UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmakologie a toxikologie

DIPLOMOVÁ PRÁCE

HODNOCENÍ NOVÉ SÉRIE [1,2,4]TRIAZOLO[4,3-*a*]CHINOXALINŮ A PŘÍBUZNÝCH SLOUČENIN: VYSOCE ÚČINNÝCH ANTAGONISTŮ ADENOSINOVÝCH RECEPTORŮ

vypracováno na:

Univerzita Bonn
Institut farmacie
Oddělení farmaceutické chemie
Bonn, Německo

Školitelé: Prof. Dr. Christa Elisabeth Müller
PharmDr. Marie Vopršalová, CSc.

Hradec Králové 2010

Irena Krpelíková

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADCI KRÁLOVÉ

Department of farmacology a toxicology

DIPLOMA THESIS

EVALUATION OF A NOVEL SERIES OF [1,2,4]TRIAZOLO[4,3-*a*]QUINOXALINES AND RELATED COMPOUNDS: HIGHLY POTENT ADENOSINE RECEPTOR ANTAGONISTS

performed at:

University of Bonn
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Hradec Králové 2010

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I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, all of them are given in the list of used literature and they are quoted in text regularly.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány.

…..……

Irena Krpelíková

Acknowledgement

I would like to thank to my supervisor, Prof**.** Dr. Christa Elisabeth Müller**,** for her kind acceptance, advice and suggestions of this thesis.

Next, I would like to thank to Bernt Alsdorf and Nicole Florin for their assistance, patience and friendship during my stay in Germany.

Finally, I would like to thank to my Czech supervisor, PharmDr. Marie Vopršalová, CSc., for her advice to this work.

Ráda bych poděkovala vedoucí diplomové práce, Prof. Dr. Christě Elisabeth Müllerové, za její vlídné přijetí, rady a připomínky k této práci.

Dále bych ráda poděkovala Berntu Alsdorfovi a Nicole Florinové za jejich pomoc, trpělivost a přátelství po celou dobu mého pobytu v Německu.

V neposlední řadě děkuji mé české školitelce, PharmDr. Marii Vopršalové, CSc., za její cenné rady při vzniku této práce.

Content

Souhrn

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové, Katedra farmakologie a toxikologie, Česká republika

Vypracováno na: Univerzita Bonn, Institut farmacie, Oddělení of farmaceutické chemie, Německo

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Název diplomové práce: **Hodnocení nové série [1,2,4]triazolo[4,3-*a*]chinoxalinů a příbuzných sloučenin: vysoce účinných antagonistů adenosinových receptorů**

Adenosin je lokální modulátor, který je zapojen do mnoha různých biologických procesů. Svých účinků dosahuje působením na buňky přes adenosinové receptory (ARs). Až dosud byly popsány čtyři typy ARs- A_1, $A_{2A,}$ A_{2B} a A_3, které patří do skupiny receptorů spřažených s G-proteinem. ARs jsou důležitým cílem nově vznikajících léčiv.

Předkládaná diplomová práce se zabývá studiem skupiny nových [1,2,4]triazolo[4,3-*a*]chinoxalinů a jim podobných sloučenin jako potenciálních antagonistů adenosinových receptorů.

Prezentované výsledky byly získány použitím metody vazebného radioligandu.

Skupina chinoxalinonů a tetrazolochinoxalinů je selektivní k lidskému (h) A_3 receptoru, nicméně afinita tetrazolochinoxalinů je nízká a u chinoxalinonů střední. Největší skupinu testovaných látek tvoří triazolochinoxaliny. Substituent v poloze 1 (R_1) určuje afinitu sloučeniny zatímco substituent v poloze 4 (R_4) selektivitu. Sloučeniny bez R_4 jsou neaktivní, naopak nejaktivnější jsou sloučeniny s objemným substituentem s delším (C_3) lipofilním řetězcem. N-alkyl substituovaný pyrrol-2-yl v poloze 4 znatelně snižuje afinitu, ovšem jeho kombinací s fenylpyrrolem v poloze 1 vznikají aktivní a zároveň selektivní ligandy potkaního (r) A_1 receptoru. Tyto jsou N4-ethyl-pyrrol-2-yl-1-fenylpropyl[1,2,4]triazolo[4,3-*a*]chinoxalin (K_i rA_1 = 4,80 nM) a N4-propyl-pyrrol-2-yl-1-fenylpropyl[1,2,4]triazolo[4,3-*a*]chinoxalin (K_i rA_1 = 15,2

nM). Další látky hA_3 selektivní je N4-methyl-pyrrol-3-yl-1-benzyl[1,2,4]triazolo[4,3-*a*]chinoxalin (K_i hA_3 = 5,91 nM).

Klíčová slova: adenosinové receptory, [1,2,4]triazolo[4,3-*a*]chinoxaliny, metoda vazebného radioligandu, vztahy mezi strukturou a aktivitou

Summary

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Title of diploma thesis: **Evaluation of a novel series of [1,2,4]triazolo[4,3-*a*]quinoxalines and related compounds: highly potent adenosine receptor antagonists**

Adenosine, a local modulator, acts in many diverse biological processes. Its effects are mediated through adenosine receptors (ARs). Four types of ARs have been described, A_1, $A_{2A,}$ A_{2B} and A_3, which belong to the superfamily of G protein-coupled receptors. AR ligands are being developed as new drugs.

This thesis deals with the study of a novel series of [1,2,4]triazolo[4,3-*a*]quinoxalines and related compounds as potential adenosine receptor antagonists.

The presented results were obtained using radioligand binding assays.

Series of quinoxalinones and tetrazoloquinoxalines exhibit significant selectivity for human (h) A_3 ARs but their affinity is relatively low for tetrazoloquinoxalines and moderate for quinoxalinones.

Triazoloquinoxalines comprise the largest group of this series. Generally, the R_1 substituent determines the affinity, while the R_4 substituent confers the selectivity. Compounds without any R_4 substituent are not active, conversely compounds with bulky substituents with a lipophilic chain (C3) are the most potent ones. N-alkyl-(C_2-C_3) substituted pyrrol-2-yl at position 4 dramatically decreases affinity but combination with phenylpropyl at position 1 gives very promising and selective ligands at rat (r) A_1 ARs. These are N4-ethyl-pyrrol-2-yl-1-phenylpropyl[1,2,4]triazolo[4,3-*a*]quinoxaline (K_i at rA_1 = 4.80 nM) and N4-propyl-pyrrol-2-yl-1-phenylpropyl[1,2,4]triazolo[4,3-*a*]quinoxaline (K_i at rA_1 = 15.2 nM). Another potent and hA_3 AR selective compound is N4-methyl-pyrrol-3-yl-1-benzyl[1,2,4]triazolo[4,3-*a*]quinoxaline (K_i at hA_3 = 5.91 nM).

Key words: adenosine receptors, [1,2,4]triazolo[4,3-*a*]quinoxalines, radioligand binding assays, structure-activity relationships

List of abbreviations

ADA	Adenosine deaminase
AR	Adenosine receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CADO	2-Chloroadenosine
CHO cells	Chinese hamster ovary cells
DMEM F12	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham
DMSO	Dimethyl sulfoxide
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GTP	Guanosine triphosphate
NECA	N-Ethylcarboxamidoadenosine
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
TNF	Tumor necrosis factor
TRIS	Tris(hydroxymethyl)aminomethane

1. Introduction

Adenosine, a major constituent of nucleic acids or ATP, is involved in many physiological and pathological processes including Parkinson´s disease, inflammation, asthma bronchiale, neuropathy etc. Its effects are mediated through adenosine receptors (ARs) which occur abundantly in the human body and in most species.

