Area:

Chemical and Pharmaceutical Sciences and Biotechnology

Curriculum:

Pharmaceutical Sciences

Ph. D. Thesis in

ROLE INVESTIGATION OF ADENOSINE RECEPTOR LIGANDS ON NEUROINFLAMMATION AND NEURODEGENERATIVE DISEASES

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XXXII cycle

Al meu pare que m'il·lumina
a la meva mare que em guia
a la meva germana que m'acompanya

Summary

Abbreviation List

2’-dCCPA – 2-chloro-N6-cyclopentyl-2’-deoxyadenosine

A_1AR – A_1 adenosine receptor

A_{2A}AR – A_{2A} adenosine receptor

A_{2B}AR – A_{2B} adenosine receptor

A_3AR – A_3 adenosine receptor

AA – amino acids

AC – Adenylyl cyclase

AD – Alzheimer’s disease

ADA – Adenosine deaminase

Ado – Adenosine

ADP – Adenosine diphosphate

AK – Adenosine kinase

AMP – Adenosine monophosphate

AP – Anterior-Posterior

AR – Adenosine receptor

ATP – Adenosine triphosphate

Aβ –Amyloid beta

BBB – Blood brain barrier

C16 – Compound 16

C6 – Compound 6

C7 – Compound 7

cAMP – Cyclic adenosine monophosphate

Casp-3 – Caspase-3

CCPA – 2-chloro-N^6-cyclopentyl-adenosine

CHA - N^6-cyclohexyl-adenosine

CHO – Chinese hamster ovary

CK – Cytokine

Cl-IB-MECA - 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide

CNS – Central nervous system

CPA - N^6-cyclopentyl-adenosine

cpm – count per minute

Cyt C – Cytochrome C

DD – Death domain

DI – Discrimination index

DMEM - Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DPCPX - 1,3-dipropyl-8-cyclopentylxanthine

DV – Dorsal-Ventral

EC_{50} - Half maximal effective concentration

EL – Extracellular loop

ERK - Extracellular signal-regulated protein kinases

FBS – Fetal bovine serum

G1 – Group 1

G2 – Group 2

G418 – Geneticin

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

Glu – Glutamate

GPCR – G-protein coupled receptor

GRK – GPCR kinases

GTP – Guanosine triphosphate

h – Human

HBSS – Hank's Balanced Salt Solution

HD – Huntington disease

HEMADO - 2-hexyn-1-yl-N^6-methyladenosine

HENECA - 2-Hexynyladenosine-5'-N-ethylcarboxamide

I.C.V. – Intracerebroventricular

I.P. – intraperitoneally

IB-MECA - N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide

IC_{50} – Half maximal inhibitory concentration

IFN-γ – Interferon gamma

IL – Interleukin

IL – Intracellular loop

IL-1β – Interleukin 1 beta

iNOS – Inducible nitric oxide synthase

K_i – Constant inhibitory binding

KO - Knock-out

L-DOPA – Levodopa, L-3,4-dihydroxyphenylalanine

LPS - Lipopolysaccharides

MAPK - Mitogen-activated protein kinase

ML – Medial-Lateral

mRNA – Messenger ribonucleic acid

MS – Multiple sclerosis

NECA - 5'-(N-Ethylcarboxamido)adenosine

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA – N-methyl-D-aspartate

NS- Nervous system

OD – Object displacement

OF – Open field

PBS – Phosphate-buffered saline

PD – Parkinson's disease

PI – Propidium iodide

PKA – Protein kinase A

PKC – Protein kinase C

PS – Pattern separation

PVDF – Polyvinylidene fluoride or polyvinylidene difluoride

RLU – Relative luminescence unit

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

R-PIA - N^6-(L-2-Phenylisopropyl)adenosine

rpm – revolutions per minute

RT – Room temperature

Sal – Saline

SAR – Structure-activity relationship

SCH58261 – 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SE – Standard error

TLR – Toll- like receptor

TM – Transmembrane Domain

TNF-α – Tumor necrosis factor alpha

Tris - 2-Amino-2-(hydroxymethyl)-1,3-propanediol

TSPO – Mitochondrial Translocator Protein

UDP – Uridine diphosphate

UTP – Uridine triphosphate

Veh – Vehicle

vs – versus

WB – Western Blot

ZM241385 – 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

1. ABSTRACT

Neuroinflammation is a complex and chronic response of the nervous system (NS) against infections, oxidative stress, neurodegenerative diseases or injuries. This pathological condition is produced by an aberrant activation of microglia and astrocytes, which represent the neuroimmune brain cells. Nowadays adenosine receptors (ARs) are emerging as potential and attractive targets to treat several diseases like cardiovascular problems, sleep disorders, ischemia, cancer, immune pathologies, neuroinflammation and neurodegenerative diseases. ARs belong to G-protein coupled receptors and are divided into A_1, A_{2A}, A_{2B}, and A_3, among them, A_1AR and A_{2A}AR play the major roles in neuroinflammation modulation. In fact, it has been discovered that the activation of A_1AR and the blockade of A_{2A}AR could conferee beneficial effects to neuroinflammatory pathologies like Alzheimer's or Parkinson's disease. For these reasons, the synthesis and development of new ligands for these receptors are of great interest for the discovery of new potential drugs.

The scope of this work was to study the pharmacological profile of a series of A_1AR agonists (CPA derivatives) present in the chemical library and new potential A_{2A}AR antagonists substituted at 2, N^6, 8, or 9 positions. The investigation has been centred in their role in protection and/or recovery from neuroinflammation both in *in vitro* and in *in vivo* models of neuroinflammation experiments.

After their biological characterization through binding and cAMP assay, all the A_1AR agonists and the three most potent and selective A_{2A}AR antagonists (Compound **6**, **7** and **16**) were selected for further *in vitro* assays. These compounds were tested on mixed glial cell cultures in presence or absence of proinflammatory cytokine cocktail (TNF-α, IL-1β and IFN-γ) through viability assay, Griess technique and Hoechst staining. The two best neuroprotective ligands, the A_1AR partial agonist 2'-dCCPA, (endowed with submicromolar affinity versus A_1AR) and the A_{2A}AR antagonist compound **7** (possessing subnanomolar affinity versus A_{2A}AR), were subsequently tested in other cell lines to ensure and provide solider results of their positive effects. 2'-dCCPA and compound **7** were then studied on N13 (mouse microglial cells) and primary neuronal cell culture, resulting in the reaffirmation of their protective and restoring effects against cytokine inflammation. Since these compounds showed good biological effects at cell level the study continued on *in vivo* rat neuroinflammation model in order to test their *in vivo* potential. Animals were treated with lipopolysaccharides (LPS) in presence or absence of the partial agonist (2'-dCCPA) or antagonist (compound **7**). Both of the ligands demonstrated not to alter the velocity, distance and permanence time of the animals in Open Field. On the other hand, both ligands ameliorate the animal's memory

in Y Maze after the treatment with LPS, allowing the rat to remember which arm was closed and which one was opened. Lastly, despite not showing significant differences in Object Displacement, animals treated with 2'-dCCPA or compound **7** were able to elucidate which object was moved, while LPS treated animals did not.

Collectively, these data provide new evidence that the use of selective and potent A_1AR partial agonists and A_{2A}AR antagonists result as a promising therapeutic approach to improve the functional recovery of patients with neuroinflammation or associated pathologies.

2. INTRODUCTION

2.1. NEUROINFLAMMATION

Inflammation comprises a wide range of biological and cellular responses to counteract injuries, traumas, autoimmune disease, aging, pollution, smoking, biological agents or infections that can affect cells or tissues **(**Patraca I. et al., 2017**).** Inflammation is a process that is activated for eradicate pathogens and initiate the healing procedure. However, in the brain, the inflammation process could bring to very important negative impacts and start and/or worsen brain disorders. Unlike many other organs, the brain is isolated from biological agents by an almost impenetrable structure, the blood-brain barrier (BBB). Due to the presence of this structure, the brain has his own protective system constituted by the glial cells included microglia and astrocyte. The brain inflammatory process is called neuroinflammation. Neuroinflammation is a chronic pathological condition caused by the activation of the neuroimmune cells from the brain, mainly microglia and astrocytes. Microglia can be activated in presence of several agents like a damage, a strange stimulation, neurotoxins, infections or pathogens. In normal conditions, microglia develop some biological actions such as nourishment of neurons, protection or synthesis of anti-inflammatory molecules, but this response needs to be controlled and lasts for a short period; otherwise, it may to be an unleashed factor for major neurodegenerative and psychiatric disorders. Neuroinflammation is mediated by the production of cytokines (CK), chemokines, reactive oxygen species, and secondary messengers. In addition, microglia start to proliferate, migrate, present antigen to T-cells and to activate the transcription of several genes like COX-1, COX-2 or iNOS and neurotoxic compounds. These agents not only damage the attacker agent but also the healthy neurons by phagocytosis or by pro-apoptotic molecules provoking synaptic deterioration, leak of synapses and neuronal death. (Figure 1).

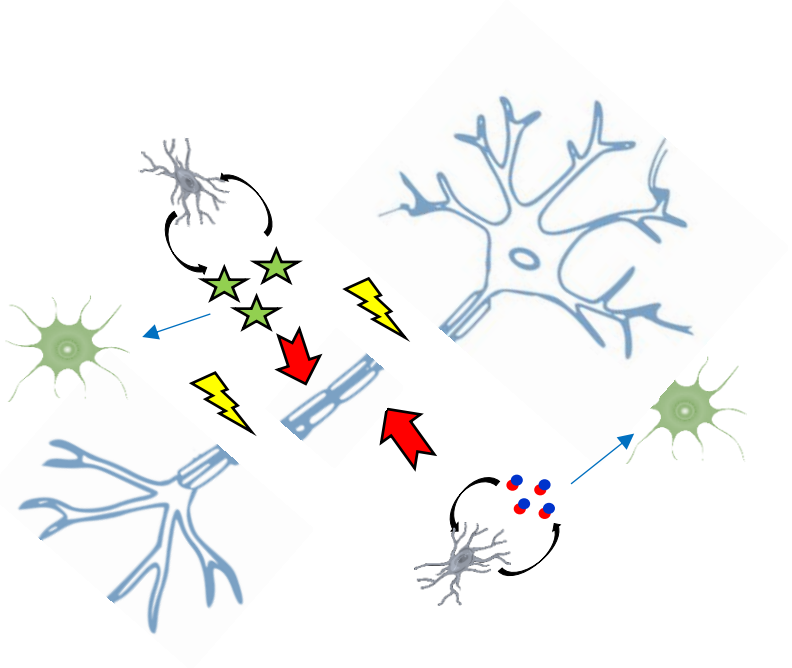


Figure 1. When microglia cells are activated, start to synthetize proinflammatory CK (green stars), ROS and RNS (blue and red dots). These elements activate also the astrocytes, increase the activation of other microglial cells (feedback) and create an insult against neurons causing their apoptosis (yellow lightning).

Neuroinflammation is a positive feedback process which, once started, becomes stronger and stronger. During microglia activation, proinflammatory CK, ROS and RNS are produced, which they are able in turn to induce activation of new microglial cells. This vicious circle causes the neuronal death, neurodegeneration and pathological conditions (Figure 2). In neuroinflammation the balance between the positive effects and the proinflammatory agents must be maintained in order to avoid injuries or alterations in the CNS. In fact, it has been demonstrated that neuroinflammation plays a crucial role in many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease or multiple sclerosis.

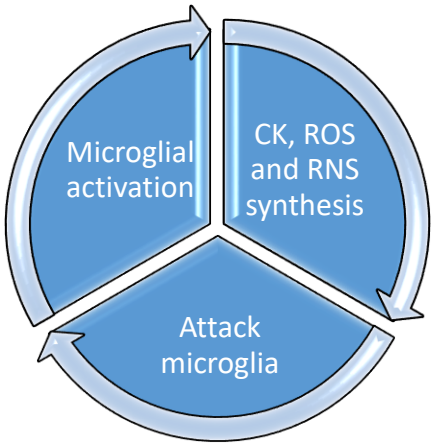


Figure 2. Feedback circle of neuroinflammation.

The initial factor that triggers the neuroinflammation is not yet clear due to several elements are implicated. Deterioration of vascularization in brain could be the key aspect. In fact, in Alzheimer's disease the beta-amyloid proteins accelerate capillary deterioration causing a low oxygen and glucose flux to the brain. The brain deprivation of these two elements can cause the neuroinflammation and bring to the pathological side effects. The oxidative stress hypothesis is the second theory that could explain neuroinflammation trigger. Despite that the brain only weights about 1.4 Kg, it uses the 20% of the total resources from all the body and thus create a big amount of residues such as ROS. It has been discovered that patients with neurodegenerative disease or neurological disorders have high levels of oxidative stress elements. In addition, these patients showed to have lower levels of omega-3 and omega-6, two fatty acids with antioxidant profile. All these alterations bring to the neuroinflammation state in the brain. The last theory indicates that mitochondria could be the trigger of neuroinflammation. This organelle is considered the power house of the cell, producing energy in form of ATP, crucial for cells. During this production, inoffensive levels of free radicals are created, but if there are some mutations in the mitochondrial DNA or alterations in the electron transport chain (ETC), the quantity of these radicals increase as well as in neuroinflammation. In addition, it has been shown that patients with peripheral inflammation or chronic inflammation due to obesity are more prone to suffer from neuroinflammation.

2.2. AFFECTED STRUCTURES

Nervous System (NS) is the main system affected by neuroinflammation. Despite being one of the smallest system it has a very important role and its dysfunction causes several and important disorders (Tortora G.J. et al., 2016). The major impact of neuroinflammation is produced in Central Nervous System (CNS) that include the brain and the spinal cord. The brain has an extra protection element, the BBB that regulate CNS homeostasis, which is critical to allow for proper neuronal function, as well as protect the CNS from toxins, pathogens, inflammation, injury, and disease. The restrictive nature of the BBB provides an obstacle for the immune cells passage. Hence, in the brain there are cells with several immune and protective effects named glial cells. Glial cells can be divided in macroglia (oligodendrocytes and astrocytes) and microglia (Figure 3). One of the main differences between neurons and glia, is that glia has functions in support, nutrition or defence but no direct synaptic transmission.

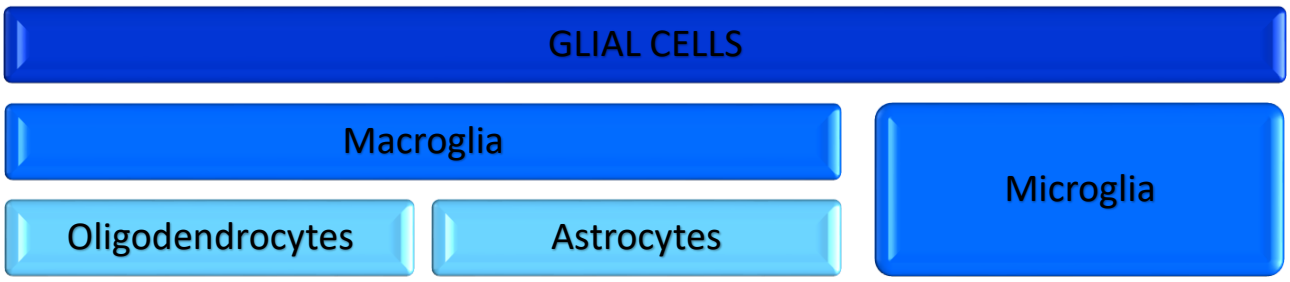


Figure 3. Glial cells classification.

2.2.1. Macroglia

Macroglia is subdivided in astrocytes (or astroglia) and oligodendrocytes, astrocytes play the most critical role in neuroinflammation and immune response. It has been demonstrated that they can be associated with the blood vessels or cover the outside surface of the brain (Tabata H. et al., 2015). Astrocytes are a major glial cell type, which extend polarized cellular processes that ensheath either neuronal processes or blood vessels. They also connect with the blood vessels and enwrap them creating a barrier, the BBB. For these reasons, any lesion on astrocytes could affect the BBB structure and bring to a neuroinflammation process. Depending on the stimuli that activates astrocytes, they could exert positive or negative functions (Colombo E. et al., 2016). In the next table are summarized some functions (Table 1):

Table 1. Astrocyte's function.

POSITIVE	NEGATIVE
Neuronal Survival	Neuronal death
Reduce astrogliosis	Demyelination
Immunosuppression	Immune cell recruitment
Clinical improvement	Oxidative stress
Anti-inflammatory cytokines release	Pro-inflammatory cytokines release

2.2.2. Microglia

Microglia, despite having some common functions with other glial cells, also participate in unique actions in the CNS. As immune cells, they are crucial role players in the communication of the nervous system with the immune system, due to they are in the front line of action and defence *versus* damaged tissue or pathogens (Sousa C. et al., 2017). Microglia cells are the responsible for controlling the state of the brain environment thank to their capacity to be highly active even in their resting state (Nimmerjahn A. et al., 2005). In fact, they have the ability to proceed rapidly to

any change or disruption in the CNS like pathogens that arrived to the CNS, traumas, strokes or changes in genetic, function or morphology that cause neuronal or brain dysfunction (Keshavarz M. et al., 2016). In addition, they have the capacity to present antigens (such as the detection MHC antigens, T- and B- lymphocyte markers among other antigens), to migrate to the affected areas, phagocytize and proliferate, ion homeostasis, and control of extracellular levels of glutamate (Vilar A. et al., 2014). Moreover, microglia release crucial components for the efficient operation such as neurotropic factors, chemokines and CK (Sousa C. et al., 2017). Microglia is regulated through the activation of pro- and anti-inflammatory cytokines receptors. These CK (pro- and anti-) are produced by the glial cells in CNS like tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interferon gamma (IFN-γ) among others (Colonna M. et al., 2017). Besides all these roles and actions that take place in the adult brain, microglia are crucial and decisive for neuronal development and neurogenesis (Sousa C. et al., 2017). Several studies demonstrated that microglia are necessary for the neuronal networks and in prenatal development, because they are the first glial cells that migrate to the CNS. They also act as guides for neurons and their axons to create the neuronal circuits necessary for the correct functioning of the CNS (Squarzoni P. et al., 2015). It is important to highlight that microglia can be considered as a neuroprotective and/or neurodestructive agents once that they are activated and can be considered as one of the main indicator of neuroinflammation and neurodegenerative disorders.

2.3. NEURODEGENERATIVE DISEASES

Neurodegenerative disorders have different origins such as genetic mutations, infections, trauma or protein aggregations, but all are characterized by a neuronal damage associated with chronic activation of an innate immune response. Some examples of these pathologies are Alzheimer's disease (AD), Parkinson's disease (PD), Multiple Sclerosis (MS) or Huntington's disease (HD).

2.3.1. Alzheimer's disease

AD was first proposed and investigated by the German neurologist Aloïs Alzheimer in 1906 who identified the symptoms and the neuropathological characteristics. This pathology is the first cause of dementia worldwide, and 60-65% of dementia cases are due to AD. It is a slow and progressive disease that attacks the neurons and destroys the brain. Since AD mainly affects elderly people (mostly over 65 years) the early symptoms like memory problems or the loss of cognitive abilities can be unrecognized, but they become more recognizable with the progression of the disease

interfering with the day-to-day life (https://www.alzheimer-europe.org/). Dementia is different compared to AD, the first one is characterized by the death of neurons in brain regions that take care in cognitive function like memory, problem solving or language, while in AD the damaged area is not limited to one region but it also spread along the whole brain. Post mortem studies found that brains from AD patients had extreme shrinkage areas and ventricles severely enlarged (Hane F.T. et al., 2017). Actually, there is not a unique theory explaining the appearance and progression of AD, but three of them are the most accepted. The oldest one is the beta-amyloid and neurofibrillary tangles (NFTs) theory defending that the accumulation of Aβ in the extracellular matrix and the presence of NFTs in the cytoplasm create important changes in the brain function causing the destruction and damage on neuronal cells. This attack provokes all the effects and symptoms that AD shows. Aβ plaques exert their toxic role by obstructing the interneuronal communication, while NFTs impede the transport to neurons of crucial molecules such as nutrients. These two causes bring to neuronal and glial cell death (Association As. 2016). The second theory is based on the cholinergic hypothesis asserting that patient's brain are not able to produce enough acetylcholine. This neurotransmitter is used by neurons in NS and it is particularly implicated in the memory circuits. This theory defends that the AD patients have a decrease on neurons that produce acetylcholine in the brain regions related with cognition and memory causing the pathological symptoms. The last theory is the neuroinflammatory hypothesis. This theory is founded on a deregulation of microglia and astrocytes together with an aberrant production of proinflammatory CK (such as TNF-α, IL-1β or IFN-γ) and an increased level of reactive oxygen and nitrogen species (ROS and RNS) bring to a chronic inflammatory brain state, causing all the symptoms of this neurodegenerative disease (Patraca I. et al., 2017). Despite the hard research work of the scientific community, there is no effective treatment for the patients (Figure 4). Some investigations discovered treatments that could help to attack AD through the Aβ plaques and NFTs. Some of them are focused on the inhibition of β- and γ-secretase avoiding the production of Aβ plaques (Mitani Y. et al., 2012.); others used anti-Tau antibodies in 2 animal models (JNPL3 and P3018) demonstrating a reduction in markers and a decline in disease progression (Chai X. et al., 2011).

In the same field anti-Aβ plaques antibodies are under study (Adolfsson O. et al., 2012). Other drugs are based on the inhibition of acetylcholinesterase and cholinesterase (enzymes that hydrolyse acetylcholine) such as: tacrine, galantamine, rivastigmine, donepezil or memantine. Other studies bring to light some biological molecules or components with interesting profiles, like thioflavin T that avoid Aβ aggregation and increment lifespan, curcumin as an anti-inflammatory (May P.C. et

al., 2011) or the use of a decoy peptide from ABAD, Aβ peptide binding alcohol dehydrogenase, to avoid the interaction of this mitochondrial enzyme and the Aβ segments (Yao J. et al., 2011). In addition, AD is related with risk factors that cause cardiovascular diseases, for this reason some investigations found a possible role of an oral anticoagulant, dabigatran, that ameliorate the animal state with Alzheimer's symptomatology (Cortes-Canteli M. et al., 2019).

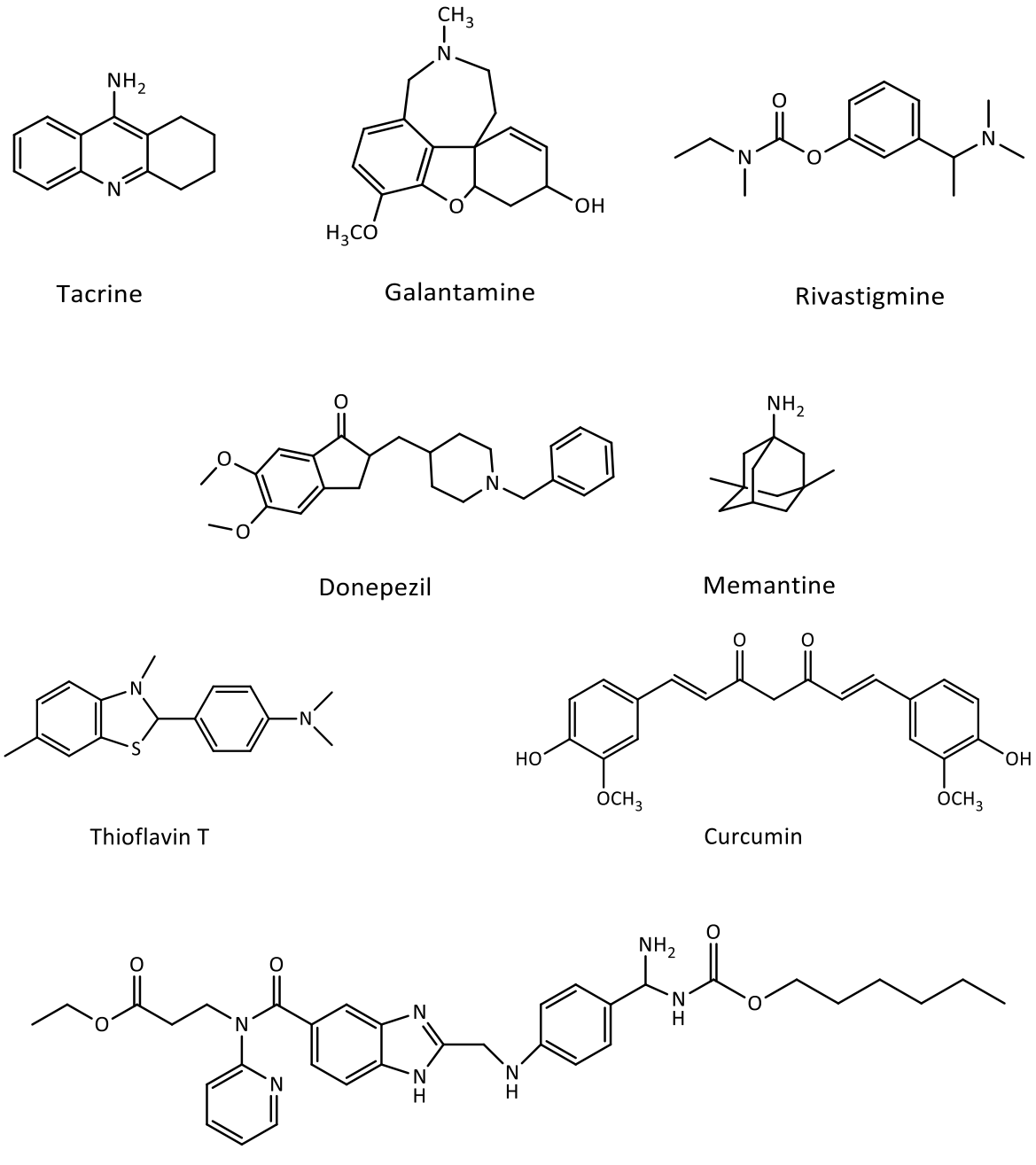
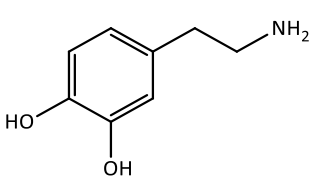


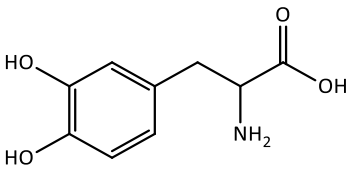
Figure 4. Alzheimer's disease drugs.

2.3.2. Parkinson's disease

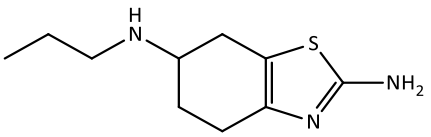
PD takes its name thanks to James Parkinson, and English doctor who studied and analysed the symptoms in 1817. PD is a long-term degenerative disorder of the CNS that mainly affects brain cells that are responsible of body movements. Symptoms start gradually, making this pathology a progressive condition with fatal ending. They often start with a slight tremor in one hand and a feeling of stiffness in the body. Nowadays, it affects about 10 million people (the second most extensive neurodegenerative disease after AD) and the prevalence is 1.5 times higher among men than women. Most of the cases of PD appears after 60 years but even though some cases appear at earlier ages. PD has a wide range of symptoms but the main are rigidity, slowness, tremors, pain depression or anxiety (European Parkinson's disease Association, www.epda.eu.com). PD is the most studied pathology among all synucleinopathy diseases being characterized by the presence of Lewy bodies in the brain. Lewy bodies are the aggregation in a non-normal way of alpha-synuclein inside the cells from the nervous system. This aggregation together with the loss of nigrostriatal dopamine neurons and the activated microglia characterize this pathology (Pujols J. et al., 2018). Dopamine plays a vital role in regulating the movement of the body. A reduction in dopamine is responsible for many of the PD symptoms. Some studies demonstrate the anti-inflammatory profile of 2-(5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl)-N-(2-hydroxyethyl)-2 oxoacetamide (CDMPO) against activated microglia by LPS. (Kim B. et al., 2019). Despite all the investigations, there is no effective cure for PD. Several drugs with different targets are available in the market like levodopa (L-DOPA, a precursor of dopamine, noradrenaline and adrenaline), pramipexole and rotigotine (both of them dopamine agonists), tolcapone and entacapone (COMT inhibitors, avoiding the methylation of L-DOPA and dopamine), rasagiline (MAO-B inhibitor, blocking the breakdown of neurotransmitters like dopamine), amantadine (releasing dopamine agent) or rivastigmine (acetylcholinesterase inhibitor) (Van Bulck M. et al., 2019) (Figure 5).



Dopamine



L-DOPA



Pramipexide

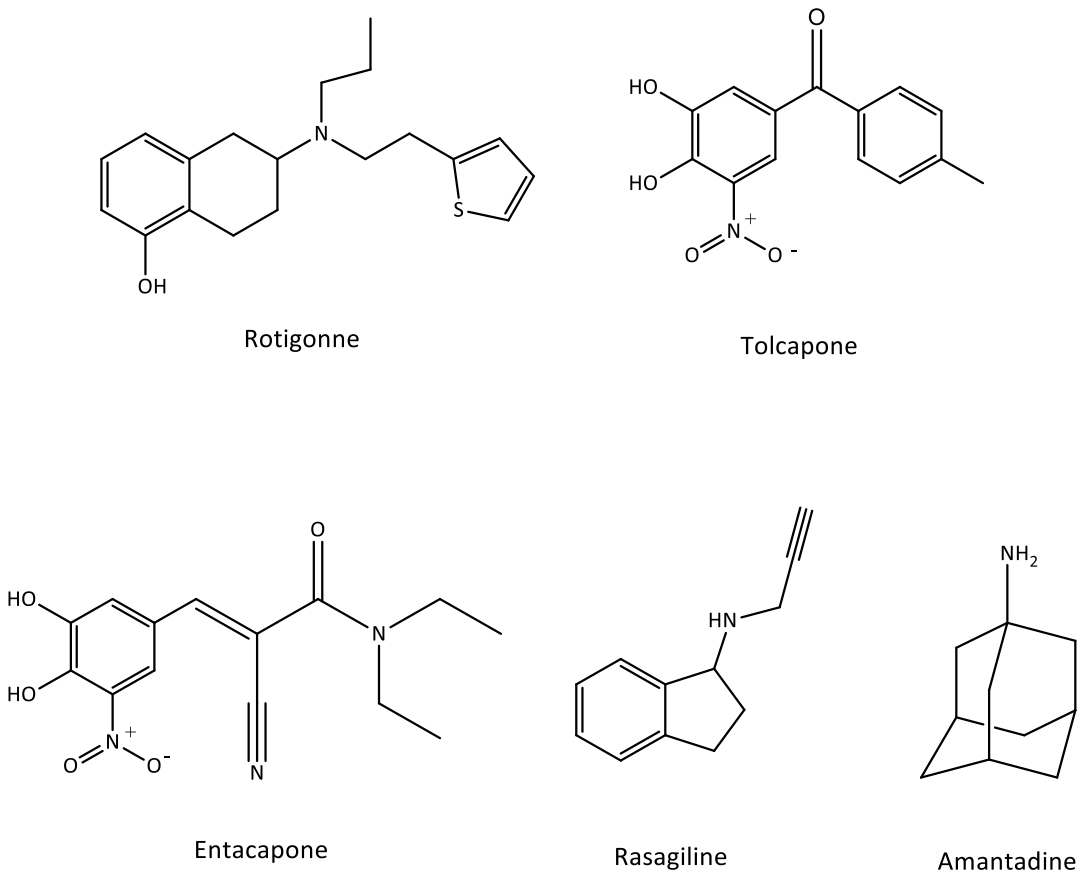


Figure 5. Parkinson's disease drugs.

2.3.3. Multiple Sclerosis

MS was first defined as a pathological condition by the French doctor Jean-Martin Charcot, baptizing it as "*sclérose en plaques disséminées".* He was able to establish a relationship between all the clinical and anatomical findings, making a correlation among the pathology changes. In addition, he elucidated a considerable number of isolated progressive motor symptoms that included rigidity, contractures or bulbar involvement and death from respiratory failure (Waraich M. et al., 2018). Nowadays MS affects 2.5 million in the entire planet, being higher in Northern Europe and America, and lower in Japan (Howard J. et al., 2016). Most of the patients could live longer than 20 years with the disease, even if it's virtually unheard of to survive for 50 years (Glasser A. et al., 2019). MS is a chronic, inflammatory neurodegenerative disease, which is characterized by demyelination and remyelination, and neuronal damage. In MS, the immune system attacks the protective sheath, myelin, that covers nerve fibres and causes communication problems between brain and the rest of the body. It is defined as a demyelinating and inflammatory pathology that disturbs the correct functioning of the CNS. Until now the exact and precise mechanisms that produce the disease are

not fully understood, but seems to be due to an interaction between environment and genetics factors. Scientific studies establish that the cognitive deterioration and other early stages disabilities are caused by an axonal and neuronal depletion (Katz Sand I. et al., 2015). MS is not only considered as a neurodegenerative condition but also a pathology with inflammatory processes. In fact, it has been demonstrated that people affected by MS with a progressive phenotype suffer from inflammatory activity. Many theories were done to explain the appearance and progression of this pathology. One of them affirms that everything is guided by an inflammatory event, causing and creating a surrounding climate that promotes the retention of inflammatory cells. Another hypothesis asserts that MS could start as an inflammatory pathology, but over the years, a neurodegenerative cascade starts and independently of the inflammation becomes the main actor of the disease progression. The third theory hypothesizes that MS is primary a neurodegenerative disease with a secondary phase of inflammation that exacerbate the negative state (Correale J. et al., 2017). In addition, exist some factors that could help the disease progression. One example is the ectopic formation of follicle-like structures, which create an environment in the CNS that helps the B cells interaction and influence the humoral and cell mediated immunity. This triggers the secretion of proinflammatory CK, CXCL13, cytotoxic factors and the recruitment and maturation of B cells. Furthermore, the astrocyte activation produces a BBB breakdown and brings to the synthesis of TNF-α or IL-1β, chemokines as CCL2, CCL5 or CXCL12, ROS and RNS and increase the glutamate toxicity. This astrocyte activation could provoke the activation of microglia, which in turn decrease the expression of factors that will help the immunosuppression like factalkine-CXCR1 and CD200-CD200R. At its turn, the activated microglia will increase the production of proinflammatory CK, chemokines, ROS and RNS. Another mechanism of neurodegeneration and axonal dysfunction is the mitochondrial injury, the powerhouse of cells. Alterations in the correct function bring to serious and several problems such as neurological problems, dementia and movement disorders. Some effects are the impaired activity of complexes I, III and IV, failure of Na^+/K^+ ATPase and excess of intra-axonal Ca^{2+} that brings to energy deficits and oxidative stress (Correale J. et al., 2019). For this pathology, also, there is no effective cure. Several molecules are present for the treatment of MS (Figure 6). One of the drugs that was studies and used for MS is mitoxantrone. A double-blind phase II study was conducted with this drug and results led to its approval by the US FDA. Other molecules, under study in phase III of clinical trials, are IFNβ1a and IFNβ1b that enhances the production of anti-inflammatory cytokines IL-4 and IL-10 and decreases the production of the proinflammatory cytokine, respectively (Andersen O. et al., 2004). Immunosuppressive drugs as cyclophosphamide,

azathioprine and methotrexate have been also tested in MS, giving some therapeutic effects that could help alleviating the progression of the disease (Schwartzman R.J. et al., 2009). Another approach is the inhibition of Na^+ and Ca^{2+} channels-mediated activators, which are used as therapeutic targets as they could help to counteract axonal degeneration and delay the permanent disability of people with MS (Waxman S.G. Et al., 2006). It has been shown that the administration of Na^+ channel blockers, anticonvulsants like carbamazepine, lamotrigine or phenytoin (Bechtold D.A. et al., 2006) and the administration of flecainide (Bechtold D.A. et al., 2004)**,** diminishes the neurological impairment.

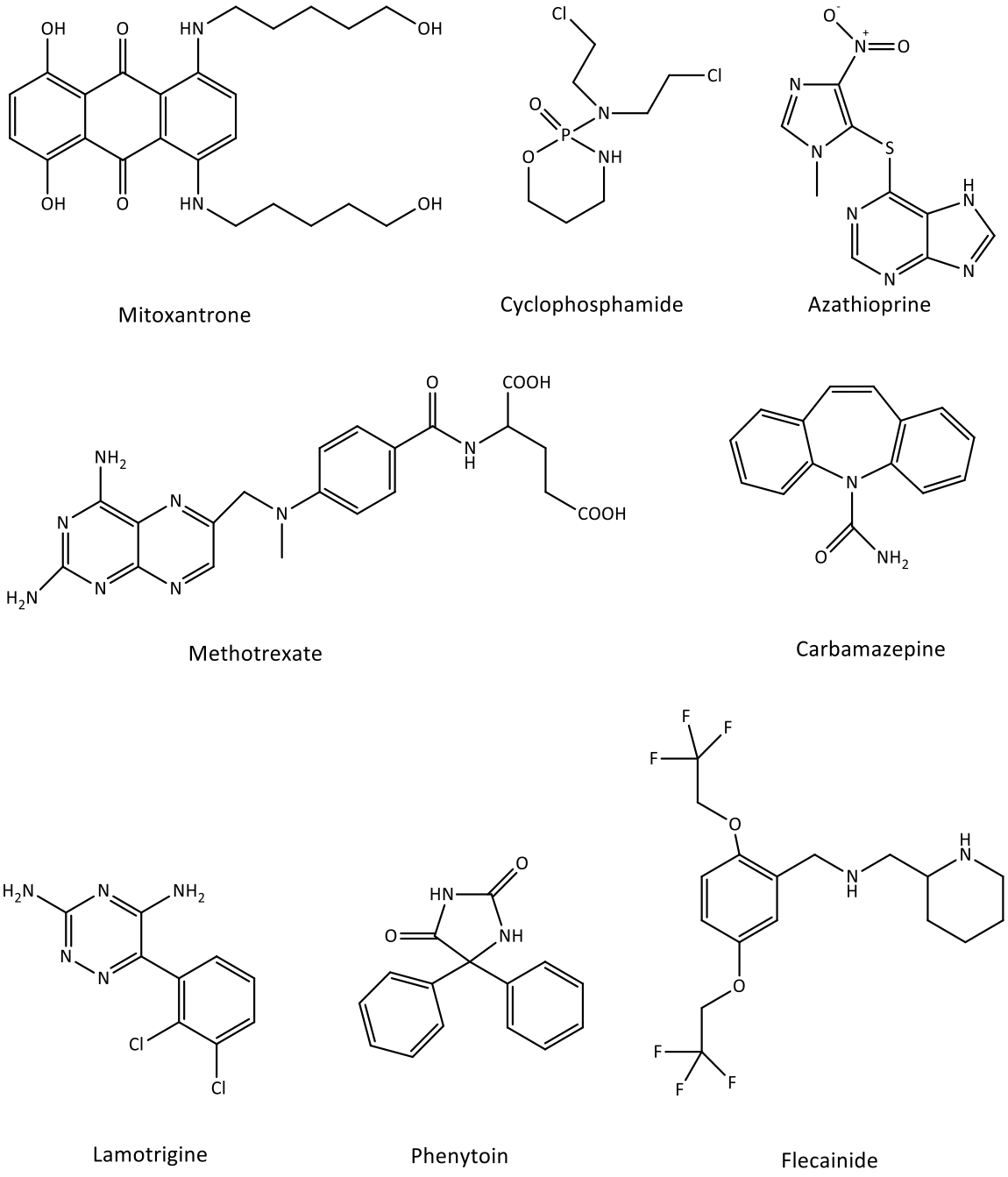


Figure 6. Multiple Sclerosis drugs.

2.3.4. Huntington's disease

Huntington's disease (HD), which can be also named Huntington's chorea (HC), was initially named "Saint Vitus's dance" because it was considered a curse sent by this Saint. Medically, it was first characterized by the American Charles Oscar Waters in 1841, but it was not until 1872 when the physician George Huntington fully described the disease and discovered their genetically causes. (Vale T.C. et al., 2015). HD is an inherited disease that causes the progressive degeneration of nerve cells in the brain provoked by a defective gene. The prevalence of the disease varies worldwide, for example in Western countries it is 10.6–13.7 individuals per 100 000, while if we look at populations like Taiwan, Hong Kong or Japan, the prevalence value is reduced until 1–7 per million. Despite this, the lowest prevalence rates were shown to be in South Africa, particularly in black population. HD is a neurodegenerative disease produced by the CAG (cytosine-adenine-guanine) trinucleotide repetition on chromosome 4, precisely in the huntingtin (HTT) gene. This gene is inherited following an autosomal dominant pattern, which means that it is easier to suffer from HD if a person only has one affected parent (Figure 7).

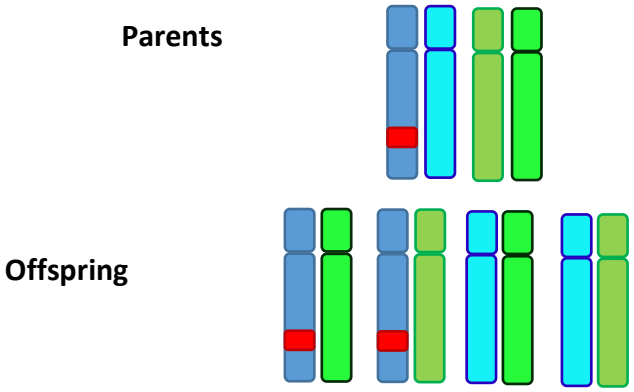


Figure 7. Autosomal dominant pattern of HD. Parent 1 (blue) has one chromosome with the affected gen (red box) and suffer from HD, while parent 2 (green) is not affected. When they pass the chromosomes to their offspring, all of them with the parent 1 chromosome affected will suffer from HD (red box).

The result of this gene mutation (mHTT) is translated in a protein with a long repetition of glutamine (Gillian-Bates. et al., 2002). When there are more than 39 CAG repetitions in the gene, the HD is known to occur, whereas when the repetition is between 36 and 39 the chance to suffer from the disease is reduced. mHTT causes the formation of aberrant aggregates affecting the normal activity of axonal transport, transcription, translation, cellular proteostasis or even mitochondrial and synaptic function (Bates G.P. et al., 2015). In addition, two other gens located on chromosome 15 demonstrated to have also an effect on HD progression. The FAN1 expression (Fanconi anemia

FANC1/FANCD2 associated endonuclease) produces a slower evolution of the disease, highlighting this gene as a possible protecting factor against a multiplied CAG repetition (Goold R. et al., 2019). The other gen, which demonstrated to be tightly involved in inositol-phosphate signalling and with cognitive function loss and working memory, is the MTMR10 (myotubularin related protein 10) (Wright, G.E.B. et al., 2019). Other 2 genes located on chromosome 8 showed to be related with the disease progression, they are RRM2B (a subunit of DNA damage p53-inducible ribonucleotide reductase M2 B) and URB5 (an HECT domain E3 ubiquitin-protein ligase).

