell line. It was concluded that kinetin-3-glucoside, *cis*-zeatin riboside, and N^6-isopentenyladenosine showed neuroprotective abilities in salsolinol model. In glutamate model, *trans*-, *cis*-zeatin and kinetin showed antioxidative abilities as they were able to significantly reduce formation of superoxide radicals.

Screening of antioxidative activity of 8-azapurine derivatives compared to R-LA showed, that only MARK 15 at 10 μM and MARK 26 at 1 μM and 10 μM were able to decrease superoxide radical formation as effectively as antioxidant R-LA. As already mentioned, cytokinins are potent antioxidants, therefore, the result corresponds with available literature.

Data provided by Mgr. Gruz, Ph.D. (unpublished) measured the activation of the Nrf2-ARE pathway. The results are presented in Table 3. The measured signals were compared to R-LA, whose activity = 1. The results show that only MARK 11 was able to increase the activity Nrf2-ARE pathway, however, it did not exhibit potent neuroprotective activity in the screenings. On the other hand, MARK 26 exhibited the most potent neuroprotective effect, but it did not upregulate the activation of Nrf2-ARE pathway. MARK 26 is an analogue of *para*-topolin, which is a potent direct antioxidant. Therefore, it is thought that MARK 26 protects the cells from ROS the same way, by directly reacting with the molecules of ROS.

Table 3 – Measured activity of activation of Nrf2-ARE pathway by selected substances compared to the activity of R-LA (R-LA = 1)

Substance	R-LA
MARK 22	0.6
MARK 29	0.0
MARK 23	-0.3
MARK 11	10.7
MARK 27	0.6
MARK 25	-0.7
MARK 28	0.5
MARK 26	0.5
MARK 24-o-BAP	-0.1
MARK 15	0,21
MARK OEt	0,51

6. Conclusion

Theoretical part of the thesis characterized NDDs and their treatment approved by the FDA. Molecular mechanisms, as well as glutamate toxicity in NDDs were summarized as well. Experimental part was focused on primary screening of 8-azapurine derivatives inspired by natural cytokinins and selective inhibitor of necrosis IM-54 used as a standard on glutamate-induced oxidative damage on neuronal SH-SY5Y cell line. Cytotoxicity of the derivatives was tested and it was concluded, that none of the compounds was cytotoxic. The compounds were tested on 160mM glutamate model at concentrations of 0,1 μM, 1 μM and 10 μM. The main aim of the screening was to select compounds that are able to decrease cell death induced by glutamate by at least 10 %. Cell death was assessed by PI and cell death induced by glutamate was normalized to 100 %. MARK 11, 15, 26 and 29 were selected from 160mM screening for further co-treatment with 100mM glutamate, as they were able to sufficiently decrease cell death (to 93 – 90 %), however, they were not as effective as 0.1 – 1 μM IM-54 (90 – 74 %). 100mM glutamate model was used in order to test the neuroprotective abilities of the derivatives in less toxic conditions.

Co-treatment with 100mM glutamate exhibited sufficient decrease in cell death by all of the derivates at 10 μM. The effect corresponded with protective abilities of the standard IM-54 at lower concentrations (88 – 68 % and 85 – 79 %). However, the most potent ability to decrease cell death was exhibited by MARK 26 at 10 μM (68,64 %). MARK 11, 15, 26 and 29 were further selected for the screening of their antioxidative abilities, which were assessed by DHE on 160mM glutamate model. R-LA was chosen as a positive control for its antioxidative abilities and the activity of the compounds was compared to it. Only MARK 15 at 10 μM (79 %) and 26 at 1 μM (86 %) and at 10 μM (87,5 %) exhibited antioxidative abilities similar to R-LA at 50 μM (81 %).

It was concluded that IM-54 is able to reduce cell death induced by glutamate, therefore, it is thought that glutamate induces cell death by caspase-independent necrosis, not only by apoptosis. Furthermore, it was concluded that MARK 26 have the most potent neuroprotective and antioxidative ability. MARK 26 is an analogue of *para*-topolin, which is proven to be a potent antioxidant. MARK 11 was the only compound that was able to increase the activation of Nrf2-ARE pathway, however, it did not exhibit neuroprotective abilities. In addition, it is suggested that future studies are focused on the synthesis of more *para*-topolin derivatives, as well as on the interaction between MARK 11 and the Nrf2-ARE pathway.

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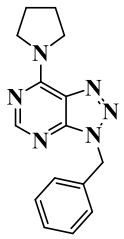
Appendices

Structures of the tested 8-azapurine derivatives (3,7-disubstituted 1,2,3-triazolo [4,5-d] pyrimidine derivatives) synthesized by Mgr. Gonzalez, Ph.D.



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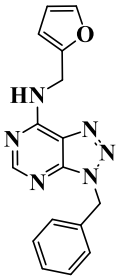

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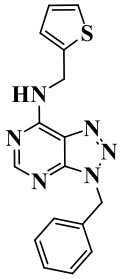

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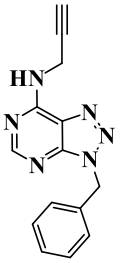

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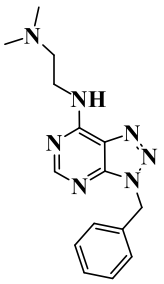

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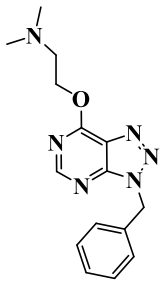

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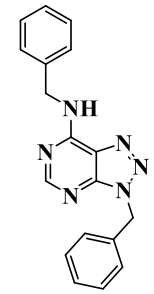

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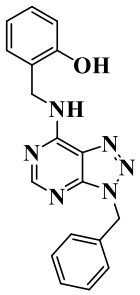
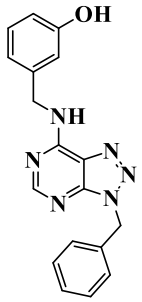


MARK 24-o-BAP



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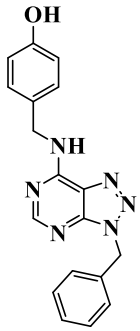

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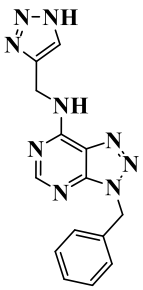

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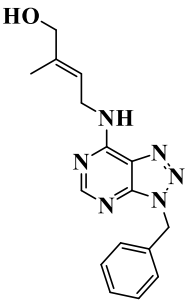

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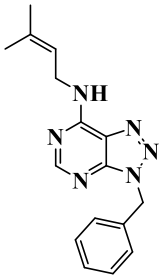

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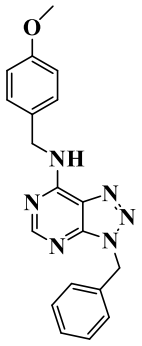

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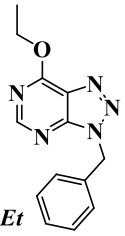

MARK OEt