Due to widespread functions of adenosine and its receptors, efforts have been carried out to develop potent and selective agonists and antagonists of ARs for several decades. Until 2008, around 2000 compounds have been patented as AR ligands and at least 20 clinical trials are going on currently. In 2008, Lexiscan® was approved by the US Food and Drug Administration (FDA) for medical use as the first synthetic adenosine receptor ligand. Lexiscan® (regadenoson, chemically 2-[4-[(methylamino)carbonyl]-1H-pyrazol-1-yl]adenosine monohydrate) is an A_{2A} adenosine receptor antagonist. It is a pharmacological stress agent indicated for radionuclide myocardial perfusion imaging (MPI) in patients unable to undergo adequate exercise stress[1)].

The main research of AR ligands has been focused on modifications of natural ligands themselves, i.e. xanthines such as caffeine or theophylline. Nowadays some approaches have been directed toward the study of different tricyclic systems. Among them, [1,2,4]triazolo[4,3-*a*]quinoxalines and related compounds were widely investigated by alterations on position 1 and 4 of the tricyclic scaffold. Next studied series are tetrazoloquinoxalines and quinoxalin-1-ones. They were supposed to have selective A_3 antagonist effects. The advanced compounds could be used for following trials.

The aim of this project was:

1) To test effects of a novel series of [1,2,4]triazolo[4,3-*a*]quinoxalines and related compounds at all four adenosine receptors

2) To determinate K_i values of active compounds

3) To find new selective ligands of adenosine receptors (especially A_3)

2. Theoretical background

2.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) comprise the largest group of cell-surface receptors in the body. They facilitate cellular communication and mediate many responses to signals from other cells such as hormones, neurotransmitters or local mediators. Despite of the large size of this group of proteins and the diversity of ligands, all GPCRs have a similar structure.

They consist of a single polypeptide chain that crosses the phospholipid membrane seven times. At the inner face of the cytoplasmatic membrane, it couples GTP-binding protein, called G protein. G proteins consist of three subunits – α, β and γ. A signal induces change of the receptor conformation and thereby activates the G protein. In the inactive state, the α-subunit binds GDP. When the GPCR is activated by an agonist, the α subunit releases GDP and binds GTP in its place. This process induces a large conformational change in the G protein and activates it in this way. Subsequently the α-subunit is dissociated from the βγ-subunit having different targets for their interactions. GTPase activity of the α subunit causes the hydrolysis of GTP to GDP. This way it becomes in(n)active. The activity of G protein depends on the speed of the GTP hydrolysis. It is usually accelerated by the binding of the α-subunit to a second protein, a regulator of G protein signaling (RGS)[2].

There are several different kinds of G proteins divided into following major groups:

- $G\alpha_{\mathbf{t}}$, also called transducin, is crucial for the visual perception. $G\alpha_t$ activates cyclic GMP phosphodiesterase, which hydrolyses cyclic-GMP and in this way allows the cyclic-GMP-sensitive channels to close. The subsequent hyperpolarisation and decrease in Ca^{2+} concentration results in an electric signal of the visual centre of perception.

- $G\alpha_{\mathbf{olf}}$, an olfactory-specific G protein, which stimulates adenylyl cyclase in odorant epithelium. The resulting increase in cyclic AMP activates cyclic-AMP-gated cation channels and it leads to the depolarisation of the olfactory receptor neuron. It initiates a nerve impulse which is conducted into the olfactory centre in the brain.

- $G\alpha_s$ stimulates adenylyl cyclase and in this way increases cyclic AMP concentration. Except optic and olfactory organs it ubiquitously acivates cyclic-AMP-dependent protein kinase (PKA). The binding of cyclic AMP to the PKA alters its conformation and the activated PKA phosphorylates specific target proteins.

- $G\alpha_i$ inhibits adenylyl cyclase.

- G_q activates the plasmamembrane-bound enzyme phospholipase C-β (PLCβ). The phospholipase cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into two products: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from the endoplasmic reticulum (ER) and the cytosolic concentration of Ca^{2+} is so rapidly increased. It initiates a cascade process of phosphorylation. In this way the cell regulates multiple cell functions, for instance antiapoptosis cell regulation. The other product of the PIP_2 hydrolysis, diacylglycerol activates different isoenzymes of protein kinase C (PKC). It can be also used in the synthesis of eicosanoids.

- G_o is able to interact directly with ion channels

- $G\alpha_{12}$ and $G\alpha_{13}$ activate a monomeric GTPase of the Rho family via the guanine exchange factor (GEF), which regulates the cytoskeleton contraction[3)].

2.2. Characterization of adenosine

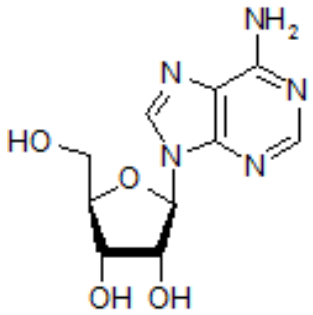


Figure 1: Chemical figure of adenosine

Adenosine is an important signalling molecule which has many regulatory functions mediated through adenosine receptors. It is also a building block of many important molecules such as ATP and nucleic acids[4]. Adenosine is not considered to be a classical neurotransmitter but a neuromodulator. It can be released directly from cells (it is stored in no special vecicles), or by degradation of ATP by extracellular enzymes. Adenosine can be metabolized by two pathways: mainly by phosphorylation to AMP (under normal conditions) or by deamination to inosine by adenosine deaminase.

In a human body adenosine has diverse effects. It induces sedation in brain through release inhibition of excitatory neurotransmitters. In the heart it has negative inotropic, chronotropic and dromotropic as well as antihypertensive effects. Further it has antidiuretic effects and it also influences the immune system.

The concentration of extracellular adenosine can be enhanced under some pathological conditions – ischemia, hypoxia, trauma, stress, convulsions or inflammation[5].

Adenosine is the ligand of adenosine receptors, which belong to the family of purine receptors.

2.3. Adenosine receptors

The family of purine receptors consists of two main groups: P1 (adenosine) receptors and P2 (nucleotide) receptors.

There are four subtypes of adenosine receptors: A_1, A_{2A}, A_{2B}, A_3. All of them are G protein-coupled. A_1 and A_3 couple to the $G\alpha_i$ protein, inhibiting thus cyclic AMP**;** whereas A_{2A} and A_{2B} couple to the $G\alpha_s$ protein[6)].

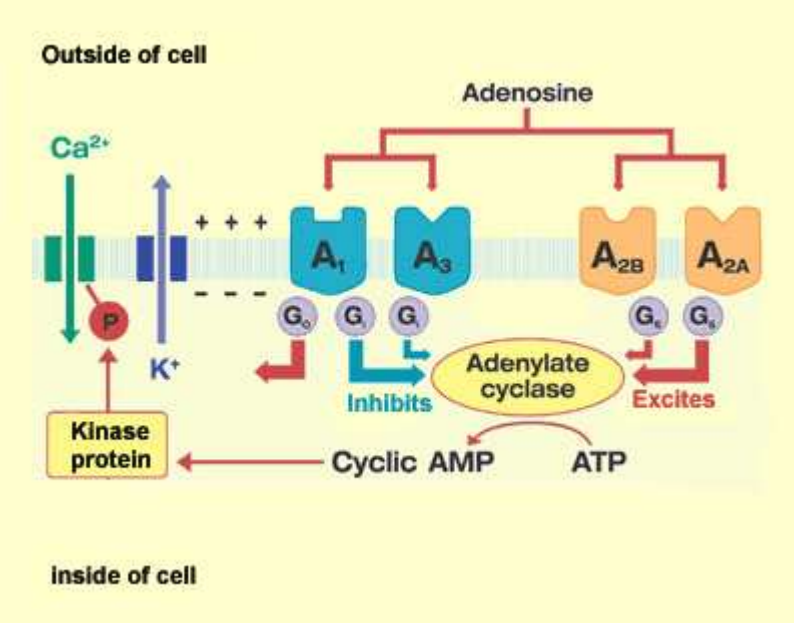


Figure 2: Adenosine receptors: mechanisms of effects[7)]

2.3.1. Structure

Adenosine receptors have seven transmembrane (TM) domains of hydrophobic amino acids like other G protein-coupled receptors. TM domains are connected by three extracellular and three intracellular hydrophilic loops of different lenght. The N terminal is located outside of the cell and the C terminal inside of the cell. Transmembrane domains are crucial for the ligand binding and for the receptor specificity. The ligand binding site is created by a pocket of the transmembrane regions.