It has been shown in post-mortem patients with HD an atrophy presence of several brain zones like putamen or caudate, together with a decay of dorso-ventral, medio-lateral and caudo-rostral gradient. In a small measure are also damaged the globus pallidus and nucleus accumbens (McColgan P. et al., 2018). All this aggressions, mutations and damaged areas have an ample list of symptoms like involuntary spasmodic movements, muscular problems, rigidity, dystonia, posture and equilibrium negatively affected, slow or abnormal movements and even difficulty to swallow. Moreover, neuropsychiatric symptoms such as anxiety, irritability (13% of cases), depression (13%), obsessive-compulsive behaviour (13%), psychosis (1%) or apathy (28%) can be present (van Duijn E. et al., 2014). The body movement disorder such as dystonia, bradykinesia or walking disturbance are associated with the CAG length repetition and disease duration (Dorsey E.R. et al., 2013). The only drug that is actually approved by the FDA is tetrabenazine (TBZ) a reversible inhibitor of the vesicular monoamine transporter 2 (VMAT-2), which is useful for the treatment of chorea in HD since is able to prevent dopamine, norepinephrine and serotonin uptake into presynaptic vesicles. The inhibitory action of this drug brings to the degradation of these neurochemicals leading positive effects in chorea. TBZ is effective in counteracting the symptoms related with neurotransmitters decrease like anxiety, due to the dopamine decrease, and depression due to the reduction of serotonin and norepinephrine. The other drugs used to treat the symptoms of HD are the typical antipsychotics such as perphenazine, fluphenazine, haloperidol or clozapine that showed a positive effect on chorea. Risperidone and quetiapine demonstrated good effects improving the involuntary movements and psychosis (Figure 8) (Wyant K.J. et al., 2017). Another target that is attracting the scientific community are the “dopamine stabilizers” called dopidines. The pharmacological effect of dopidines is related to dopamine level, in fact when the dopamine tone is elevated they behave as dopamine receptor antagonists, like antipsychotics, while when the dopaminergic tone is low they stimulate the transmission (Lundin A. et al., 2010). Actually some compounds are under study, like the metal protein-attenuating compound, better known PBT2 (5,7-Dichloro-2-

[(dimethylamino)methyl]quinolin-8-ol). This drug candidate reduce the metal induced aggregation of mHTT and it is actually in phase III of clinical trial (Investigators AloopSGRH, 2015). Another target to explore is the phosphodiesterase 10A (PDE10A, reduced in striatum of HD), in fact, the inhibition of this enzyme helps to restoring the basal ganglia functions in animal models (Beaumont V. et al., 2016). Antisense oligonucleotides (ASOs), that reduce the synthesis of mHTT even to 80% in animal models, are in phase I and phase II (Kordasiewicz H.B. et al., 2012). Recent studies demonstrate that CRISPR/Cas9 could be a good tool to attack and treat this disease, but even if some studies with fibroblast of HD patients have shown promising results, more investigation is needed (Shin J.W. et al., 2016; Yang S. et al., 2017). However, not all treatments use drugs, in fact some studies used the technique known as deep brain stimulation (DBS). These investigations sustain that deep brain stimulations with bilateral internal globus pallidi (GPi) stimulations concede beneficial effects to the patients, both at short- and long-term. (Gonzalez V. et al., 2014).

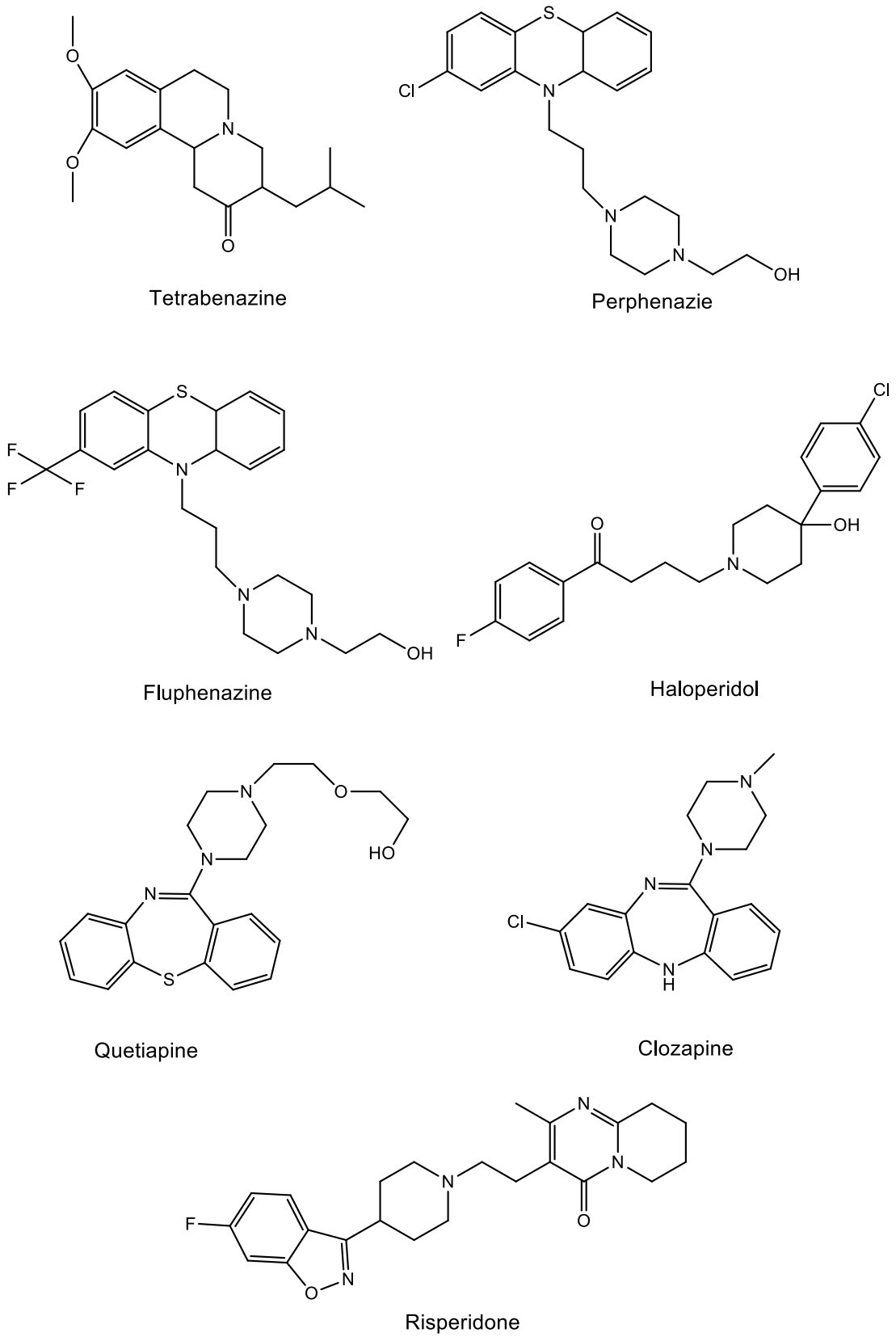


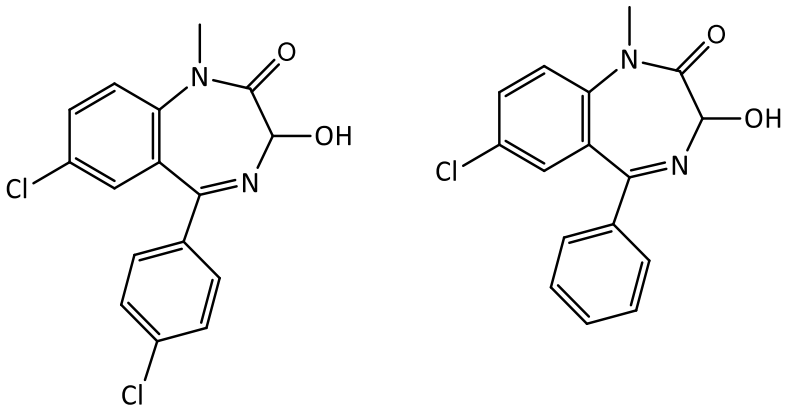
Figure 8. Huntington's disease drugs.

2.4 RECEPTORS

In neuroinflammation, several receptors are involved in the initial, progression and maintenance phase. The main receptors are the translocator protein, the Toll-like, the cytokine and the glutamate receptors (Schain M. et al., 2017; Shabab T. et al., 2017).

2.4.1. Mitochondrial Translocator Protein

The Mitochondrial Translocator Protein (TSPO) is a transmembrane receptor with a molecular weight of 18 KDa and it is localized in the outer membrane of the mitochondria. This protein is related with the cholesterol transport, steroid and bile acid synthesis, regulates the heart rate and the contractile force. In healthy states, TSPO is expressed at low levels in immune cells, leukocytes, macrophages, astrocytes and microglia, but once that a stimulus activates the glial cells, there is an overexpression of TSPO. This increment in the expression of TSPO is present in several diseases and pathological conditions like multiple sclerosis, HIV, cancer, Alzheimer's or Parkinson's disease. In these pathological cases, it has been shown that it can induce the apoptosis through the reduction of transmembrane potential. This receptor demonstrated the ability to bind diazepam, a benzodiazepine with relaxing effects, and Ro-4864 a derivative of diazepam with sedative and neuroprotective effects (Figure 9).



Ro-5-4864 Diazepam

Figure 9. Ro-5-4865 and Diazepam structures.

2.4.2. Toll-like receptors

Lipopolysaccharide (LPS) is a toxin present in outside membrane of the Gram-negative bacteria that can induce the inflammation after the binding to its receptors, the toll-like receptor (TLR) (Figure 10). TLR represents one of the main defence lines against outsider pathogens that can attack the

human body. Structurally, they possess conserved regions to bind ligands and an extracellular leucine-rich repeat region that recognize pathogens. In addition, they have a Toll/IL-1 receptor (TIR) domain, which is the responsible for the signal pathway. Once that LPS binds to TLR4 on the microglia surface it activates a cascade signalling that culminates with the activation of NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells). NF-kB is a protein complex being part of a transcription elements family. NF-kB structure is a dimer between P50-P65 (NF-kB1/RelA), which is necessary for the DNA binding. The C-terminal of the protein is the responsible for the nonspecific contact with the phosphate group of the DNA and the dimerization, while the N-terminal is responsible for DNA binding. The activation of NF-kB influences in the synthesis of chemokines, inducible nitric oxide synthase (iNOS), COX-2, pro-inflammatory cytokines, etc.

2.4.3. Pro-inflammatory cytokines and neuroinflammation pathway

Microglia immune functions are tightly related with two pro-inflammatory cytokines, the TNF-α and the IL-1β. In the case of TNF-α it has been demonstrated that the level of apoptotic cells, in particular neurons in hippocampal regions, is associated with the levels of TNF-α mRNA. These increased levels are required for the induction of the apoptosis process. Via the activation of its receptor (TNFR1), TNF-α exerts its pro-inflammatory effects (Figure 10). Structurally is characterized for possessing in the extracellular part cysteine-rich domains (CRD) and a death domain (DD) in the intracellular space. DD is a conserved element that allows the interaction with other proteins. The second important ligand and receptor is the IL-1β and its receptor, the IL-1R (Figure 10). IL-1β is a key role player in the development of neurological disorders and neurodegenerative diseases like stroke, ischemia, AD, PD or brain injury. IL-1R possesses three extracellular domains named immunoglobulin (Ig)-like domains. These domains are called D1, D2 and D3 being the first two linked while the last one is completely separate.

2.4.4. NMDA receptor

N-methyl-D-aspartate receptor (NMDAR) is an ion channel of glutamate (Glu) present in several nervous systems (Figure 10). Potassium and glutamate are crucial for maintaining the normal levels of this element in the brain, because excessive amounts of glutamate can have important and severe impacts such as cell death. In fact, the toxicity caused by glutamate it has been demonstrated to take part in several neurodegenerative pathologies such as Alzheimer's, Parkinson, Huntington disease, and amyotrophic lateral sclerosis. When Glu binds to its receptor causes excitotoxicity and neuronal damage due to an abnormal Ca^{2+} influx. This increase in calcium levels brings to the

activation of protease, cell damage, mitochondrial malfunction, ROS and at the end apoptotic events. As mentioned above, glia plays a crucial role in neuroimmune response and its activation affects the neuroinflammation response. It has been demonstrated that with the age, glial cells suffer from senescence and affect the well function of the brain. During this decline process, they lose their capacity to function perfectly affecting the potassium and glutamate transport (Limbad C. et al., 2020). Concretely, β-amyloid peptides increment the Glu release and inhibits Glu uptake, bringing to excitotoxicity, process that leads to neuroinflammation state in the brain. Nowadays some therapies exist that act in this target like Riluzole and Ceftriaxone (Figure 11). These drugs focus on increasing the levels of glutamate transporters and ameliorate the memory function.

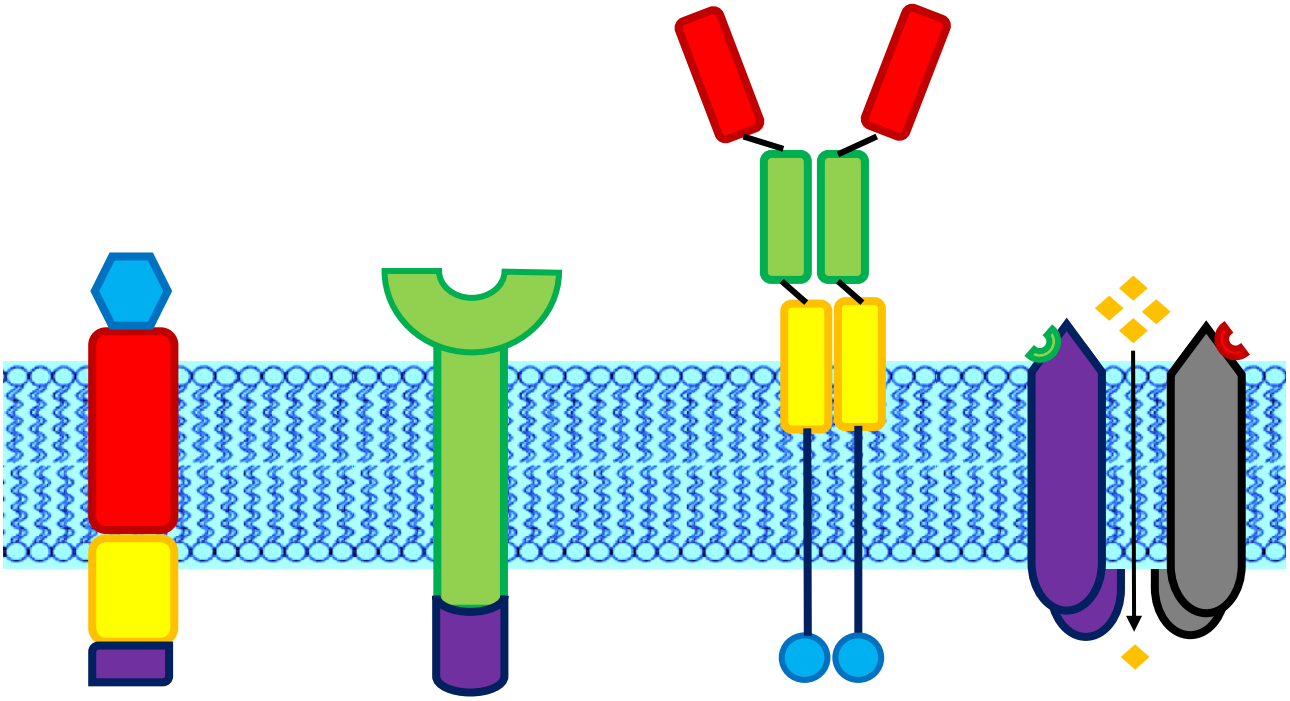


Figure 10. The main four receptors involved in neuroinflammation. The first image from the left represents the TLR, in blue the LPS, in red the leucine-rich region, in yellow the intracellular Toll/IL-1R (TIR) and in purple the MyD88, a critical signalling ligand for neuroinflammation pathway. The second image is the TNFR1, in green is represented the transmembrane and extracellular region and in purple the tumor necrosis factor receptor 1-associated death domain protein (TRADD). The third image is the IL-1R, the boxes in red, green and yellow represent the domains 1, 2 and 3 respectively, while the blue circles the Toll/interleukin-1 receptor (TIR) homology domain. Lastly in purple and grey are represented the four domain of the NMDAR, in green the glutamate binding site, in red the glycine binding site and the yellow diamonds represent the calcium.

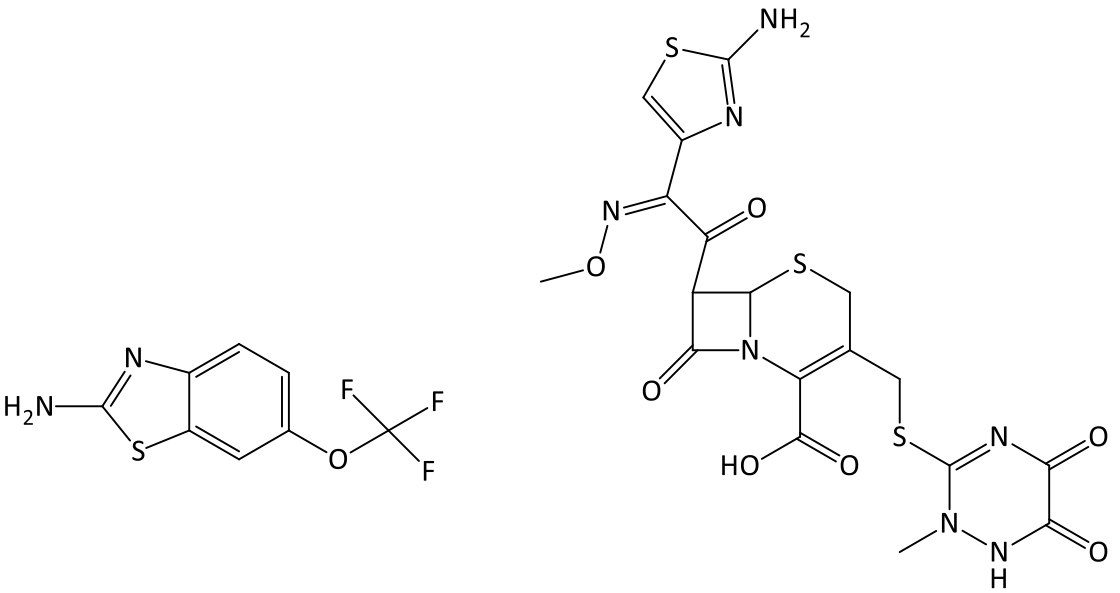


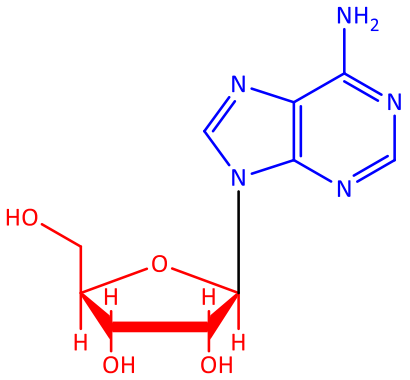
Figure 11. Riluzole and Ceftriaxone structures.

2.5. ADENOSINE SYSTEM

Recently, the adenosine receptors (ARs) are emerging as an attractive therapeutic target for modulating brain injury in a variety of animal models of neurological disorders. Adenosine (Ado) is an endogenous nucleoside widely distributed throughout the body where it regulates many functions (Haskó G. et al., 2008). Through the modification of the adenosine or adenine core, investigators obtained potent and selective agonists useful as anti-inflammatory (A_{2A}AR and A_3AR), cardioprotective (A_1AR and A_3AR), antinociceptive (A_1AR) or selective antagonists with great impact on kidney defence (A_1AR), neuroprotection (A_{2A}AR) or against glaucoma (A_3AR).

2.5.1. Adenosine

Adenosine is a ubiquitous purine nucleoside produced in physiological processes by breakdown of ATP (Layland J. et al., 2014). Structurally, adenosine is composed of the nitrogenous base adenine and a ribose sugar, concretely ribofuranose. These two elements are linked via a β-N^9-glycosidic bond (Figure 12).



Adenosine

Figure 12. Adenosine structure. Ribofuranose is represented in red, adenine in blue and the bond in black.

Ado is structurally and metabolically correlated with several coenzymes like coenzyme A, flavin adenine dinucleotide (FAD) or nicotinamide adenine dinucleotide (NAD). On the other hand, biologically it is related with other active nucleotides such as cyclic AMP (cAMP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), the energy molecule carrier of the cell, and S-adenosyl-L-methionine (SAM) a methylating agent.

Ado is constitutively present at low concentrations intracellularly as well as extracellularly and it is a product of complete dephosphorylation of adenine nucleotides. Ado intracellular production is mediated either by an intracellular 5'-nucleotidase, cytoplasmic 5'-nucleotidase (cN-I), that breaks down AMP to adenosine (Phillips E. et al., 1979; Zimmermann H. et al., 1998; Sala-Newby G.B. et al., 1999) or by hydrolysis of S-adenosyl-L-homocysteine (SAH) by the activity of the S-adenosyl-L-homocysteine hydrolase (SAHH) (Broch O.J. et al., 1980). Within cells, adenosine is an intermediate for the synthesis of nucleic acids and adenosine 5'-triphosphate (ATP). Adenosine generated intracellularly is transported into the extracellular space mainly via specific bi-directional transporters through facilitated diffusion that efficiently evens out the intra- and extracellular levels of adenosine. In some tissues such as the kidney, there is a concentrative nucleoside transport protein capable of maintaining high adenosine concentrations against a concentration gradient.

Under basal conditions, adenosine extracellular levels are in nanomolar range, from 30 to 200 nM (Ballarín M. et al., 1991) and the maintenance of these concentrations depends on a balance between the release/removal of the nucleoside by membrane transporters/enzymes and its formation from ATP due to the activity of ecto-nucleotidases. In the extracellular space, adenosine is produced via enzymatic hydrolysis of extracellular ATP. The half-life of adenosine in the extracellular space is about of 10 s. Under physiological conditions, most of the extracellular Ado

appears to re-enter cells through equilibrative transporters. A small fraction can be irreversibly converted into inosine and its derivatives (hypoxanthine, xanthine, uric acid) by ADA and xanthine oxidase. Extracellular Ado can also be metabolized by ecto-kinases to regenerate AMP, ADP, and ATP (Eltzschig H.K., 2009).

Extracellular levels of Ado can rise from low nanomolar to micromolar concentrations in response to increased metabolic demand, injury or stress, such as ischemia and inflammation, mainly by the breakdown of ATP released by either lysis or non-lytic mechanisms from multiple cell types (Boison D. et al., 2019) (Figure 13).

At extracellular levels, the conversion starts with the discharge of ATP that is stored inside the cells. This release is produced due to cell disturbance or through connexin hemichannels (Cx) and vesicular exocytosis (VE). ATP is hydrolyzed into ADP by nucleoside triphosphate diphosphohydrolase-1 (NTPDase-1/CD39, which also can transform ATP to ADP) or into AMP by the enzyme nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1). The enzyme ecto-50-nucleotidase/CD73 converts AMP into Ado, which is deaminated to inosine (Ino) or transported inside the cell by nucleoside transporter (ENT) and concentrative nucleoside transporter (CNT). At cytosolic level, the synthesis of adenosine depends on the SAH cycle reaction. S-adenosylmethionine (SAM) is used as a donor of a methyl group to obtain SAH thorough a transmethylation reaction catalyzed by methyltransferases (MT). SAH is hydrolyzed by S-Adenosylhomocysteine hydrolase (SAHH) obtaining Ado and homocysteine (HCy).

It is important to highlight that the intracellular Ado can be restored into ATP by the activity of three enzymes: cytoplasmic form of adenosine kinase (ADK-S), adenylate kinase-1 (AK1) and nucleotide diphosphokinase (NDPK).

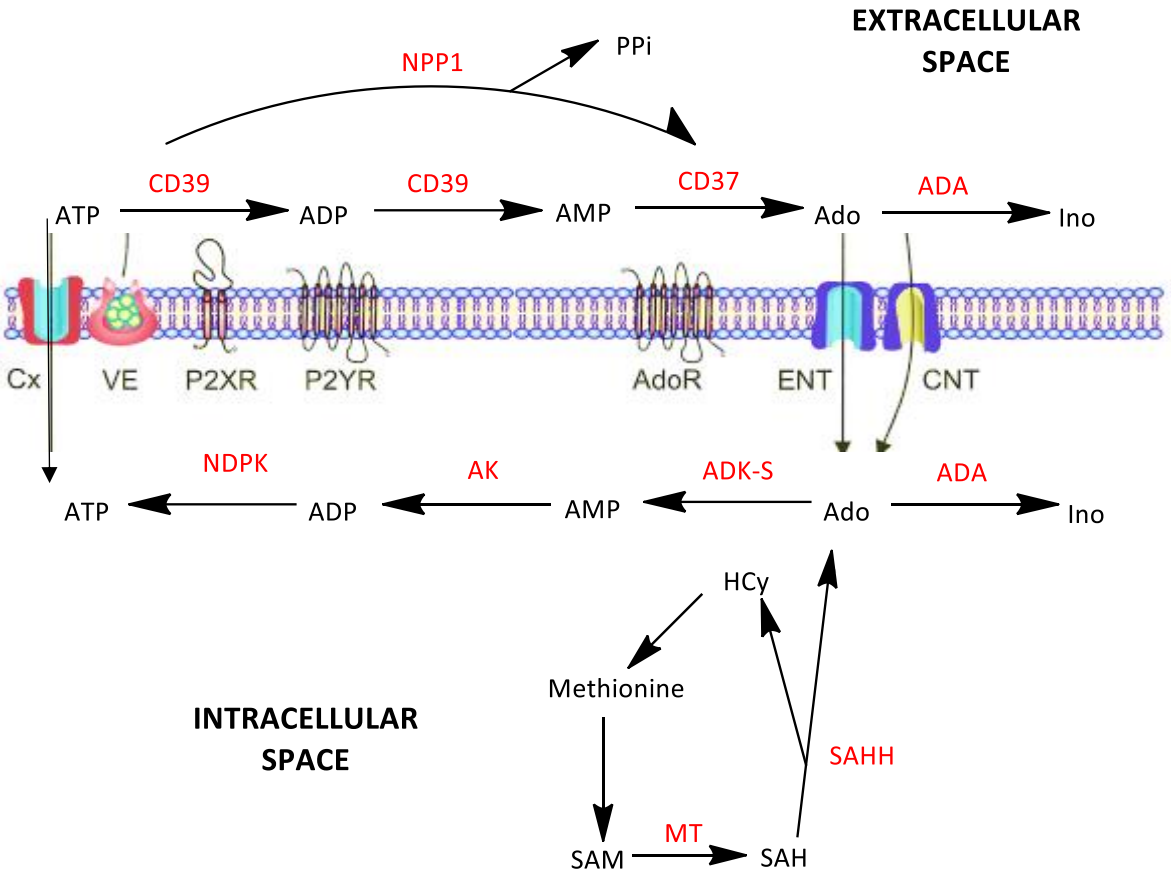


Figure 13. Adenosine biosynthesis. In red are written the enzymes and in black the molecules.

The termination of extracellular adenosine signalling occurs by uptake or degradation of this nucleoside, achieved by nucleoside transporters and ecto-adenosine deaminase, respectively.

Two other actors play an important role in this scenario. The first one is the mitochondria, which due to it deeply connection with ATP production it is also linked with adenosine homeostasis. The second one is the nucleus, where adenosine is one key role player of the transmethylation pathway. This signalling pathway allows the increase of DNA and the methylation of the histones (Figure 14).

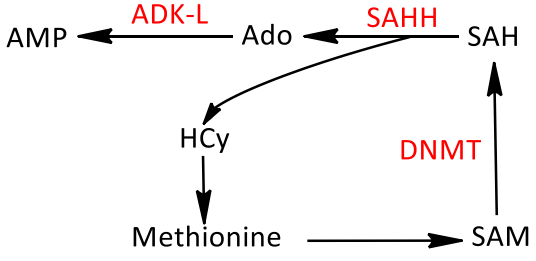


Figure 14. Adenosine pathway in the nucleus. In red are written the enzymes and in black the molecules.

2.5.2. Adenosine receptors

Ado signalling is evoked through activation of four distinct and widely expressed receptors: A_1, A_{2A}, A_{2B} and A_3 ARs. All four subtypes are members of the superfamily of G-protein-coupled receptors (GPCRs) which transfer signals by activating heterotrimeric G proteins (Ralevic V. et al., 1998). Burnstock proposed that there are distinct receptors that bind adenosine or ATP, designated P1 and P2 receptors, respectively (Burnstock G., 1972 and 1976). Families of both P1 and P2 receptors have been described since then, and adenosine receptors are now identified as purinergic receptors, consisting of P1, while ATP receptors are designated as P2 receptors and consist of P2X and P2Y subtypes (Dal Ben D. et al., 2011). Seven P2X ($P2X_{1\text{-}7}$) and eight P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13}$ and $_{14}$) receptor submembers were described (Figure 15).

Adenosine receptors display the typical topology of GPCRs, characterized by a common central core domain consisting of seven transmembrane helices numbered from 1 to 7 that are composed of 20-27 amino acids and that are largely α-helical, with an extracellular amino-terminus and an intracellular carboxy-terminus (Ralevic V. et al., 1998). First evidence for the existence of Ado receptors was in 1965 when DeGubareff and Sleator observed that adenosine action on heart could be antagonized by caffeine; subsequently, in 1970, Sattin and Rall showed that adenosine regulates cell function via occupancy of specific receptors on the cell surface. To date, adenosine receptors have been cloned, and the signal transduction mechanisms have been described. Classically, the first effector molecule of adenosine receptors is the enzyme adenyl cyclase (AC) which is either stimulated or inhibited, increasing or decreasing cyclic adenosine monophosphate (cAMP) levels according to the receptor subtype triggered.

On the other side, seven P2X ($P2X_{1\text{-}7}$) and eight P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13}$ and $_{14}$) receptor submembers were described (Figure 15).

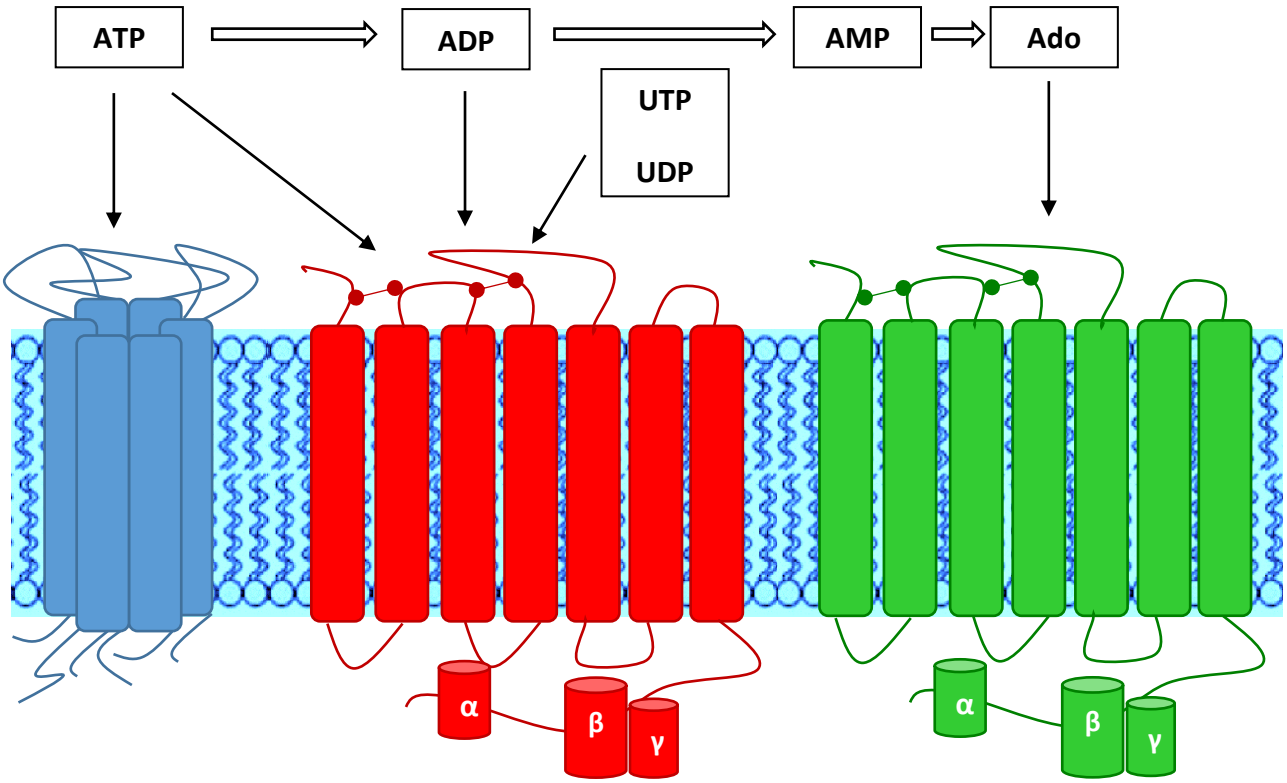


Figure 15. Adrenergic receptors structure and classification. In blue are designed the P2X receptors, in red the P2Y receptors and in green the P1 receptors.

A difference between P1 and P2 receptors is a different sensitivity to xanthinic antagonists. For example, caffeine, theobromine and theophylline have an inhibitory activity on P1 receptors while in P2 receptors are entirely inactive (Sawynok J. et al., 2007) (Figure 16).

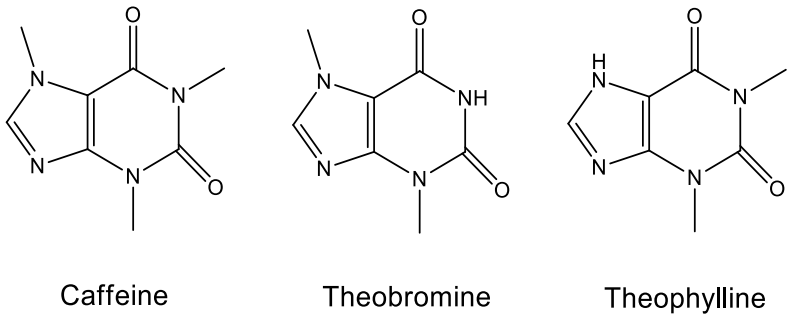


Figure 16. Caffeine, theobromine and theophylline structures

2.5.2.1. A_1 adenosine receptor (A_1AR)

A_1 Ado receptor (A_1AR) is coupled to G proteins belonging to the family of the G_i/G_q. Activation of A_1AR inhibits AC activity through activation of pertussis toxin-sensitive G_i proteins, it decreases PKA activity and phosphorylation of the cyclic AMP response element binding (CREB) protein (Van Calker et al., 1979). This receptor is constituted by 326 amino acids (AA). From all the 7TM, 3 extracellular loops (EL) and 3 intracellular loops (IL), the TM3 and EL2 have strong conserved sequences necessary for ligand binding (Jespers W. et al., 2018). Some studies demonstrate that the orthosteric site is in the TM with and implication of the EL2 in the ligand affinity and in the signal transduction (Nguyen A.T. et al., 2016a). However, the presence of EL2 is also important for the formation of the allosteric site (Nguyen A.T. et al., 2016b). Among all the four subtypes of adenosine receptors, A_1AR is the most abundant receptor subtype and largely expressed in the CNS in a great manner in the hippocampus, cerebellum, neocortex and the dorsal horn of the spinal cord. Outside the nervous system, it can be found in adipose tissue, heart and inflammatory cells (Townsend-Nicholson A. et al., 1995). In cardiac muscle and neurons, A_1AR can activate pertussis toxin-sensitive K^+ channels, as well as K-ATP channels, and inhibit Q-, P- and N-type Ca^{2+} channels. Furthermore, activation of A_1AR leads to increased intracellular Ca^{2+} levels due to the stimulation of phospholipase C (PLC), which in turn promotes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Klinger M. et al., 2002). Moreover, the enhancement of intracellular calcium can activate certain enzymes, such as protein kinase C (PKC), phospholipase D (PLD), phospholipase A2 (PLA2)

One of the major functions of A_1AR is the regulation of energy balance at cellular level. Particularly, in the heart, they reduce heart rate and force of contraction. In kidneys, the glomerular filtration diminishes and consequently reduces the need of energy. When A_1AR is activated on postsynaptic neurons it causes a postsynaptic and neurochemical release inhibition. In addition, the brain administration of Ado causes sleep induction. It is implicated also in locomotor activity, pain or memory. The neuroprotective effect of Ado, in pathological conditions, is explicated by the activation of hA_1AR, while A_1AR antagonist aggravate the negative events (Fredholm B.B. et al., 2005 a and b).

In Alzheimer's disease it has been demonstrated the reduction of A_1AR receptors and that their activation could bring to β-amyloid peptides into a more soluble structures. Additionally, in physiological and/or pathological conditions, adenosine, by A_1AR activation, reduced the astrocyte proliferation and induced the release of nerve growth factor (NGF) (Burnstock G. et al., 2011).

2.5.2.2. A_{2A} adenosine receptor (A_{2A}AR)

A_{2A} Ado receptor (A_{2A}AR) couples to G_s protein in peripheral tissues or G_{olf} protein in the brain (Kull B. et al., 2000). Increased cAMP level stimulates cAMP-dependent kinase (protein kinase A, PKA) which, in turn, activates several pathways through calcium channels, potassium channels, CREB, MAPK and PLC activation. Activation of A_{2A}AR can also promote activation of PKC in a cAMP-dependent and independent mechanism (Sheth S. et al., 2014). There is evidence that the A_{2A}AR can occur as receptor homodimer as well as heterodimers with other receptors such as dopamine D_2 receptor (Fredholm et al., 2007). Moreover, recent studies have shown that there are several proteins interacting with the cytoplasmic tail of the activated A_{2A}AR resulting in G-protein-independent signalling pathways (Fredholm BB. et al., 2007).

A_{2A}AR is constituted by a number of AA that varies between 409 and 412 among species but with a fix length of 412 AA in humans. This receptor is characterised by an intracellular end point longer compared with the other subtypes. It has been seen the presence of disulphide bounds between two cysteine in TM3 and EL2 and the existence in TM1 and TM7 of a glutamic acid and a histidine, respectively, with high positive impact in agonist binding. A structurally peculiarity of this subtype is the presence of a short TM8 domain facing the intracellular membrane (de Lera Ruiz M. et al., 2014). Once that the ligand binds to the receptor a conformational change is done, particularly in TM3, TM5, TM6 (the responsible of G protein activation) and TM7 (Jespers W. et al., 2017). Despite the well-known binding site of the receptor it has been identified 2 cholesterol-binding sites that are only available with the presence of an inverse agonist (Rouviere E. et al., 2017). It has been demonstrated that the presence of the sugar structure in the agonist have stabilizing proprieties on the intermediate-active state (Carpenter B. et al., 2017). Outside the nervous system, this receptor is present in blood vessels and have vasodilator proprieties. A_{2A}AR are expressed majorly in neurons on the brain striatum area but also in low levels on neurons and glial cells outside the striatum. In addition, it is expressed in the nucleus accumbens, caudate putamen and olfactory tubercle (Boison D. et al., 2012). It has been demonstrated that the function of this receptor neurons, in particular on caudate putamen, is to modulate the neurotransmission of several molecules such as acetylcholine, glutamate or γ-aminobutyric acid (Matasi J.J. et al., 2005). It is worth noting that A_{2A}AR expression in microglia increases as a result of brain insults, and this overexpression lead to signal transductions that do not occur in cells with the receptors expressed at a normal level such as facilitating the release of cytokines (Minghetti L. et al., 2007). The use of A_{2A}AR antagonists suppress microglia activation, *in vitro*, and *in vivo* studies (Gomes C. et al., 2013; Aires I.D. et al.,

2019). On the other hand, the activation of this subtype reduce the locomotion by the activation of the $G\alpha_s$ paralogue, $G\alpha_{olf}$ and could strengthen the neuronal damage promoting the neurotransmitter release. For these reasons many efforts are focusing on the research of potent and selective A_{2A}AR antagonists (Diener H.C. et al., 2005; Volpini R. et al., 2009a)

2.5.2.3. A_{2B} adenosine receptor (A_{2B}AR)

Activation of A_{2B} Ado receptor (A_{2B}AR) can stimulate AC and PLC through activation of G_s and G_q proteins, respectively (Feoktistov I. et al., 1995). The A_{2B}AR has also been described to be involved in the extracellular signal-regulated kinase (ERK) ERK1, ERK2 (Schulte G. et al., 2003) and p38 MAPK in mast cells. A_{2B}AR are composed by 328 amino acids. Some studies showed the TM3, TM5, TM6 and TM7 are crucial for the binding of both agonist and antagonist (Aherne C.M. et al., 2011). A characteristic of these receptors is the longer sequence of the EL2 with four cysteine (Cys) that constitute the disulphide bonds (C154, C166, C167, C171). Despite the structurally importance of these Cys and disulphide bonds only the one present between C171 in EL2 and C78 in TM3 is critical for ligand recognition and receptor transport through the cell membrane (Seibt B.F. et al., 2013). The His280 and Asn282 AA stabilize the binding site by hydrogen bounds; Trp247, Val250 and Ser279 are essential for Ado binding; Leu81, Asn186, and Val250 are important for xanthine antagonists (Thimm D. et al., 2013). A_{2B}ARs are present in blood vessels, colon, mast cells, lung, gastrointestinal tract and bladder but they can be find in different concentrations in all the tissues (Haskó G. et al., 2009). A_{2B}AR showed low affinity for adenosine.

MAPK are secondary agents of several and important cell signalling pathways that are present in various diseases like AD. In pathological conditions the A_{2B}AR activation on mast cells by adenosine contributes to inflammatory airways disease or the synthesis of pro-inflammatory agents. Outside the mast cells they are also important for the regulation of vascular tone. The A_{2B}AR is considered as an “emergency” receptor that is mainly activated under stressful conditions.

2.5.2.4. A_3 adenosine receptors (A_3AR)

A_3 Ado receptor (A_3AR) couples to classical second-messenger pathways such as inhibition of AC, stimulation of PLC and D and calcium mobilization (Zhou Q.Y. et al., 1992; Abbracchio M.P. et al., 1995). A_3AR can regulate the activity of PLC via a pertussis toxinsensitive G protein in rat basophilic leukemia cells or by direct coupling to G_q protein. A_3AR is architected with 318 amino acids. The C-terminal region presents 6 serines and threonines and is quickly phosphorylated by GPCR kinases

(GRK) during a rapid desensitization process. This rapid desensitization could be explained with the presence of Cys residues on the C-terminal domain in A_3AR receptors, which is indispensable for GRK activation (Jacobson K.A. et al., 2018). Some studies showed that A_3AR receptors need the Trp (W648) in TM6, a highly conserved AA, crucial for signal transduction activation, interaction with β-arrestin 2 and the internalization of the receptor (Stoddart L.A. et al., 2014). It has been widely identified in several tissues and cell type, including lung, mast cells, neutrophils, eosinophils, testis, kidneys, heart, and brain cortex (Sachdeva S. et al., 2013). In addition, in animal models of allergic response A_3AR is implicated in mast cell degranulation. A_3AR is also involved in inflammatory states including asthma, due to the presence in macrophages and eosinophils. A_3AR agonists were studied for the treatment of several pathological conditions like rheumatoid arthritis, ocular hypertension, glaucoma, psoriasis, hepatocellular carcinoma. On the other hand, antagonists could be useful on the treatment of other pathologies like inflammatory bowel disease and glaucoma.

Table 2. Adenosine receptors summary. Expression is indicated with three different symbols depending on their levels, (++) indicates high expression, (+) is for medium expression and (-) low expression.

	A_1AR	**A_{2A}AR**	**A_{2B}AR**	**A_3AR**
AA length	326 AA	412 AA	328 AA	318 AA
Chromosome	1q32.1	22q11.23	17p12	1p13.2
Adenosine affinity	0,3-3 nM	1-20 nM	10 µM	+1 µM
Expression	Cortex (++), hippocampus (++), eye (++), adrenal gland (++), liver (+), kidney (+), adipose tissue (+), pancreas (-), lungs (-)	Thymus (++), olfactory bulb (++), blood platelets (++), heart (+), lung (+), peripheral nerves (+), other brain regions (-)	Colon (++), bladder (++), cecum (++), eye (+), mast cells (+), lung (+), adipose tissue (-) adrenal gland (-), kidney (-)	Testis (++), lung (++), mast cells (++), cerebellum (+), hippocampus (+), thyroid (-), kidney (-), heart (-)
Effects	Energy balance, neurotransmitters inhibition	Modulation of neurotransmission, anti-inflammatory	Potentiate calcium release	Mast cell degranulation
Target disease or pathologies	Alzheimer's disease, atrioventricular node block, cardiac transplant reactions	Parkinson's disease, headache pain, reperfusion injuries	Inflammatory airway disease, allergic reactions, asthma	Glaucoma, rheumatoid arthritis, arrhythmias, asthma
Molecules with affinity	L-PIA	NECA		HEMADO

2.5.3. Adenosine and neuroinflammation

Ado has been recognised as an endogenous signalling molecule that is able to signal inflammation as well as modulate the inflammatory response (Haskó G. et al., 2013). Ado is one of the many biomolecules that accumulate in the inflammatory milieu conferring pleiotropic effects which can be beneficial or harmful (Driver A.G. et al., 1993). Ado effects are the outcome of receptor activation and may be pro-inflammatory or anti-inflammatory depending

1) on the tissue or cells where receptors are expressed,

2) on the affinity between adenosine and a specific receptor and

3) on the extent of tissue receptor expression under pathological conditions (Bours M.J. et al., 2006).

In physiological conditions, extracellular adenosine level is around nanomolar concentration. After brain injury the level is forcefully increased, even if many of adenosine effects protect neuronal integrity, it, in same events, exacerbates neuronal injury by promoting inflammatory processes (Haskó G. et al., 2005). The main receptors involved in the neuroinflammation modulation are A_1AR and A_{2A}AR. The A_1AR is expressed in microglia and plays an important role on microglia activation (Luongo L. et al., 2014). It was demonstrated that in A_1AR knockout mice there is an increase in neuroinflammation and microglia activity (Synowitz M. et al., 2006). This finding suggests that the A_1AR activation produces a neuroprotective effect, in pathological conditions. Additionally, in physiological and/or pathological conditions, adenosine, by A_1AR activation, reduced the astrocyte proliferation and induced the release of nerve growth factor (NGF) (Sheth S. et al., 2014). In summary, A_1AR is a critical endogenous physiological regulator in neurons and it may be potential therapeutic target in neuroinflammation.

The A_{2A}AR, in physiological condition, is highly expressed in striatal neurons and less in glial cells and neurons outside the striatum. Other brain regions with this receptor subtype are the nucleus accumbens, caudate putamen and olfactory tubercle (Boison D. et al., 2012). It is worth noting that A_{2A}AR expression in microglia increases as a result of brain insults, and this overexpression leads to signal transductions that do not occur in cells with the receptors expressed at a normal level such as facilitating the release of cytokines (Mingehtti L. et al., 2007) On the other hand, A_{2A}AR antagonists suppress microglia activation, *in vitro*, and *in vivo* studies (Gomes C. et al., 2013; Aires I.D. et al., 2019).

2.6. ADENOSINE RECEPTOR LIGANDS

2.6.1. A_1 adenosine receptor agonists

R-PIA (N^6-(R)-phenylisopropyladenosine) was the first molecule synthetized with a selective profile for A_1AR (Daly J.W., 1982).

Majorly, the structural modifications that bring to the discovery of new potent and selective agonists were carried out in N^6 or 2-position of the adenine moiety and in the 3', 4' or 5' positions of the ribose moiety. In additions these modifications increased the metabolic stability of these molecules, improving their therapeutically profile compared with adenosine that has a biological short half-life. Despite this, not all the agonist are structurally analogues of adenosine, the family of 3,5-dicyano-4-phenyl-6-aminopyridine demonstrate also good affinity for this receptor subtype (Jacobson K.A. et al., 2006).

The substitution at N^6 position with alkyl, cycloalkyl and arylalkyl derivatives, that are hydrophobic large groups, brings to selective and potent A_1AR agonists. Some examples of this are N^6-cyclopentyl-adenosine (CPA), N^6-cyclohexyl-adenosine (CHA), 2-chloro-N^6-cyclopentyl-adenosine (CCPA) (Figure 17) (Gao Z.G. et al., 2003). Due to their potency and selectivity, CPA and its derivative are positive pharmacological agents.

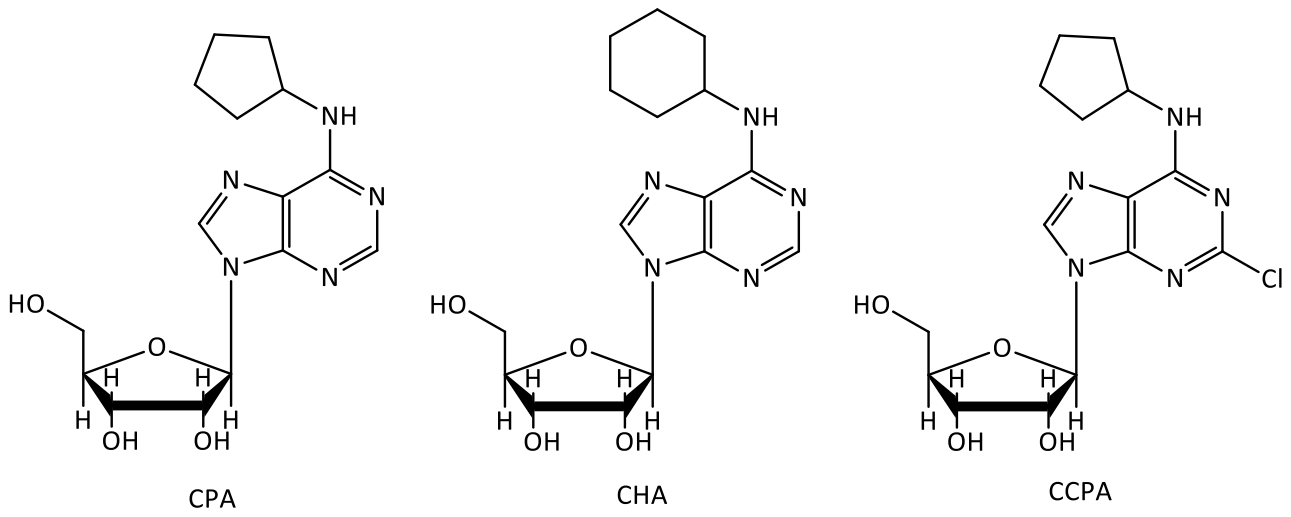


Figure 17. CPA, CHA and CCPA structures.

Other analogues with modification at N^6 position with bicyclic analogues (like S-ENBA) demonstrate to possess subnanomolar activity and selectivity for this receptor (Figure 18). Other with the substitution with cycloalkyl groups or by A_1AR-related heteroaromatic rings are Tecadenoson or

GR79236 with a 3-tetrahydrofuran and 2-hydroxycyclopentyl group respectively (Figure 18). However, these improvements not only have effects on the affinity and selectivity, but also avoid the action of the enzyme adenosine deaminase which degrades adenosine *in vivo*. Despite these modifications had given good results not all were suitable. For example it has been seen that substitution of the hydrogen at N^6 position with an N,N-disubstituted adenosine derivatives decreased the activity. This indicated that this hydrogen should be involved in the interaction with the receptor trough a hydrogen bound.

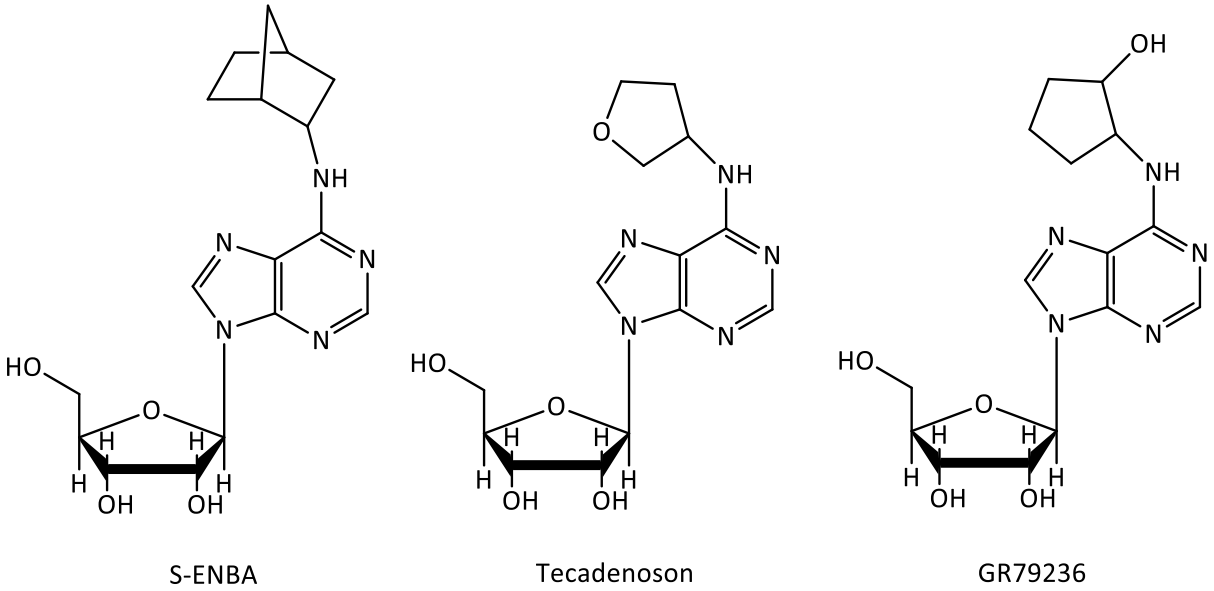


Figure 18. S-ENBA, Tecadenoson and GR79236 structures.

As mentioned above the substitution with a F or Cl atoms on the 2-position increase the affinity and selectivity for the A_1ARs and lower the affinity for A_{2A}AR, like CCPA in compare with CPA (Lohse MG. et al., 1988). Another interesting compound synthetized by the group of the professor Grifantini was the 2-chloro-2'-C-methyl-N^6-cyclopentyl-adenosine (2'-Me-CCPA) (Figure 19) (Maione S. et al., 2007). It has a lipophilic, potent and selective profile for A_1AR (K_i of 1.8 nM in bovine membranes) and antinociceptive effect in animal models. Tecadenoson was a derivative (with a K_i of 6 nM, Gao Z.G. et al., 2007) that reached clinical trials for the treatment of acute paroxysmal supraventricular tachycardia (PSVT). This potential drug on one hand demonstrate to develop a quick action and have not side effects at low dosage, and on the other hand, at elevated doses it could provoke high-degree atrioventricular block. In addition, it has an antilipolytic profile and lowers the levels of free fatty acids (FFA) in animal models. Another drug candidate with lipid-lowering activity but also glucose lowering activity demonstrated thanks to rodent insulin resistance model (type 2 diabetes) was the GR79236. Moreover, a derivate of this compound (CVT-3619) substituted with a (2-

fluorophenylthio)methyl group on the 5'-position of the ribose moiety (Figure 19) showed to be A_1AR partial agonist, similar to Tecadenoson (Dhalla A.K. et al., 2007).

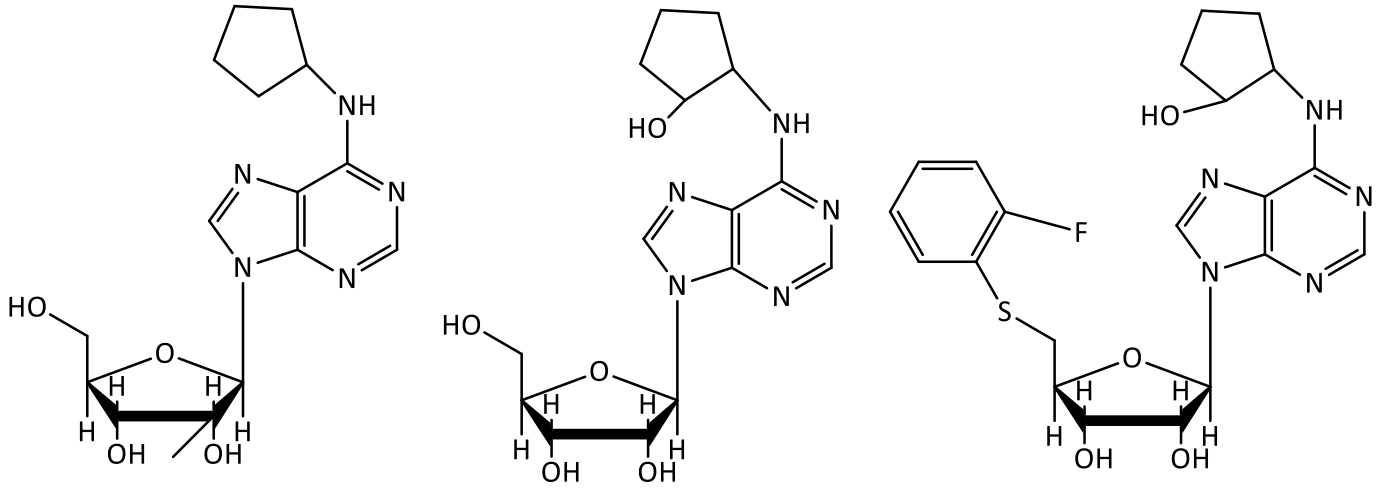


Figure 19. 2'-Me-CCPA,GR79236 and CVT-3619 structures.

One of the most important N^6-cyclopentyl substituted molecule is Selodenoson (Figure 20). This compound differs from the other classical agonists for the presence of the ethylamide substituent on the ribose moiety instead of the 5'CH_2OH group. It demonstrated to have K_i values of 1.1 and of 306 nM for A_1AR and A_{2A}AR respectively in rodent models. Despite the good effects that it showed in the first clinical trials, at the end on the phase II it was discarded due to renal toxicity (Kiesman W.F. et al., 2009).

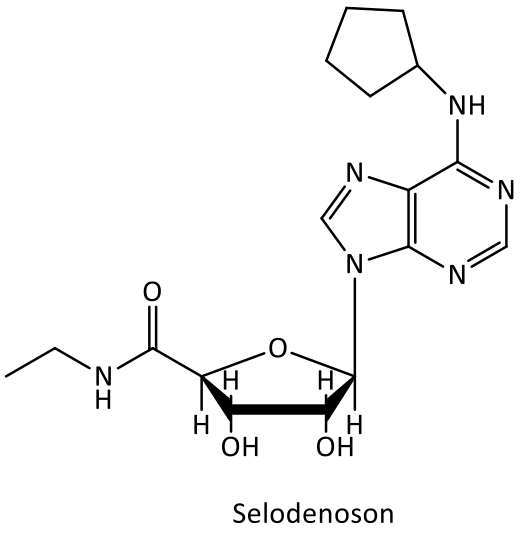
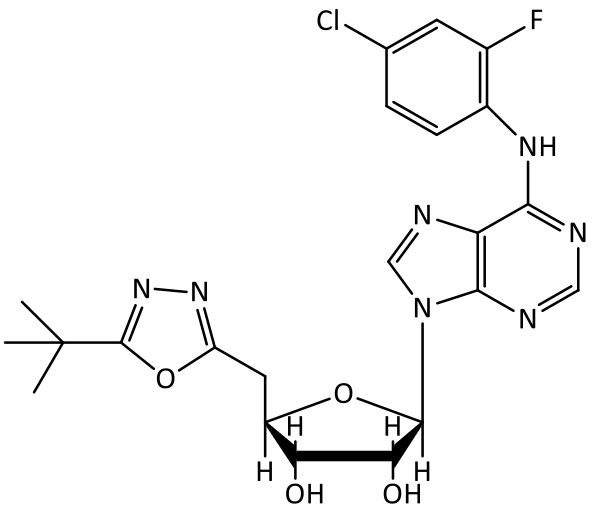


Figure20. Selodenoson structure.

Modification on 5'-hydroxyl group of the ribose brings to very potent compounds, while substitution on 2' or 3'-hydroxyl groups are less suitable for the activity. One compound substituted in 5' with a bulky t-butyl-oxadiazole group is GW-493838 (Figure 21). This A_1AR agonist showed good effects on neuropathic pain in phase II clinical studies, but the investigation was stopped due to severe side effects (Giorgi I. et al., 2008).

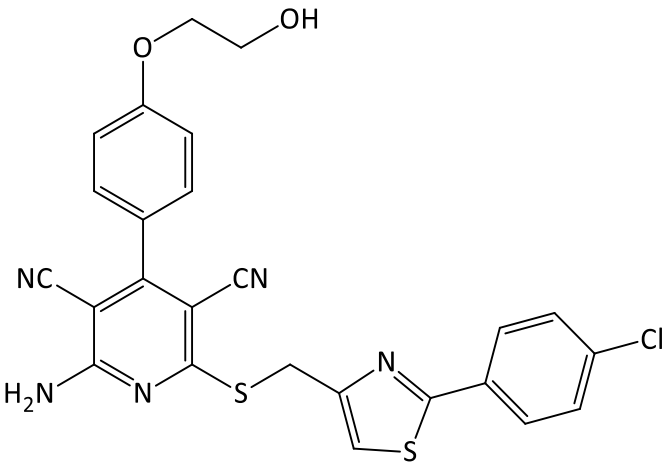


GW-493838

Figure 21. GW-493838 structure.

It has been demonstrated that the substitution of C8 with alkylamino groups and steric bulk at C2 reduce the affinity for the receptor, but there exist some compounds C2-substituted that have demonstrated to be selective against A_1AR, like 2-pyrazolyl (Elzein E. et al., 2007).

In addition of Ado derivatives, exists the non-nucleosidic agonist family. The only members of this family with good effects in human health are 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines. The main compound of this group is Capadenoson, being in phase II of clinical trial for angina and atrial fibrillation (Figure 22). Derivatives of Capadenoson substituted with a (2-hydroxyethyl)thio group exhibited an interesting pharmacological profile (Chang L.C. et al., 2006).

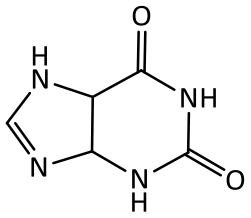


Capadenoson

Figure 22. Capadenoson structure.

2.6.2. A_1 adenosine receptor antagonists

Methylxanthines are the main molecules that have an antagonistic profile against A_1AR (Figure 23). This family of compounds like caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) or theophylline (1,3-dimethylxanthine) are widely distributed in human food such in tea, coffee or chocolate (Kolahdouzan M. et al., 2017). For this reason, their main actions take place following the ingestion. Scientific studies (Mitani T. et al., 2017) demonstrate that theobromine can supress the adipogenesis, restore the normal levels of A_1AR in AD and ameliorate the cognitive functions (Mendiola-Precoma J. et al., 2017). Caffeine showed to have great impacts on neurodegenerative disease like in PD or MS (lowering the risk to suffer of this pathologies), but less in others like ALS (Herden L. et al., 2018). These molecules are not selective for the receptor but have been useful for the discovery and synthesis of selective and potent A_1AR antagonist.



Xanthine

Figure 23. Xanthine structure.

Principally A_1AR antagonists are bicyclic or tricyclic compounds, aromatic, π-electron rich or nitrogen-containing heterocycles. In addition, it had been seen that hydrophilic substituents

transform the antagonist in insoluble compounds in water and made them intolerants for the human body, while the addition of hydrophobic substituents may enhance affinity. In xanthine derivatives, the main substitutions are at N^1, N^3, N^7 and $C8$ positions.

The substitution at C8 position of the xanthine nucleus with aryl or cycloalkyl groups leads to the synthesis of new potent and selective antagonist for this subtype like 8-cyclopentyl-1,3-dimethylxanthine (CPX) (Figure 24). This modification creates a molecule with low affinity for human receptors but high affinity for rat receptors. Structure-Activity Relationship (SAR) of this molecule lead to the synthesis of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Figure 24), a potent and selective antagonist. This antagonist demonstrated to have also several positive effects like the inhibition of phosphodiesterases or the inhibition of renal cell carcinoma proliferation *in vitro* and tumour growth *in vivo* (Zhou Y. et al., 2017).

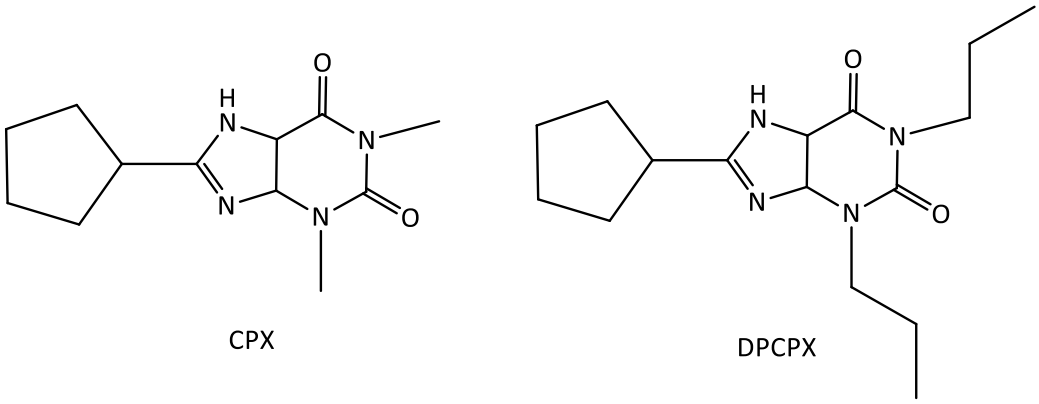
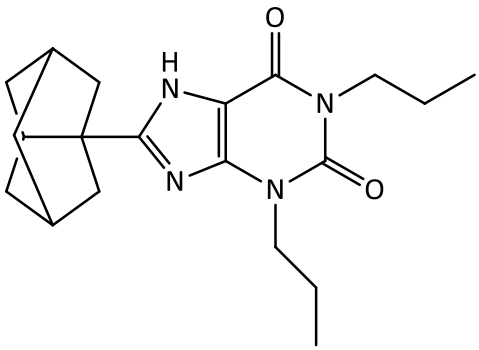
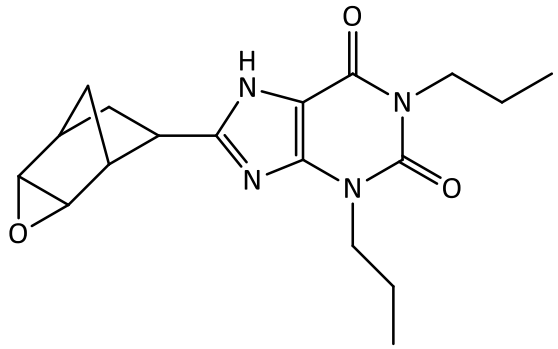


Figure 24. CPX and DPCPX structures.

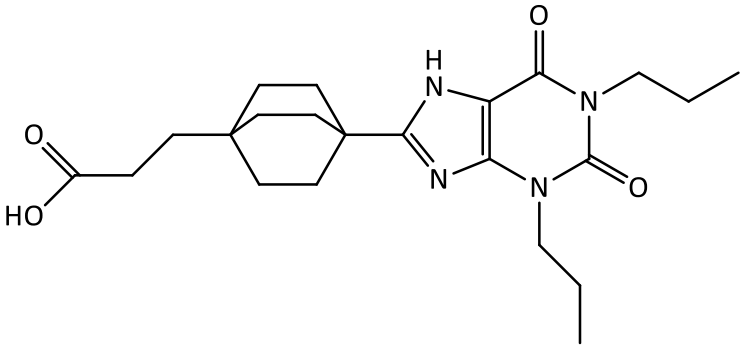
Drug candidates were synthetized with the substitution of a bicyclic group in 8 position such as 1,3-dipropyl-8-(3-noradamantyl) xanthine (Rolofylline) or 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)] xanthine (Naxifylline) (Figure 25). Some studies demonstrate the positive effects of Rolofylline to restore synaptic function and morphology on *ex vivo* assays using a model of cultured organotypic hippocampal slice expressing aggregated levels of tau protein. In addition, the same study showed the prevention of memory impairment in *in vivo* models of mice through several behaviour tests (Dennissen F.J. et al., 2016). Naxifillylline was used to treat renal dysfunction in persons that also suffer from congestive heart failure. It demonstrated to increase the urine output and ameliorate the renal functions without having negative effects on the glomerular filtration rate (Gottlieb S.S., 2008). Tonapofylline and its Imidazoline analogue showed a high affinity and selectivity *versus* hA_1AR (K_i values of 45 and 22 nM respectively) (Figure 25).



Rolofylline



Naxifylline

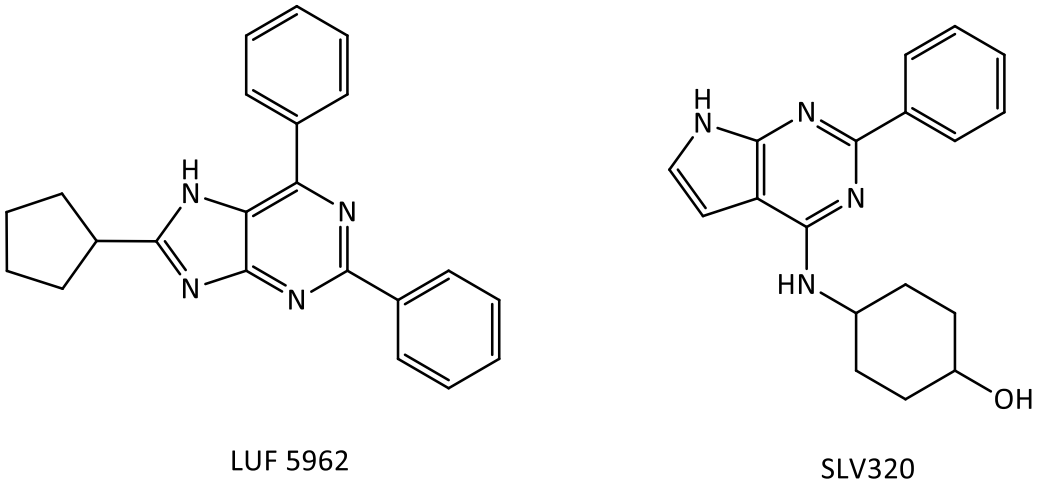


Tonapofylline

Figure 25. Rolofylline, Naxifyline and Tonapofylline structures.

Other compounds were used as radiotracers like 8-cyclopentyl-3-(3-[^{18}F]fluoropropyl)-1-propylxanthyne, [^{18}F]CPFPX. This molecule has a fluorine-18 atom on the N^3 propyl chain and demonstrated to be potent and selective against several A_1AR species (rat = 0.63, pig = 1.37 and human = 1.26 nM) (Ilas J. et al., 2005)

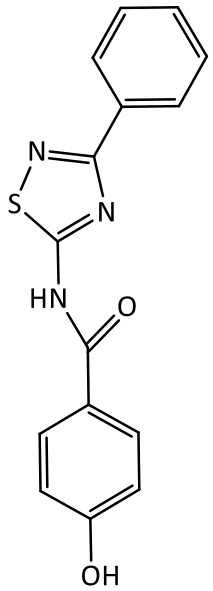
Interesting compounds were the LUF 5962 (with a C8 cyclopentyl substituent) and SLV320 (a 7-deazaadenine derivative with an N^{6-}trans-cyclohexanol side chain), which showed K_i values of 0.29 nM and 1.0 nM, respectively (Kiesman W.F. et al., 2009) (Figure 26).



LUF 5962 SLV320

Figure 26. LUF5962 and SLV320 structures.

LUF5437 (Figure 27) resulted one of the most potent compound thanks to the modification of 1,2,4-thiadiazolobenzamide group (Yuzlenko O. et al., 2009). This compound demonstrates to have a K_i of 7 nM in rat cortical membranes.

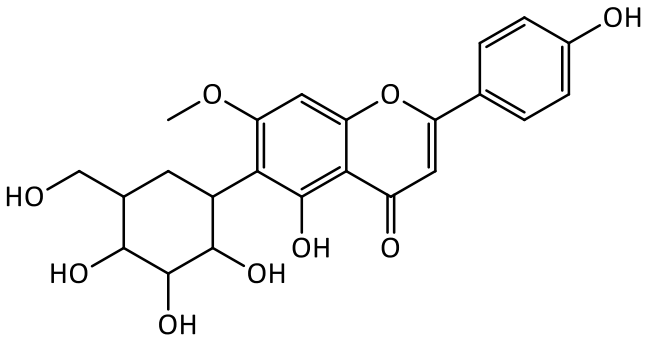


LUF5437

Figure 27. LUF5437 structure.

The discovery of new and potent non-purine A_1AR antagonists was based on virtual screening techniques of several chemical libraries, the structural studies of the receptor and other computational and *in silico* approaches. From these studies various di- and tricyclic scaffolds have been discovered such as 7-amino-5-phenylimidazo-[1,5-b]pyridazine derivative, MRS5942 that

shows a K_i of 63 nM (Rodríguez D. et al., 2015). In addition, molecules with natural origin demonstrated to have beneficial effects through the inhibition of A_1AR such like Swertisin or the methoxy flavonoids from *Orthosiphon stamineus* (Figure 28). The first one is a flavonoid present in the *Swertia japonica*, that has demonstrated to ameliorate the cognitive function in rodent's model (Lee H.E. et al., 2016), while the second ones have diuretic actions (Yuliana N.D. et al., 2009).



Swertisin

Figure 28. Swertisin structure.

2.6.3. A_{2A} adenosine receptor agonists

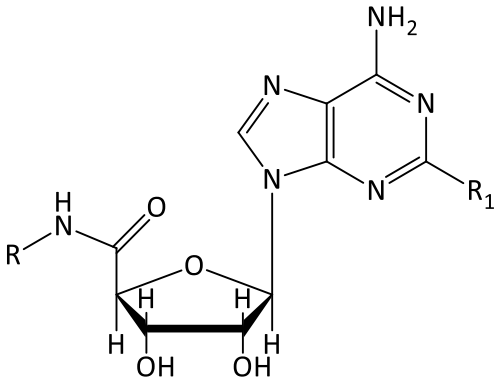
The major part of A_{2A}AR agonists are nucleoside derivatives, despite this, exist some peculiar non-nucleoside ligands without ribose or ribose mimic. Some examples are the of 3,5-dicyanopyridines and cyanopyrimidines (Lane J.R. et al., 2012). One of the main difficulties that the scientific community have been facing for the synthesis of new anti-inflammatory agonists is the high hypotension caused. Recently, drugs for the treatment of chronic obstructive bronchopathies are emerging as interesting items to study. In some cases, prodrugs possessing phosphate groups in 5' position are created because they can be activated in the inflammation site thank to the activity of the ecto-nucleotidase.

5'-N-Ethylcarboxamidoadenosine (NECA) was one of the first agonist discovered for the A_2AR. Potent A_{2A}AR agonists were discovered with the substitution with small dimension groups such as alkylamidic (Müller C.E. et al., 2011) or 5'-N-cyclopropyl (Day Y.J. et al., 2005). It has been demonstrated that the addition in 2 position of the purine nucleus of amine, ether, thioether or alkyl groups leads to the synthesis of several molecules with an increased potency and selectivity for A_{2A}AR. Taking into account the NECA structure, several compounds were developed, among them the 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-Nethylcarboxamidoadenosine (CGS21680)

was the most interesting compound (Figure 29). Studies demonstrated that the pre-treatment with CGS21680 attenuated pulmonary injury before a cardiopulmonary bypass, but it demonstrated to be less potent in humans than in rats (K_i 27 nM and 22 nM respectively) (Kong X. et al., 2019). SAR of this molecule underlined that the 2-phenylethylamine group increments the affinity and it is important for receptor binding.

The ATL146e (Apadenoson) is a molecule 50 times more potent than CGS21680 and showed a K_i of 0.5 nM. It has been entered in several clinical trials against myocardial perfusion imaging, but unfortunately the clinical development was discontinued (Rieger J.M. et al., 2001) (Figure 29). ATL-313 substituted with a cyclopropyl group in 5' position demonstrated to have beneficial effects *in vivo* in the reduction of adhesion molecules in perfused vascular endothelium. (Okusa M.D. et al., 2000) (Figure 29). In fact, it has been seen in mice models a protection against mechanical allodynia and thermal hyperalgesia after only one intrathecal injection of CGS 21680 or ATL-313 (Loram L.C. et al., 2009).

HENECA (2S,3R,5R)-5-[6-amino-2-(hex-1-yn-1-yl)-9H-purin-9-yl]-N-ethyl-3,4-dihydroxyoxolane-2-carboxamide) showed an agonist profile against $A_{2A}R$ with a small selectivity for A_3AR but not *versus* A_1AR (Figure 29). In fact, it has been demonstrated that the activation of $A_{2A}AR$ is involved in the synthesis of Aβ and neurodegeneration. Concretely, it has been shown that there is an interaction of $A_{2A}AR$ with γ-secretase, specifically with the catalytic subunit PS1. Moreover, the overproduction of Aβ and the increment of APP levels and γ-secretase activity it has been correlated with G_s protein and cAMP/PKA signal pathway after the activation of $A_{2A}AR$ (Lu J. et al., 2016). This was elucidated trough the activation of the receptor with HENECA, which increased the activity of γ-secretase and following the Aβ1–42 formation in human neuroblastoma cell line (Nagpure B.V. et al., 2014).



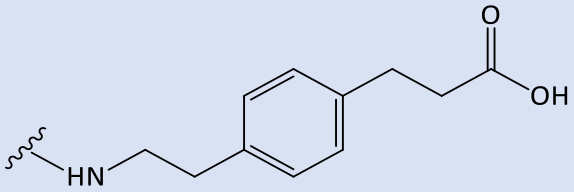
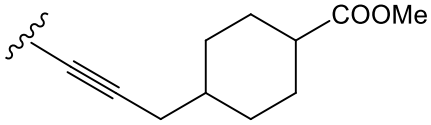
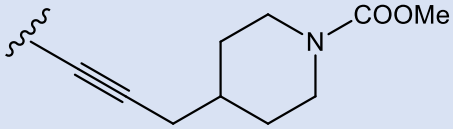
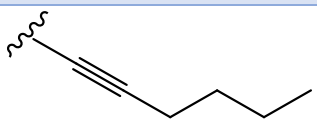
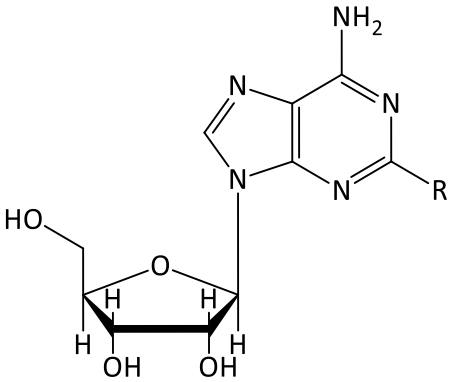
Compound	R	R_1
CGS21680	$-CH_2-CH_3$	
ATL146e	$-CH_2-CH_3$	
ATL313	cyclopropyl	
HENECA	$-CH_2-CH_3$	

Figure 29. CGS21680, ATL146e, ATL313 and HENECA structures.

There are some molecules with bulky groups in position 2, but without a group in position 5' like Regadenoson, Binodenoson and Sonedenoson (Figure 30). Regadenoson demonstrated to be well tolerated for cardiac MRI stress tests and safety for use (Bastarrika G. et al., 2019). In addition, it showed to be useful as a coronary vasodilator and a fast producer of hyperaemia handy radionuclide myocardial perfusion imaging. Due to the beneficial effects of this drug, it is actually marked by GE Healthcare. At the same time, Binodenoson causes maximal coronary hyperaemia in pharmacological stress testing but it demonstrated to have some low affinity *versus* A_1AR, $A_{2B}AR$, and A_3AR. Finally, Sonedenoson has been demonstrated to fast the wound healing process in mice, but regardless of this the Phase II clinical trials failed (Mantell S. et al., 2013).



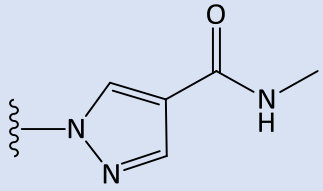
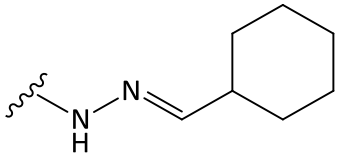
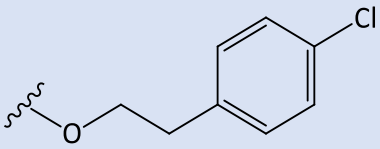
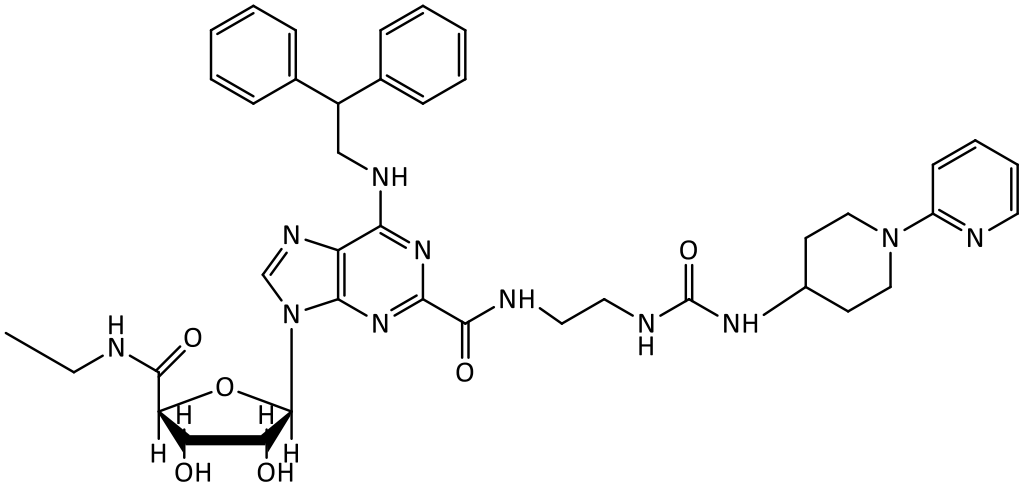
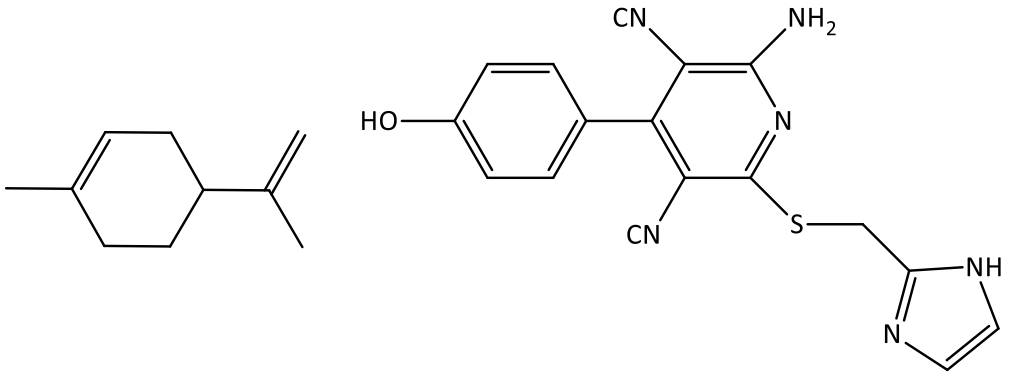
Compound	R
Regadenoson	
Binodenoson	
Sonedenoson	

Figure 30. Regadenoson, Binodenoson and Sonedenoson structures.

The UK-432097 one analogue of N^6-2,2-dipheninyladenosine ligand is a selective agonist against $A_{2A}AR$ with a K_i of 4 nM (Figure 31). Clinically it has been involved in trials for chronic obstructive pulmonary disease, but due to the lack of positive effects, investigations were closed. In addition, there are some agonists for $A_{2A}AR$ from natural compounds. One example of this is limonene (Park H.M. et al., 2011) that can be found in the citrus rind (Figure 31). It produces sedative effects due to its selective affinity for $A_{2A}AR$. Another non-nucleoside ligand that has been discovered as partial agonist is LUF5834 (6-amino-3,5-dicyano-4-phenyl-2-thiopyridines) (Figure 31) (Lane J.R. et al., 2012)



UK-432097



Limonene

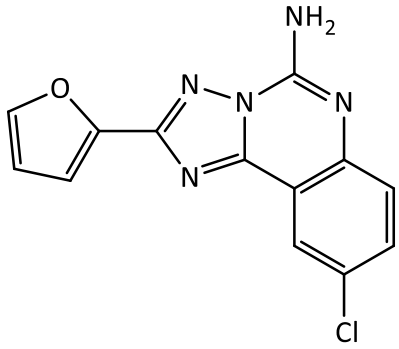
LUF5834

Figure 31. UK-432097, limonene and LUF5834 structures.

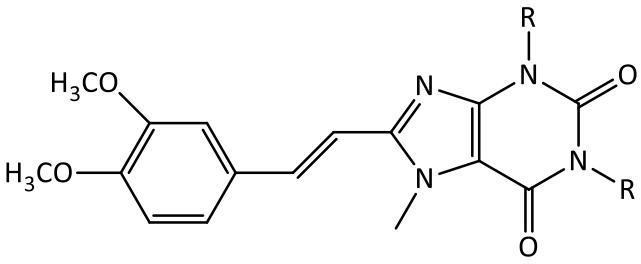
2.6.4. A_{2A} adenosine receptor antagonists

Starting from xanthine nucleus A_{2A}AR antagonists were designed and synthetized. One of the first molecule synthetized was the CGS-15943, a pyrazolo[4,3-e][1,2,4]Triazolo[1,5-c]Pyrimidine (PTP) derivative (Figure 32). Newly, a group of scientists defined that PTP antagonists were advantageous for drug screening at the A_{2A}AR (Duroux R. et al., 2017). This molecule showed some potency but was not selective against the other receptors, for this reason, many SAR were performed. Substitutions on 8 position with styrene groups lead to the discovery of ligands with a good antagonistic profile, for examples KW-6002 and KF17837 (Figure 32). Both of them have in position 8 a 3,4-dimethoxyrene but diverge in the position 1 and 3 where they have two ethyl and two propyl chains respectively. Actually, KW-6002 is approved for Parkinson's disease in Japan and in Phase III

in Europe (Oertel W. et al., 2016). It is important to highlight that this family of analogues, demonstrate to be selective for $A_{2A}AR$ *versus* the other subtypes.



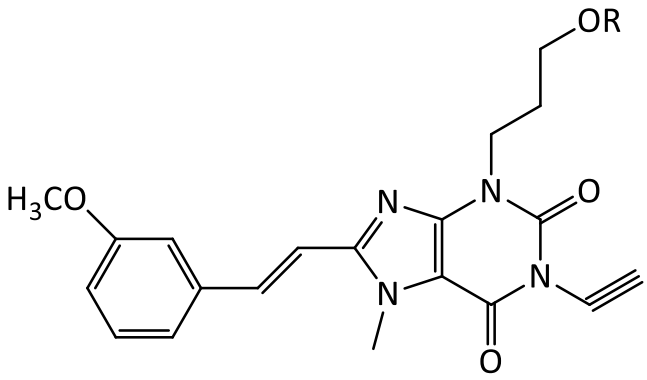
CGS-15943



R = ethyl = KW-6002
R = propyl = KF17837

Figure 32. CGS-15943, KW-6002 and KF17837 structures.