Histidine residues on TM6 and TM7 play also an important role in ligand binding. The second extracellular loop is often glycosylated. The intracellular part of the receptor interacts with the G protein[8].

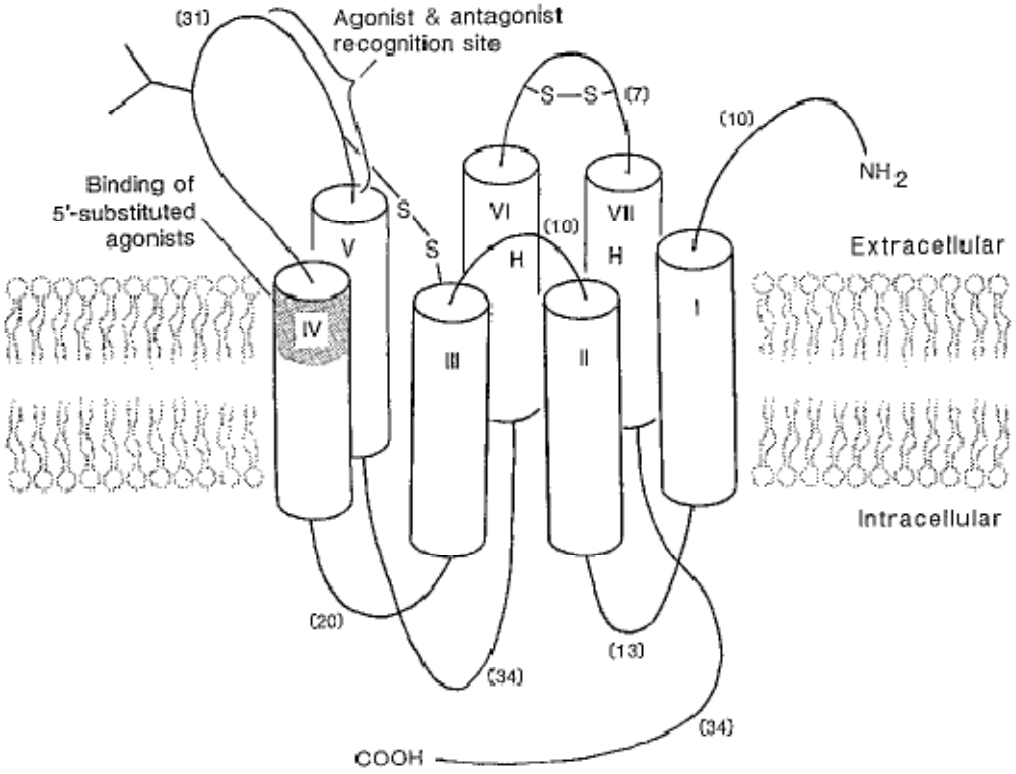


Figure 3: Schematic representation of the A_1 adenosine receptor

2.3.2. A_1 Receptor

A_1 receptors are widely distributed in the most species. They are highly expressed in most brain regions, spinal cord, adipose tissue, heart muscle and on leukocytes.

In the nervous system, A_1 receptors mediate inhibition of neurotransmision. Adenosine is released under some pathological conditions, such as hypoxia and ischemia. This mechanism of inhibition has a neuroprotective role. A_1 receptors can also influence behaviour, they have sedative, anxiolytic, anticonvulsant and locomotor depressant effects. On the other hand, A_1 antagonists (xanthines) have central stimulant effects. They also induce cardiac depression (through negative chronotropic, dromotropic and inotropic effects. These effects are used in clinical treatment of

supraventricular tachycardia and of bradyarrhytmia. In kidneys, A_1 receptors induce vasoconstriction, a decrease of glomerular filtration, and inhibition of renin secretion.

A_1 receptor agonists

Some selective A_1 agonists from N^6-substituted adenosine derivates were found, such as N^6-cyclopentyladenosine (CPA), N^6-cyclohexyladenosine and (−)-N^6-(2-Phenylisopropyl)adenosine (R-PIA). Another selective agonist – 2-chloro-N^6-cyclopentyladenosine (CCPA) has an additional substitution at C2-position.

A_1 receptor antagonists

Xanthine-based derivates with hydrophobic substituents, for example 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), are important selective A_1 antagonists.

There are also some non-xanthine selective antagonists, represented by non-fused rings (e.g. N-(3-phenyl-1,2,4-thiadiazol-5-yl)-4-hydroxybenzamide), bicyclic fused heteroaromatic systems (e.g. pyrazolo[3,4-*b*]pyridine-5-carboxylic acid ethyl ester derivates) or tricyclic fused heteroaromatic systems (e.g. 1,2,4-triazolo[1,5-*a*]quinoxalin-4-amines)[9].

2.3.3. A_{2A} receptors

Adenosine A_{2A} receptors are abundantly expressed in immune tissues, platelets, the CNS, vascular smooth muscle and endothelium[10].

The highest concentration of A_{2A} receptors is in the striatum, the nucleus accumbens and the olfactory tubercle.

Furthermore the A_{2A} receptors are highly expressed in immune tissues, eye and skeletal muscles.

A_{2A} receptors stimulate neurotransmitter release by coupling to G_s protein[8].

A_{2A}-D_2 interactions

There is a negative functional interaction between A_{2A} and dopamine D_2 receptors in the neostriatum and nucleus accumbens. It has been observed, that the stimulation of adenosine A_{2A} receptors induces a reduced affinity of dopamine agonists to dopamine D_2 receptors, while stimulation of D_2 receptors inhibits A_{2A} receptor-induced activation of adenylate cyclase[11),12)].

These antagonistic interactions regulate the function of the GABAergic encephalinergic system. This mechanism is considered to be very useful for the treatment of Parkinson´s disease. Till now the most used preparate for Parkinson´s disease has been L-3,4-dihydroxyphenylalanine (L-DOPA) combined with a peripheral DOPA decarboxylase inhibitor. During a long-time treatment with L-DOPA, the therapeutic effects decrease while the adverse side effects increase. These side effects include the progressive decline of symptomatic benefits, end-of-dose "wearing-off", "on-off" phenomenon and dyskinesia.

A_{2A} antagonists could enhance the effect of L-DOPA by the blockade of A_{2A}-D_2 receptor interactions. However, there are still no data about potential use in monotherapy[13)].

A_{2A} receptor agonists

A_{2A} receptor agonists have antiinflammatory effects, antihypertensive activity and they accelerate wound healing. They also could be used to treat psychosis and Huntington´s disease. They seem to be useful in the treatment of asthma bronchiale, chronic obstructive pulmonary disease and sepsis.

Generally**,** A_{2A} receptor agonists are adenosine derivates altered at both the purine and the sugar moiety.