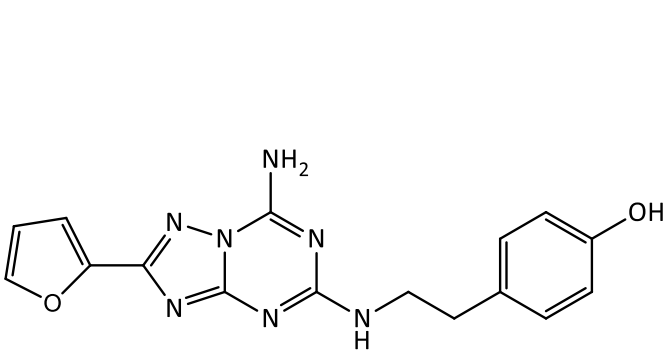
It has been seen that modifications at at the 1 and 3 positions increase the aqueous solubility (such as in MSX-2) or produce prodrugs (like MSX-3 and MSX-4 (Vollmann K. et al., 2008) (Figure 33). MSX-3 has a propylic end portion with a phosphate group, *in vivo* this phosphor group is eliminated and the molecule becomes an effective drug. MSX-4 has been shown to possess several good effects like the reversion of the locomotor suppression provoked by eticlopride in open field test, the decrease of c-Fos and pDARPP-32 after the injection of eticlopride in the nucleus accumbens and the elimination of oral tremor. For these reasons, MSX-4 is proposed as a compound that can be useful for the treatment of PD and motivational symptoms of depression (Santerre J.L. et al., 2012).



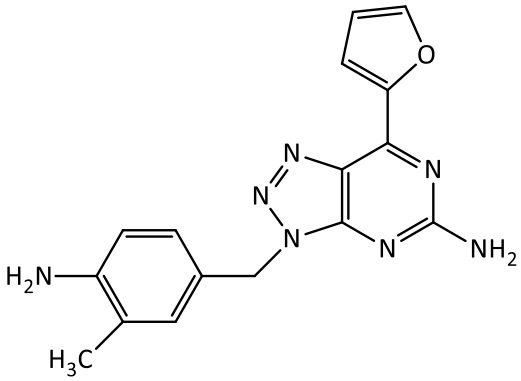
Compound	R
MSX-2	H
MSX-3	PO_3Na_2
MSX-4	L-valine ester

Figure 33. MSX-2, MSX-3 and MSX-4 structures.

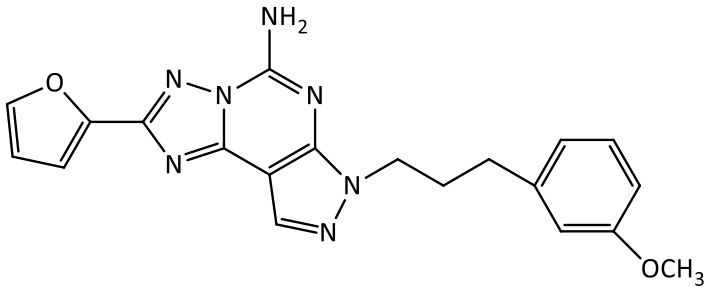
With the triazoltriazine scaffold several interesting antagonists were obtained such as ZM241385, SCH442416 or Vipadenant (Figure 34). These molecules demonstrated to have a very high affinity and selectivity for the A_{2A}AR, with the exception of ZM241385 who also binds the A_{2B}AR. Among these compounds, some of them demonstrate to be useful for the treatment of PD such as SCH420814, SYN115 (Tozadenant), and V2006 (Vipadenant) (Figure 34).



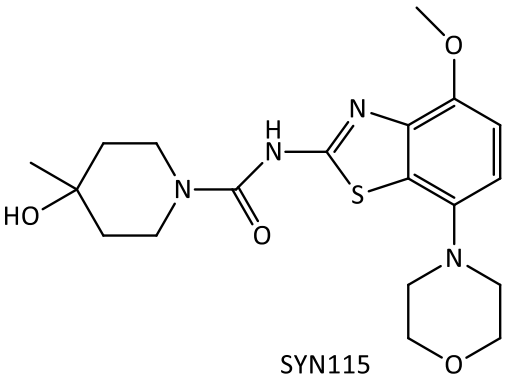
ZM241385



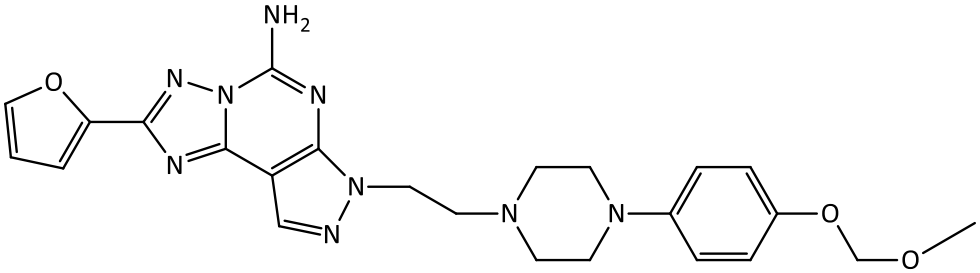
Vipadenant



SCH442416



SYN115



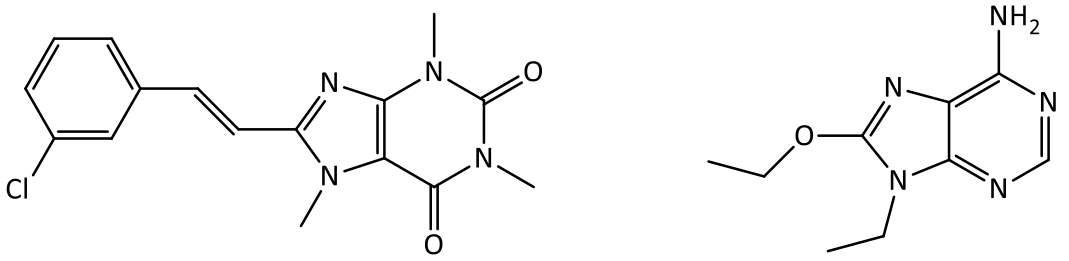
SCH420814

Figure 34. ZM241385, Vipadenant, SCH442416, SYN115 and SCH420814 structures.

The 8-(3-chlorostyryl)caffeine (CSC) demonstrated to be selective against A_{2A}AR and to possess beneficial in HD models diminishing the striatal atrophy or degeneration. In addition, this compound showed to inhibit the activity of monoamine oxidase-2 (MAO-2). For these reasons, this compound

could be beneficial for the treatment of neurodegenerative diseases (Brunschweiger A. et al., 2014). (Figure 35)

Volpini and co-workers synthetized a low molecular weight ligand known as 8-ethoxy-9-ethyladenine (ANR 94) which demonstrated to have beneficial effects in PD models of akinesia and tremor (Pinna A. et al., 2010) (Figure 35).



CSC ANR94

Figure 35. CSC and ANR94 structures.

2.6.5. A_{2B} adenosine receptor agonists

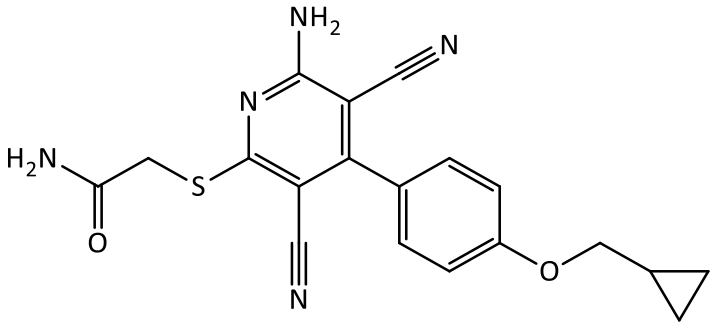
Adenosine, the natural agonist of this receptor, possesses EC_{50} values in the micromolar range, while inosine only activates the receptor at high concentrations (Doyle C. et al., 2017). A_{2B}AR ligands can be divided in adenosine-like and non-adenosine-like ligands.

Actually, not many ligands with a selective profile against A_{2B}AR exist. The main modifications were carried out in the purine ring on N^6 and C2 position or at the 5'-position of the ribose moiety. The mainly molecule considered as agonist it is NECA, that possesses a K_i of 140 nM, but it is not selective (Baltos J.A. et al., 2017).

One example of 2-aminopyridine-3,5-dicarbonitrile derivative is BAY 60-6583 (2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio) acetamide), which was described as a selective and potent (low nanomolar range) agonist *versus* this receptor (Goulding J. et al., 2018). (Figure 36). Initially it was described as a full agonist, recently it has been reported to be a partial agonist. Müller's group synthetized the BAY 60-6583 tritiated ([^{3}H]BAY-60-6583). Unfortunately, this molecule was not useful for binding assays due of its elevated non-specific binding values (Hinz S. et al., 2018).

The capacity of a ligand to perform a single receptor conformation to turn on an intracellular cascade is named "functional selectivity" or "biased agonism". Biased agonism is crucial for clinical studies because help to the design of drugs that can activate intracellular pathways avoiding the negative effects. In fact, some studies demonstrated that BAY60-6583 acts as a biased agonist,

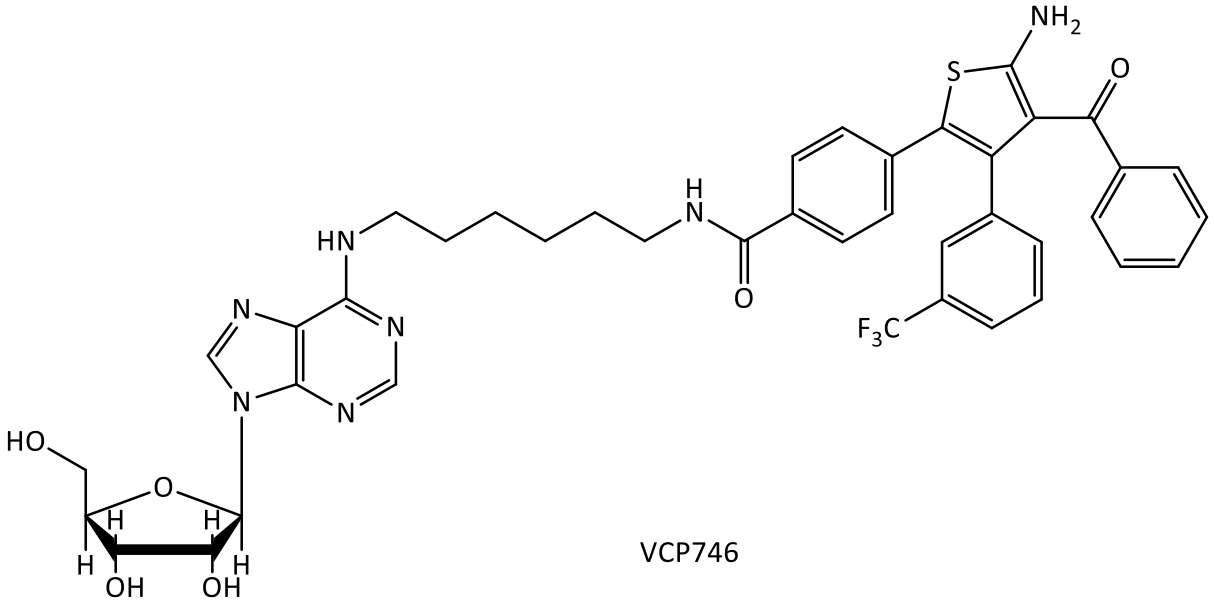
which can even act as an antagonist in mouse pancreatic β-cells expressing low levels of A_{2B}AR (Gao Z.G. et al., 2014).



BAY 60-6583

Figure 36. BAY 60-6583 structure.

Vecchio and co-workers synthetized a compound named VCP746 (Figure 37). This ligand showed the adenosine core linked by alkyl chain at position N^6 with an allosteric enhancer moiety for the A_1AR. VCP746 was able to activate A_{2B}AR with an affinity similar to NECA and BAY 60–6583 in cAMP assays (Vecchio E.A. et al., 2016a; 2016b). Despite these good results, it is unselective. Recently it has been demonstrated that VCP746 possess interesting profile in cardiac cells due to activation of A_{2B}AR like Capadenoson (Vecchio E.A. et al., 2019).

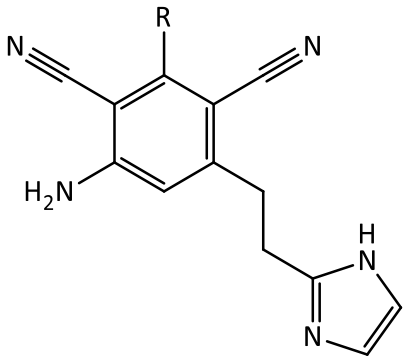


VCP746

Figure 37. VCP746 structure.

Beukers and co-workers synthetized a 2-aminopyridines series (Figure 38). This series showed a good affinity *versus* A_{2B}AR but they are in general unselective. LUF5834 (EC_{50}=12 nM) was one of the compounds from the series that took the attention of the investigators due to its potency at

A_{2B}AR and the selectivity against A_3AR. LUF5835 with an EC_{50}=10 nM demonstrates to be a powerful agonist. Two other compounds of the group that were potent against A_1AR and A_{2B}AR respectively were LUF5844 (EC_{50}=34 nM) and LUF5845 (EC_{50}=9 nM), the last one behaving as a partial agonist (Beukers M.W. et al., 2004).



Compound	R
LUF5834	*p*-OH phenyl
LUF5835	m-OH phenyl
LUF5844	*m*-OCH_3 phenyl
LUF5845	*p*-OCH_3 phenyl

Figure 38. LUF5834, LUF5835, LUF5844 and LUF5845 structures.

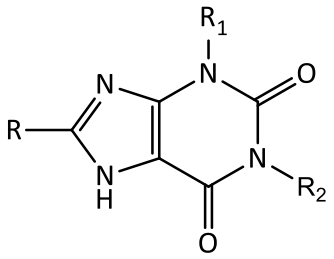
As previously mentioned, Capadenoson demonstrated to be selective against the A_1AR, but a recent work showed that this molecule could also bind with the A_{2B}AR (K_i=300 nM). In functional experiments Capadenoson exhibited an EC_{50} equal to 0.66 nM for A_1, 1,400 nM for A_{2A}AR and 1.1 nM for A_{2B}AR (Dal Ben D. et al., 2019).

2.6.6. A_{2B} adenosine receptor antagonists

A_{2B}AR antagonist can be classified into two groups, the xanthine derivatives and the non-xanthine derivatives. The first and well known A_{2B}AR antagonists are caffeine and theophylline. These compounds are not selective for this receptor but they can be used at micromolar concentrations for several purposes such as central stimulants, antiasthmatic agents, adjuvants in the treatment of cancer or even to improve lung function (Popović D.J. et al., 2018).

Various A_{2B}AR antagonists were discovered with high water solubility and good selectivity values (Figure 39). Some of them are: MRE-2029-F20, MRS1754 (with Th1-suppressive effect and an inhibition of cell growth in colon carcinoma), GS-6201 (a potent one with a pyrazole ring), PBS-1115 (with a sulphonic group useful in *in vivo* studies and to decrease inflammatory pain on mice models), PBS-603 (with high affinity in humans and avoiding cell proliferation on prostatic cancer cells) or

ATL-802. In particular, PSB-603 possesses subnanomolar affinity in *in vitro* studies demonstrating its ability to inhibit the cell growth and the transmigration. All these effects were related with the apoptotic cascade signalling and cell cycle stop (Wilkat M. et al., 2019).



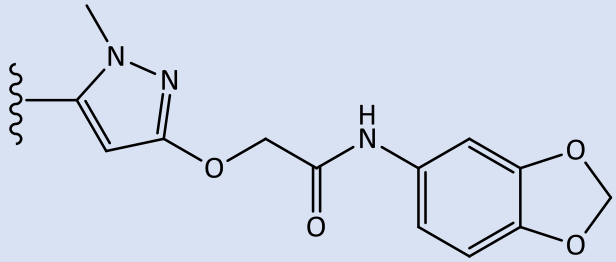
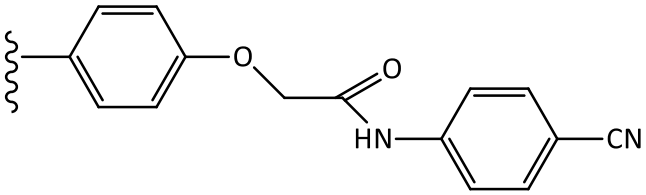
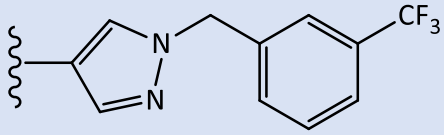
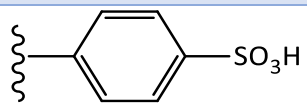
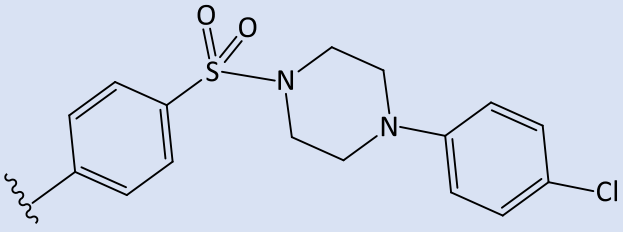
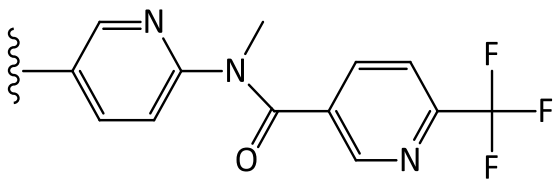
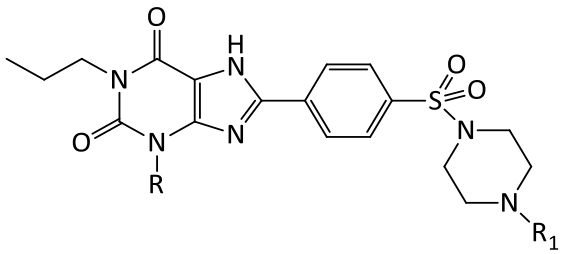
Compound	R	R_1	R_2
MRE-2029-F20		$-CH_2-CH_2-CH_3$	$-CH_2-CH_2-CH_3$
MRS1754		$-CH_2-CH_2-CH_3$	$-CH_2-CH_2-CH_3$
GS-6201		$-CH_2-CH_3$	$-CH_2-CH_2-CH_3$
PSB-1115		-H	$-CH_2-CH_2-CH_3$
PSB-603		-H	$-CH_2-CH_2-CH_3$
ATL-802		$-CH_2-CH_2-CH_3$	cyclopropyl

Figure 39. MRE-2029-F20, MRS1754, GS-6201, PSB-1115, PSB-603 and ATL-802 structures.

Müller and co-workers were able to synthetize several molecules and ligands with interesting antagonistic profiles against $A_{2B}AR$ (Jiang J. et al., 2019) (Figure 40 and Figure 41). Using compounds PSB-1115 and PSB-603 as base compounds, 8-phenylxhantive derivatives were synthetized. The major part of them demonstrated to have good affinity for $A_{2B}AR$ and some of them even with subnanomolar K_i. In addition, 1-propyl substitution demonstrated to increase the affinity while the NH group at position 7 is indispensable for the interaction with the receptor (Müller C.E. et al., 2018). The substitution at 3 position by removing the N3-H donor is preferred not to be done, nevertheless, small groups can be tolerated such as H, methyl and ethyl. Even though compounds with methyl (C**1**) and ethyl (C**2**) groups at 3 position showed to be potent and selective for $A_{2B}AR$ (K_i 1.91 and 4.31, respectively). The elimination of Cl substituent at *para* position (C**3**) reduced the selectivity while the replacement of the piperazine ring by a dimethylamine leads to a potent antagonist (C**10**) (K_i = 18.6 nM). The addition of a phenethyl substituent to the benzenesulfonyl moiety bound to the 8 position of the tetrahydro-purine core leads a potent and selective ligand (C**12**) with K_i values of 3.62 nM. Aromatic residues, N-methylation and disubstitution of sulphonamide N-atoms (C**11**) help to maintain or increase the selectivity $A_{2B}AR$.

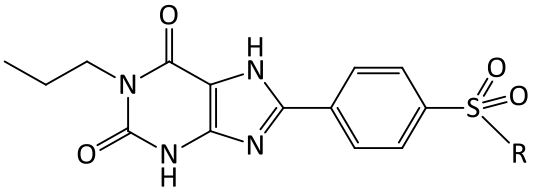
Mono-substitutions on the aromatic ring are well tolerated as long as the substitution is lipophilic. In addition, *para* substituents bulkier that F (Cl, Me, OMe…) together with rigid phenyl piperazines demonstrated to be more selective. *P*-halogen-substituted phenylpiperazine can be rank according the substituent being the most suitable Br (C**5**), I (C**6**), Cl (PSB-603) and F (C**4**) respectively. It is notably to note that compound C**5** has a K_i=0.0835 nM and a selectivity 10000-folds against the other subtypes.

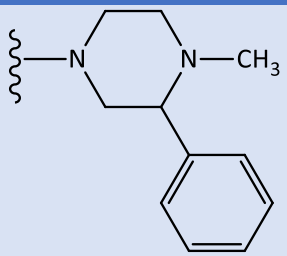
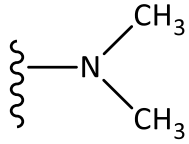
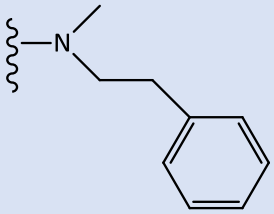
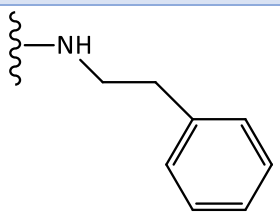


Compound	R	R_1	K_i $A_{2B}AR$ (nM)
1	Methyl	4-chlorophenyl	1.91
2	Ethyl	4-chlorophenyl	4.31
3	-H	Phenyl	0.643
4	-H	4-fluorophenyl	0.64
5	-H	4-bromophenyl	0.0835
6	-H	4-iodophenyl	0.16
7	-H	Benzoyl	5.7
8	-H	1-phenylethyl	8.3

Figure 40. Christa E. Müller's compounds and their structures.

In addition, the insertion between the aromatic residue of the phenylpiperazine and the piperazine ring (C**4**) of a methylene or ethylene group (C**13** and C**14**) it is well accepted. The potency of these compounds is inversely proportional to the length of the linker between the piperazine ring and the aromatic residue of the phenylpiperazine. In fact, compound **3,** without a linker among them, has a K_i=0.643 nM, while compound **11,** which possess a methylene linker, shows a K_i=3.6 nM and the compound **14,** with an ethylene linker, has a K_i=7.51 nM. Compound **9** (K_i=7.15 nM) also demonstrated a high potency due to the presence of phenyl group in 3-position. It is interesting to note that an insertion of a phenyl group to the piperazine *N*-atom leads to the most affine and selective compounds for A_{2B}AR. On one hand it has been seen that with a 1-methylbenzyl (C**8**) or a benzyl (C**13**) the ligand still remains potent and selective, while a carbonyl linker decreases the ratio affinity between A_{2A}AR an A_{2B}AR, like in compound **7**.



Compound	R	K_i A_{2B}AR (nM)
9		7.15
10		18.6
11		3.6
12		3.62

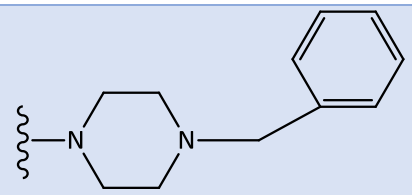
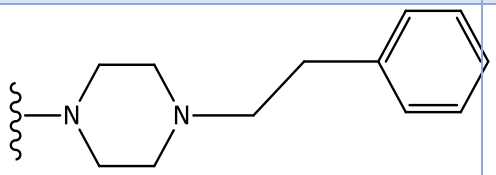
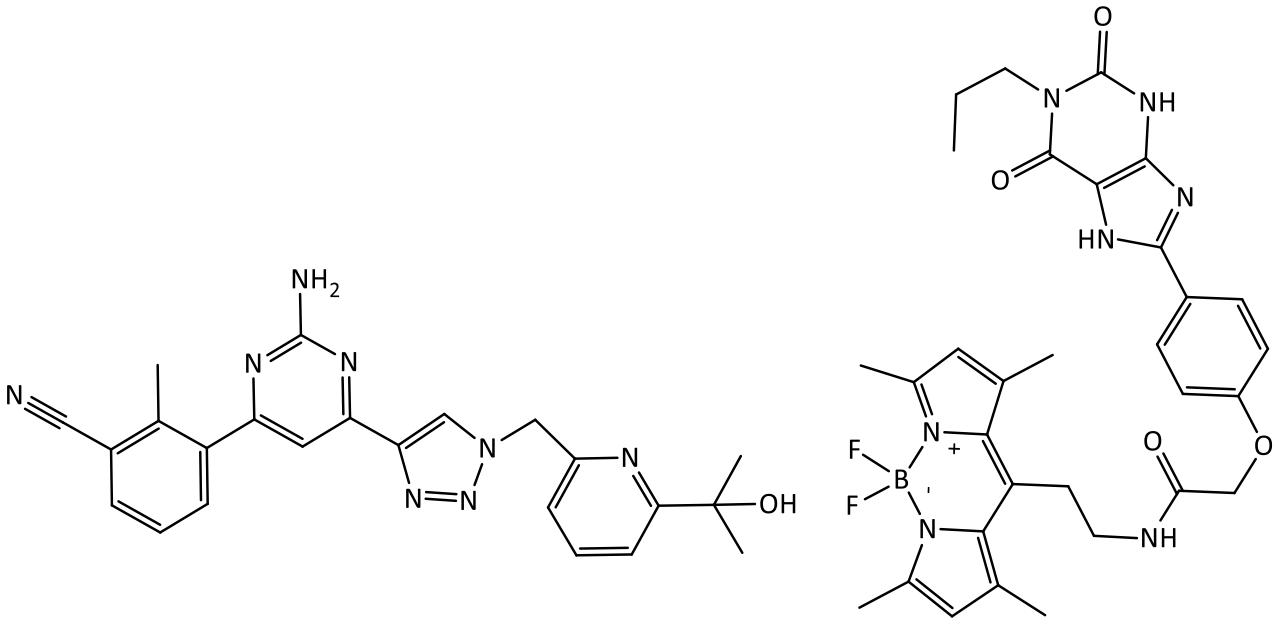
13		3.6
14		7.51

Figure 41. Christa E. Müller's compounds and their structures.

Recently, a new dual $A_{2A}AR/A_{2B}AR$ antagonist named AB928 was tested in healthy patients (Figure 42). This compound did not show harmful effects or alter any physiologic parameters. The ≥90% ARs inhibition is related with AB927 plasma levels ≥1 µM (Seitz L. et al., 2019). Another compound with an interesting profile was the xanthine derivative PSB-12105, which showed nanomolar or subnanomolar affinity for mice, rat and human receptor (Köse M. et al., 2018) (Figure 42)

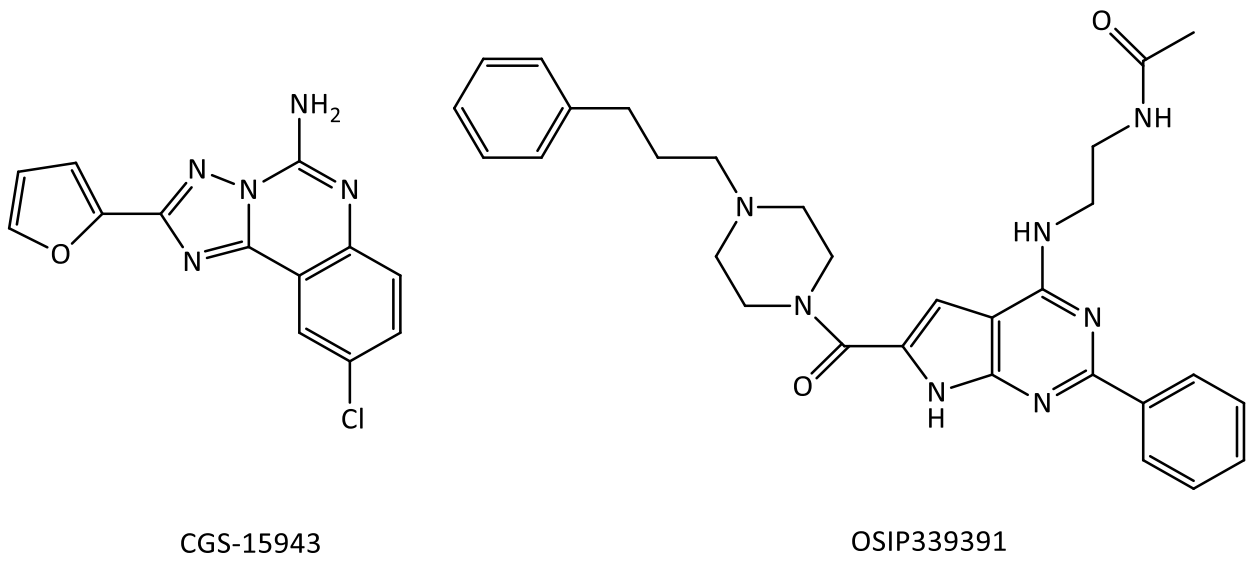


AB928

PSB-12105

Figure 42. AB928 and PSB-12105 structures.

Non-xanthine molecules similar to adenine were used to synthetize the $A_{2A}AR$ ligands but some of them demonstrated to have some affinity for $A_{2B}AR$ such as ZM241385 and CGS-15943 (Figure 43). Another ligand with antagonistic profile that was radiolabelled was OSIP339391 (Figure 43).



CGS-15943 OSIP339391

Figure 43. CGS-15943 and OSIP339391 structures.

2.6.7. A_3 adenosine receptor agonists

IB-MECA (N^6-(3-iodobenzyl) adenosine-5'-N-methyluronamide) is an A_3AR selective agonist. This was achieved by adding in N^6 of the adenosine a benzyl group with an iodine and an amide function in ribose 5' position. In addition, it has been demonstrated that the addition of N^6-benzyl was a well-tolerated substitution that maintained the affinity for the A_3AR but decreased *versus* the others subtypes making this compound selective. Starting from IB-MECA (Piclidenoson) and with a modification with a 2-chloro group, a more selective compound was synthetized, the Cl-IB-MECA (Namodenoson). IB-MECA and Cl-IB-MECA are both in clinical trials for inflammation and cancer respectively. The first one is going to start the Phase II for the treatment of rheumatoid arthritis and psoriasis, while the second one will begin Phase II for primary liver cancer. Two analogues of IB-MECA and Cl-IB-MECA were the thio-IB-MECA and thio-Cl-IB-MECA (Figure 44). The last one demonstrated to be useful for the treatment of cancer or inflammation due to the anti-angiogenic proprieties. Another interesting molecule that acts as agonists is the well-known HEMADO. This methyladenosine, carrying an ethinylhexyl group in 2 position, possesses high selectivity and affinity for the A_3AR (Volpini R. et al., 2009b).

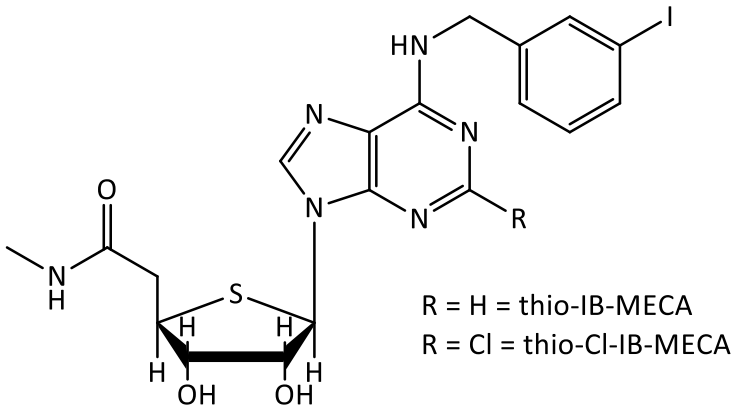


Figure 44. Thio-IB-MECA and thio-Cl-IB-MECA structures.

In addition, CP-532903 and CP-608039 are indicated to be useful for cardioprotection as anti-ischemic agents (Wan T.C. et al., 2008). These two compounds possess in 3' position of the ribose an amino group (Figure 45).

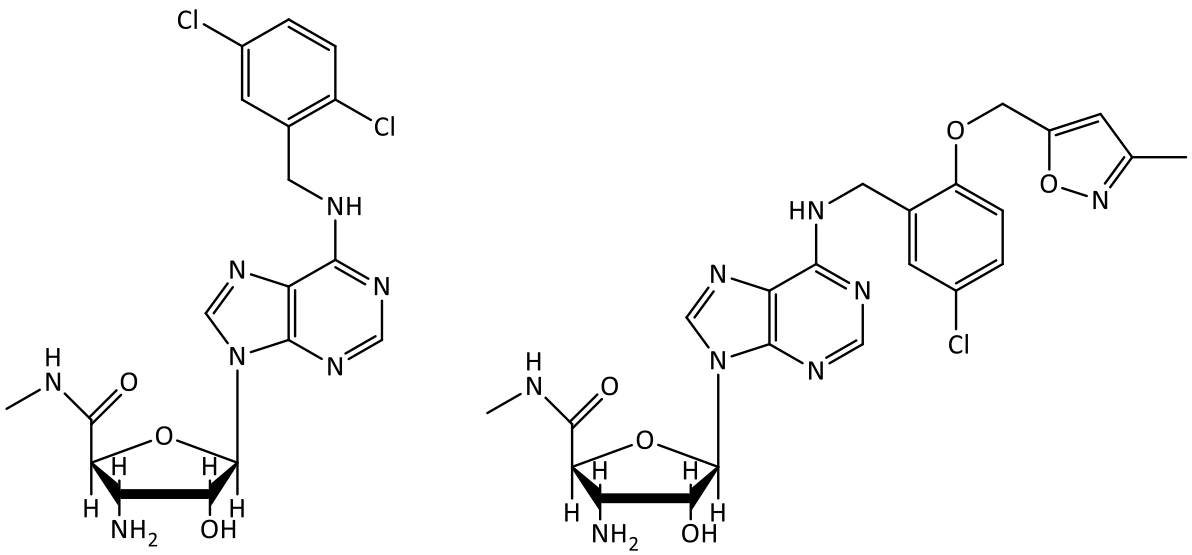
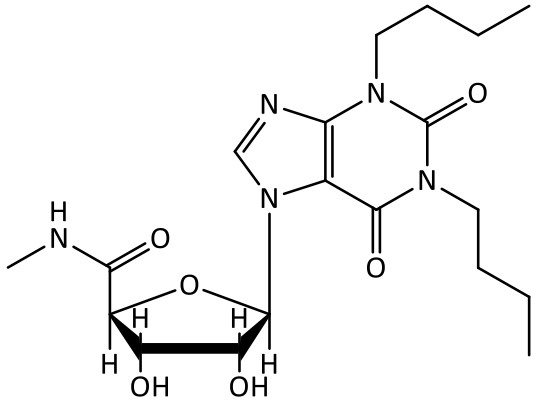


Figure 45. CP-532903 and CP-608039 structures.

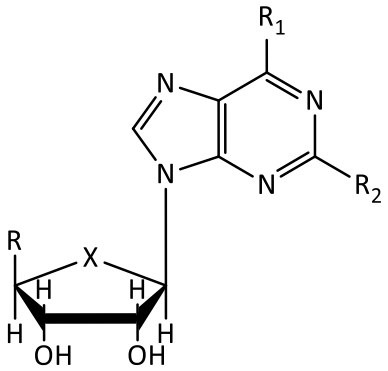
The agonist $A_{2A}AR$ known as CGS21680 also showed to possess high affinity for the A_3AR. SAR demonstrated that small alkyl or alkoxy groups could be linked to N^6; or that xantine-7-ribosides (DBXRM) lead to a good selectivity of A_3AR (Figure 46).



DBXRM

Figure 46. DBXRM structure.

Recently, Jacobson's group reported (Jacobson K.A. et al., 2018) that the position at 5'-ribose can be substituted with an analogue of 5'-methyl ether like in compound NNC53-0055 (Figure 47). The addition of an N-methanocarba group leads to useful ligands for this subtype such as MRS1898 or compounds with terminal alkyl and alkylene at C2 group like MRS5221 (Figure 47). Two other compounds, which demonstrate interesting profiles, were the MRS5698 and the MRS5841 (Figure 47). The first one is a 3,4-difluorophenyl ethynyl member with high affinity for the A_3AR receptor (K_i = 3 nM), while the second one has a sulfonate group that avoids passing the BBB, making it useful to differentiate peripheral and central impacts of the A_3AR. One modification that allowed the full activation of the A_3AR was the substitution of the NH from the adenosine with an H or CH_3, like in MRS5919 (Figure 47).



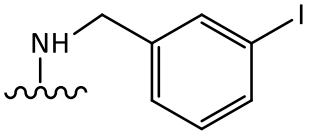
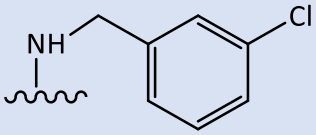
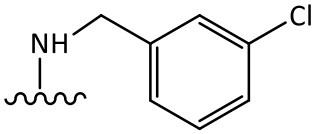
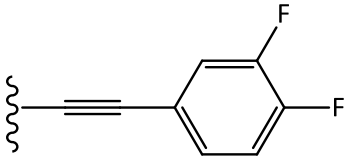
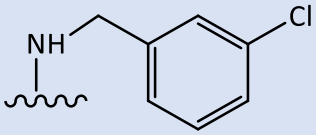
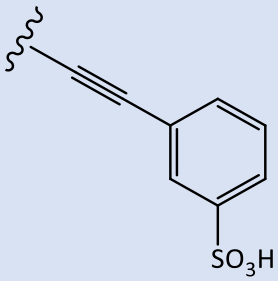
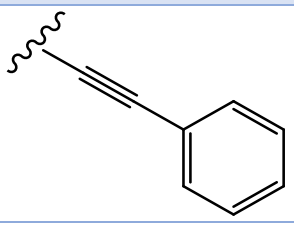
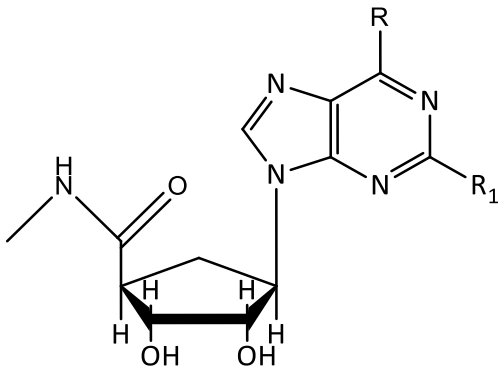
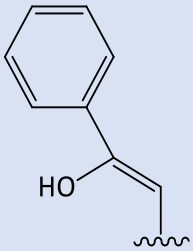
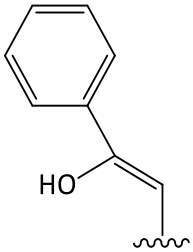
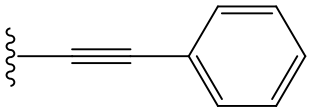
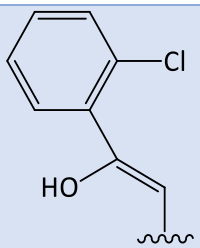
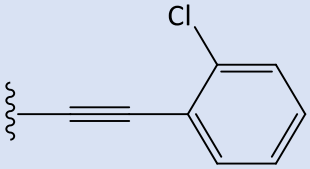
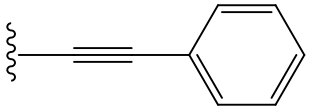
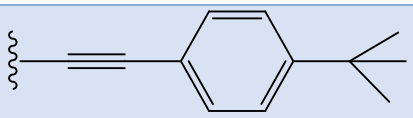
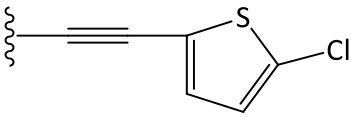
Compound	R	R_1	R_2	X	K_i A_3AR (nM)
NNC53-0055	$-CH_2OCH_3$	$-HNOCH_3$	-Cl	O	4.6
MRS1898	$-CONHCH_3$		-Cl	C	1.5
MRS5221	$-CONHCH_3$		$-C\equiv C-(CH_2)_4-C\equiv CH$	C	-
MRS5698	$-CONHCH_3$			C	3.0
MRS5841	$-CONHCH_3$			C	1.0
MRS5919	$-CONHCH_3$	$-CH_3$		C	6.0

Figure 47. Compounds synthetized by Jacobson et al.

A C6-methyl derivate that demonstrate to have a high affinity for the A_3AR with a K_i of 6.01 nM was the compound **4**. The 2-Cl, compound **1,** and 2-arylethynyl, compound **5,** belonging to the C6-alkyl or alkenyl derivates, demonstrated to be selective for the A_3AR. Compounds **2** and **3**, 2-chloro nucleosides containing in C2 a rigid extension, showed a potent agonist activity. Compounds **2** and **4** resulted to be full agonists (K_i of 78.5 and 6.01 nM, respectively). Compounds **6** and **7,** possessing

a 5-chlorothienylethynyl group, demonstrated to be A_3AR selective with a K_i of 42.2 and 60 nM, respectively (Tosh D.K. et al., 2016) (Figure 48).



Compound	R	R_1	K_i A_3AR (nM)
1		-Cl	1140
2			78.5
3			515
4	$-CH_3$		6.01
5	$-CH_3$		305
6	$-CH_3$		42.2

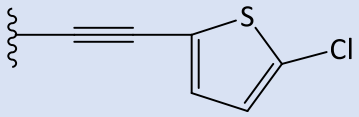
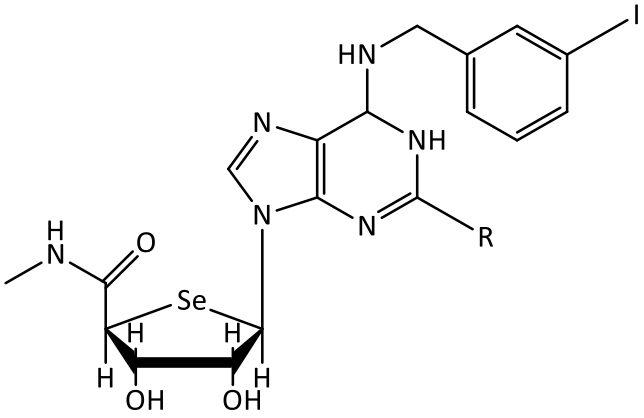
7	-		60

Figure 48. Compounds synthetized by Jacobson et al.

Another study discovered the new and surprisingly profile of novel 4'-selenonucleosides. Among all the compounds synthetized two of them showed to be quiet potent and selective *versus* the A_3AR (Figure 49). The first compound was **3d** who showed to be a full agonist with a K_i of 0.57 nM and more than 800 and 1900-fold selective *versus* A_1 and A_{2A}ARs, respectively. *In vitro* assays showed that, this compound inhibits MCP-1 induced microglial chemotaxis and have no cytotoxicity even up to 20 µM. The second compound was the 2-Cl-N^6-3-iodobenzyl analogue **3p** with a K_i of 4.20 nM. This compound had the ability in microglia and monocytes cells to inhibit chemoattractant-induced migration without causing cell death even at ≤ 50 µM. This study also concluded that these compounds could be used as novel anti-stroke agent (Yu J. et al., 2017).



Compound	R	K_i A_3AR (nM)
3d	-H	0.57
3p	-Cl	4.20

Figure 49. Compounds synthetized by Jacobson et al.

2.6.8. A_3 adenosine receptor antagonists

A_3AR antagonists with a xanthine structure demonstrated to be less active antagonists, compared to the results obtained with the same core in other receptors subtypes. For this reason, various non-xanthine ligands have been synthetized such as 1,4-dihydropyridines, pyridine and flavones. Two important potent and selective compounds with an antagonistic profile were the MRE 3008-F20 and VUF-5574 (Figure 50). The first one has demonstrated to be one of the most potent ligands. Many

efforts were spent in order to transform A_3AR agonists in potent and selective A_3AR antagonist based in the blockage of the ribose or in shortening the chain in 4' position of the sugar. Several compounds were discovered with interesting proprieties like the MRS-5147 and its analogue 3-iodine-MRS-5127 or 4'-thioadenosin, derivative LJ-1251 (Figure 50). With the addition of a butyl substituent in 5 position and a phenyl ring in 8 position of the triazolo-purine core, a new antagonist (OT-7999) was created, being useful for the glaucoma treatment (Figure 50).

Park's et al. demonstrated that the compound LJ-1888 (2R,3R,4S)-2-[2-chloro-6-(3-iodobenzylamino)-9H-purine-9-yl]-tetrahydrothiophene-3,4-diol (Figure 50) was a selective and strong antagonist with beneficial effects for the treatment of unilateral ureteral obstruction (Park J.G. et al., 2018).

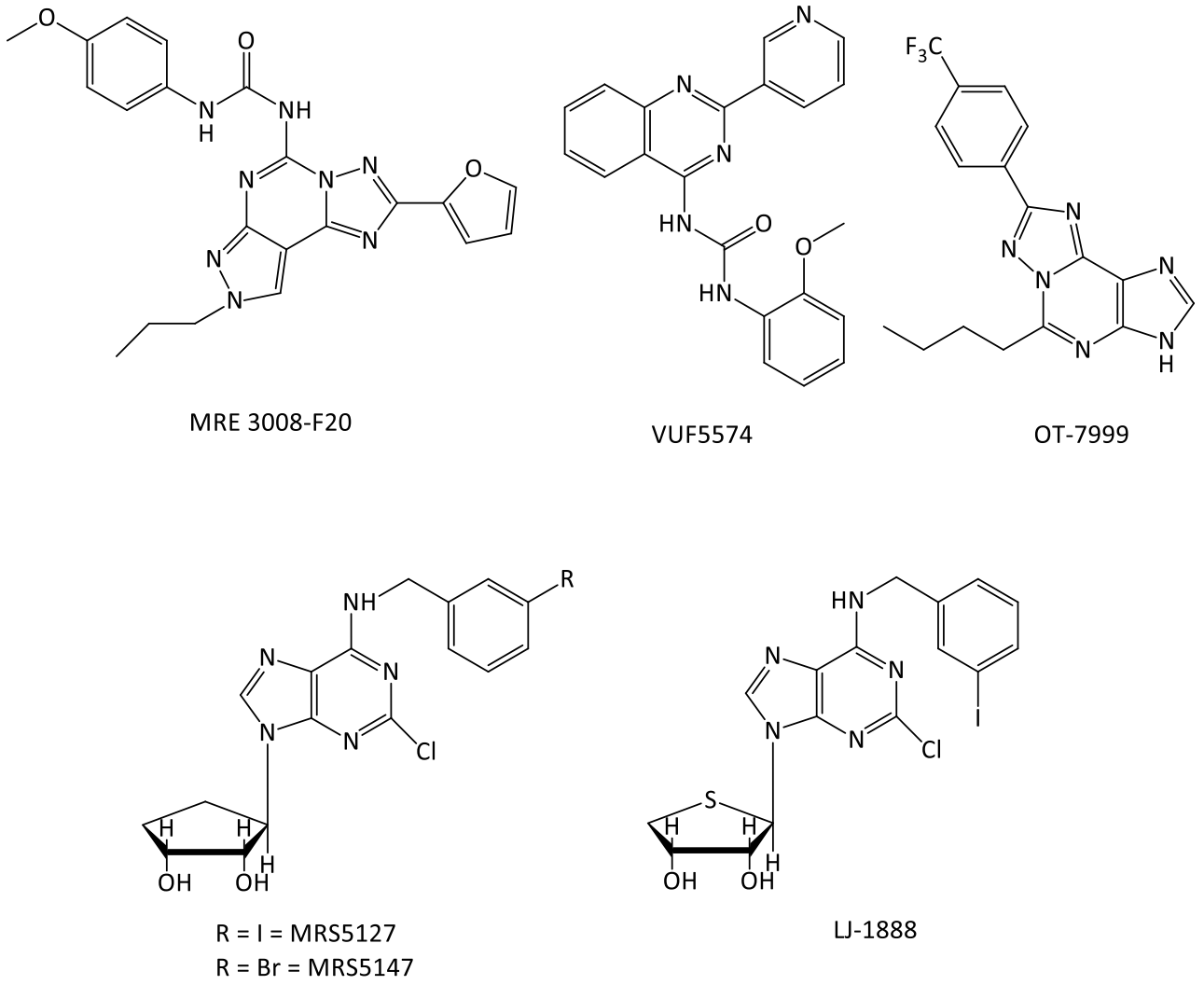


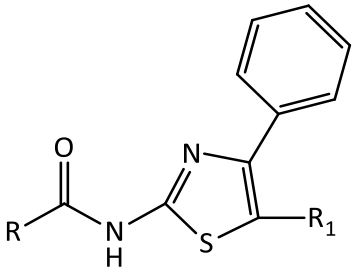
Figure 50. MRE 3008-F20, VUF5574, OT-7999, MRS5127, MRS5147 and LJ-1888 structures.

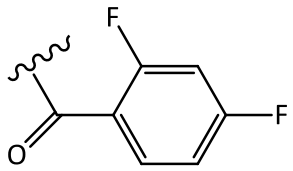
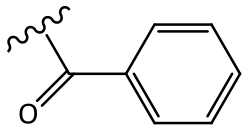
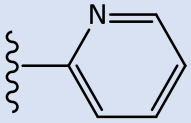
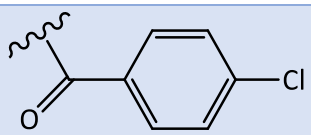
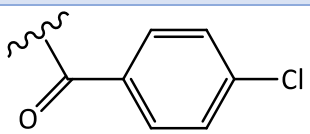
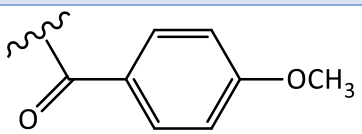
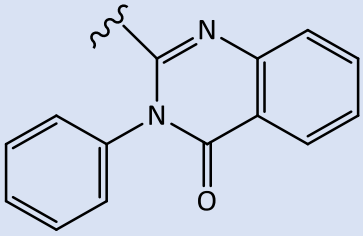
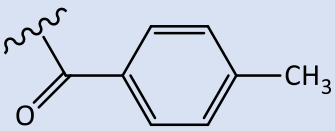
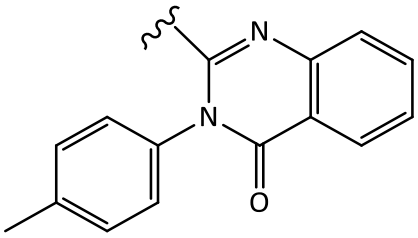
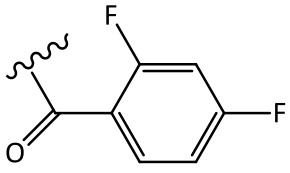
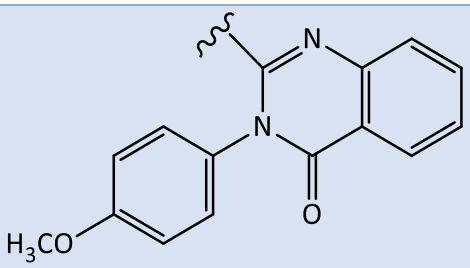
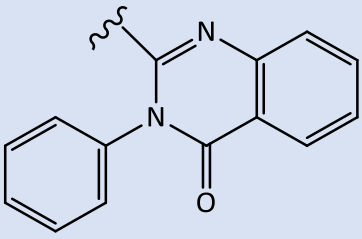
Recent investigations (Abdelrahman A. et al., 2020) reported a new series of compounds with interesting roles as antagonists against A_3AR (Figure 51). The amino group of the 2-amino-4-phenylthiazole core was modified to form an amide with benzoic acid, *p*-methyl-benzoic acid, or 2-furancarboxylic acid, respectively.

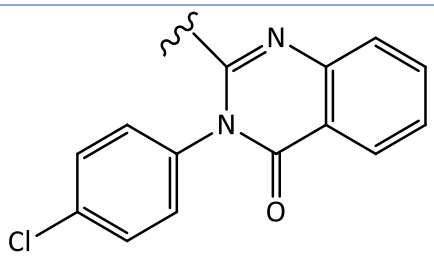
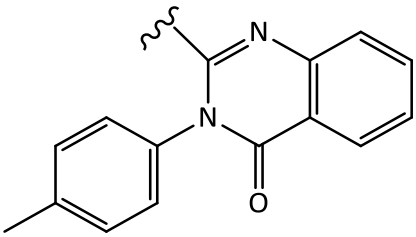
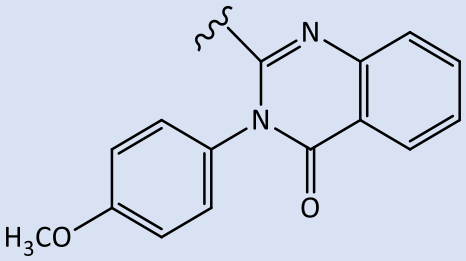
The obtained compounds were joined with distinct groups in the 5- position of the 2-amino-4-phenylthiazole core. The voluminous substituent had demonstrated to give the A_3AR selectivity and affinity. These compounds were divided in three groups according to the group adhered to the amide linker in the thiazole at 2-position. The first subgroup possessed a phenyl group (compounds **3-9**), the second one a *p*-methoxyphenyl (compounds **10-17**) and the last one a furanyl moiety (compounds **18-23**).

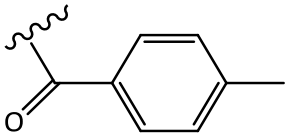
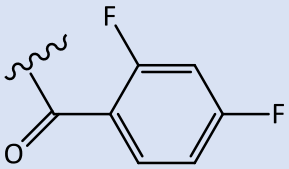
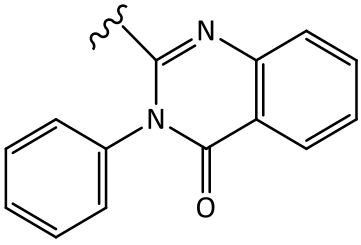
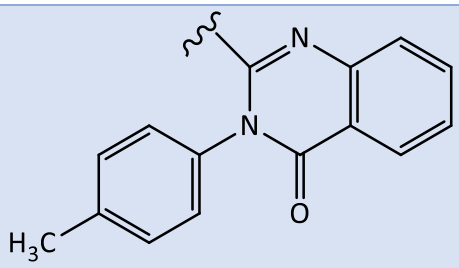
The most potent compounds from the series *versus* hA_3AR were obtained by the addition of bulky species at 5-position of the thiazole core. The K_i values of these ligands for the A_3AR were in the low nanomolar range being also selective against the other subtypes. Among these molecules compound **17** exhibits a very good affinity of A_3AR with a K_i = 4.63 nM. The 3-(p-substituted phenyl)-2-(thiazol-5-yl)quinazolin-4(3H)-one moiety is well accepted, making compounds with K_i under the micromolar value, like for compounds **7** (p-methyl), **22** (p-methoxy) and **23** (p-chloro). Compounds **10**, **16** and **23** showed a K_i in nanomolar range and the last one is the powerful ligand of the series both in rat and in human receptors.

Compound **10** and **18** demonstrated to have a dual antagonistic profile for A_1AR/A_3AR. Compound **10** was an N-(5-Benzoyl-4-phenylthiazol-2-yl)-4-methoxybenzamide (K_i =36.7/25.4 nM), while compound **18** had in 5 position a 4-methylbenzoyl and in 2 position of the thiazole a furan-2-carboxamide (K_i =17.57/42.2 nM).



R_1			R_1		
R = phenyl		K_i A_3AR (nM)	R = *p*-methoxyphenyl		K_i A_3AR (nM)
3		67.8	**10**		25.4
4		>1000	**11**		>1000
5		428	**12**		>1000
6		111	**13**		>1000
7		9.05	**14**		>1000
8		8.23	**15**		174

9		83.3	**16**		27.8
			17		4.63

R_1		
R = 2-furyl		***K_i* A_3AR (nM)**
18		42.2
19		124
20		129
21		25.4

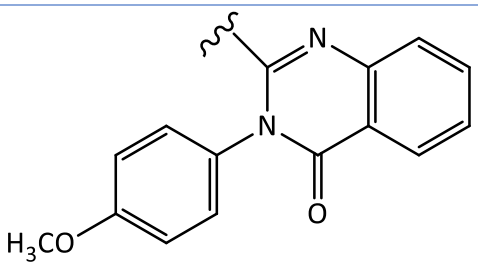
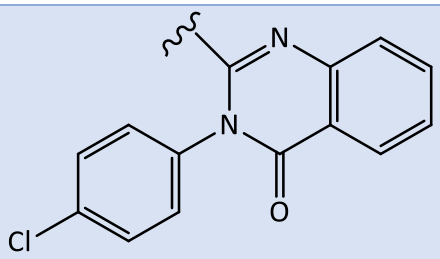
22		6.45
23		48.3

Figure 51. Compounds synthetized by C.E Müller.

3. AIM OF THE THESIS

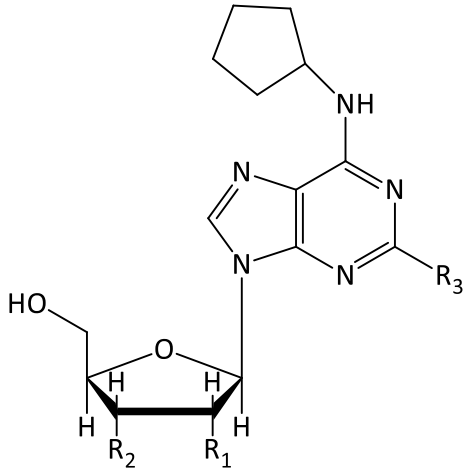
Neuroinflammation is a complex protective response of the brain against harmful agents, such as pathogens, toxins, and traumatic shocks or factors that induce neurodegeneration. Nevertheless, this response needs to be controlled and last for a short period; otherwise, it may to be an unleashed factor for major neurodegenerative and psychiatric disorders. This inflammation is mediated by the production of cytokines, chemokines, reactive oxygen species, and secondary messengers.

The most common neuropathology in almost all CNS diseases is characterized in changes in microglial morphology. In fact, the microglia are considered the immune cells in the CNS and their activation is a significant common cause of neuropathology in CNS diseases (Salter M. et al., 2017). These immune cells express adenosine receptors where adenosine plays an important immunoregulatory role. The effects on the inflammation regulation by adenosine suggest that use of selective agonists or antagonists able to activate or inactivate adenosine receptors could have important therapeutic implications in several diseases. Accordingly, the A_1AR activation produces a neuroprotective effect and A_{2A}ARs prevents neuroinflammation; the thesis work is designed to study the stimulation of A_1AR and the block of A_{2A}AR in *in vitro* and in *in vivo* models of neuroinflammation. Hence, it was studied the A_1AR full or partial agonists belonging to the 2-chloro-N^6-cyclopentyl-2' or 3'-deoxyadenosine previously synthesized (Vittori S. et al., 2000) (Table 3a) and the A_{2A}AR antagonists belonging to 9-ethyladenine substituted in 2 or in 8 positions (unpublished), which were synthesized in house (Table 3b). Among A_1AR agonists the 2-chloro-N^6-cyclopentyl-2'-deoxyadenosine (2'-dCCPA) was chosen for *in vivo* investigations since it showed a submicromolar A_1AR affinity in binding studies performed at Chinese hamster ovary cells (CHO) stably transfected with the human ARs. In addition, functional studies at CHO cells stably transfected with hA_1AR, performed by evaluating the inhibition of adenyl cyclase activity induced by forskolin, revealed that this compound is endowed with a partial agonist behavior (α=0.70) and this result was confirmed in an *ex vivo* experiment performed at mouse ileum contractility. In this model, in fact, the compound induced the 75% of maximal contractility reduction obtained with the full agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) (Martire A. et al., 2019). The partial agonist behavior could be beneficial in the treatment of acute and chronic disease due to less side effects compared to the A_1AR full agonist and due to minor receptor desensitization (Mundell S. et al., 2011). In addition, this compound showed to protect SH-SY5Y cells from oxygen-glucose deprivation (OGD) at different

concentrations representing a possible alternative for the management of cerebral ischemia and thus also for the management of neuroinflammation (Martire A. et al., 2019).

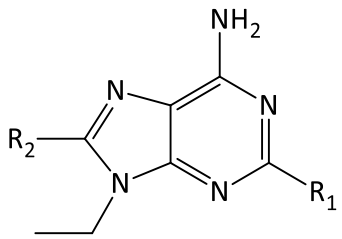
Among the series of 9-ethyladenine substituted the 8-substituted 9-ethyl-2-phenethoxyadenine was chosen for further studies, which was synthesized with the aim to improve the affinity at the $A_{2A}AR$ of its 8-bromo analogue (Lambertucci C. et al., 2007). It showed a subnanomolar affinity and a good selectivity versus $A_{2A}AR$ (K_i $A_{2A}AR$ = 0.75 nM; *Ki* A_1AR = 17 nM and K_i A_3AR = 227 nM, unpublished results).

Table 3a. Chemical structures of A_1AR agonists.



Compound	R_1	R_2	R_3
CCPA	OH	OH	Cl
2'-deoxyCCPA	H	OH	Cl
3'-deoxyCPA	OH	H	H
3'-deoxyCCPA	OH	H	Cl

Table 3b. Chemical structures of $A_{2A}AR$ antagonists.



Compound	R_1	R_2
1	Cl	Br
2	Cl	OCH_3
3	Cl	OCH_2CH_3
4	Cl	$OCH(CH_3)_2$
5	Cl	2-Furyl
6	H	$O(CH_2)_2Ph$
7	OCH_2CH_2Ph	Cl
8	Cl	OCH_2CH_2Ph
9	OH	OCH_2CH_3
10	Cl	OH
11	Cl	$NHCH_2CH_3$
12	CH_3	OCH_2CH_3
13	OCH_2CH_3	OCH_2CH_3
14	I	Br
15	I	OCH_2CH_3
16	2-Furyl	OCH_2CH_3

4. MATERIAL AND METHODS

4.1. CONTINUOUS CELL LINE CULTURES

4.1.1. CHO cell culture

Experiments were performed using Chinese hamster ovary (CHO) cells stably transfected with human adenosine receptors and biosensor (kindly given by Prof. Klotz K.N., Institute of Pharmacology and Toxicology, University of Wuerzburg, Germany). They were grown in DMEM/F12 enriched with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2,5 µg/ml of amphotericin B, 1 mM of sodium pyruvate, 0,1mg/ml of geneticin (G418) and 10% of fetal bovine serum (FBS). Cells were kept in humidified environment at 37 ºC and with 5% CO_2/95% air. When cells reached the desired confluence, they were splited into new flasks.

4.1.2. N13 cell culture

N13 are microglia cells from mice. They were grown in RPMI 1640 media with 2mM of glutamine and enriched with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % FBS. They were seeded in plates and splited as needed. Cells were maintained in humidified environment at 37 ºC and with 5% CO_2/95% air.

4.2. MEMBRANE PREPARATION

Crude membranes for radioligand binding experiments were prepared by collecting cells (CHO stably transfected with hA_1, hA_{2A} and hA_3 ARs) in ice-cold hypotonic buffer (5mM Tris/HCl, 2mM EDTA, pH 7.4). Briefly, 2 ml of this buffer (Lysis Buffer) were added per plate to detach cells, which were scraped and collected. Cell suspension was homogenized with an Ultra-Turrax 2 x 20 sec at full speed and the homogenate was centrifuged for 10 min (4 °C) at 3200 rpm. The pellet was eliminated and the supernatant was centrifuged for 45 min at 37000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended with the specific binding buffer (hA_1ARs: 50mM Tris/HCl buffer pH 7.4; hA_{2A}ARs: 50mM Tris/HCl, 50mM $MgCl_2$ pH 7.4; hA_3ARs: 50mM Tris/HCl, 10mM $MgCl_2$, 1mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 2–4 mg/ml and stored at −80 °C.

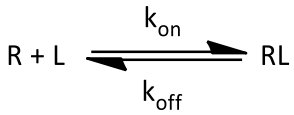
4.3. RADIOLIGAND BINDING ASSAY

Radioligand binding assay is based in the incubation of a mix made of cell membranes containing the desired human receptor, the chosen radioactive agonist and a ligand under study at different concentrations (in competition experiments). The radioligands used were for hA_1 the [^{3}H]CCPA, for hA_{2A} the [^{3}H]NECA and for hA_3 the [^{3}H]HEMADO. These molecules were used for their high affinity with the respective receptors. In saturation and competition assays, the mixture was performed and incubated in a 96-well plate. It was also necessary to evaluate the non-specific binding of each radiolabelled agonist. For [^{3}H]CCPA it was used 1 mM of theophylline and for [^{3}H]NECA and [^{3}H]HEMADO was used 100 µM of *N*^{6}-phenylisopropyladenosine (R-PIA). The mixture was incubated for 3 h and the plates filtered using a 96-well microplate filtration system called Microbeta Filtermat 96 Cell Harvester (PerkinElmer). This process helps to separate the bound fractions from the free fractions. The filter plates were washed three times with 200 µl of ice-cold binding buffer (hA_1ARs: 50 mM Tris/HCl buffer pH 7.4; hA_{2A}ARs: 50 mM Tris/HCl, 50 mM $MgCl_2$ pH 7.4; hA_3ARs: 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25) and then let them dry. Then 20 µl of scintillation cocktail were added to each well and the bound radioactivity was obtained using a Perkin Elmer Microbeta^2 scintillation counter (Falsini M. et al., 2017). To calculate all data from binding experiments non-linear curve fitting with Prism 5.0 programme (GraphPAD Software, San Diego, CA, USA) was used.

Radiolabelled binding experiments at A_{2B}AR were not performed due to the lack, in the cells used, of sufficient receptors to obtain a satisfying signal; for this reason, the affinity of A_{2B}AR ligands was studied by adenylyl cyclase experiments.

4.3.1. Kinetic Experiments

To elucidate the rate constant (k_{on}) and dissociation rate constant (k_{off}) of each radiolabelled ligand with its specific receptor (R) kinetic experiments were performed. The specific buffer for each receptor subtype, 10 µg of cell membranes expressing the specific receptor and one concentration of the radiolabelled agonist (L) composed the mixture. As previous mentioned, the mixture was incubated and filtered at different time points. The filtermat was dried and the scintillation cocktail was added. Plate was evaluated through a scintillation counter, the results were analysed using GraphPad software and k_{off}, and k_{on} calculated.



4.3.2. Saturation Experiments

Once determined the values of k_{on} and dissociation rate constant k_{off} the following step was to calculate the tendency of the receptor-ligand complex to dissociate (K_D) and the concentration of binding sites in the assay (B_{max}). The K_D value was calculated according to the following formula:

$$K_D = \frac{k_{off}}{k_{on}}$$

The saturation binding experiments were performed incubating for 3 h at RT a mixture made with the membranes containing the receptor under study, the specific binding buffer and the radioligand at increasing concentrations. Subsequently, the plate was filtrated and counted in a scintillation counter. With data acquired it was constructed a curve for each radiolabeled compound under study. In the graphs increasing radioligand concentrations were represented on X axis and the count per minute (cpm) obtained on Y axis. The point in which the curve reaches a plateau is considered the B_{max}.

4.3.3. Competition Experiments

The compounds under study were tested through competition experiments on radioligand binding assays. For these tests, CHO membranes expressing the receptor of interest (hA_1, hA_{2A}, and hA_3 ARs) were used. The inhibitory constant (K_i) was calculated through the Cheng–Prusoff transformation. K_i value indicates the affinity of the ligand under study for the receptor of interest. In competitions experiments it was used a fixed concentration of the radioligand ([^{3}H]CCPA, 1 nM for A_1AR; [^{3}H]NECA, 10 nM for A_{2A}AR; and [^{3}H]HEMADO, 1 nM for A_3AR), 10 µg of membranes, increasing concentrations of ligands under study and the specific binding buffer. The non-specific binding (NSB) was evaluated as previously described, adding 1 mM theophylline for hA_1AR or 100 µM R-PIA in the case of hA_{2A} or hA_3 ARs. After preparing the mix, the 96-well plate was incubated, filtered and the radioactivity in the filtermat counted as previous mentioned. The specific binding (SB) was calculated subtracting from cpm values acquired the values of NSB. This allowed to create a sigmoid curve with increasing concentrations of the ligand under study on X axis and the cpm values on the

Y axis. K_i value is a parameter to determine if a compound has a good (low K_i) or poor affinity (high K_i) for the receptors.

4.4. GLOSENSOR™ cAMP ASSAY

GloSensor™ cAMP assay is a non-radioactive method with an extremely sensitive and easy-to-use tool to study GPCRs. This experiment is based on the intracellular levels of the second messenger cAMP. This powerful approach uses a mutant form of *Photinus pyralis luciferase* into which it has been inserted a cAMP binding protein moiety. When the biosensor become active, through the interaction with cAMP, it suffers a conformational change and interacts with luciferin (its substrate), which in turn is converted it into oxyluciferin emitting light at 562 nm (Buccioni M. et al., 2011). This increase of light allows studying the activity of the desired molecules for the receptor under study (Figure 52).

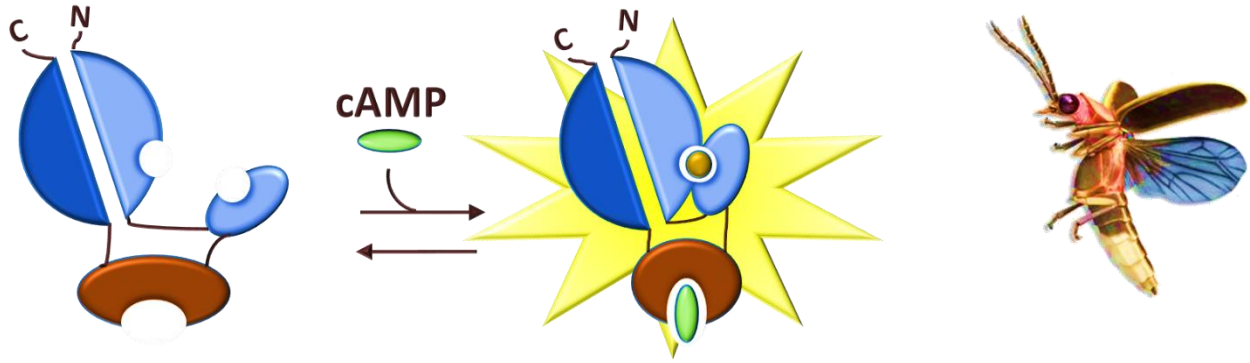


Figure 521. Principle of GloSensor™ cAMP Assay.

4.4.1. Stable transfection with biosensor

CHO stably transfected with ARs were cultured as mentioned in the first section. $3x10^5$ cells per well were seeded on a 6-well plate and after 24 h they were transfected with pGloSensor™-22F cAMP plasmid encoding for the genetically modified form of the luciferase (Figure 53). This plasmid allows the expression of biosensor in mammalian cells. The process was carried out using the TurboFect Transfection Reagent (ThermoFisher), a polymer able to create positively-charged complexes with plasmid DNA. These complexes assure the DNA from degradation and simplify the plasmid delivery into CHO cells. Positive clones were selected with 1 mg/ml hygromycin B and single clonal lines were isolated by limiting dilution. Medium was changed the next day and then every 2 days decreasing the quantity of antibiotic up to reach the final concentration of 200 µg/ml. Expression of the biosensor was verified using the GloSensor cAMP assay.

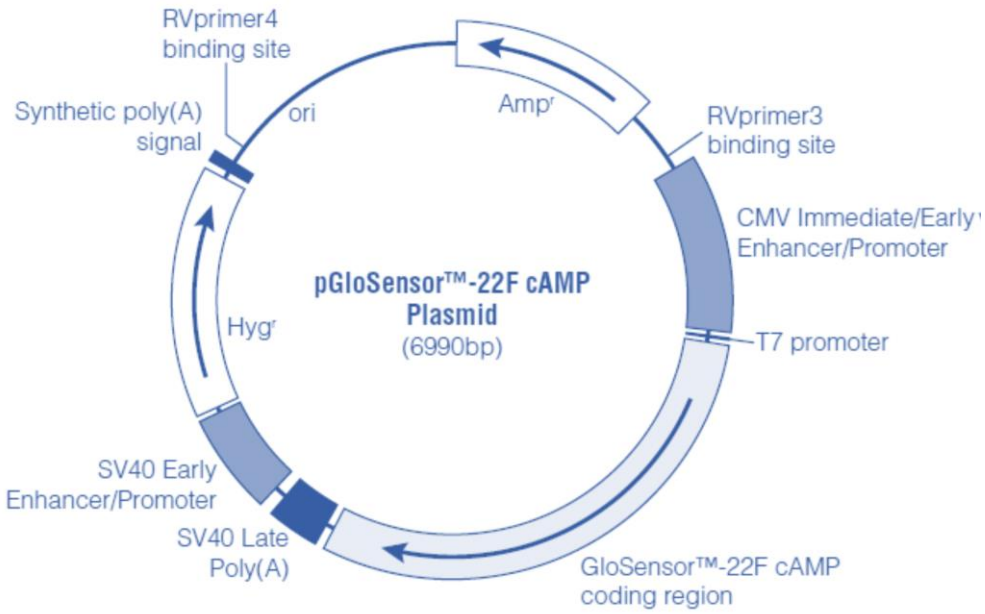


Figure 532. pGloSensor™-22F cAMP plasmid.

4.4.2. GloSensor™ cAMP Assay

CHO cells stable transfected with both ARs and biosensor were collected using CO_2-independent medium and counted in a Neubauer chamber. Subsequently, the required quantity of cells was incubated for 2 h in equilibration medium composed by 3% v/v GloSensor™ cAMP reagent stock solution, 10% FBS, and 87% CO_2 independent medium.

- To study the G_s coupled receptors ($A_{2A}AR$ or $A_{2B}AR$), cells were placed into the wells of a 384-well plate and basal signal was obtained. Then the reference agonist (NECA) or the ligands under study were added at different concentrations. If new ligands did not stimulate the production of cAMP, they were studied as antagonists. The antagonistic biological profile was investigated determining their ability to counteract an agonist-induced increase of cAMP level. Cells were incubated 10 minutes at RT with different concentrations of ligands under study and later treated with a single concentration of NECA (1 µM in the case of $A_{2A}AR$ and 10 µM in the case of $A_{2B}AR$). After 10 minutes of incubation, several luminescence reads were done at different incubation times.
- To study G_i coupled receptors (A_1AR or A_3AR), cells were incubated for 10 minutes with different concentrations of agonists under study and after that, a fixed concentration of forskolin (10 µM) was adjoined to all wells. Forskolin is used to stimulate the production of cAMP at intracellular levels and appreciate the effect of agonists acting decreasing the cAMP

production. If new ligands did not reduce the production of cAMP, they were studied as antagonists.

The antagonistic profile was explored by determining their ability to counteract the agonist-induced decrease of cAMP level. Cells were incubated 10 minutes at RT with different concentrations of ligands under study and later treated with a single concentration of NECA (1 µM) for 10 minutes. Subsequently a single concentration of forskolin (10 µM) was added and kinetic or end-point measurements of luminescence were acquired after 10-30 min post-forskolin addition.

Results were expressed as percentage of the maximal relative luminescence units (RLU). Concentration-response curves were constructed with the Prism 5.0 programme (GraphPAD Software, San Diego, CA, USA). To measure the agonist under study or NECA potency it was calculated the EC_{50} value. This value indicates the concentration of agonist necessary to cause the 50% of the maximum effect. On the contrary, to study the antagonist profile it was calculated the IC_{50} value, which indicates the amount of antagonists that cause the 50% inhibition of the agonist maximum effect. Each compound concentration was tested three-five times in triplicate and the values are given as the mean ± standard error.

4.5. PRIMARY CELL CULTURES

4.5.1. Mixed glial cell culture

All animal experiments were carried out according to the principles and procedures outlined in the European Community Guidelines for Animal Care, DL 26/2014, application of the European Communities Council Directive, 2010/63/EU.

Newborn male Wistar rats between 3 and 5 days old were sacrificed and mixed glial cell cultures obtained from the cerebral cortices. Briefly, cerebral cortices were dissected and meninges were cleared away. Tissues were treated with trypsin solution (Trypsin 0.05% (w/v) trypsin-EDTA 5 mM) for 25 minutes at 37 °C. The tissue was fully disaggregated by pipetting and the dissociated cells were seeded with Dulbecco's modified Eagle's/High glucose medium enriched with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS. Fresh medium was changed the next day and then every 3 days decreasing the quantity of FBS. All experiments were carried out after 7–9 days of plating (Vilar A. et al., 2014).

4.5.2. Primary neuronal cell cultures

Neurons were obtained from hippocampus or cortex of embryos. Briefly, pregnant rat was sacrified and the uterus removed. Embryos were taken out from the uterus and brains were obtained (Figure 54). After removing the meninges, hippocampus and cortex were dissected and homogenated with tweezers. The result was incubated with trypsin for 15 minutes at 37 °C and then centrifuged 2 minutes at 1000 rpm. The pellet was resuspended with 15 ml of HBSS/Glucose/30% FBS and centrifuged as before. The pellet was resuspended with 15 ml HBSS/Glucose and re-centrifuged. The process was repeated again. Finally, the pellet was resuspended with 15 ml of Neurobasal media supplemented with 25 µM of Glutamine. The result was filtered and plated in 24 well plate at a concentration of 10^5 cells/well (Ferreira, D.G. et al., 2017). The concentration used of each compound under study was: 2'-dCCPA 5500 nM, Compound **7** 10 nM, CK 20 ng/ml, SCH58261 50 nM and DPCPX 100 nM.

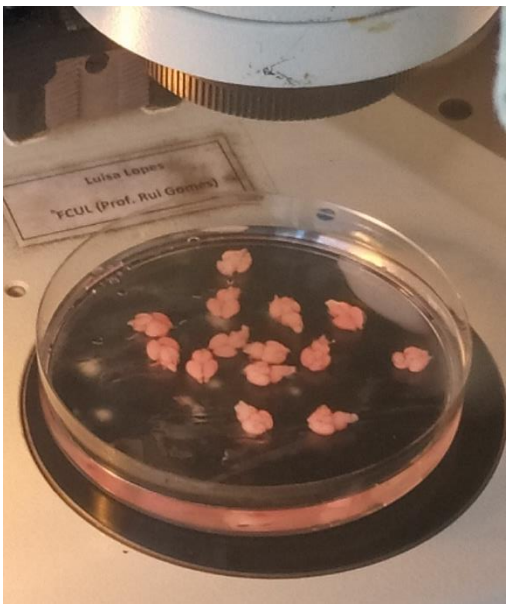


Figure 543. Brains dissected from the embryos.

4.6. IMMUNOFLUORESCENCE

A_1AR and A_{2A}AR presence was investigated using an immunofluorescence technique. A_1AR presence was checked using the Adenosine A_1 Receptor Polyclonal Antibody ALEXA FLUOR®488 Conjugated, while A_{2A}AR was studied by Adenosine A_{2A} Receptor antibody ALEXA FLUOR® 594 Conjugated. Briefly, media was aspired and cells fixed with Fixative Solution for 15 minutes (high purity 4% formaldehyde in PBS, pH=7.3). After that, they were washed 3 times with PBS and permeabilized with a Permeabilization Solution (0.5% Triton X-100) for 15 minutes. They were washed again with

PBS and incubated for 1 hour with Blocking Buffer (3% of BSA, fraction V de-lipidated in PBS). Finally, it was proceeded with the antibody labelling. Mixed glial cells were seeded in a 6 well plate at $6x10^5$ cells per well while N13 were plated in a 6 well plate at $3x10^5$ cells per well.

4.7. CELL TREATMENT

Mixed glial cell cultures were divided into 2 groups, one to study A_1AR agonists and another for A_{2A}AR antagonists (Table 4). Cells from the first group were pre-treated with 2 µl at 3 different concentrations of A_1AR agonist for 15 or 30 minutes and then treated for 48 h with a pro-inflammatory cocktail of cytokines (TNF-α, 20 ng/ml; IL-1β, 20 ng/ml; IFN-γ 20 ng/ml). Cells from the second group were pre-treated with the same CK cocktail for 48 h and then treated with 2 µl of A_{2A}AR antagonist at three different concentrations for 15 or 30 minutes. ZM241385, a well know A_{2A}AR antagonist, was used as reference compound. Furthermore, ligands (either agonist or antagonist) and CK were tested alone for 15 or 30 minutes and 48 h respectively.

Table 44. Cell treatment.

	Control	Aggression	Protection	Combination	Reference compound
Treatment for A_1 receptor	Media	CK 48 h	A_1AR agonist 15' or 30'	A_1AR agonist + CK	CCPA for 15 or 30 min
Treatment for A_{2A} receptor			A_{2A}AR antagonist 15' or 30'	CK + A_{2A}AR antagonist	ZM241385 15 or 30 min

4.8. PROLIFERATION ASSAY

Cell viability assay was performed to see the cell-state after the different treatments performed (control, positive control, pro-inflammatory cytokines or ligands alone, A_1AR agonist + CK cocktail and pre-treatment of CK + A_{2A}AR antagonist). Seventeen thousand mixed glial cells and $1x10^4$ N13 cells were cultured in 98 µL of the specific medium in a 96 well plate overnight. Shortly, after all treatments 20 µl of CellTiter 96® AQ_{ueous} One Solution Reagent were added to each well. Absorbance was read at 490 nm using the plate reader GENiosPro. Cell viability was calculated as a percentage using the following formula:

$$\text{Cell viability} = \frac{\text{OD mean of treated cells}}{\text{OD mean of control cells}} \text{ x } 100$$

An untreated control (only media), a positive control (well-known agonist or antagonist) and a control with water were performed. All experiments were done in triplicate (Antognoni F. et al., 2017).

4.9. GRIESS ASSAY

To test the antioxidant capacity of these new molecules Griess assay was performed. The Griess Reagent System is based in the conversion of sulphanilamide into an azo-compound in presence of N-1-napthylenediamide dihydrochloride (NED) under acidic condition (Figure 55). Seventeen thousand mixed glial cells were seeded in 98 µL of the specific medium in a 96 well plate overnight. Shortly, the media from each well of the cell culture was transferred into a 96 well plate and then 50 µl of the Sulphanilamide Solution was added to each well. After 5-10 minutes of incubation at RT, 50 µl of the NED Solution were dispensed to all wells and they were incubated at RT for other 5-10 minutes. Absorbance was measured within 30 minutes in the plate reader GENiosPro with a filter between 520 nM and 550 nM. This experiment was performed in triplicate. Concentration of NO_2^- in each sample was determined by comparison to a Nitrite standard reference curve (Wang Z. et al., 2002).

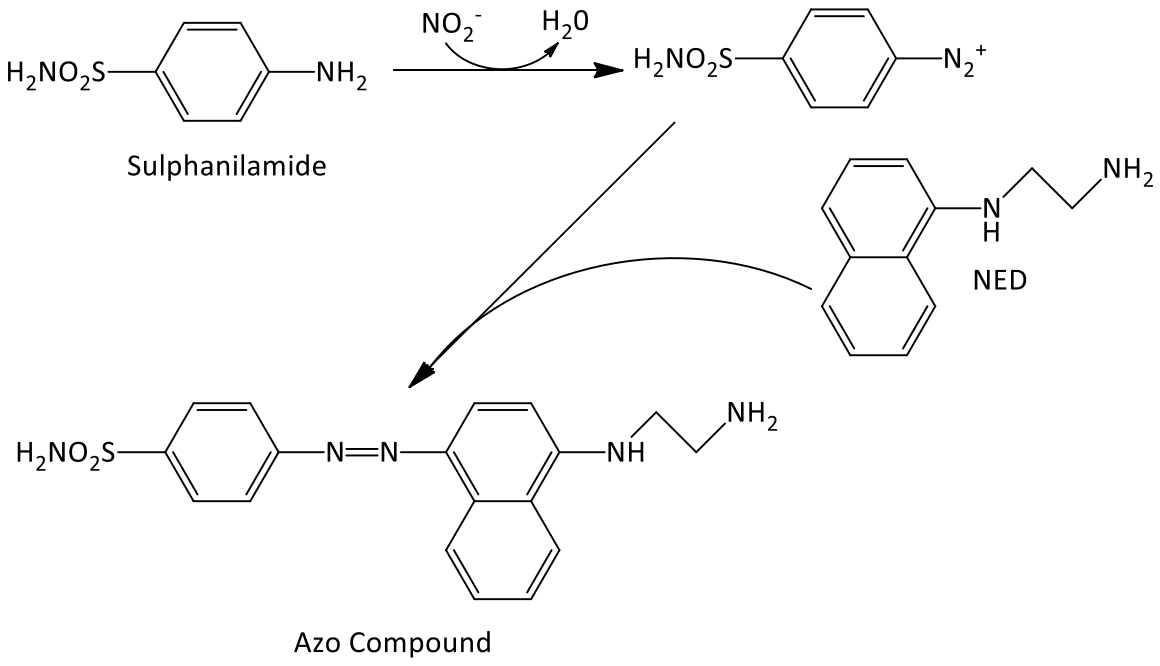


Figure 555. Griess reaction.

4.10. HOECHST ASSAY

Hoechst was accomplished to check the antiapoptotic effect of the compounds under study. This assay was carried out using Hoechst 33258. Mixed glial cells were seeded in a 6 well plate at $6x10^5$ cells/well while N13 were plated at $3x10^5$ cells/well. After 24 h the media was eliminated and cell cultures washed with PBS. They were then washed with acetic acid/methanol solution 50:50, again washed with PBS and incubated 10 minutes with a Fixative Solution. After this, cells were washed with distilled water and incubated light protected for 30 minutes at RT with Hoechst (50 ng/ml). Finally, the dye was discarded and cells washed with water. Glycerol solution was added and cells were observed under the microscope. The cell area and circularity were measured using ImageJ as image analyzing software (Fezai M. et al., 2017). Area and circularity were calculated as percentage using the following formula:

$$\text{Area or circularity} = \frac{\text{area or circularity of treated cells}}{\text{area or circularity of control cells}} \times 100$$

4.11. WESTERN BLOT

Western Blot is a useful technique that allows studying changes in specific protein levels. This technique is based in the migration of protein trough an acrylamide matrix once that an electric field is applied. This allows to separate the proteins according to their relation charge/mass.

Gels were prepared separately and polymerized between two glass plates. Gels are composed by two different types of gels: the upper one or resolving gel and the lower one or stacking gel (Table 5). The percentage of the stacking gel is always fix (5%) but the upper gel can vary. In this case 12% resolving gel was suitable for the experiment (Mahmood T. et al., 2012).

Table 56. Resolving and stacking gel composition.

	Resolving gel 12%	Stacking gel 5%
ddH_20	6.6 ml	4.1 ml
30% Acrylamide Mix	8 ml	1 ml
1.5 M Tris HCl pH=8.8	5 ml	-
1.5 M Tris HCl pH=6.8	-	0.75 ml
10% SDS	0.2 ml	0.06 ml
10% APS	0.2 ml	0.06 ml
TEMED	0.008 ml	0.006 ml

4.11.1. Sample preparation and quantification

The media from the cell cultures was removed and cells were washed with PBS. Then, PBS was removed and cells were scrapered with 150 µl of Complete RIPA (RIPA + Protease Inhibitor Cocktail + Phosphatase Inhibitors). Result was passed to an eppendorf, syringed and kept at 4 °C until use.