C2-substituted adenosine derivatives with bulky substituents have been introduced (e.g. 2-phenylethynyladenosine or 2-(hexyn-1-yl)adenosine).

The most potent agonists with sugar moiety alterations are the 5´-*N*-ethyl-, 5´-*N*-methyl- and 5´-*N*-cyclopropylcarboxamido substituted compounds[14)].

A_{2A} receptor antagonists

A_{2A} receptor antagonists are promising compounds to treat or to palliate the symptoms of Parkinson´s disease, mainly the motor symptoms, dyskinesia, dystonia, and depression.

Some selective A_{2A} receptor antagonists were developed: for example 8-(3-chlorostyryl)caffeine (CSC), 1,3-dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthine (TMSX) or 3,7-dimethyl-1-propargylxanthine derivates (DMPX)[8),26)].

2.3.4. A_{2B} receptors

A_{2B} receptors are the least explored adenosine receptors. They practically occure on every cell but in small number. Relatively higher expression can be found in the caecum, large intestine and urinary bladder.

Both, agonists and antagonists could have beneficial effects in asthma. Activation of A_{2B} receptors inhibits the TNFα release from monocytes and it has also supressive effects on the expression of pro-inflammatory cytokines in macrophages whereas A_{2B} receptors induce bronchoconstriction in airways.

The blockade of A_{2B} receptors has antinociceptive effects. Caffeine has been used as an adjuvant analgesic drug for a long time but the finding about its crucial role of A_{2B} receptor blockade is relatively new.

A_{2B} receptor agonists

No selective A_{2B} agonists were introduced but A_{2B} receptors seem to prefer C2-substituted adenosine derivatives.

A_{2B} receptor antagonists

There are some compounds of xanthine-derived structure which have high affinity and good selectivity in humans. Typically, they have small alkyl substituents at

the 1- and 3-position. Aryl or heteroaryl at the 8-position selectively enhances A_{2B} receptor affinity (e.g. 1,3-dipropyl 8-pyrazolyl derivatives).

From non-xanthine derived A_{2B} receptor antagonists, there are some pyrimidine-2-amines or substituted thiazoles (e.g. pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines) among others[15].

2.3.5. A_3 receptors

A_3 receptors are largely distributed in the most human tissues, above all in testes, lungs, kidneys, placenta, heart, brain, spleen, uterus, bladder, jejunum, proximal colon, and eye.

A_3 receptors are also expressed on mast cells of rats, but not on human mast cells, which predominantly express A_{2B} receptors. Activation of them can cause bronchoconstriction, facilitate the release of allergic mediators and is linked to the inflammation and asthma.

A_3 receptor agonists

Structurally, A_3 receptor agonists are adenosine derivates. There are some profitable modifications: at the adenine moiety, the substitution at the N^6 amine moiety (e.g. N^6-(3-iodobenzyl)-5´-(N-methylcarbamoyl)adenosine (IB-MECA)) gives selective and potent derivatives. Selectivity can be increased by parallel alteration in position 2 (e.g. 2-chloro-IB-MECA). More potent are also 4´-thio derivatives (e.g. 2-chloro-N^6-(3-iodobenzyl)-4´-thioadenosine-5´-methyluronamide).

A_3 receptor antagonists

A series of aminothiazole (e.g. 3-(4-methoxyphenyl)-5-amino-7-oxo-thiazolo[3,2]pyrimidine) derivates are thought to treat chronic obstructive pulmonary disease, as adjuvants together with antiinflammatory, bronchodilatory or antihistaminic drugs.

A series of 4-(pyrazolo-3-yl)[1,2,4]triazolo[4,3-a]quinoxalines were proposed for the treatment of renal and cardiac diseases, as well as asthma or bronchitis.

2.4. [1,2,4]triazolo[4,3-*a*]quinoxalines

These compounds were investigated in the past as potential benzodiazepine and/or adenosine receptor ligands.

Many compounds, structurally quite different from benzodiazepines, are able to bind competitevely to the benzodiazepine site of the $GABA_A$ receptor. [1,2,4]triazolo[4,3-*a*]quinoxalines together with [1,2,4]triazolo[1,5-*c*]quinazolin-5(6*H*)-ones, [1,2,4]triazolo[4,3-*b*]pyridazines and imidazo[1,5-*a*]quinoxaline derivates belong to these compounds.

Adenosine receptor antagonists are typically aromatic nitrogen-containing heterocyclic compounds, such as xanthines, pyrazolo[1,5-*a*]pyridines, pyrrolo[2,3-*d*]pyrimidines, pyrazolo[3,4-*d*]pyrimidines, [1,2,4]triazolo[1,5-*a*]quinoxalines and [1,2,4]triazolo[4,3-*a*]quinoxalines[16].

2.5. Radioligand Binding Studies

Radioligand binding assays are relatively simple but very exact and reliable methods for studying receptors. They are used to determine the interactions of hormones, neurotransmitters, growth factors, and related drugs with the receptors. They may be used to analyse the affinity of an investigated drug for a receptor or binding site, and the density (B_{max}) of a receptor in a given tissue[17]. A radioligand is a ligand labeled with a radioactive isotope (usually ^3H or ^{125}I) incorporated into the molecule.

A radioligand can bind to a receptor, transporter, enzyme or to other proteins[18].

Ideally, it should have the following properties:

1) High affinity (K_d of 1 nM or less) to minimize the non-specific binding
2) Low non-specific binding – to consider an assay as sufficient. At least 50% of total binding must be specific, 70% is good and 90% is ideal
3) High specific activity (30 Ci/mmol or higher) to detect low receptor densities[20]

2.5.1. Assay conditions

Time - The incubation time is theoretically exactly the time necessary to reach the equilibrium. This is practically impossible and it is sufficient to reach steady state conditions.

Buffer – Tris(hydroxymethyl)aminomethane (TRIS) buffer is commonly used but it is not the only possible one. More important is the pH value. It should be in the physiological range, between 7-8.

Radioligand concentration – It depends on the type of binding experiment. The higher the radioligand concentration the higher the non-specific binding. On the other hand, a higher concentration results in higher numbers of cpm (count per minute) bound and thus lower counting error. The radioligand concentration is often chosen approximately equal to the K_d of the radioligand for binding to the receptor.

Receptor concentration – The higher the receptor concentration the better the binding. It is based on the assumption that the concentration of ligand is constant (<10 % of the radioligand is bound)[21].

2.5.2. Separation of bound radioligand from the free radioligand

When steady-state conditions have been reached, the bound radioligand is separated from the free radioligand. The most widely used separative techniques are filtration and centrifugation.

It is important to minimize the dissociation of the bound radioligand during this procedure. If significant dissociation happens, the measured binding values will be too low. This can be avoided by reducing the temperature with the ice cold wash buffer, thus slowing down the dissociation rate. This separation must be done as rapidly as possible (till 10 seconds).

For membrane binding assays, vacuum filtration through glass fiber filters is the most convenient. It is based on the principle that the filter retains radioligand-receptor complex and free radioligand passes through the filter. This filter is made of glass fibres twenty times thinner than human hair and it is characterized by very high efficiency[22]. To minimize the non-specific binding of radioligand onto the filter, the filter should be presoaked in appropriate solutions (e.g., 0.1% aqueous polyethylenimine) for a certain time.

The filtration is accomplished by a cell harvester-type filter machine. This instrument also enables rapid rinsing to remove residues of free radioligand[21].