Sample were quantified using the Bradford Assay. Briefly, a standard curve was done using several concentrations of BSA (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml). Samples were diluted 1:4 and regents for the assay were added to all wells. After 45' the absorbance was read at 750 nm.

Samples to charge the gel were made of 10 µl of Sample Buffer 5x, 40 µg of cell lysate and the volume of water necessary to reach 50 µl. All samples were heated for 10 minutes at 95 °C previously to the charge.

4.11.2. Electrophoresis

Gels were disposed into an electrophoresis chamber in presence of migration buffer and samples and standard molecular weight marker were loaded on the acrylamide gel. The chamber was connected to a power source at 70 V until proteins were arrived to the second gel, then it was upped to 95 V (Figure 56).

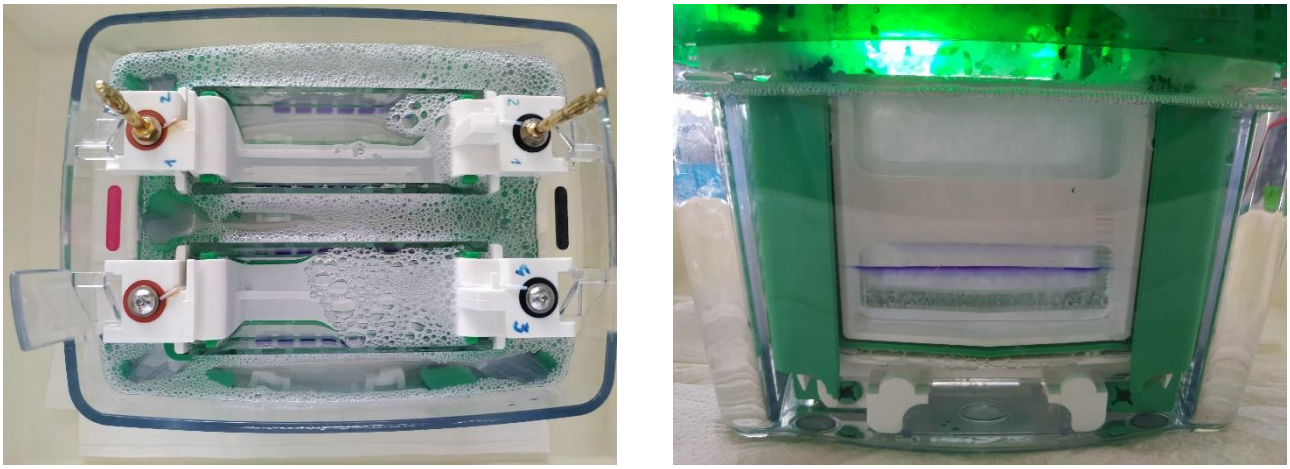


Figure 56. Western blot assembling. Image on the left shows the correct position of the glass plates and the gel. The image on the right shows a moment during the electrophoresis running. Purple line indicates the zones were the proteins were.

4.11.3. Transfer

After protein migrations, gels were transferred into a PVDF membrane. Shortly, membranes were activated with methanol and then a sandwich structure was constructed (Figure 57). The transfer was achieved by preparing a sandwich on a support in which a sponge, Whatman® paper, PVDF membrane, gel, Whatman® paper and finally another sponge was successively placed.

Subsequently, an electric field was applied allowing the transference from the gel to the PVDF membrane. Transference took place at 350 mA, 90 V for 90 minutes. The transference was carried out on ice since speed and efficiency of the transfer are inversely proportional to the temperature.

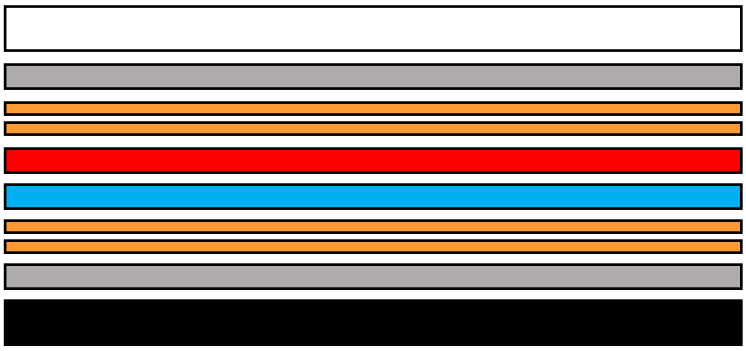


Figure 57. Transference scheme. In order from top to bottom: Anode (white), sponge (grey), Whatman® paper (orange), PVDF membrane (red), gel (blue), Whatman® paper (orange), sponge (grey) and cathode (black).

Subsequently, membranes were dyed with Ponceau solution to check the presence of transferred proteins and check the efficiency of the transfer (Figure 58). Finally, the PVDF membranes were washed with water and TBS-T until they were white again.

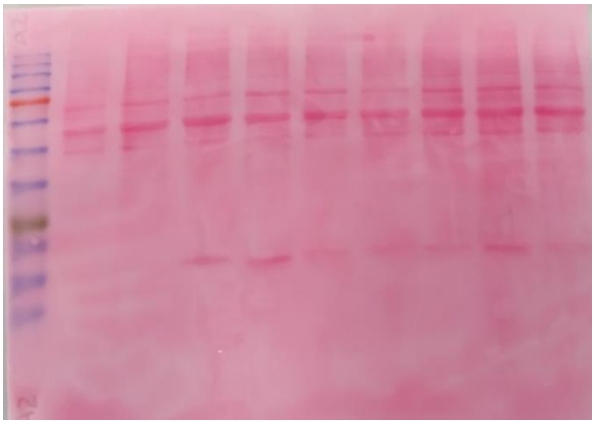


Figure 58. Membrane dyed with Ponceau stain. The first column was the Molecular Weight Marker; the other bands are protein samples.

4.11.4. Immunodetection

Membranes were blocked with 3% BSA TBS-T for 1 hour and later were incubated with the desired primary antibody overnight in a cold room. The day after membranes were washed with TBS-T and incubated with the secondary antibody for 1 hour at RT (Table 6). Then membranes were washed again and incubated for 4 minutes with ECL Western Blotting Substrate.

Table 67. Antibodies used, their dilution factor and molecular weight.

Antibody	Dilution	Molecular Weight
iNOS	1:400	135 KDa
Cyt C	1:1000	14 KDa
Caspase-3 (Pro and Active)	1:1000	33/19 KDa
GAPDH	1:1000	35 KDa
Mouse anti-rabbit HRP	1:4000	-

Membranes were read using Amersham Imager 680. Chemiluminescence measurements values were obtained using the corresponding Image Lab software and the results were expressed as a percentage compared to the control.

4.12. PROPIDIUM IODIDE AND SYTO-13 LABELLING

This technique allows, by two different nucleic acid stains, to assess the cell viability. Syto-13 is able to enter inside the living cells and emits at 509 nm when it is excited at 488 nm. On the other hand, Propidium Iodide (PI) can enters into the cells only if the membrane is disrupted, absorbing at 535 nm and emitting at 617 nm. Ten thousand cells/well were seeded on a coverslip in a 24 well plate and after 24 h washed with 1 ml of KREBS solution. A solution with 40 µl of Syto-13 100 µM, 5 µl 1 mg/ml of PI and 955 µl of KREBS was prepared. One drop (20 µl) of this solution was placed in the top of a piece of parafilm paper and covered with the coverslip with cells facing down. Coverslips were incubated 3 minutes in dark. After that, coverslips were placed in a microscope slide and four arbitrary photos were shot (Rebola N. et al., 2005).

4.13. ANIMALS

Animal procedures were performed in accordance with the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (DL 113/2013), and approved by the Instituto de Medicina Molecular Internal Committee and the Portuguese Animal Ethics Committee (Direcção Geral de Veterinária).

Environmental conditions were kept constant: food and water *ad libitum*, 21 ± 0.5 °C, 60 ± 10% relative humidity, 12 h light/dark cycles, 3-5 rats per double-decker ventilated cage. Male Sprague Dawley rats 8 weeks old were divided into the following groups (Table 7):

Table 7. Experimental groups 1 (G1) and 2 (G2): LPS (liposaccharide) was delivered intracerebroventricularly (i.c.v.) using stereotaxic coordinates, 10ug total was injected, unless otherwise noted. 2'-dCCPA was delivered i.c.v., while compound **7** (C**7**) was delivered intraperitoneally (i.p.). Control groups included i.c.v. saline delivery and vehicle, either i.c.v. or i.p., depending on compound delivery route.

	Drugs (delivery route)	LPS (i.c.v.)	Saline (i.c.v.)
G1	Compound 1 (i.c.v.)	LPS + 2'-dCCPA	Saline + 2'-dCCPA
	Vehicle (i.c.v.)	LPS + Veh	Saline + Veh
G2	Compound 2 (i.p.)	LPS + C7	Saline + C7
	Vehicle (i.p.)	LPS + Veh	Saline + Veh

4.14 SURGERY

Animals were anesthetized with a cocktail of 75 mg/kg ketamine + 1 mg/kg dexmedetomidine. Once rats were deeply anesthetized, head was fixed into a stereotaxic frame (Figure 59.A) and an incision made on the scalp to expose the skull (Figure 59.B). Coordinates for injection were AP -1.0, ML ± 1.5 mm, DV -3.7 (Figure 60).

With a high-speed drill two holes for the injection were performed. The solution was inserted at a flow ratio of 1 µl/min (Figure 59.C). After the injection, 5 minutes were waited before removing the needle and inject in the other hole. After both injections, rats were sutured, awaked and brought back to the cage (Figure 59.D).

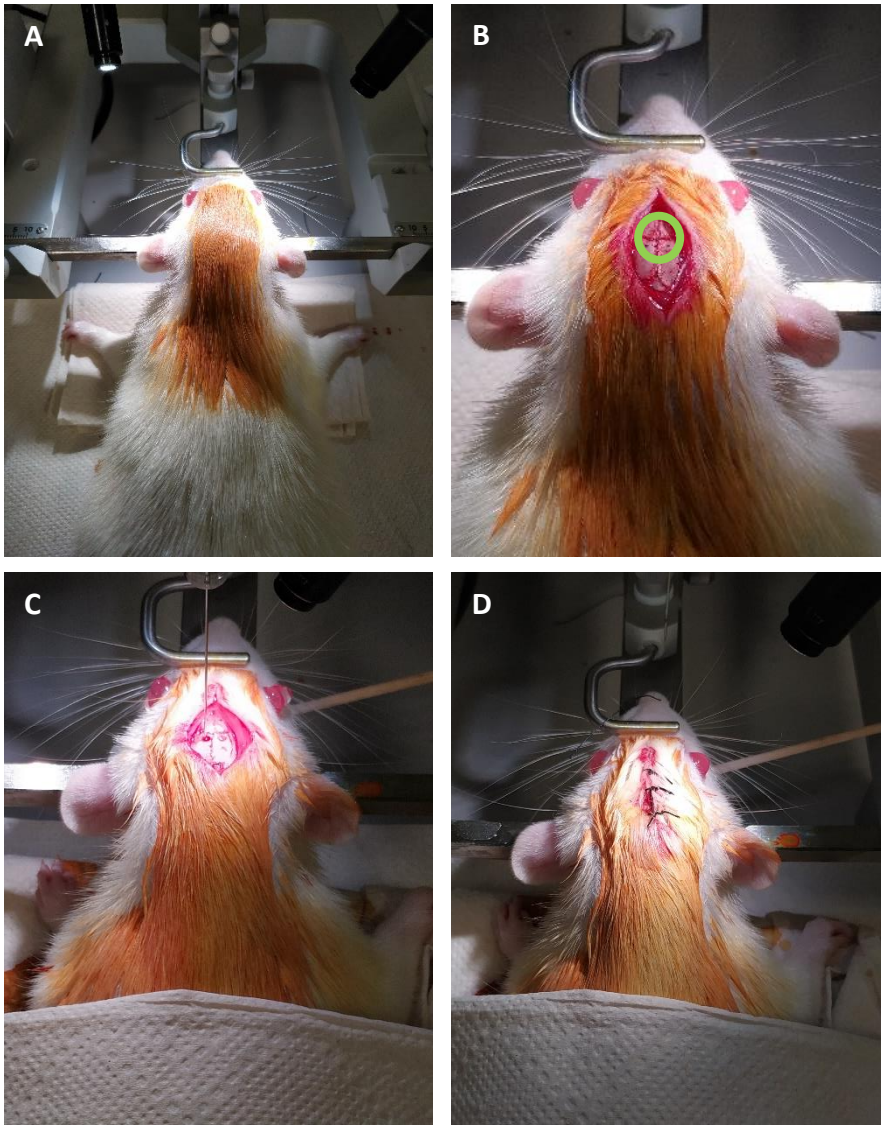


Figure 59. Surgery procedures. Image A shows the correct position of the anesthetized rat; image B shows the incision and the bregma indicated with a green circle; image C shows the injections and their positions; and D the suturing.

For G1, LPS was delivered bilaterally 5 µl of a 1 µg/µl LPS solution per hemisphere, and 2'-dCCPA was delivered also i.c.v. 15 minutes before the LPS injection, 5 µl of a 7 µg/µl solution. Flow rate was kept at 1 µl/min, using an automatic injector coupled to a 10 µl hamilton syringe gauge 30. After each injection, 5 minutes were allowed for diffusion of the drugs before slowly removing the needle. After the procedure, rats were sutured, administered atipamezole (1mg/kg) to reverse anesthesia and brought back to a heated cage until fully recovered. For G2, rats were injected i.p. with a 2.5 mg/kg solution of C**7**, 30 min after the i.c.v. injection of LPS 5µg/5µl per hemisphere, in total of 10

µg. Rats were monitored for sickness behaviour (body temperature, general activity) and given access to gel food in the first 48 h.

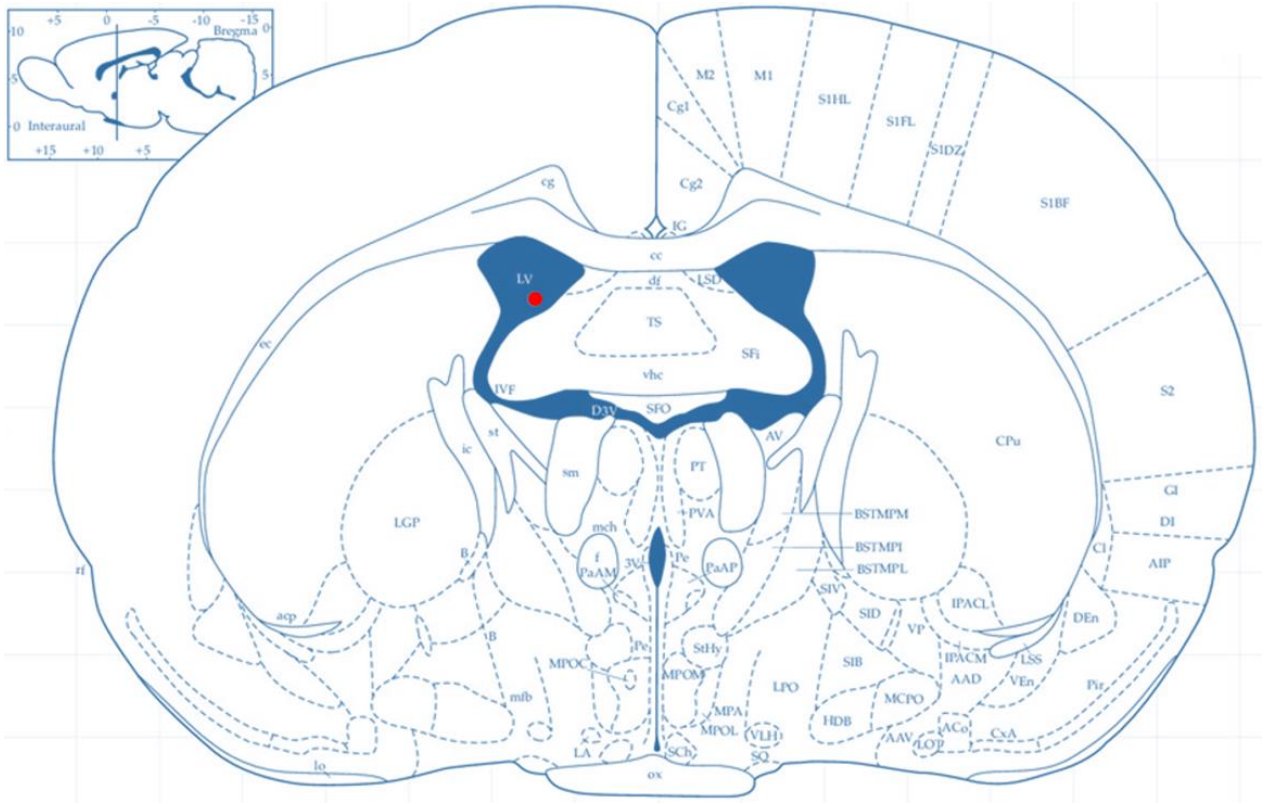


Figure 60. Rat brain region. The red spot indicates where the injection was performed (anteroposterior -1.0, mediolateral ± 1.5 and dorsoventral -3.7) and the blue area represents cerebrospinal fluid in cerebral ventricles.

4.15. *IN VIVO* ASSAYS

4.15.1. Open Field

Two days after the neuroinflammatory insult, Open Field (OF) was performed. This experiment uses the natural exploratory behaviour of the animal when placed in a novel environment to measure general locomotor activity and exploratory behaviour. It was used to determine whether normal exploratory activity was recovered at the time of the behaviour tests, to avoid possible confounds due to sickness behaviours during memory test performance. Animals were placed in an arena (40x40x40) for 5 minutes in a dim-lighted room and activity monitored using smart video tracking software. Parameters measured included distance covered, velocity and permanence time in

periphery *vs* center area of the arena. In figure 61 is reported the OF experiment box which is digitally divided in two squares, the outside one (red) and the inside one (yellow). The permanence time is the time that the animal spends in each zone. If the animal is fine and has no stress problems naturally prefers the red square compared to the yellow one. This behaviour could be attributed to natural animal attitude to feel safer along the walls, while when the rat has neuronal or motor problems this biological attitude is altered (Coelho, J.E. et al., 2014).

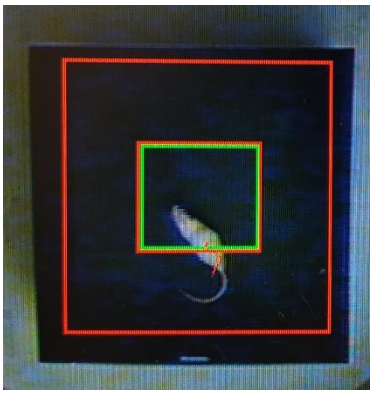


Figure 618. Image of an OF experiment.

4.15.2. Y Maze

Y Maze is a behaviour test useful to measure short-term spatial working memory. Animals are introduced in the maze always in the same starting position, at the end of the start arm (Figure 62). An initial habituation phase of 8 minutes, animals freely explore 2 of the arms, the 3rd being closed. Sixty minutes after the habituation period animals are placed again in the maze for 5 minutes this time with all 3 arms available to exploration. Using the Smart video tracing software, each animal's route was registered. The time spent in the arm initially closed, which for the animal is the novel, unexplored environment, is calculated in relation to the time spent in the already familiar arms. (Ribeiro M. et al., 2019).

Figure 629. Image of an OF experiment.

4.15.3 Object Displacement

Object Displacement (OD) or Pattern Separation (PS) is a short-term memory test that take cares of the animal ability to recognise familiar and novel object positions. In this experiment, the animal was placed into the pool with identical object in a fixed position. The animal was let to explore the pool with the objects for three minutes. Then, the animal was taken for 1 hour and the position of one object was changed (Figure 63). The animal was placed again into the pool for three minutes and the ability to recognize the new object was measured (van Goethem N.P. et al., 2018).

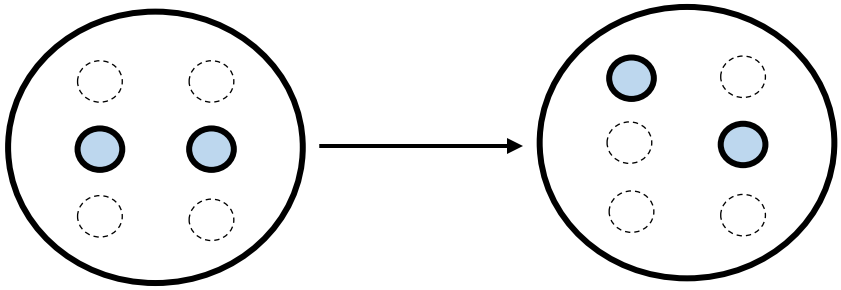


Figure 6310. Scheme of an OD experiment.

To evaluate the recognizing ability, it was calculated the discrimination index (DI) of each animal. The formula is:

$$\text{Discrimination Index} = \frac{\text{Time in familiar - Time in new}}{\text{Time in familiar + Time in new}}$$

4.16. STATISTICAL ANALYSIS

Results were represented as mean of 3-5 replicates ± standard error (±SE) for all experiments. Biological data were analysed using Prism 5.0 programme (GraphPAD Software, San Diego, CA, USA). Statistical analysis was performed using one-way ANOVA or two-way ANOVA in the case of Y Maze and Open Field. A p-value <0.05 was considered to indicate a significant difference.

5. RESULTS AND DISCUSIONS

5.1. BINDING AND FUNCTIONAL STUDIES

A new series of 9-ethyladenines, potential A_{2A}AR antagonists (synthetized by Professor Volpini's teamwork), substituted at 2 and/or 8 positions was investigated (Table 3b). This series was synthesized with the aim at improving the affinity at the A_{2A}AR of its 8-bromo analogue. The newly synthesized 9-ethyladenines **1-16** were biologically evaluated to determine their affinity and selectivity for the hA_{2A}AR *versus* the other ARs. The obtained data, reported in Table 8, show that compounds **5** and **7**, belonging to the set containing in 8 position a chlorine atom and in 2 position a 2-Furyl and phenethoxyl, respectively gave the best results, since they possess nanomolar affinity for the desired AR subtype (K_i = 6 and 0,8 nM) and also good selectivity. However, good hA_{2A}AR affinity was observed also for derivatives **1-4,** and **13**-**16**. Derivatives **6** and **9**-**12** target preferentially the hA_{2A}AR, compared to the other AR subtypes, but their affinity values fall in the medium-high nanomolar range. The most selective of the series, especially compared to A_1AR, was compound **16.** This high selectivity was due to the loss of affinity to A_1AR.

Table 8. Affinity or biological activity of the 9-ethyladenines, compounds 1-16 hARs[a]

Compound	[b]hA_1 (K_i nM)	[c]hA_{2A} (K_i nM)	[d]hA_{2B} (IC_{50} nM)	[e]hA_3 (K_i nM)	A_1/A_{2A}	A_3/A_{2A}
1	397 ± 79	54 ± 11	3160 ± 155	2938 ± 593	7	55
2	1121 ± 211	32 ± 6.6	2880 ± 700	1218 ± 265	35	38
3	353 ± 84	25 ± 4.5	5740 ± 516	391 ± 68	14	16
4	204 ± 43	23 ± 4.5	>30000	1024 ± 201	9	46
5	29 ± 5.7	6 ± 1.2	2510 ± 497	102 ± 19	5	17
6	890 ± 115	150 ± 23	7590 ± 213	>30000	6	>200
7	17 ± 4.9	0,8 ± 0.29	3960 ± 505	227 ± 55	21	284
8	21 ± 4.2	22 ± 6.1	>30000	1382 ± 341	1	63
9	6850 ± 1365	204 ± 43	>30000	6 ± 1.5	34	-
10	712 ± 141	437 ± 87	>30000	2312 ± 461	2	5
11	6129 ± 1221	359 ± 69	>30000	2073 ± 411	17	6
12	4996 ± 999	261 ± 49	>30000	2040 ± 408	19	8
13	4247 ± 842	32 ± 6.3	>30000	948 ± 187	134	30
14	588 ± 117	43 ± 8.5	>30000	447 ± 89	14	10
15	1050 ± 210	68 ± 13	>30000	3031 ± 606	15	44
16	>30000	24 ± 5	>30000	769 ± 153	>1600	32

[a]Data (n= 3-5) are expressed as means ± standard errors. [b]Displacement of specific [^{3}H]-CCPA binding at hA$_1$AR expressed in CHO cells. [c]Displacement of specific [^{3}H]-NECA binding at hA$_{2A}$AR expressed in CHO cells. [d]IC$_{50}$ values of the inhibition of NECA-stimulated adenylyl cyclase activity in CHO cells expressing hA$_{2B}$AR. [e]Displacement of specific [^{3}H]-HEMADO binding at hA$_3$AR expressed in CHO cells.

This series was evaluated with a functional study using the GloSensor™ cAMP assay. As expected results revealed that these compounds behave as antagonists in all subtypes (data not shown). Since compounds **6**, **7** and **16** were the most potent and selective of the series were chosen for further studies. In addition, the A$_1$AR full or partial agonists, belonging to the 2-chloro-N^6-cyclopentyl-2'or 3'-deoxyadenosine previously synthesized, were studied (Vittori S. et al., 2000).

5.2. *IN VITRO* STUDIES IN MIXED GLIAL CELL CULTURES

5.2.1. Immunohistochemistry

Primary mixed glial cells acquired from newborn rat pups were selected as a potent and robust tool for analysing the relationship between A$_1$AR and A$_{2A}$AR and the glia response during the inflammatory processes in CNS (Figure 64).

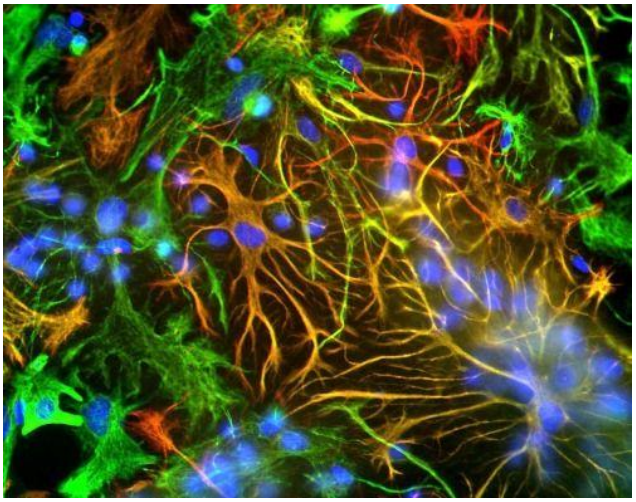


Figure 64. Mixed glial cells

First step was to elucidate the presence of A$_1$AR and A$_{2A}$AR in mixed glial cell cultures through a direct immunocytochemistry with A$_1$AR polyclonal antibody ALEXA FLUOR® 488 and A$_{2A}$AR antibody ALEXA FLUOR® 594 Conjugated. Results showed that in all these cell cultures both receptors are present in suitable amounts to make the experiments with A$_1$AR and A$_{2A}$AR ligands (Figure 65):

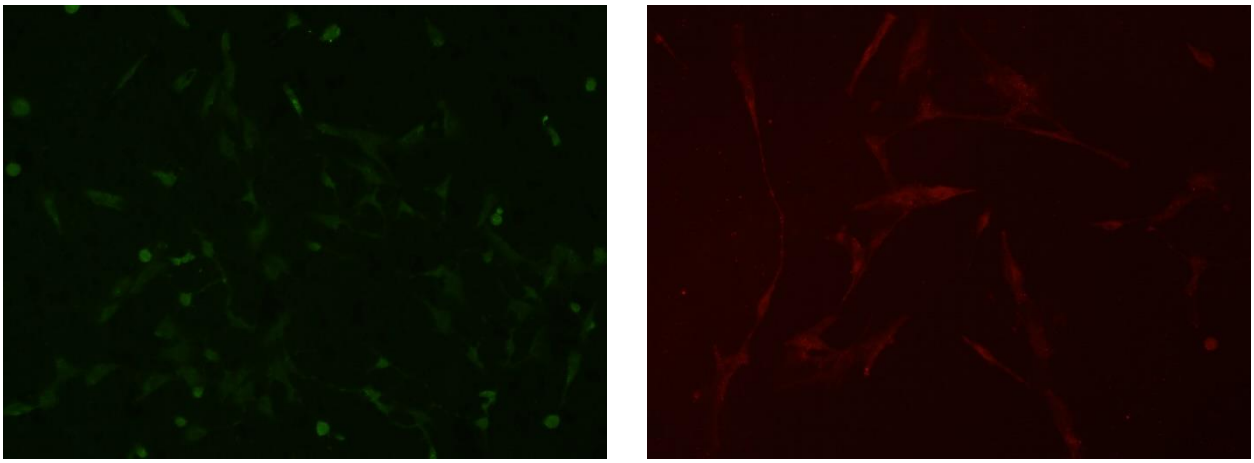


Figure 65. Direct immunohistochemistry of mixed glial cell cultures. Cells were treated with Adenosine A_1 Receptor Polyclonal Antibody ALEXA FLUOR® 488 Conjugated (green) and Adenosine A_{2A} Receptor antibody ALEXA FLUOR® 594 Conjugated (red).

5.2.2. Cell viability

After the presence assertion of both receptors in mixed glial cell cultures, viability assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay was performed. First step was to evaluate the toxicity of the ligands. Each ligand was tested at three different concentrations according to the K_i values obtained in Binding Assays in comparison with referent compounds CCPA and ZM241385 (Table 9a and b). The concentrations used were:

Table 9a. Concentrations of A_1AR agonists used.

	Concentration 1 (nM)	Concentration 2 (nM)	Concentration 3 (nM)
CCPA	5	10	60
2'-dCCPA	2000	5500	30000
3'-dCPA	50	100	600
3'-dCCPA	50	100	600

Table 9b. Concentrations of A_{2A}AR antagonists used.

	Concentration 1 (nM)	Concentration 2 (nM)	Concentration 3 (nM)
ZM241385	5	10	60
Compound 6	500	1500	6000
Compound 7	5	10	60
Compound 16	100	250	1500

All three concentrations were testes at 15, 30, and 60 minutes. Results obtained at 60 minutes were similar to those collected at 30 minutes (data not shown). The results are shown in the following graphs (Figure 66):

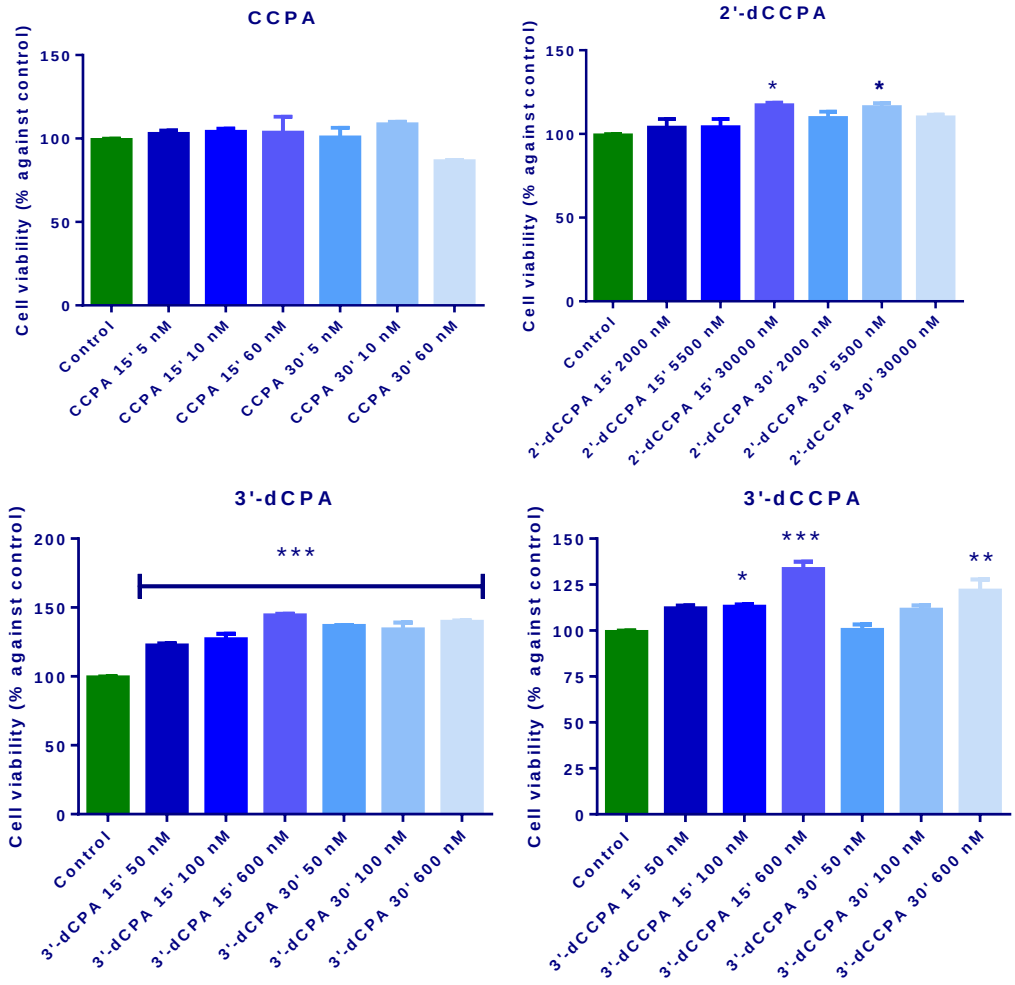


Figure 66. Effects of CCPA (reference compound) and A_1AR agonists on the viability of mixed glial cell culture. Percentage of cell proliferation after treatment with the ligand during 15 or 30 minutes. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against control

In general, the compounds demonstrate to possess no toxic effects on mixed glial cell cultures. The major part of the values are as control or a little bit higher indicating an amelioration of the cell state. The best effect was showed by 3'-dCCPA at 600 nM for 15 minutes (133 ± 3.1). The same experiment was performed for the A_{2A}AR antagonist, compounds **6**, **7** and **16** (C**6**, C**7** and C**16** respectively). Results are reported in the following graphs (Figure 67):

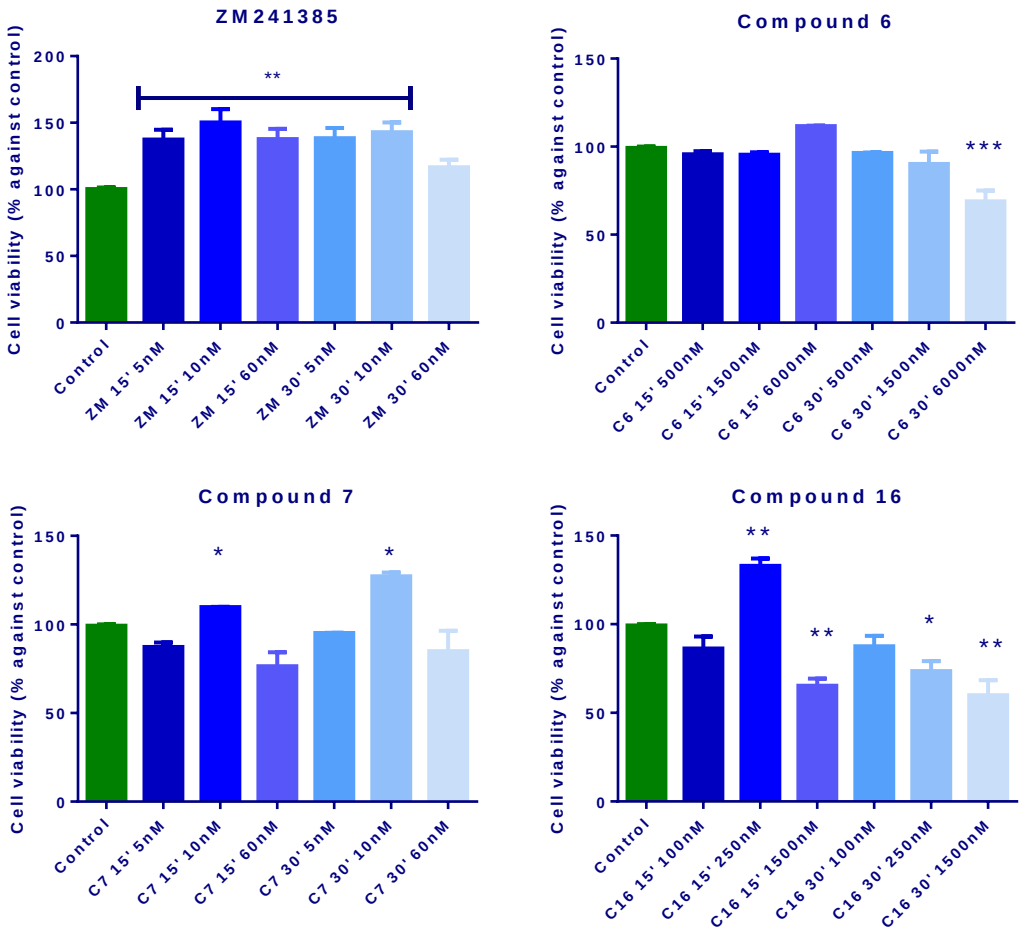


Figure 67. Effects of ZM241385 (reference compound) and A_{2A}AR antagonists on the viability of mixed glial cell culture. Percentage of cell proliferation after treatment with the ligand during 15 or 30 minutes. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against control

Compound **6** demonstrated to be safe and to have no negative effects except for the highest concentration at 30 minutes (70 ± 3.4 *vs* control) with values lower than the control. Compound **7** demonstrated to have good effects on cell viability at 10 nM even at 15 and 30 minutes (110 ± 2.7 and 127 ± 3.1 *vs* control, respectively) and only lowers levels than control at high doses but without being statistically significant (values nearly 80%). Lastly, compound **16** showed interesting beneficial effects at 250 nM at 15 minutes (133 ± 2.7 *vs* control), but despite this it showed harmful effects at highest doses and time being statistically significant against the control. ZM241385, an A_{2A}AR antagonist used as reference compound, demonstrated not be toxic at all concentrations or times tested (being the best dose at 15 minutes at 10 nM, 135 ± 3.5 *vs* control). Once it was asserted that these A_1 agonists and A_{2A} antagonists were not harmful, it was tested whether these compounds could provide protection in an inflammatory model. For this mixed glial cell cultures were exposed to a pro-inflammatory cocktail of cytokines (CK) constituted of TNFα, IL-1β and IFN-γ 20 ng/ml for

48 h. The choice of these CK was supported by several evidence. In many studies, as a pro-inflammatory stimulus, IFN-γ and TNF-α were used in *in vitro* neuroinflammation model since they bind to receptors on microglia and other brain cells (Benveniste E.N. et al., 1995; Liu X. et al., 2018; Miu M. et al., 2009; Suk K. et al., 2001). In addition, the use of IL-1β for microglia activation is usually related to neuroinflammation. In neurodegenerative disorder the level of all these cytokines are elevated and this chronic high level is implicated in initiating and/or maintaining glial activation in an *in vivo* experimental model of Parkinson's disease (Barcia C. et al., 2011; Reale M. et al., 2015). As expected, when the mixed glial cells were treated with a pro-inflammatory cocktail of cytokines at 20 ng/ml for 48 h the cell viability decreased (Figure 68).

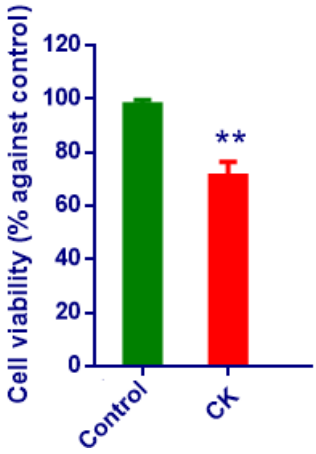


Figure 68. Effects of CK cocktail on the viability of mixed glial cell culture. Percentage of cell proliferation after treatment with the CK cocktail for 48 h. Results represent the average of three independent experiments. **P<0.01 of treated cells against control.

Results showed that CK cocktail concentration used is enough to create an aggressive insult to consider cells as in a neuroinflammation state (70 ± 3 *vs* control), being the cell viability difference between control and inflamed cells statistically significant. The aggression caused by CK cocktail alone in mixed glial cell cultures are in concordance with those obtained by Patraca and co-workers (Patraca I. et al., 2017). In order to study the neuroprotective effect of the compounds, different experiments were performed. Some evidence suggest that the reduction of A_1AR in microglia, macrophage, and neurons leads to state of neuroinflammation that occurs by enhancement of pro-inflammatory response and the release of cytokine (Tsutsui S. et al., 2004; Cunha R.A. 2005). On this bases the experiments with A_1AR agonists, which produces an A_1AR activation, were performed using the compound before the aggression by cytokine cocktail. On the other hand, the overexpression of A_{2A}AR associated to aging and chronic stress, combined with evidence that glial

$A_{2A}AR$ participates in neurodegeneration induced by $A_{2A}AR$ stimulation (Coelho, J.E. et al., 2014; Saura J. et al., 2005), directed the experiments towards an administration of $A_{2A}AR$ antagonists after the aggression caused by cytokine cocktail. Hence the cells were pre-treated with different concentrations of A_1AR agonists for 15 or 30 minutes and then with the CK cocktail. In the other experiment cells were pre-treated with the CK cocktail and then with the different concentrations of antagonists for 15 or 30 minutes. Results obtained with A_1AR agonists are shown in Figure 69.

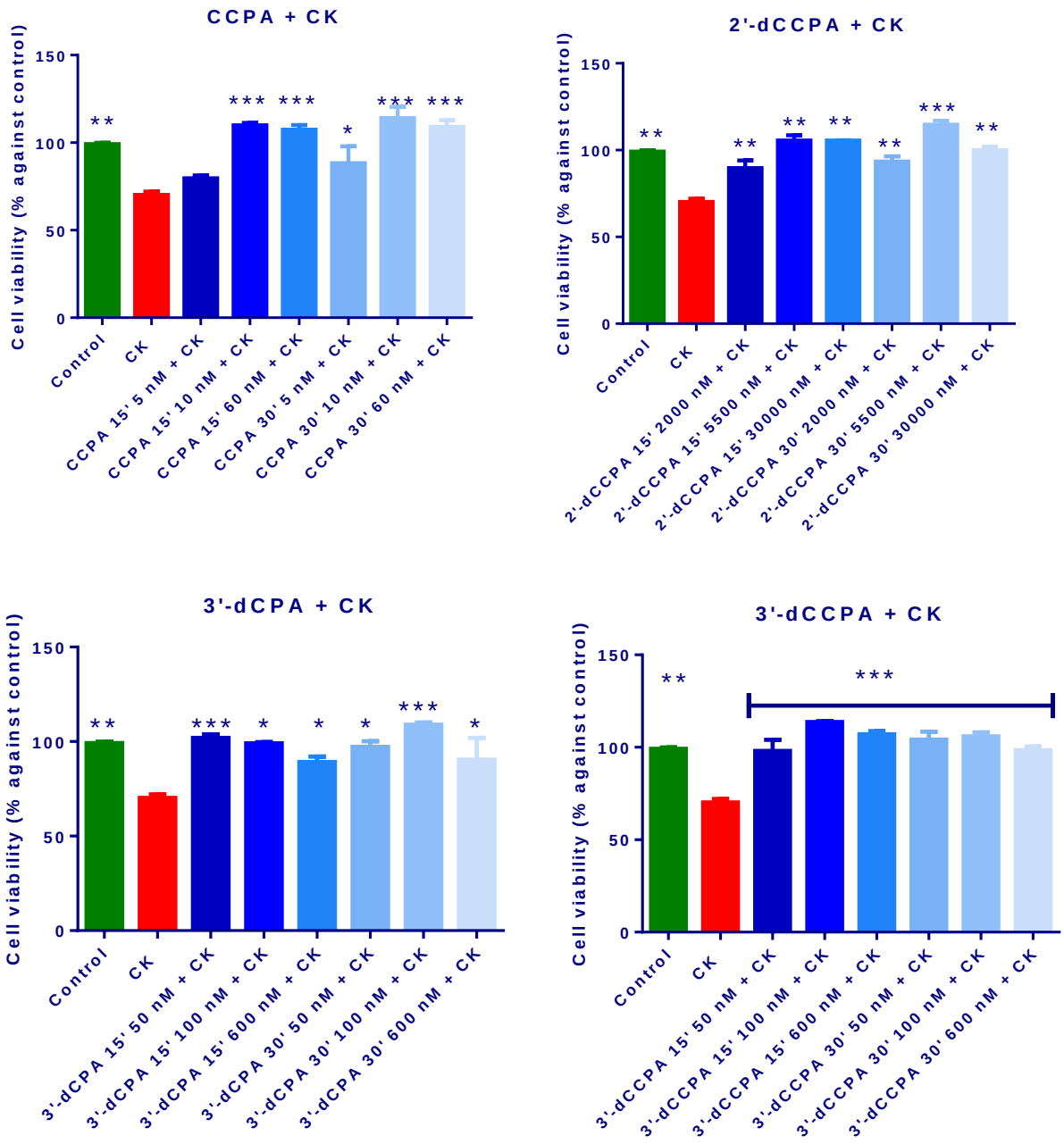


Figure 69. Protective effects of CCPA and A_1AR agonists against CK aggression on mixed glial cell culture. Percentage of cell proliferation after treatment with the CK cocktail for 48 h. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against CK.

Referent compound CCPA pre-incubation at lowest dose for 15 minutes showed no effect in preventing the CK aggression (80 ± 1.9 *versus* control), while all the other doses an incubation times showed values similar to the control or slightly higher such as the highest doses at 15 or 30 minutes. All agonists showed values similar to the control and thereof higher that CK, demonstrating that they protect cells against the insult. Results obtained with the pre-treatment with CK followed by the treatment with A_{2A} antagonists and referent compound ZM241385 are reported in Figure 70.

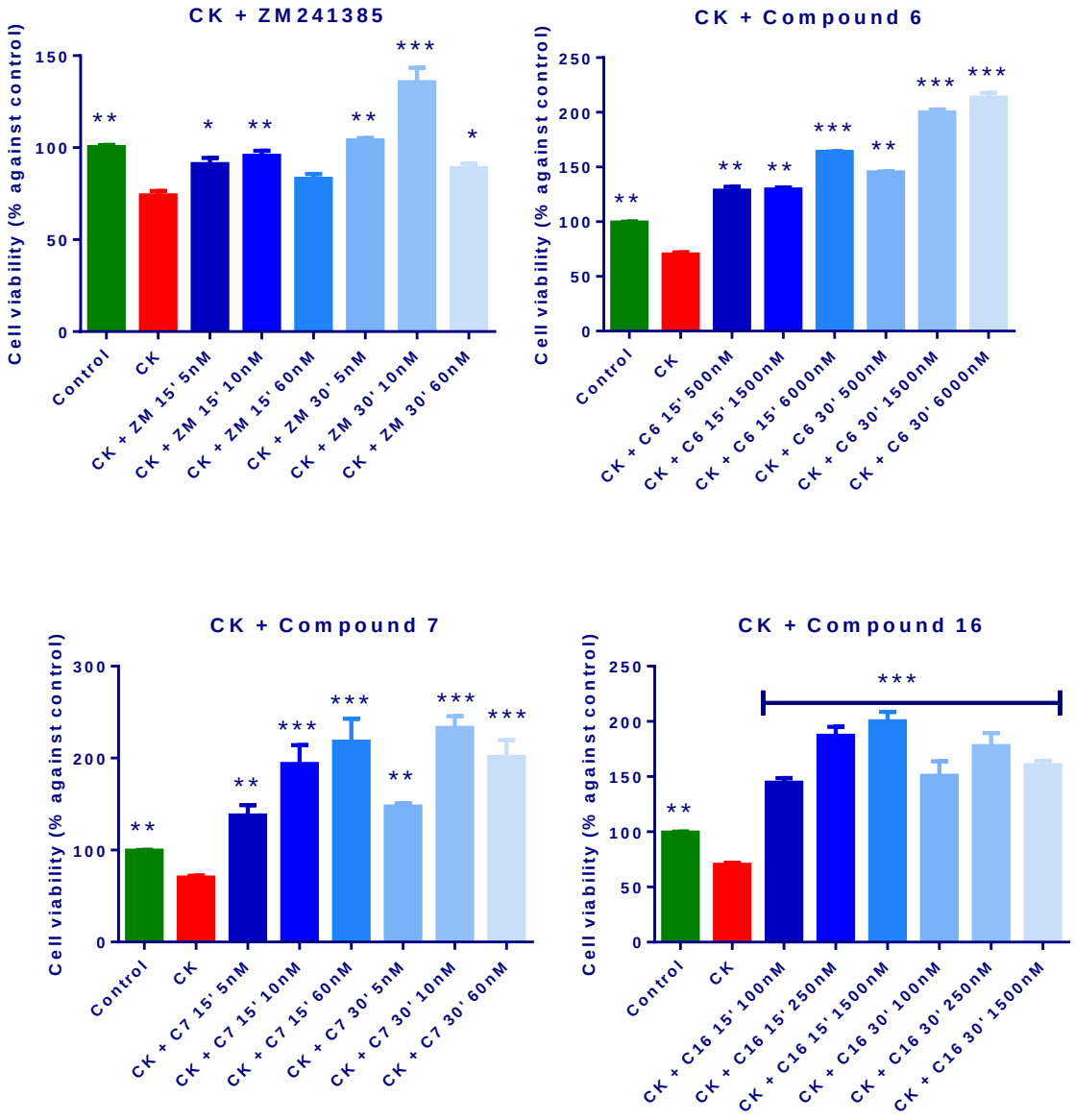


Figure 70. Restoring effects of ZM241385 and A_{2A}AR antagonists against CK aggression on mixed glial cell culture. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against CK.

These A_{2A}AR antagonists were tested in comparison with the reference compound ZM241385. The reference compound demonstrated to possesses good viability characteristics at 10 nM for 30 minutes (135 ± 3.4 *vs* control). All tested compounds independently of time and dose demonstrated to be effective after the aggression of the CK cocktail. Compound **6** at doses of 1500 nM and 6000 nM at 30 minutes showed high levels of cell viability (199 ± 2.2 and 213 ± 2.6 *vs* control respectively). The best compound among the series was compound **7** at 10 nM or 60 nM independently of the incubation time (all of them near or over 200%). In fact, this compound at 10 nM for 30 minutes of incubation demonstrated to improve cell viability up to 200%. Lastly, compound **16** also demonstrated good cell viability effects at 250 nM and 1500 nM majorly at 15 minutes (186 ± 3.1 and 200 ± 3.3 6 *vs* control respectively).

5.2.3. Antioxidant profile

In brain physiological conditions, in order to preserve cell functions, the immunological responses counteract the formation of reactive oxygen (ROS) and nitrogen (RNS) species. To preserve cell functions in the brain, the normal formation of reactive oxygen and nitrogen species is controlled by immunological responses. On the other hand, in pathological conditions, it was demonstrated a cross-talk between pro-inflammatory and oxidative signals that can lead to neuronal damage and subsequent neurodegeneration (Aguilera G. et al., 2017). The antioxidant capacity of compounds was tested at same concentrations and times reported in the viability assay. This assay was performed with Griess Reagent System. The neurodegenerative diseases are characterized by neuronal death and progressive neuroinflammation. The exact mechanisms of neuronal death remain elusive but seems that the programmed cell death (PCD) apoptosis plays a critical role. Neuroinflammation and glial activation are implicated in apoptosis process and compounds under study showed an anti-apoptotic effect reducing the aggression of pro-inflammatory CK cocktail *in vitro*. Compounds were tested alone and with a combination of CK. Results obtained with A_1 ligands are reported in the following graphs (Figure 71).

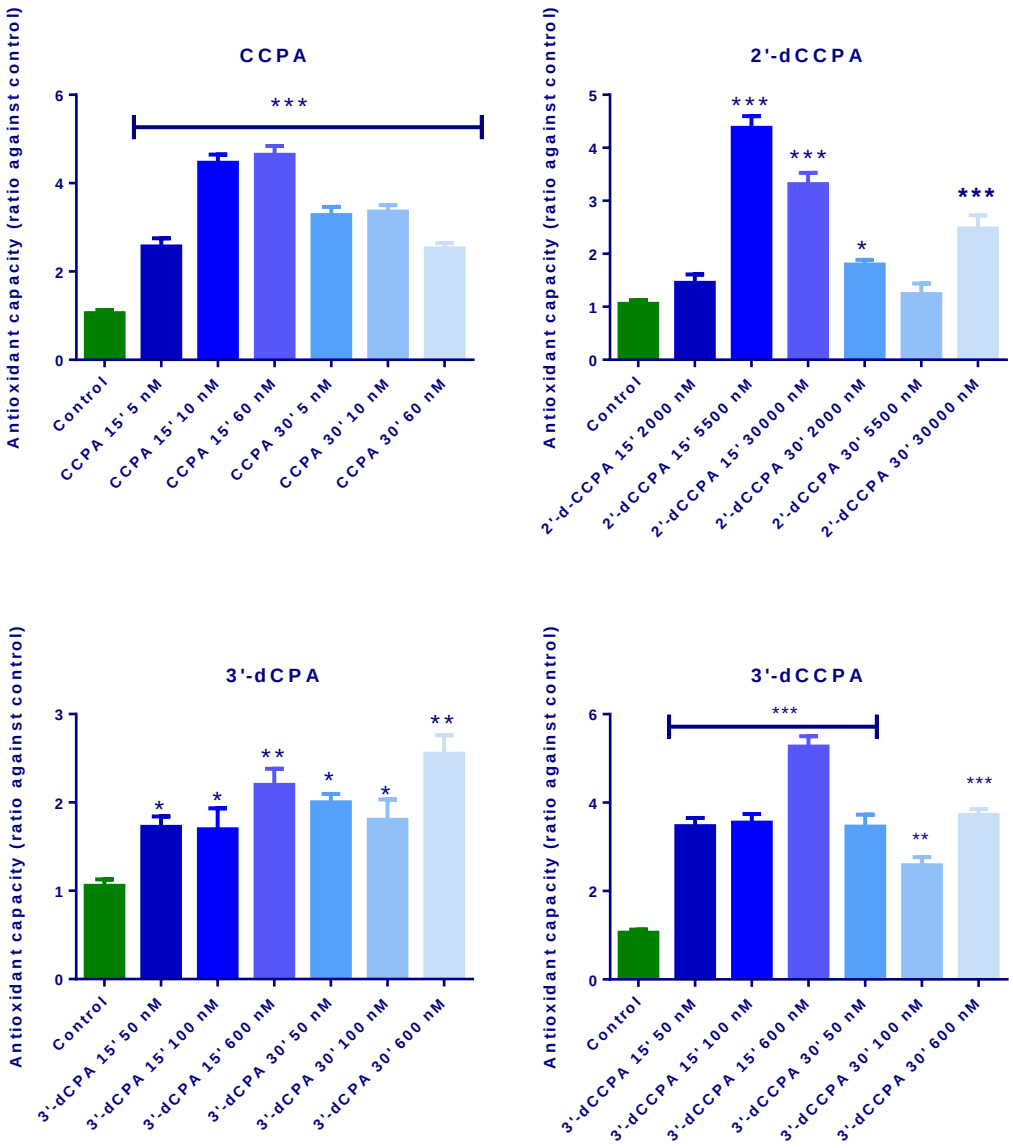


Figure 71. Antioxidant effects of CCPA and A_1AR agonists on mixed glial cell culture. Ratio of NO_2^- production after treatments. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against control.

No one of them at any concentration or incubation time demonstrated to possess antioxidant activity. The same response was obtained in presence of CK cocktail. Results obtained with the pre-incubation of the agonist followed by the CK aggression are reported in Figure 72.

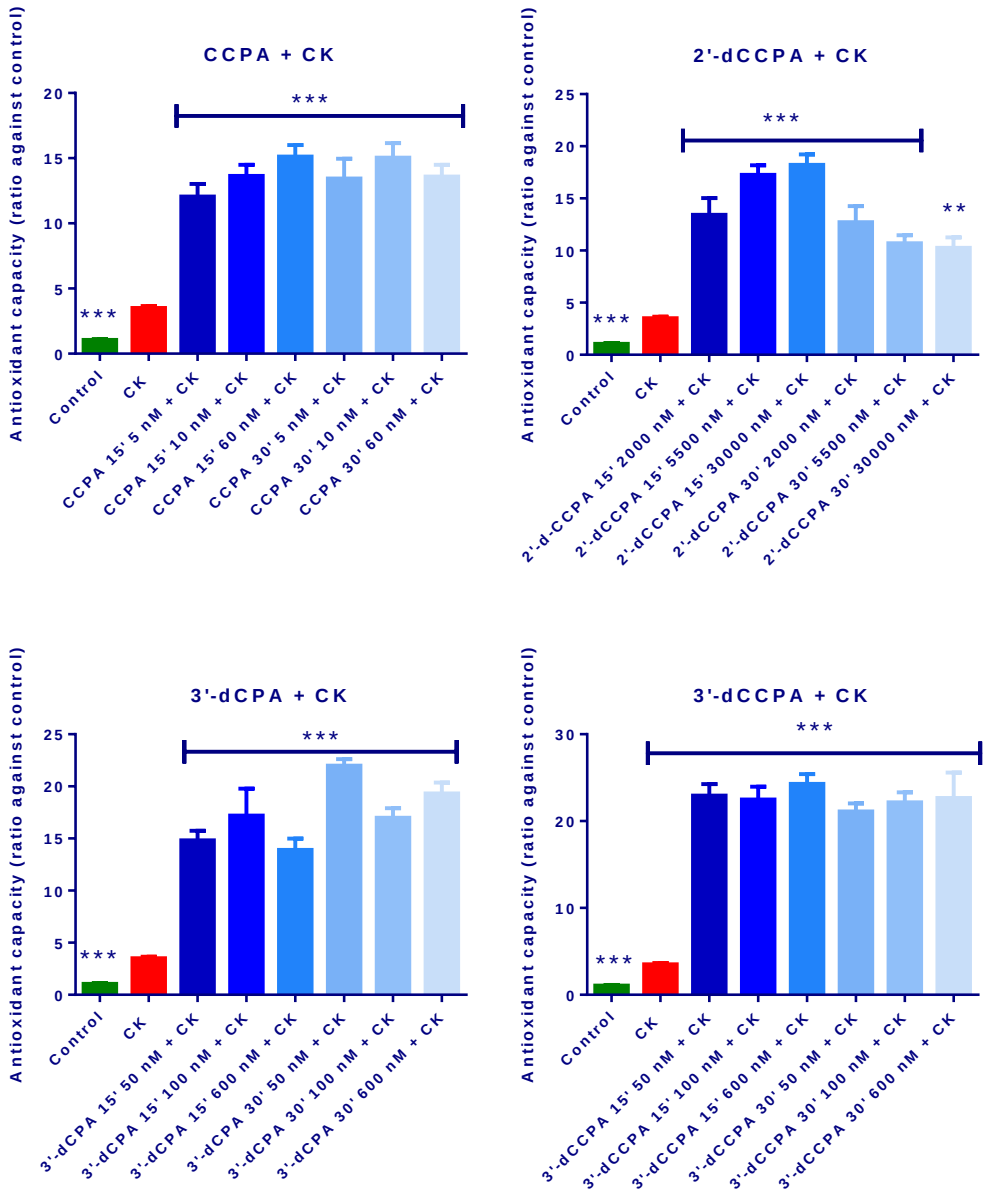


Figure 72. Antioxidant effects of CCPA and A_1AR agonists against CK aggression on mixed glial cell culture. Ratio of NO_2^- production after treatments. Results represent the average of three independent experiments. **P<0.01, *** P<0.001 of treated cells against control.

Experiments conducted by Minelli and co-workers (Minelli A. et al., 2010) demonstrated the presence of adenosine A_1 receptor in the mitochondria suggesting an important role for this adenosine receptor as a mitochondrial vulnerability factor. They discovered through primary brain mixed cell cultures from WT and A_1KO rodents that animals without the presence of A_1AR are less responsive to ROS and more resistant to insults created by the aggressive agent. These data point out that adenosine A_1 receptor could function as a mitochondrial vulnerability factor. These evidence could explain the results obtained on the lack of antioxidant activity of A_1AR agonists.

Experiments with selected $A_{2A}AR$ antagonists were performed in order to evaluate their antioxidant activity. Initially it was evaluated the antioxidant activity of these compounds alone, results were reported in Figure 73.

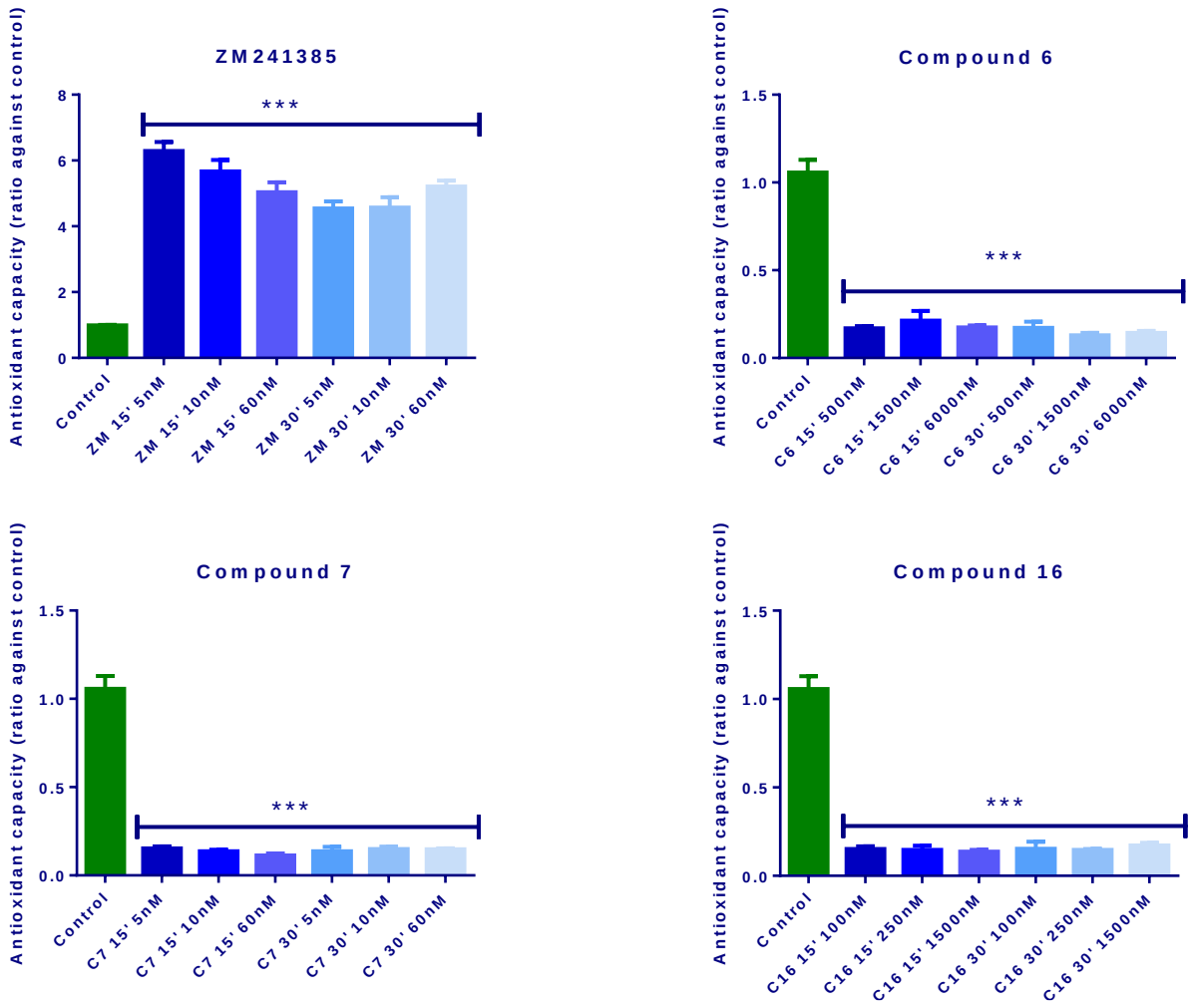


Figure 73. Antioxidant effects of ZM241385 and $A_{2A}AR$ antagonists on mixed glial cell culture. Ratio of NO_2^- production after treatments. Results represent the average of three independent experiments. *** P<0.001 of treated cells against control.

In general, these molecules showed to possess an important antioxidant capacity. In fact, as reported in Figure 73, the levels of nitrite produced were even lower than those obtained with the control, demonstrating that these compounds could be useful as antioxidant agents. In addition, the referent compound did not demonstrate any antioxidant activity. At this point it was evaluated their antioxidant activity after CK insult. Cells were pre-incubated with the CK cocktail and then with the antagonists (Figure 74).

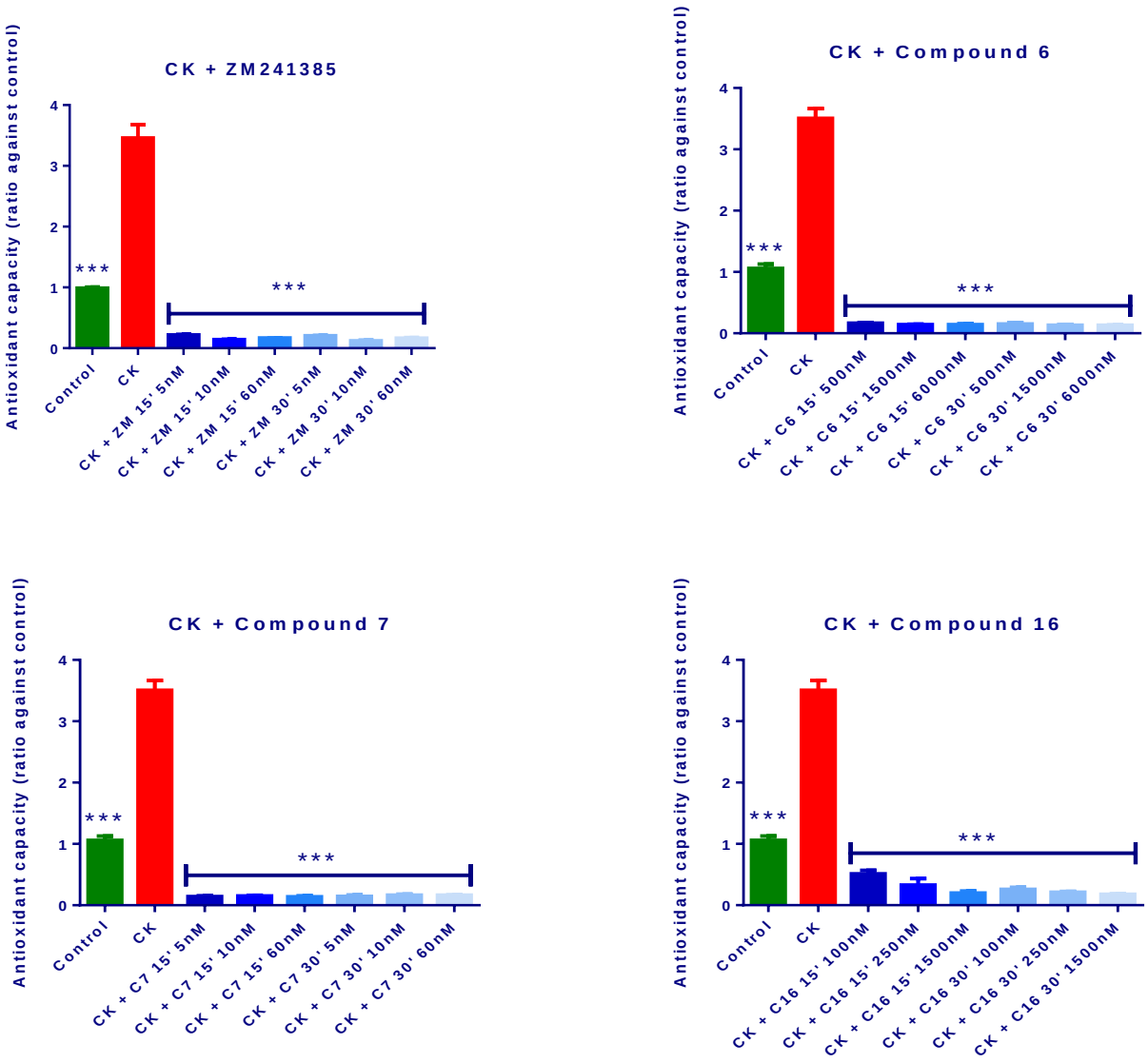


Figure 74. Antioxidant effects of ZM241385 and A_{2A}AR antagonists after pre-treatment with CK cocktail on mixed glial cell culture. Ratio of NO_2^- production after treatments. Results represent the average of three independent experiments. *** P<0.001 of treated cells against CK.

Also in this case compounds **6**, **7** and **16** demonstrated to have antioxidant profiles with a high decrease of nitrite levels induced by the CK cocktail. The major activity was shown by compounds **6** and **7**. It is worth noting that in this condition also the ZM241385 was able to counteract the increase of the formation of reactive oxygen RNS species induced by CK. In summary, these experiments confirmed the good capacity of these antagonists as antioxidant agents. The antioxidant capacity of these A_{2A} antagonists to reduce reactive species levels can be supported by the results obtained by Hosny and co-workers. They showed the antioxidant and anti-Inflammatory efficacy of caffeine in neuroinflammation models (Hosny EN. Et al., 2019).

5.2.4. Antiapoptotic capacity

In the CNS redox activity is essential for cell metabolic processes and functions. Normally, brain functions are preserved by immunological responses, which avoid the aberrant formation of ROS and RNS. Under pathological conditions, it has been seen a synergic action between the pro-inflammatory and oxidative signals that provokes neuronal damage and the associated neurodegeneration (Aguilera G. et al., 2017). In addition, mitochondrial ROS induce the activation of mitochondrial apoptotic proteins, leading to cellular apoptosis and organ damage. TNF-α, and IFN-γ have been shown to synergistically induce apoptosis via the induction of nitric oxide (Li X. et al., 2019). In addition, IL-1β can induce cell death by apoptosis (Tricarico P.M. et al., 2018). Generally, anti-apoptotic properties that lead to a great neuron survival involve reduction in apoptosis mediators as well as oxidative substances, such as superoxide dismutase and hydrogen peroxide. As explained before, functional studies in hA_1AR stably transfected at CHO cells demonstrated that 2'-dCCPA acts as a partial agonist (α = 0.70), result proved in an *ex vivo* experiment performed at mouse ileum contractility. In that study it was demonstrated by 75% of maximal contractility reduction obtained with the full agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) (Martire A. et al., 2019). In addition, the partial agonist profile could conferee beneficial effects in the treatment of acute and chronic disease due to the less side effects compared to the A_1AR full agonist. On the basis of binding assays, Cell viability and Griess assays results, the best A_1AR agonist and the best $A_{2A}AR$ antagonist were chosen for further experiments. The A_1AR agonist was the 2'-dCCPA at 5500 nM while the $A_{2A}AR$ antagonist was Compound **7** at 10 nM. In these experiments, the effects of 2'-dCCPA and compound **7** were compared with referent compounds CCPA and ZM241385, respectively. In both referent compounds, the best effects were observed at 10 nM after 30 minutes of treatment. The anti-apoptotic property of compounds was assessed in mixed glial cell cultures by Hoechst assay. In this experiment, the cell circularity was determined as a structural element (Figure 75).

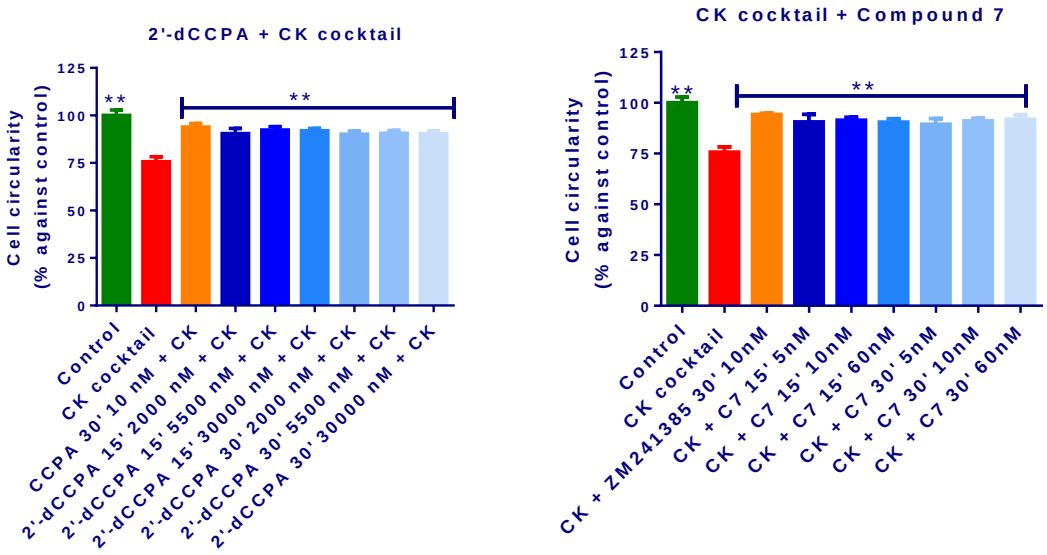


Figure 75. Cell circularity after the pre and post treatment with CCPA and 2'-dCCPA or ZM241385 and C**7** respectively in presence of the CK cocktail. The results represent the average of three independent experiments. **P<0.01 of treated cells against CK.

When the mixed glial cell cultures were treated with CK cocktail there was a decrease of cell circularity until 25% compared to the control cells (P-values < 0.05), indicating DNA degradation and a cells apoptotic state. The pre-treatment with 2'-dCCPA blocked the CK cocktail effect and cells circularity was the like of the control. The treatment with compound **7** counteract the damage of cells induced by CK cocktail and also in this case cells circularity was the like of the control, little differences are not statistically significant. Another parameter studied with Hoechst was the area occupied by cells after each treatment (Figure 76).

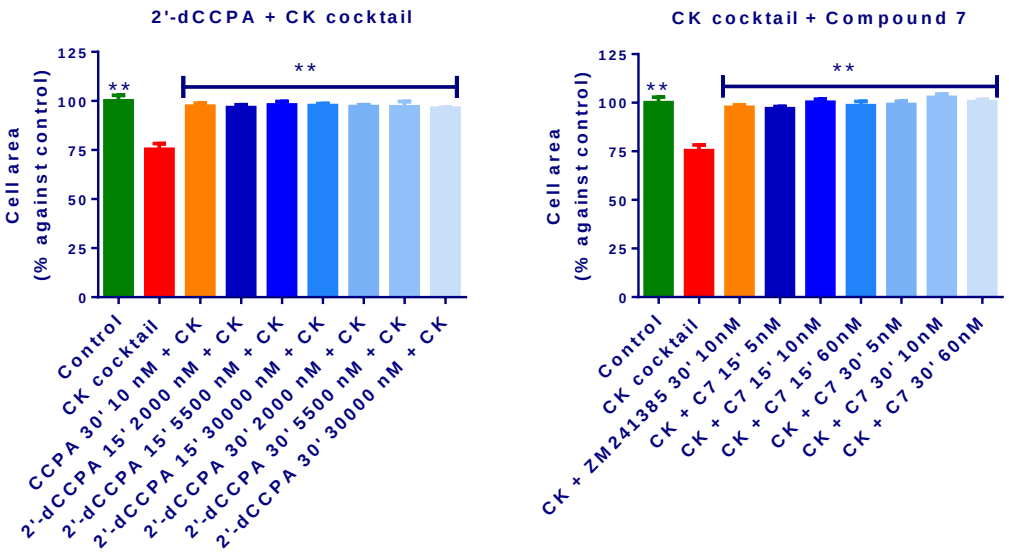


Figure 76. Cell area after the pre and post treatment with CCPA and 2'-dCCPA or ZM241385 and C**7** respectively in presence of the CK cocktail. The results represent the average of three independent experiments. **P<0.01 of treated cells against CK.

As results obtained before, when cells were treated with CK alone, the area decreased under the 80%. When cells were incubated with agonist or antagonist plus de CK, the area returned to control values, indicating that this compound have beneficial effects against the apoptosis. These results in decreased cell area and circularity after the CK treatment and the maintenance or recovery levels after the agonist or antagonist treatment can be explained by Fezai's experiments. Fezai and co-workers demonstrated that cells under aggression, with decreased cell viability levels, possessed a lower cell circularity and area compared to control (Fezai M. et al., 2017). This diminishment in cell area and circularity is tightly correlated with the apoptosis cell process. It is worthwhile to note that this results correlate positively and significantly with those obtained by cell viability assay (R = 0.988, P-value = 0.049). Effects obtained with 2'-dCCPA and compound **7** in pre and post treatment, respectively, indicated that they have a protective effect and contrast the CK aggression in a statistically significant way, consequently demonstrating to have anti-apoptotic properties.

5.3. *IN VITRO* STUDIES IN N13 CELL CULTURES

Primary cell cultures are not immortal and hence the number of cells available for experiments is much more limited. In addition, recent studies have pointed out that microglia cell lines differ both genetically and functionally from primary microglia and *ex vivo* microglia (Butovsky et al., 2014; Das et al., 2016; Melief et al., 2016). In addition, microglial cell lines obtained from neonatal or

embryonic CNS sources are unlikely to reflect the phenotype of adult or elderly microglia. For these reasons the studies were performed also in immortalized microglial cells N13.

5.3.1. Immunocytochemistry

The experiment performed in mixed glial cell cultures were repeated in N13 cell cultures. The presence of A_1AR and A_{2A}AR was checked by direct immunocytochemistry. The presence of both receptors were confirmed as showed in Figure 77:

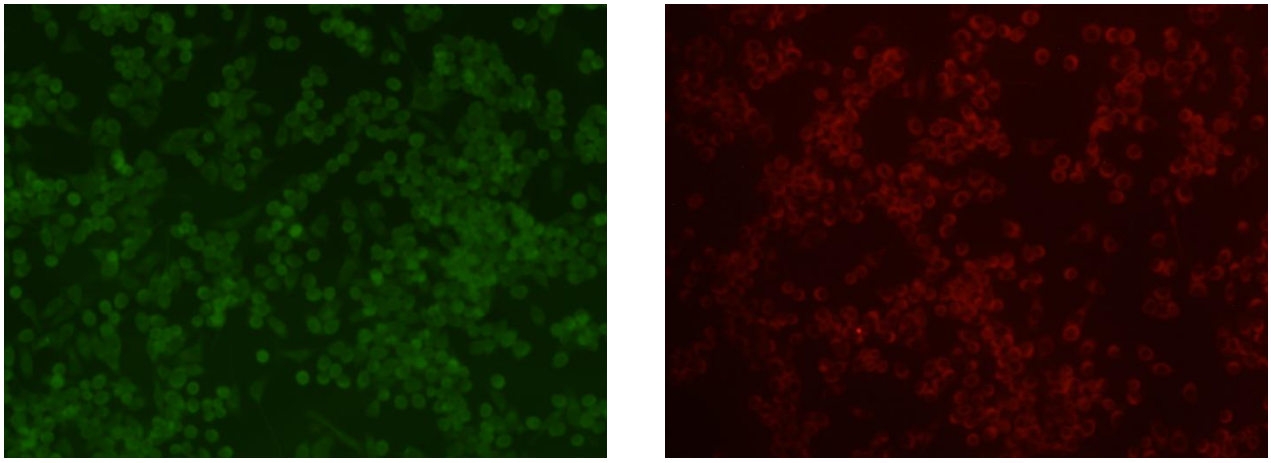


Figure 77. Direct immunohistochemistry of N13 cell cultures. Cells were treated with Adenosine A_1 Receptor Polyclonal Antibody ALEXA FLUOR® 488 Conjugated (green) and Adenosine A_{2A} Receptor antibody ALEXA FLUOR® 594 Conjugated (red).

5.3.2. Cell viability

Initially, the selected compound 2'-dCCPA and compound **7** were tested in N13 cell cultures and results showed that in general cells maintain or enhance their viability compared to control, except for the higher dose of 2'-dCCPA (86 ± 1.8 *vs* control, Figure 78).

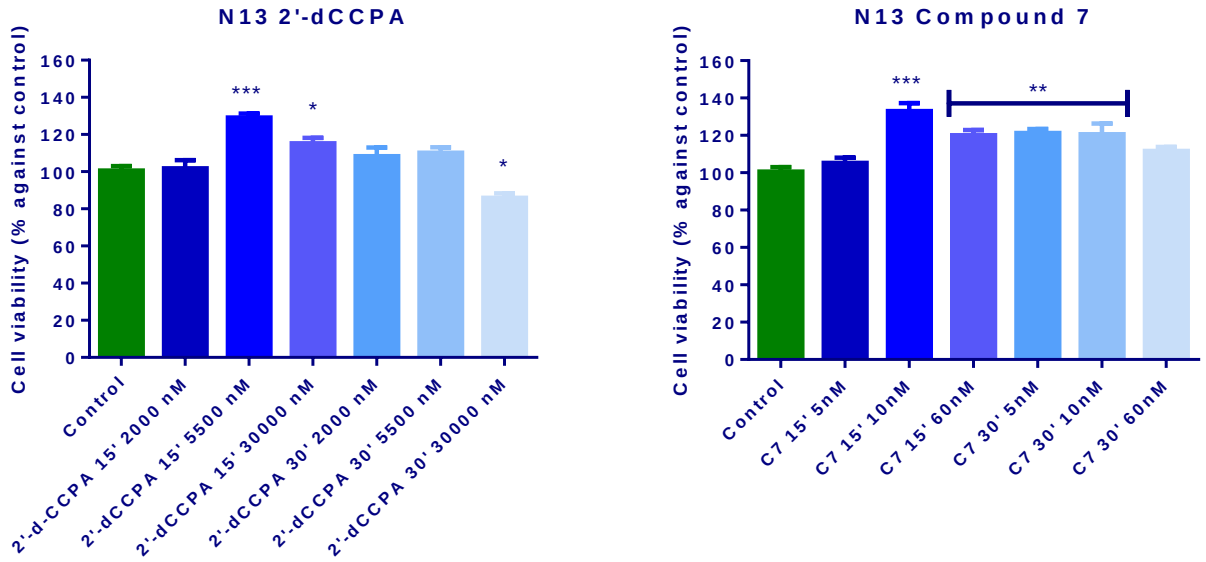


Figure 78. Protective effect of 2'-dCCPA and restoring effect of C**7** against CK aggression on N13 cell culture. Percentage of cell proliferation after treatment with the CK cocktail for 48 h. Results represent the average of three independent experiments. *P<0.05, **P<0.01, *** P<0.001 of treated cells against control.

The effects of 20 ng/ml CK cocktail for 48 h in N13 cells were assessed. The cells were in very suffer conditions, more than those mixed glial. For this reason, the time of incubation was changed maintaining the concentration 20 ng/ml. It was proved that after 40 h of incubation the effects was the same obtained with mixed glial cells during 48 h of incubation (69 ± 2.4 *vs* control; Figure 79).

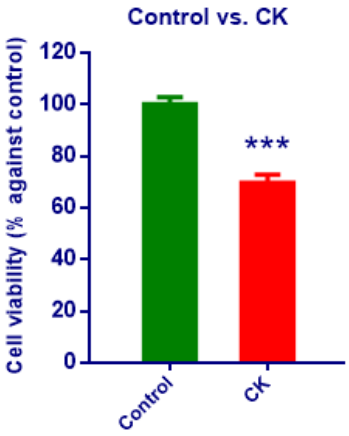


Figure 79. Effects of CK cocktail on the viability of mixed glial cell culture. Percentage of cell proliferation after treatment with the CK cocktail for 48 h. Results represent the average of three independent experiments. *** P<0.001 of treated cells against control.

After asserting the best CK time incubation, it was performed the pre-incubation with 2'-dCCPA followed by CK cocktail and the pre-incubation with CK cocktail followed by compound **7**. Results are similar to those obtained in mixed glia cells, in fact A_1AR agonist and A_{2A}AR antagonist possess beneficial effects for cell surviving before and after the aggression respectively (Figure 80). It is

worthwhile to note that in both cases the best time and concentration are the same used in mixed glial cell cultures: 5500 nM for 2'-dCCPA and 10 nM for compound **7**, both at 30 minutes of incubation (151 ± 1.4 and 156 ± 1.7 *vs* control, respectively).

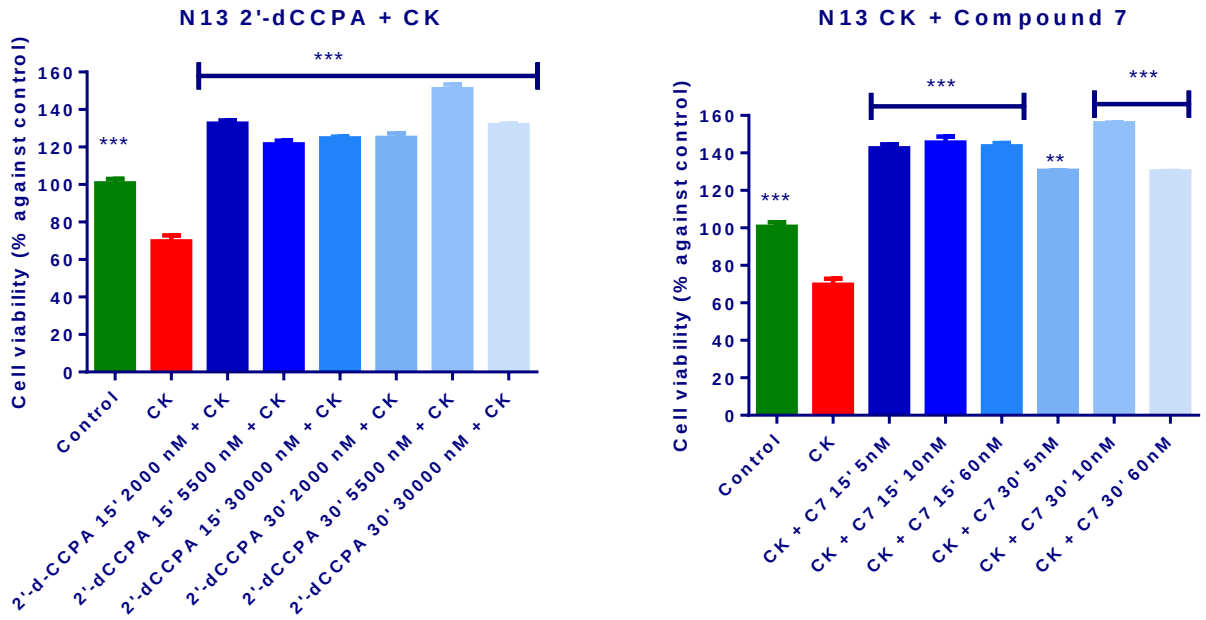


Figure 80. Effects of 2'-dCCPA and Compound **7** with CK treatment on the viability of N13 cell culture. Percentage of cell proliferation after treatment 2'-dCCPA and Compound for 15 or 30 minutes and CK for 40 h. Results represent the average of three independent experiments. **P<0.01, *** P<0.001 of treated cells against CK.