2.5.3. Principle of Liquid scintillation counting

Liquid scintillation counting (LSC) is the typically used method for counting of organic radioactive compounds. LSC also detects short path lenght of soft β emission in contrast to solid scintillation. Liquid medium enables the close contact between the scintillator and the isotop atoms, which is impossible in solid scintillation. The Liquid scintillation cocktail absorbs the energy emitted by radioisotops and changes it into light flashes. A scintillation cocktail usually contains an organic solvent as well as primary and secondary scintillators. It accomplishes the two actions-absorption and re-emission. The solvent comprises the majority of the total scintillation cocktail. The emitted energy is captured in aromatic rings of solvent and is conducted to primary scintillator. This captured energy is transformed and emitted as light. Secondary scintillators are included in scintillation cocktail to transfer the fluorescence energy to longer optimal wave length. One molecule of scintillator gives off just one photon on activation. The amount of photons corresponds to the path length of the β particle. The scintillation counter evaluates each pulse of photons on the basis of the amount of photons in the pulse which is proportional to the single β emission event. The intensity of radiation is expressed as counts per minute (CPM), which is proportional to the amount of radioisotope in a sample[23].

2.5.4. The law of mass action

The principle of radioligand binding assays is based on a simple model – the law of mass action (Equation 1), where the ligand (L) binds to (a) the free receptor (R) to form a ligand-receptor complex (LR). K_{on} is the association rate constant and k_{off} is the dissociation rate constant.

$$\text{Ligand (L)} + \text{Receptor (R)} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{Ligand-Receptor Complex (LR)} \qquad [1]$$

Equilibrium occurs when the association and dissociation rates are equal (Equation 2)

$$[L] \cdot [R] \cdot k_{on} = [LR] \cdot k_{off} \quad [2]$$

The equilibrium dissociation constant K_d can be expressed by rearranging the equation (Equation 3)[24)]

$$\frac{[L][R]}{[LR]} = \frac{k_{off}}{k_{on}} = K_d \quad [3]$$

Two basic experimental methods for binding assays are described: saturation and competition binding.

2.5.5. Saturation binding

Saturation experiments are performed to determine receptor affinity ($1/K_d$) and density (B_{max}).

K_d [mol/l] is the dissociation equilibrium constant of a drug for a receptor. It is defined as the ratio of the dissociation rate constant (k_{off}) of the Ligand-receptor complex to the association rate constant (k_{on}). Smaller K_d means that the receptor has a higher affinity for the ligand, respectively larger K_d means that the receptor has a lower affitnity for the ligand.

B_{max} [mol/g] is the maximum density of receptors occupied at saturation concentration of radioligand.

In saturation experiments, the receptor concentration stays constant, while the radioligand concentration varies.

<u>The data analysis</u>

The parameter measured is the amount of radioactive ligand specifically bounded to the receptor. However, most radioligands are able to bind to more binding sites. The non-specific binding sites can be other receptors, other parts of tissues, or just

sites on test tubes or glass fiber filters. Total binding is the amount of radioligand bound to the receptor in the absence of unlabeled ligand. Non-specific binding is the binding in the presence of unlabeled ligand. It is necessary to measure both total and non-specific binding and the difference is the specific binding.

The non-specific binding is direct proportional to the radioligand concentration.

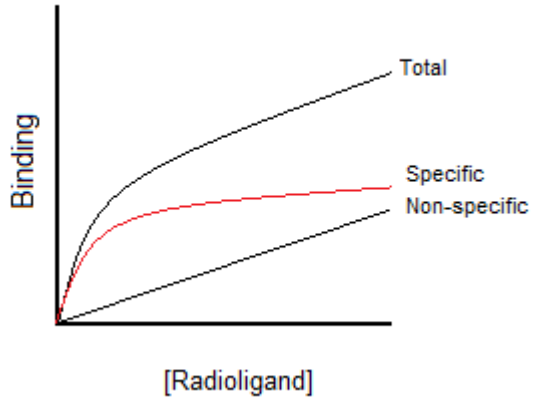


Figure 4: Graphical presentation specific binding-illustration of the total and the non-specific binding. The red curve symbolizes the specific binding.

The most suitable way to analyse the data from the saturation experiments is nonlinear regression using computer programs as GraphPad, San Diego, CA.

Another alternative for visualization of the data is to use a hyperbolic or a sigmoidal curve analysis.

Before the nonlinear regression programs were widely available, it had often been required to transform the data into a linear form and then to analyse them with linear regression.

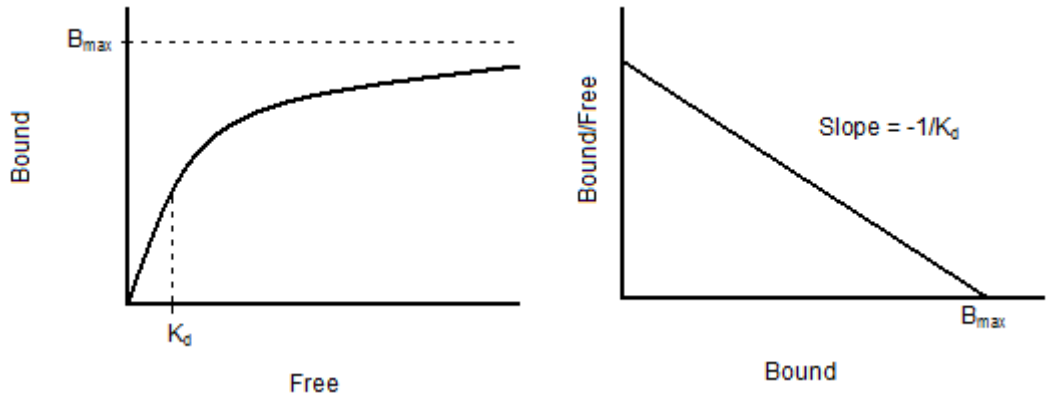


Figure 5: Scatchard (more accurately called Rosenthal) plots

2.5.6. Competition binding (IC_{50} measurements)

This method is used to determine the concentration of a drug required to inhibit binding of the standard radioligand by 50% (IC_{50} values). The affinity of a drug of interest is determined indirectly, by measuring its ability to compete with a radioligand of known parameters at binding to the same receptor.

The K_i value, the equilibrium dissociation constant for a competitive inhibitor of the receptor, is proportional to the IC_{50} value. It can be obtained using the Cheng-Prusoff equation (Equation 4), where L is the concentration of the radioligand and K_d determines the affinity of the radioligand for the receptor.

$$K_i = \frac{IC_{50}}{1+\frac{L}{K_d}} \qquad [4]$$

The next step is the determination of the selectivity of a drug for receptor sub-types.

In competition experiments, the radioligand concentration is constant and the unlabeled ligand concentration varies. The higher concentration of the unlabeled ligand the lower binding of the radioligand[17].

Data analysis

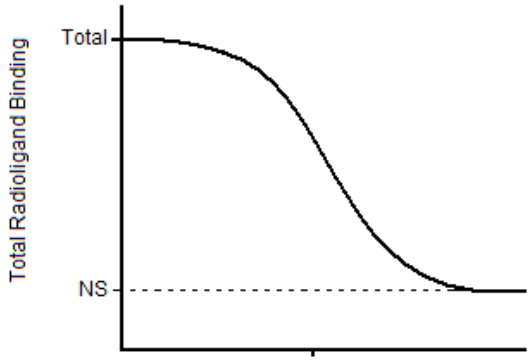


Figure 5: Competition plot

The high plateau of the curve equals the total radioligand binding in the absence of unlabeled ligand. The low plateau of the curve equals the non-specific (NS) binding. The difference between high and low plateaus is the specific binding. The IC_{50} is the concentration of unlabeled ligand that results in 50% inhibition of the radioligand. The Y axis is typically expressed in cpm, fmol bound per miligram protein, number of binding sites or in %[18)].

3. Experimental

3.1. Materials

3.1.1. Chemicals

ADA	Roche, Germany
BSA	Roth, Germany
CADO	Fluka, USA
DMSO	Riedel, Germany
DPCPX	Tocris, USA
EDTA	AppliChem
HCl conc.	Riedel de Haen, Germany
Isopropanol	Uni Bonn, Germany
LumaSafe Szintillationscocktail	Perkin Elmer, USA
NECA	Sigma, USA
PEI	Sigma, USA
Sucrose	Roth, Germany
TRIS	Roth, Germany

3.1.2. Radioligands

[^{3}H]CCPA	Quotient Bioresearch, UK
[^{3}H]MSX 2	Amersham, UK
[^{3}H]PSB-603	YE-Healthcare, UK
[^{3}H]PSB-11	Amersham, UK

3.1.3. Equipment

Autoclave	Varioklav 75T H+P
	3850ELV Systec
Balance	Competence CP Sartorius
	SBC 42 SCALTEC
	KERN 440- 47N
Centrifuge	Beckman Avanti, J-201
Incubator	IG 650 Jouan
Filter	GF/B Glass fibre cuts
Hamilton syringe	Roth, Germany
Harvester	Brandell M24, Gaithersburg,
	MD, USA
	Brandell M48, Gaithersburg,
	MD, USA
Laminar-air flow	Microflow Biological safety
	cabinet Nunc
Magnetic stirrer + hotplate	RCT Basic IKA Labortechnik
pH-Meter	691 pH Meter Metrohm
	WTW pH 197
Photometer	Beckman DU 530
Pipettes	Eppendorf
Ultraturrax	T25 basic IKA Labortechnik

Vortexer	MS 2 Minishaker IKA
	Labortechnik

3.1.4. Computer programs

GraphPad Prism 4.0

ISIS/Draw 2.4

3.2. CHO cell culture

3.2.1. Materials

human A_{2B} CHO cells	Prof. Müller, University of Bonn
human A_3 CHO cells	Prof. Klotz, University of Würzburg

Medium

DMEM F12, Mixture 1:1	Invitrogen

Supplements

10% FCS (0.1 µM)	Sigma
1% PenStrep (1000 U/ml Penicillin, 10 mg/ml Streptomycin	Invitrogen
0.4% G418 (50 mg/ml)	Calbiochem

Store at 4˚C

Trypsin

PBS buffer	500 ml	
EDTA (0.5 M, pH 7.6)		Roth

120˚C, 20 min., autoclave

Trypsin 2.5% (sterile)	10 ml	PAN
Phenol red 0.5% (sterile)	1.5 ml	Biochrom

Store at -20˚C

PBS buffer

NaCl	150 mM		Roth
KCl	2.5 mM		Sigma
Na_2HPO_4 x $2H_2O$	7.5 mM		Applichem
KH_2PO_4	1.5 mM		Applichem

pH 7.2, store at 4˚C

TRIS/EDTA pH 7.4

TRIS	5 mM	0.303 g	Roth
EDTA	2 mM	0.372 g	Roth
Aqua dest.		ad 500 ml	

Store at 4˚C

The cell suspension was transferred into the T175 cell culture flask, prepared with 25 ml medium. The cells were incubated at 37˚C till the cell density was about 70% (3 to 4 days). Then the cell suspension was transferred into ten T175 flasks. Each flask was prepared with 25 ml medium. The old medium was removed and the cells were washed with 5 ml PBS. Using trypsin solution (5 ml, 37˚C, 1 min) the cells were removed from the bottom of the flask. Medium (5 ml) was added to the cell suspension to neutralize trypsin and it was divided into ten prepared flasks. When the cells were 70% confluent, they were transferred into 180 dishes vessels. The dishes were prepared with 25 ml of complete medium. The medium was removed from the flask. The flask was washed with 5 ml PBS followed by 5 ml trypsin to detach the cells from the bottom, and the flask was incubated for 1-2 min at 37˚C. Medium (15 ml) was added into the flask and the cell suspension was divided in 20 dishes (1 ml per dish).

3.3. Membrane preparation

When the cells were 70% confluent, the medium was removed and each dish was washed with approximately 5 ml of PBS buffer. The dishes were immediately frozen at -80˚C and then thawed and 1-2 ml of 5 mM TRIS/2 mM EDTA was added. Using a rubber wiper the cells were collected from the bottom of the dish. Subsequently, the cells were homogenized using an Ultraturrax (twice for 15 seconds) and centrifuged for 10 min. at 1000 g and 4˚C. The supernatant was subsequently centrifuged for 60 min at 37 000 g and 4˚C. The resulting pellet was resuspended with 18 ml TRIS buffer (50 mM) and once more homogenized. The homogenisate was divided in 1 ml vials and shock-frozen in liquid nitrogen. Vials were stored at -80˚C.

3.3.1. Striatal membrane preparation

Material: Rat brains Pel Freeze®, USA

All the following procedures were carried out on ice. Rat striatum was dissected and kept in TRIS (50 mM) solution on ice. The suspension was homogenized with an Ultraturrax (max. 10 s) and centrifuged twice at 37 000 g, 15 min, 4˚C. The pellet was resuspended in TRIS buffer, homogenized with an Ultraturrax and stored at -80˚C.

3.3.2. Cortex membrane preparation

Material: Rat brains Pel Freeze®, USA

All the following procedures were carried out at 4˚C (on ice). Rat cortex was dissected and kept in sucrose (0.32 M) solution on ice. The tissue was homogenized with a glass Potter and centrifuged four times. First, it was centrifuged at 1000 g for 10 min, 4˚C (to remove nuclei and unbroken cells) and then three times at 37 000 g, 1 h, 4˚C with subsequent exchange of the buffer (from sucrose via water to TRIS). The pellet was resuspended in TRIS buffer, homogenized with an Ultraturrax and stored -80˚C.

3.4. Protein determination – Lowry's method

Reagents

A	Na_2CO_3	2%	Sigma
	NaOH	0.1 M	University of Bonn
B	Cu_2SO_4 . 5 H_2O	0.5%	Applichem
	Sodium tartrate	1%	University of Bonn

C Always new made: 50 parts of reagent A + 1 part of reagent B

D Folin & Ciocalteau's Phenol Reagent Working Solution:

18 ml Folin reagent (F-9252) Sigma

ad 90 ml aqua bidest.

This method is based on the reaction between protein and copper in alkaline solution as the first step and the subsequent reduction of the Folin reagent as the second step which results in a turn of colour to intensive blue. Four samples with increasing concentration of protein (0, 1:50, 1:20, 1:10) in TRIS buffer were prepared. Reagent C (1000 µl) was added into each sample and the tubes were mixed and incubated for 20 min at room temperature. After that 100 µl of reagent D was added, samples were mixed and incubated for 30 min. The samples were measured in a spectrophotometer at 500 nm. The protein concentration was determined by comparison to a standard curve[25)].