5.3.3. Antiapoptotic capacity

The circularity and the area of N13 cells after the treatments were evaluated through Hoechst assay. Results showed that also in this case the treatment with 2'-dCCPA prevents the effect of the CK and compound 7 counteract it. These results underline again the antiapoptotic effects of these selected ligands (Figure 81).

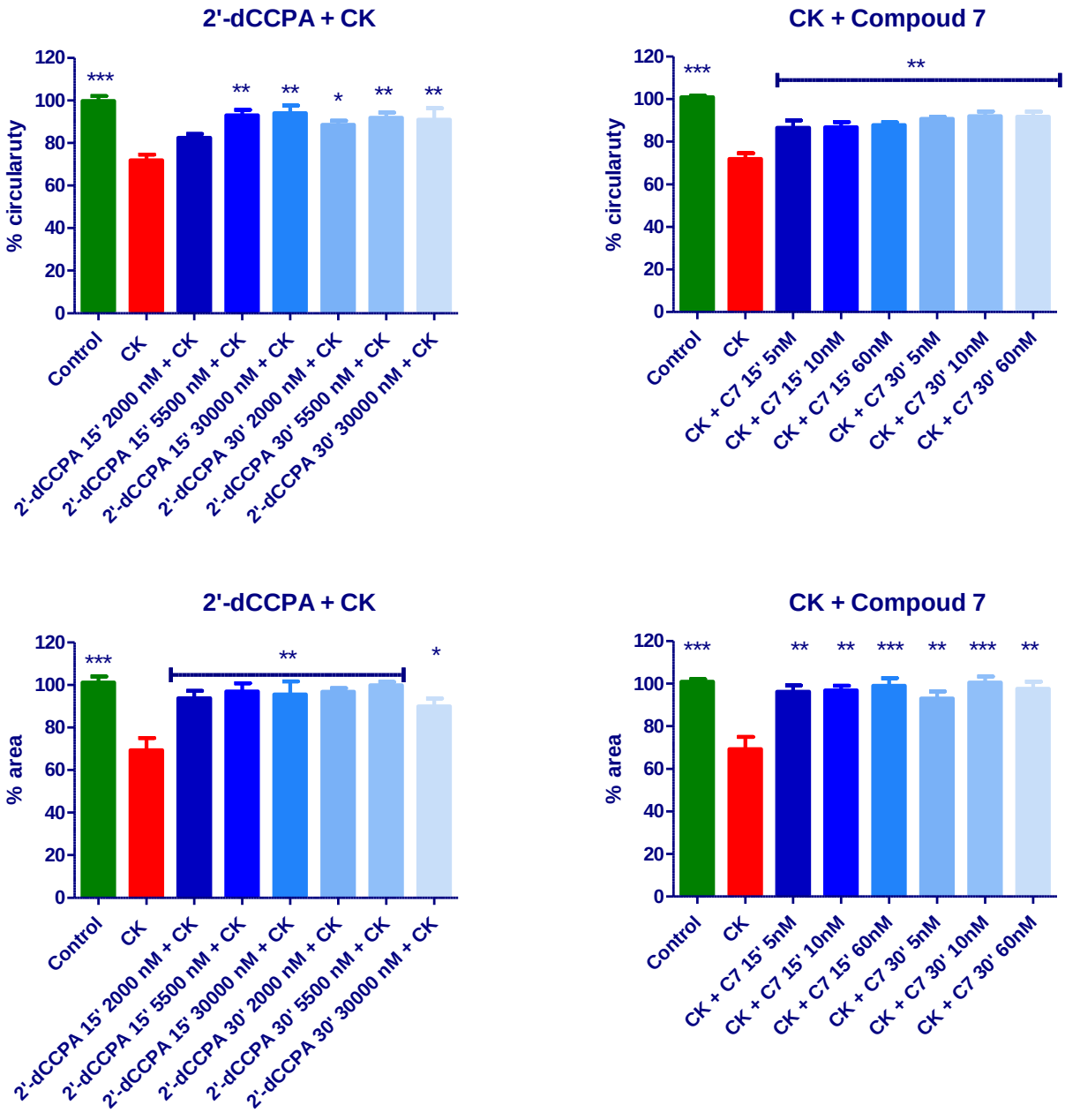


Figure 81. Cell area and circularity after the pre and post treatment with 2'-dCCPA and compound **7** respectively in presence of the CK cocktail. The results represent the average of three independent experiments. **P<0.01, *** P<0.001 of treated cells against CK.

During my PhD course I spent a period in *Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Universidade de Lisboa* under supervision of Professor Luísa Lopes. In this period, I performed experiments in *in vitro* and in *in vivo* models of neuroinflammation using 2'-dCCPA and compound **7**.

5.4. *IN VITRO* STUDIES IN PRIMARY NEURONAL CELL CULTURES

5.4.1. SYTO-13/PI fluorescence

In order to validate the results obtained in mixed glia cell culture viability experiment was performed on primary neuronal cell culture (Figure 82). These cells maintain the characteristics of their origin tissue, making them a biologically and physiologically relevant tool for the study of neuroinflammation.

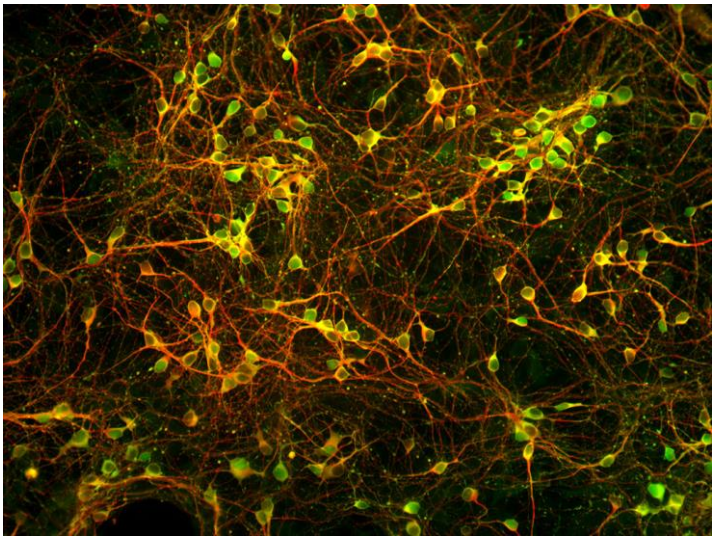


Figure 82. Primary neuronal cells.

Neurons were obtained from rat embryos and were seeded until confluence. They were treated, using the best dose (2'-dCCPA 5500 nM and compound **7** 10 nM) and incubation time of 30 minutes, with each ligand alone and with combination of CK cocktail. In addition, parallel experiments were performed treating neurons with a well-known selective A_1AR antagonist DPCPX to check if the activity of the partial agonists 2'-dCCPA is due to A_1AR, and with a selective A_{2A}AR antagonist SCH58261 to check if compound **7** could act through A_{2A}AR receptors. The concentration used of DPCPX is 100 nM and SCH58261 50 nM in accordance to their K_i 3.9 nM and 1.2 nM. Neurons treated with 2'-dCCPA and compound **7** with and without CK demonstrated to restore cell health after the aggression with CK in a statistically significant way (115 ± 3.2 and 118 ± 3.8 *vs* control respectively). In both cases, values were higher than in CK and very similar to control (Figure 83).

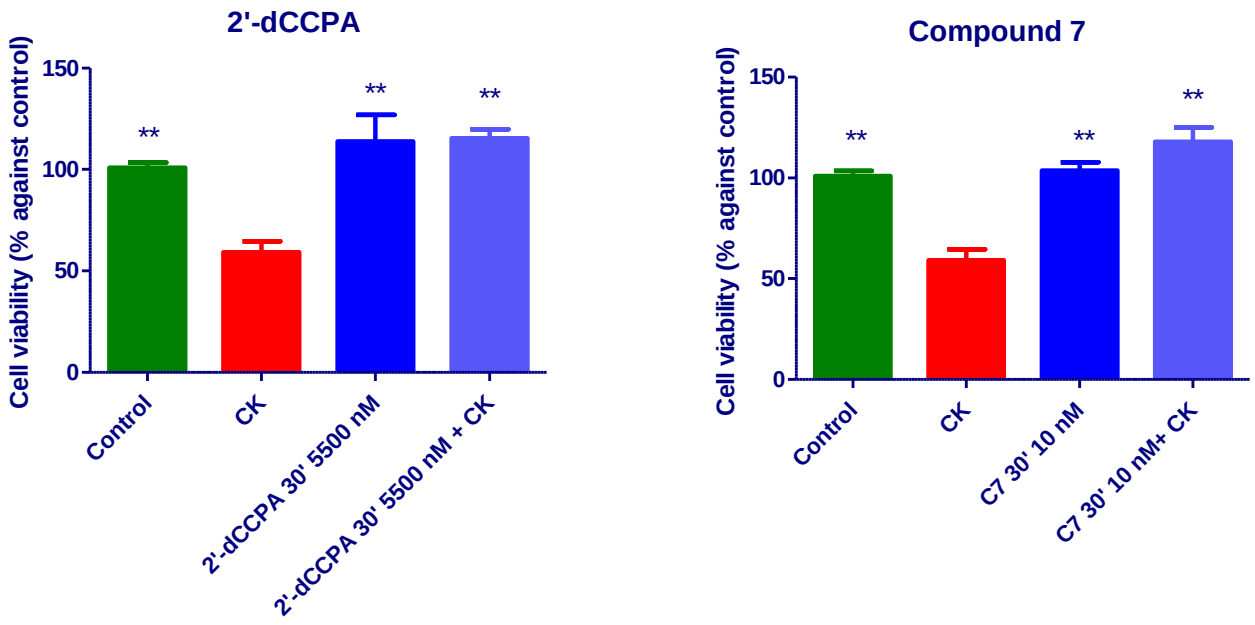


Figure 83. Syto-13/PI assay treated with 2'-dCCPA or compound **7** with and without CK aggression in primary neuronal cell cultures. The results represent the average of three independent experiments. **P<0.01 of treated cells against CK.

Results with DPCPX at 100 nM and with SCH58261 at 50 nM are reported in (Figure 84):

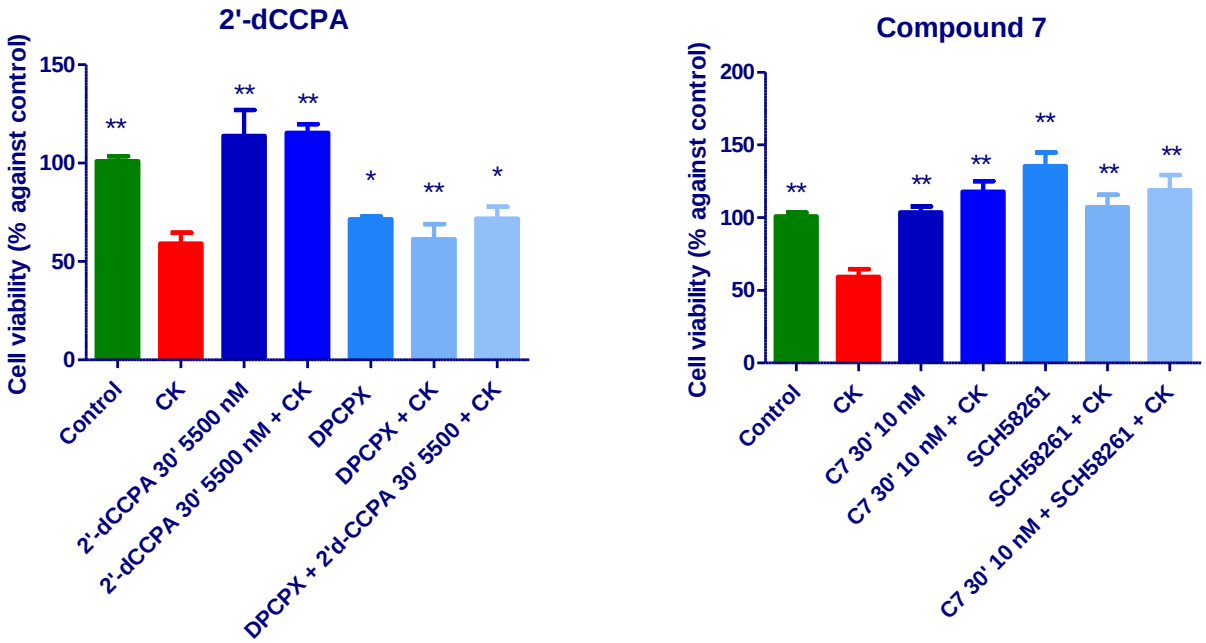


Figure 84. Syto-13/PI assay treated with 2'-dCCPA and DPCPX or compound **7** and SCH58261 with and without CK aggression in primary neuronal cell cultures. The results represent the average of three independent experiments. **P<0.01 of treated cells against CK.

Results demonstrated that the addition of DPCPX produces a damage to neurons blocking the 2'-dCCPA action (72 ± 3.4 *vs* control). In fact, the cell survival decreases compared to control and 2'-dCCPA+CK (115 ± 3.2 *vs* control) reaching the same CK value (60 ± 3.7 *vs* control). These results demonstrated that the effect of 2'-dCCPA is via A_1AR. On the other hand, when compound **7** was incubated with SCH58261 at 50 nM (119 ± 3.6 *vs* control), no synergic effects were found, demonstrating that compound **7** competes with the SCH58261 to bind with the same receptor. This result is explained by the different concentrations used of these antagonists since SCH58261 (50 nM) is 5 folds higher than C**7** (10 nM) but their A_{2A}AR affinity are similar (SCH58261 $K_i A_{2A}$AR = 1.2 nM and C**7** $K_i A_{2A}$AR = 0.75 nM). In these conditions, receptors were mainly bound by SCH58261 and the addition of C**7** is unable to produce a synergic effect.

In conclusion, it has been demonstrated that both compounds had beneficial effects in protecting neurons against CK insult and this effect was linked to A_1AR activation and A_{2A}AR blockade.

5.4.2. Western Blot

To elucidate the pathway that could be enrolled after receptor activation of 2'-dCCPA and compound **7**, some intracellular molecules were checked by western blot. The study investigates the involvement of cytochrome C, inducible nitric oxide synthase and caspase-3.

Cytochrome C is a small protein that works as electrical transporter inside the mitochondria. It plays the main role between the respiratory complex III and IV, but under aggression or stress environments, it could be released into the cytosol activating the apoptosis pathway. When the Cyt C is in cytosol, it binds to the apoptotic protease activating factor-1 (APAF1), which suffers a conformational change allowing the formation of apoptosome and activation of caspase-9. This signalling pathway leads to the cell apoptosis. As expected when primary neuronal cells were treated with CK the levels of Cyt C increase in comparison to the control (134 ± 4.1). It is interestingly to note that both compounds under study, alone (52 ± 3.8 for 2'-dCCPA and 58 ± 3.2 for C**7** *vs* control) or in combination with the CK (53 ± 2.9 for 2'-dCCPA and 62 ± 2.7 for C**7** *vs* control), allowed a reduction of this Cyt C release, indicating protective effects for the mitochondria and antiapoptotic roles (Figure 85).

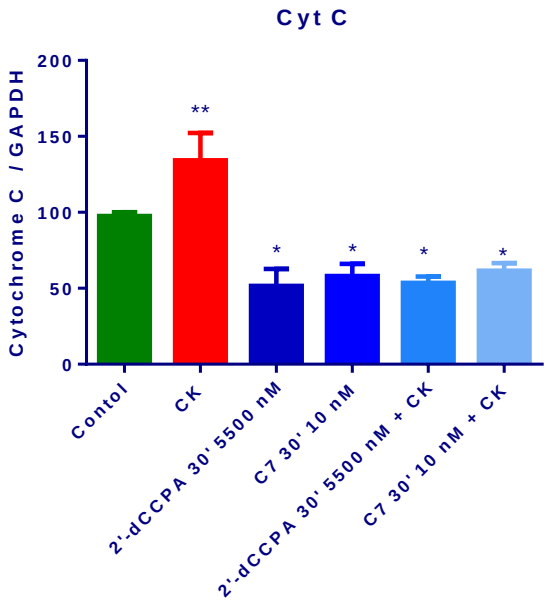


Figure 85. Cyt C expressions levels after the treatment with 2'-dCCPA or compound *7* in presence or absence of CK cocktail. The results represent the average of three independent experiments. *P<0.05, **P<0.01 of treated cells against control.

The second molecule studied was the inducible nitric oxide synthase (iNOS). This catalytic enzyme, after activation, produces a large amount of NO. The production of these reactive species leads to production of other reactive elements creating an inflamed state. After neurons treatment with CK there is an increment of iNOS levels (132 ± 4.3 *vs* control), indicating an activation of iNOS and thereof RNS production. Results showed that only compound **7** at 10 nM for 30 minutes with and without CK (72 ± 3.3 and 73 ± 2 *vs* control, respectively) demonstrated a significant decrease compared to control, but both compounds showed a reduction of iNOS levels compared to CK (81 ± 2.7 and 82 ± 3 *vs* control, respectively; Figure 86).

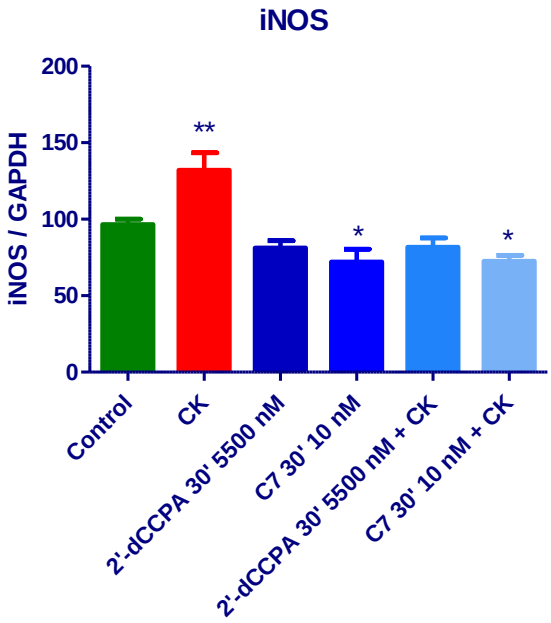


Figure 86. iNOS expressions levels after the treatment with 2'-dCCPA or compound *7* in presence or absence of CK cocktail. The results represent the average of three independent experiments. *P<0.05, **P<0.01 of treated cells against control.

Caspase-3 is a pro-apoptotic element that can be activated by an intrinsic or extrinsic pathway. Its activation is mediated through the exit of Cyt C, activation of Apaf1 and caspase-9. In addition, the activation of caspase-3 is due to external elements that activate also caspase-8. Western blot of this enzyme was calculated by the ratio of (active caspase-3)/(inactive caspase-3). As it is demonstrated by the graphic, CK leads to a higher activation of caspase-3 (1.37 ± 0.17 *vs* control), while the A_1 agonist and A_{2A} antagonist decrease this activation (0.62 ± 0.085 0.56 ± 0.091 *vs* control, respectively). When these ligands were incubated with the CK cocktail, only compound **7** is able to reduce the activated caspase-3 levels (0.83 ± 0.12 *vs* control; Figure 87).

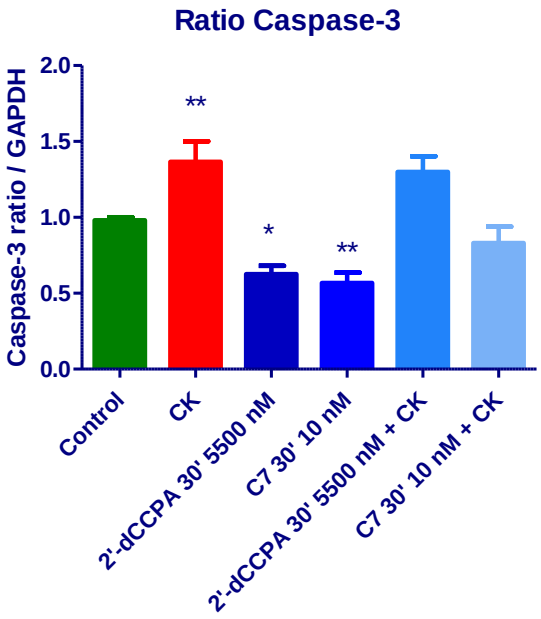


Figure 87. Caspase-3 ratio expressions levels after the treatment with 2'-dCCPA or compound *7* in presence or absence of CK cocktail. The results represent the average of three independent experiments. *P<0.05, **P<0.01 of treated cells against control.

In pathological conditions and programmed cell death, cytochrome C binds to Apaf-1, forming the apoptosome, activating capsase-9 and caspase-3. This last enzyme is responsible of the apoptosis process. Taking into account these results it could be affirmed that both 2'-dCCPA and compound **7** are able to avoid the release of cytochrome C from the mitochondria, preventing the union to Apaf-1, caspase-9 and caspase-3 activation and apoptosis.

5.5. *IN VIVO* STUDIES IN RAT MODELS

Since these compounds showed good biological effects in *in vitro* studies, the investigation continued on *in vivo* rat neuroinflammation model in order to test their *in vivo* potential. Rats were divided into two groups to test the effects of the agonist and antagonist. It was administrated the aggression factor (LPS) or the ligand alone or both. A group using a saline solution was used as control. Three different experiments were performed to see how these molecules affected the progression of neuroinflammation: Open Field (OF), Y Maze and Object Displacement (OD).

5.5.1. Open Field

To test the *in vivo* efficacy of these compounds, it was chosen a neuroinflammatory model, using LPS delivered directly into the CNS, which it enabled to circumvent the peripheral effects of systemic

inflammation. Nonetheless, in the first 24 h post-surgery animals displayed the characteristic sickness behaviour with increased body temperature, and lethargy that subsided after 48 h. Open Field experiment allowed to check animal health state, if they suffered from anxiety or stress or even if they had mobility alterations or motor problems. Rats were placed in an open arena and observed for 5 minutes. Their general behaviour was evaluated and both velocity and distance travelled measured. No significant differences were observed between the various groups. In fact, when animals were tested in the OF post 48 h, locomotor activity, was indistinguishable between control and LPS insult. However, when tested for short-term memory the LPS group presented impaired performance, indicating that neuroinflammation had resulted in altered brain function. Results showed with 2'-dCCPA, compound **7**, saline, LPS or they respective combinations treatment that there was not any alteration in the distance covered and in the velocity of the animals. These results indicated that animals were fine and did not have anxiety or mobility problems (Figure 88).

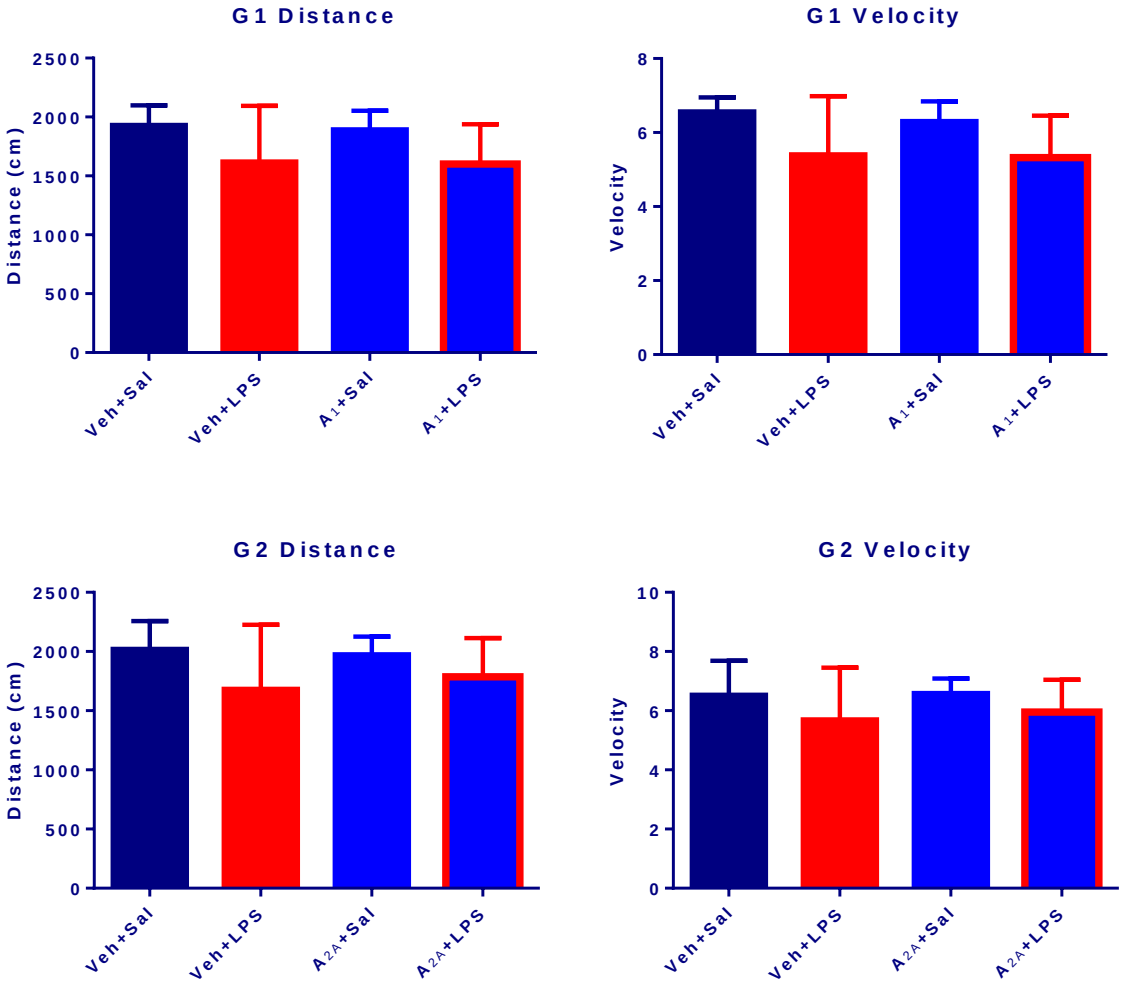


Figure 88. Effects of treatment in distance and velocity performed by rats in Open Field. Saline (sal), Vehicle (veh, vehicle used for the ligand dilution).

The percentage of time that animals spent in the peripheral *vs* central areas in the arena was also calculated. Naturally, rodents feel safer near the walls (periphery), since these provide safety from any predators, but also have a biological drive to explore new environments, and should spend sometime in the arena centre. In OF all animals independently of the treatment or insult spent similar amounts of time between the periphery and the centre area, suggesting that no changes in anxiety-like behaviours results from any of the insult/treatment pairing (Figure 89).

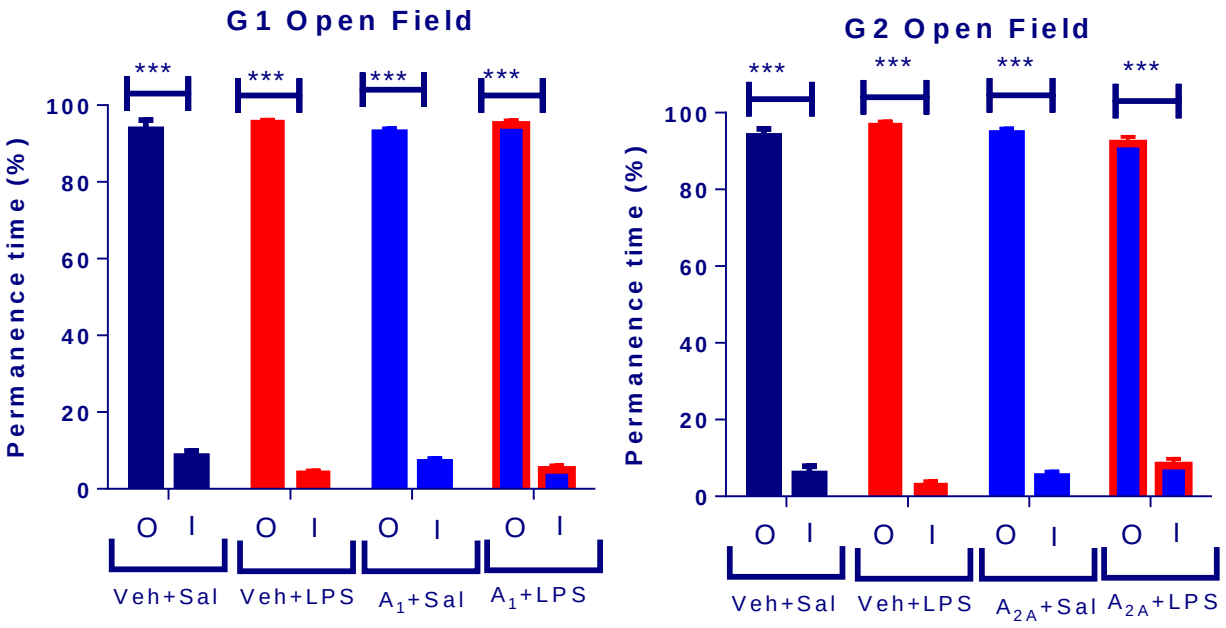


Figure 89. Effects of treatment in permanence time in the outside area (O) and inside area (I) in Open Field. Saline (sal), Vehicle (veh, vehicle used for the ligand dilution). *** P<0.001 of inside area against outside area. Inside areas did not demonstrate to be statistically different among them.

2'-dCCPA alone also induced a slight impairment in short-term memory performance. This is in accordance with its A_1AR activation, that produces generalized inhibition of cellular function, and could thus, in the absence of an insult, result in deleterious consequences, at least when directly injected into the CNS. A_1AR agonists are well known to afford neuroprotection when administered prior to an insult, namely stroke models in which pre-conditioning with an A_1AR agonist is well described to decrease the severity of the damage. This aspect is related to the increase in extracellular endogenous adenosine levels in response to any kind of cellular stress. Accordingly, blockade of A_{2A}AR is more relevant to stop the deleterious effects of A_{2A}AR over activation in these situations. Since 2'-dCCPA was delivered directly into de brain, it is clear that it acted in the affected brain areas, while compound **7**, administered by i.p. injection was also able to provide rescue from the CNS inflammatory insult, suggesting it readily crosses the BBB.

5.5.2. Y Maze

After assessing that locomotor behavior was preserved and not difference between the groups was relieved, it was evaluated short-term memory using the Y Maze. In this paradigm, animals are left to explore the maze with one of the Y maze arms closed off, for 8 minutes. After 1 hour, the animal is reinserted in the maze, but this time all arms are available for exploration. The animal's natural behavior is to spend more time exploring the previously closed off arm, since this is a new environment, and animals have a natural preference for novelty. The arms that were accessible from the beginning are considered the "other arms" (O), while the "new unexplored arm" is the novel arm (N). Control groups (Vehicle+Saline), as expected, showed a preference for the novel arm (Figure 90). Upon a neuroinflammatory stimulus with LPS, animals showed no preference for the novel arm, as can been seen in the Veh+LPS groups, demonstrating that this insult produces impairment in short-term memory performance. This phenotype was rescued by administration of either 2'-dCCPA or compound **7**, suggesting that these compounds are able to neuroprotect cells from the effects of the inflammatory insult.

2'-dCCPA alone also induced a slight impairment in short-term memory performance. It is well known that A_1AR agonists produce a marked depression of cellular activity and a generalized inhibition of cellular function, in fact acute i.p. administration of A_1AR agonists produces marked effects on locomotor activity, dependent of CNS-mediate effects (Martire A. et al., 2019). This is in accordance with A_1AR activation that produces, in the absence of an insult, deleterious consequences, at least when directly injected into the CNS. This general inhibitory role of cellular functions is one of the best known effects of adenosine, in situations of damage in CNS, acting as an energetic sensor that promotes marked inhibition of all synaptic activity. It is also well established that preconditioning tissues with an exposure to an A_1 agonist, provides neuroprotection to a subsequent insult or damage, and the outcome is an improved cellular function and behavior (Cunha R.A. 2016). Accordingly, blockade of A_{2A}AR is more relevant to stop the deleterious effects of A_{2A}AR over activation in these situations. Since 2'-dCCPA was delivered directly into de brain, we are confident that it acts in the affected brain areas, while C**7**, which was administered by i.p. injection, was also able to provide rescue from the CNS inflammatory insult, suggesting it readily crosses the BBB.

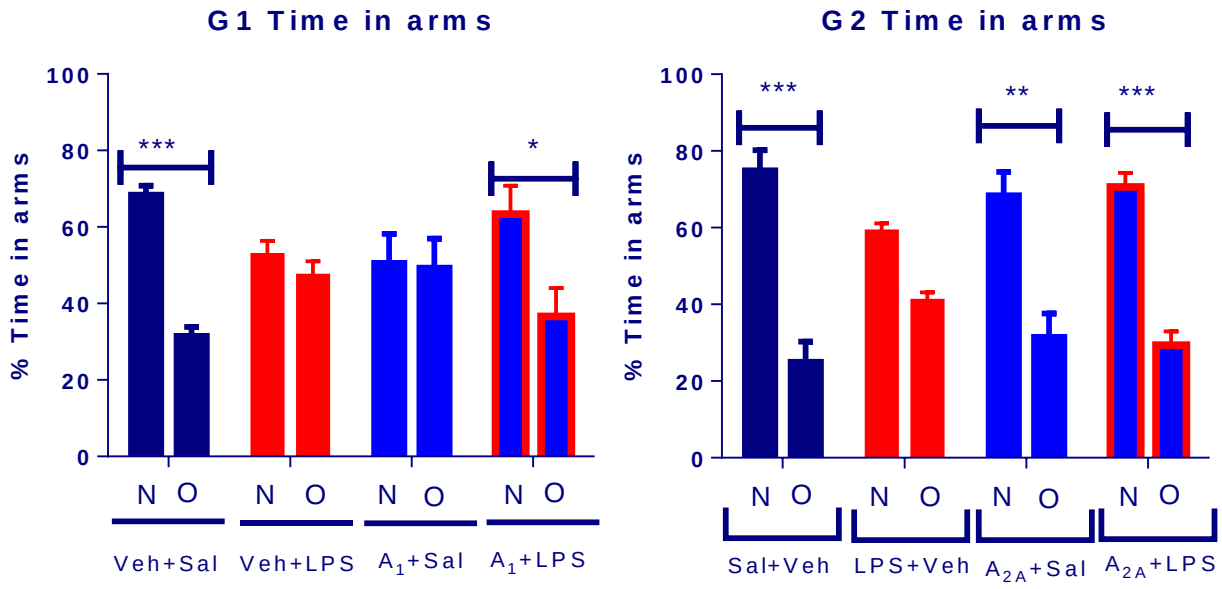


Figure 90. Effects of treatment in time spent in novel (N) and old (O) arms. Saline (sal), Vehicle (veh, vehicle used for the ligand dilution). **P<0.05, **P<0.01, *** P<0.001 of novel arm against old arm.

5.5.3. Object Displacement or Pattern Separation

Object Displacement (OD) or Pattern Separation (PS) allowed to check the short-term memory of the animal to recognise familiar and novel object positions. OD is a useful tool to understand if the animal is able to recognize the new object position and it is calculated by the Discrimination Index (DI). When the DI is positive means that the animal is able to recognize the new position while when it is negative the animal is not able (Figure 91). In the experiments performed, animals treated with Vehicle+Saline showed positive DI, indicating that they were able to recognize the new position, while after treatment with LPS alone the ID is negative, not recognising the moved object. The treatment with A_1 ligand or A_{2A} antagonist alone leads positive DI or values very close to zero, also the treatment with the ligand together with the LPS showed positive DI, indicating that animals were able to differentiate the position changes. While these results do not reach statistical significance, together with the Y maze results, they reinforce the neuroprotective effects of 2'-dCCPA and compound **7** against a neuroinflammatory stimulus.

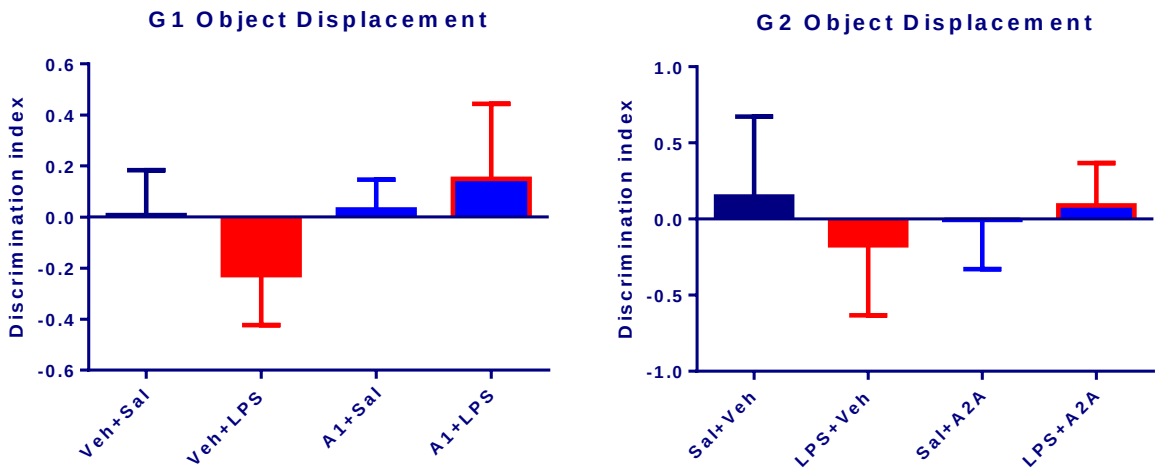


Figure 91. Effects of treatment in novel object recognition position. Saline (sal), Vehicle (veh, vehicle used for the ligand dilution).

Analysing the *in vivo* results it can be affirmed the beneficial effects of A_1AR agonist (2'-dCCPA) and A_{2A}AR antagonist (compound **7**) to treat neurodegeneration caused by LPS neuroinflammation. In particular, compound **7** seems to be more potent and efficient to treat the neuropathology since the performance in Y Maze provides better results compared to 2'-dCCPA. These affirmations of using an A_{2A}AR antagonist to treat neuropathologies are in accordance with several previous studies. Various scientific works underwrite that the blockade or genetic deletion of A_{2A}AR prevents memory or synaptic impairments in transgenic mice (Temido-Ferreira M. et al., 2019). In addition, other studies defend that the use of well-known A_{2A}AR antagonists like caffeine, KW6002 (oral administration), SCH58261 (intraperitoneally) or ZM241385 ameliorate memory spatial impairments, prevent the development of amyloidogenesis, Tau hyperphosphorylation, neuronal and synaptic proteins loss, decreased inflammatory cytokine levels in the hippocampus, the dysfunction of synaptic plasticity, and the clinical etiology of AD among others (Faivre E. et al., 2018; Silva AC. et al., 2018). Moreover, the administration of A_{2A}AR antagonist reduced the probability to develop PD, attenuated microglia reactivity, prevented BBB disruption, and provided neuroprotection against MS avoiding the lymphocyte infiltration (Madeira MH. et al., 2017) (Figure 92).

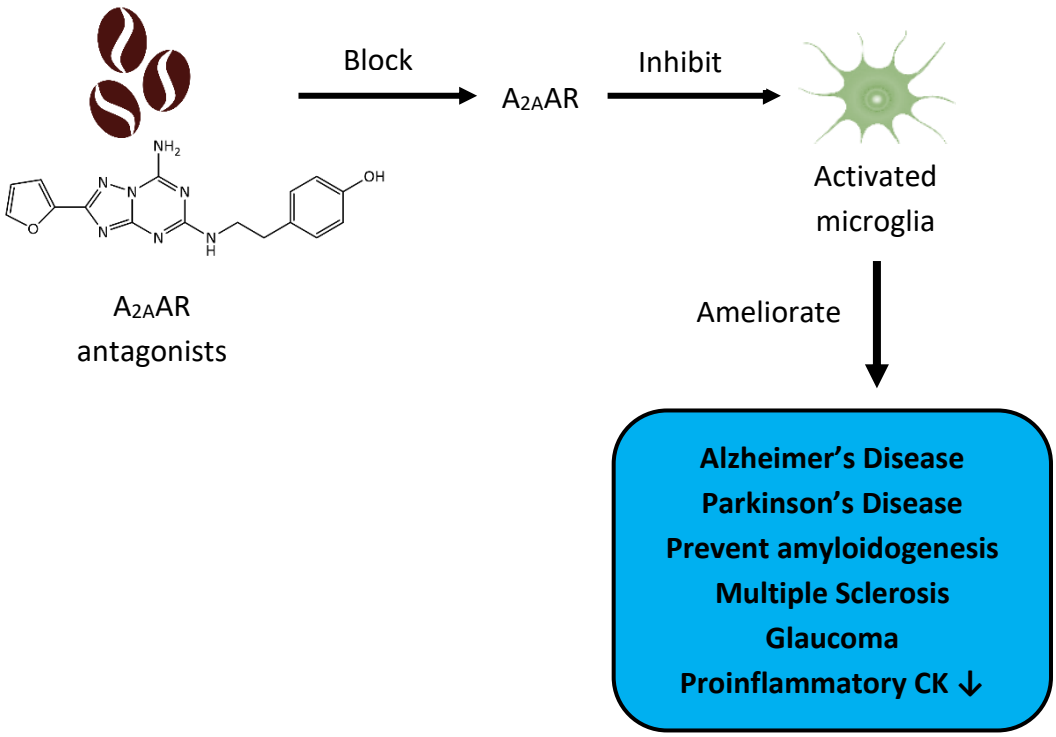


Figure 92. Schematic representation of $A_{2A}AR$ antagonist application on activated microglia and the impact in several pathologies.

6. CONCLUSIONS

The biological and pharmacological profiles of a new series of A_{2A}AR antagonists belonging to 9-ethyladenine substituted in 2 or in 8 positions, which were synthesized in the University of Camerino, were studied. *In vitro* experiments (viability, antioxidant and Hoechst assay) with A_1AR full or partial agonists belonging to the 2-chloro-N^6-cyclopentyl-2' or 3'-deoxyadenosine previously synthesized (Vittori S. et al., 2000) and the new series of A_{2A}AR antagonists belonging to 9-ethyladenine substituted in 2 or in 8 positions were performed in different cell lines. In addition, the effect of the best A_1AR agonist and A_{2A}AR antagonist were evaluated in *in vivo* neuroinflammation rat model (Open Field, Y maze and Object Displacement).

The study through binding and cAMP assay demonstrated the affinity and selectivity of all compounds investigated for each receptor. 2'-dCCPA showed to be an A_1AR partial agonist ($\alpha = 0.70$) while the three best A_{2A}AR antagonist of the new series were compound **6**, **7** and **16**. Among all the compounds tested, only one A_1 agonist (2'-dCCPA) and one A_{2A} antagonist (compound **7**) showed notable effects on cell viability in mixed glial cell culture, N13 and neurons. In addition, all the tested antagonists demonstrated a notably antioxidant profile in mixed glial cell cultures, being compound **6** and **7** the most powerful. The *in vivo* experiments confirmed that not 2'-dCCPA or compound **7** altered velocity, distance and permanence time in OF experiments, while both ligands helped to the protection or recovery of an LPS injury in Y maze. In the light of the foregoing, this data provides evidence that pro-inflammatory CK cocktail promotes neuroinflammation and cellular apoptosis, which can be alleviated by 2'-dCCPA and compound **7**. 2'-dCCPA was able to prevent the inflammatory effect induced by CK while compound **7** has both anti-inflammatory and antioxidant properties, preventing both neuroinflammation in mixed glial cells in an animal model of neuroinflammation. This suggests compound **7** as a potential candidate for neuroinflammation therapy. Collectively, data obtained provide novel evidence to use potent and selective A_1AR partial agonists and A_{2A}AR antagonists as a promising therapeutic approach to improve the functional recovery of patients with problems associated with an oxidative stress and age-associated diseases.

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