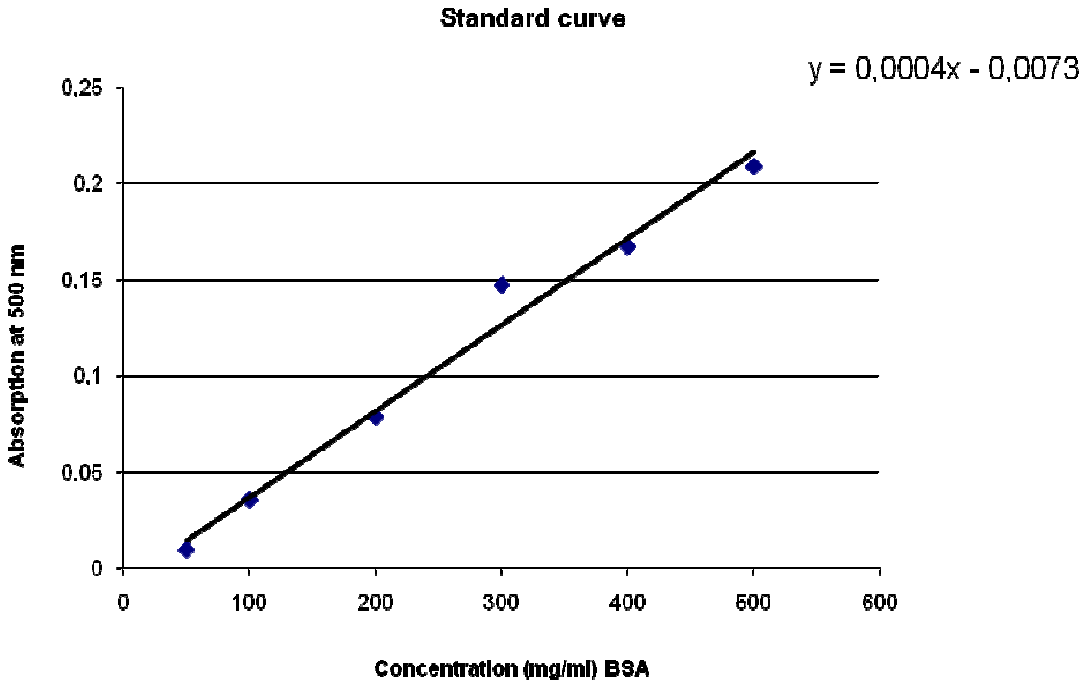


Figure 6: Example of a standard curve with BSA

3.5. Radioligand binding assays

The assays were carried out in assay tubes. Each tube included the same amount of radioactively labeled ligand, protein and TRIS buffer. Using human protein, assays were carried out in duplicates, other assays in triplicates. The first two tubes contained untested compound to determinate the total binding. The second two tubes contained an unlabeled ligand whith high affinity to determinate the non-specific binding.

3.5.1. A calculation of the necessary amount of a radioligand and a protein

Radioligand

$$\text{Ligand [µl]} = \frac{\text{(Number of tubes + 6) x Specific activity [Ci/mmol] x final conc. Ligand [nM] x Total volume [ml]}}{\text{1000 x conc. radioligand [Ci/L]}}$$

Buffer [ml] = (Number of tubes) x 0.1 ml

Human protein

$$\text{Protein [ml]} = \frac{\text{(Number of tubes + 6) x final conc. Protein [µg]}}{\text{conc. radioligand Protein [µg/ml]}}$$

Buffer [ml] = (Number of tubes + 6) x 0.1 ml – Protein [ml]

$$\text{ADA [µl]} = \frac{\text{(Number of tubes + 6) x final conc. Protein [µg] x µl ADA}}{\text{1000 µg}} \qquad \text{(2U ADA/mg Protein)}$$

Rat protein

The protein suspension was diluted 1:4 with 50 mM TRIS buffer (pH 7.4) and mixed with ADA (2 U/ml protein solution).After defined incubation time, it was filtered using a cell harvester. The harvester was pre-washed with cold 50 mM TRIS buffer. Subsequently, the GF/B filter was put in the harvester and the tested samples were filtered. The filter was washed three times to reduce the non-specific binding. Filter strips with bound protein and radioligand were cut out and put into scintillation tubes, and 2.5 ml of scintillation cocktail was added. After 6 hours of incubation time they were measured in Liquid Scintillation Counter (LSC). Results were analyzed with GraphPad Prism 4.0.

3.5.2. A_1 binding assays

A_1 receptors for this assay natively expressed in rat brain cortical membranes were utilized. [^{3}H]CCPA (specific activity: 42.6 Ci/mmol, final concentration: 1 nM) was used as radioligand. Unlabeled CADO (final concentration: 10 μM) was used to determine non-specific binding. Each data point was determined in triplicate assay tubes. The incubation time was 90 min at room temperature.

3.5.3. A_{2A} binding assays

Rat brain striatal membranes were used as a source of A_{2A} receptors. The radioligand for these assays was [^{3}H]MSX-2 (specific activity: 84 Ci/mmol, final concentration: 1 nM). NECA (final concentration: 50 μM) was used to determine non-specific binding. Each data point was measured in triplicate. The incubation time was 30 min at room temperature. GF/B-filters had to be soaked in 0.3% PEI solution for 30-60 min before the filtration. This was an important step to minimize the non-specific binding on the filter.

3.5.4. A_{2B} binding assays

Human A_{2B} receptors were recombinantly expressed in CHO cells (stably expressing cell line). [^{3}H]PSB-603 (specific activity: 73 Ci/mmol, final concentration: 0.3 nM) was the specific radioligand for A_{2B} binding assays and DPCPX (final concentration: 10 μM) was used to determine non-specific binding. Each sample was carried out in duplicate assay tubes. The incubation time was 75 min at room temperature. 50 mM TRIS washing buffer + 0.1% BSA was used to minimize the non-specific binding on the filter.

3.5.5. A_3 binding assays

Human A_3 receptors were stably expressed CHO cells. [^{3}H]PSB-11 (specific activity: 53 Ci/mmol, final concentration: 1 nM) was used as a radioligand, *R*-PIA (final concentration: 100 μM) served to determine non-specific binding. Each sample was carried out in duplicate assay tubes. The incubation time was 45 min at room temperature.

Table 1: Radioligands used

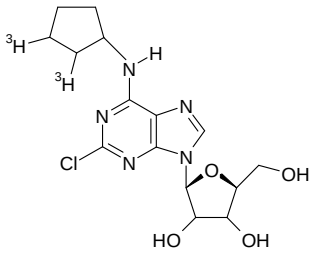
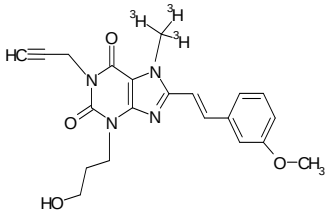
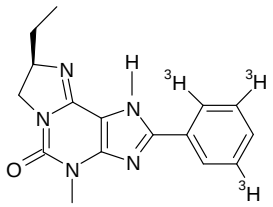
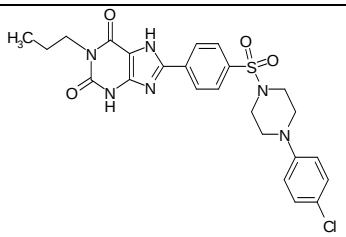
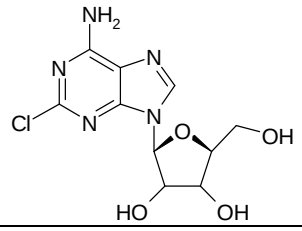
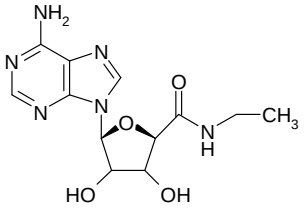
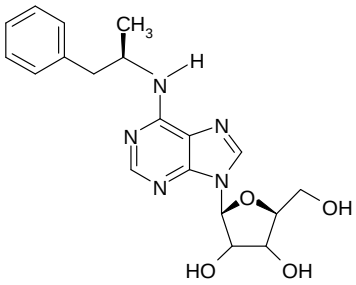
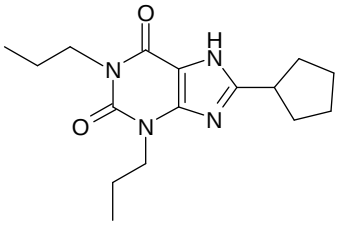
Radioligand	Chemical name	Chemical figure	Specific activity [Ci/mmol]
[^{3}H]CCPA	2-Chloro-N^6-cyclopentyladenosine		58
[^{3}H]MSX-2	3-(3-Hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine		84
[^{3}H]PSB-11	2-Phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8,-tetrahydro-1*H*-imidazo [2,1-*i*]purine-5-one		53
[^{3}H]PSB-603	8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine		73

Table 2: Unlabeled ligands used

Ligand	Chemical name	Chemical figure
CADO	2-Chloroadenosine	
NECA	N-Ethylcarboxamido-adenosine	
R-PIA	*R*-N^6-Phenylisopropyl-adenosine	
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine	

4. Results and discussion

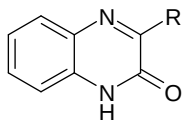
The ribose moiety was proven to be crucial for agonistic effects of the majority of AR ligands. Therefore, this presented novel series is assumed to be antagonistic at adenosine receptors since they lack the ribose moiety[26].

At first the compounds were screened at a concentration of 1μM. Promising derivatives (more than 35% inhibition at 1 μM concentration) were subsequently tested to get curves showing dependency between the concentration of the ligand and % specific binding of radioligand. To obtain reliable curves, it was necessary to measure seven different concentrations of the tested compound. These curves allow to determine the K_i values as the main parameter for deduction of structure-activity relationships.

4.1. Structure-Activity Relationships

The tested series can be divided into three groups according to their structure: quinoxalinones, tetrazoloquinaxolines and triazoloquinaxolines.

4.1.1. Quinoxalinones



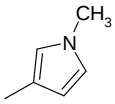
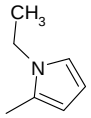
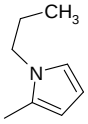
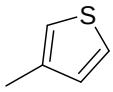
All tested quinoxalinones show affinity to A_3 adenosine receptor. The substituent in position 2 influences the affinity of a compound. The results obtained (Table 3) indicate that the pyrrolo-substituted quinoxalinones are the most potent compounds from the series. A lipophilic chain (C_2-C_3) at the N atom of the pyrrol moiety leads to further increase in A_3 affinity.

The rank order of affinity increases according to the nature of the substituents: phenyl (MD066) < fur-2-yl (MD025) < N-methylpyrrol-3-yl (IW 13) < thiophen-3-yl (MD068) < thiophen-2-yl (MD044) < N-ethylpyrrol-2-yl (MD020) < N-propylpyrrol-2-yl (MD022).

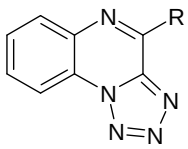
The lactame group of quinoxalinones determines their selectivity. Except for the fur-2-yl substituted derivative (MD025) the novel quinoxalinones show selective

affinity at A_3 adenosine receptors. MD025 shows affinity at A_3 as well as at A_{2A} and A_{2B} receptors.

Table 3. Quinoxalinones

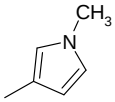
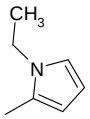
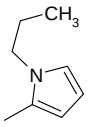
Compd.	R	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
IW 13		(13 ± 1)	(16 ± 2)	(-10 ± 5)	**130** ± 25 (59 ± 2)
MD020		(1 ± 2)	(4 ± 11)	(11 ± 10)	**25.0** ± 5.5 (84 ± 13)
MD022		(4 ± 0)	(-15 ± 13)	(-7 ± 10)	**16.6** ± 6.1 (84 ± 13)
MD025		(19 ± 2)	**212** ± 27 (50 ± 3)	(62 ± 7)	**144** ± 63 (65 ± 2)
MD044		(28 ± 0)	(8 ± 5)	(33 ± 3)	**33.8** ± 8.0 (70 ± 5)
MD068		(18 ± 2)	(10 ± 13)	(58 ± 3)	**93.8** ± 16.3 (67 ± 4)
MD066		(17 ± 0)	(22 ± 2)	(-8 ± 17)	**232** ± 31 (42 ± 5)

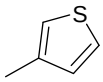
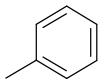
4.1.2. Tetrazoloquinoxalines



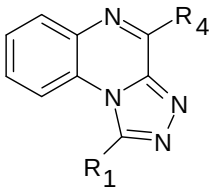
Most of the tetrazoloquinoxalines exhibit selectivity to A_3 receptors. Only MD054 with a fur-2-yl residue has similar affinity at $A_{2A,}$ A_{2B} and A_3 receptors. The affinity of most compounds at A_3 receptors is just moderate (> 30 nM).

Table 4. Tetrazoloquinoxalines

Compd.	R_4	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
IW 15		(**31** ± 1)	(15 ± 8)	(7 ± 6)	**94.3** ± 43.3 (59 ± 4)
MD037		(5 ± 3)	(10 ± 8)	(-1 ± 3)	(35 ± 4)
MD029		(12 ± 4)	(-19 ± 7)	(-10 ± 5)	**32.8** ± 8.2 (44 ± 3)
MD054		(30 ± 1)	**179** ± 19 (50 ± 7)	(56 ± 0)	**243** ± 59 (50 ± 3)
MD051		(19 ± 3)	(15 ± 9)	(30 ± 8)	**64.9** ± 6.4 (68 ± 4)

MD079		(15 ± 6)	(15 ± 3)	(22 ± 1)	**96.8** ± 21.4 (58 ± 4)
MD070		(32 ± 1)	(17 ± 7)	(-11 ± 4)	**135** ± 38 (47 ± 3)

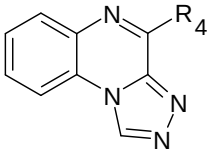
4.1.3. Triazoloquinoxalines



Substituted triazoloquinoxalines comprise the most extensive group of this series. They were divided into seven subgroups according to their R_1 substituent (i.e. phenyl, fur-2-yl, N-methylpyrrol-3-yl, N-ethylpyrrol-2-yl, N-propylpyrrol-2-yl, thiophen-3-yl, thiophen-2-yl).

The R_1 substituent is very important for the affinity of triazoloquinoxalines. Compounds without any R_1 substituent have no or just mininal affinity for all adenosine receptor subtypes (Table 5). Compounds with a bulky lipophilic substituents with longer (C_3) carbon chain show the highest affinity at adenosine receptors but no selectivity (table 6). For the selectivity the R_1 substituent seems to be the determinant.

Table 5. Nonsubstituted [1,2,4]triazolo[4,3-*a*]quinoxaline derivatives



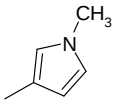
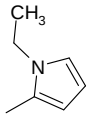
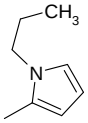
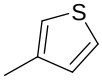
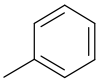
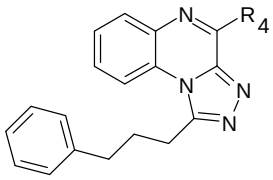
Compd.	R_4	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
IW 17		(**21** ± 4)	(-2 ± 11)	(-11 ± 8)	**580** ± 282 (30 ± 4)
MD031		(16 ± 1)	(-12 ± 14)	(20 ± 2)	(19 ± 7)
MD030		(0 ± 5)	(-17 ± 12)	(-9 ± 2)	(16 ± 4)
MD058		(24 ± 1)	**100** ± 21 (56 ± 2)	(41 ± 0)	(37 ± 2)
MD055		**363** ± 54 (34 ± 3)	**357** ± 94 (28 ± 5)	(23 ± 2)	(23 ± 2)
MD080		(**21** ± 1)	**447** ± 116 (26 ± 9)	(28 ± 0)	(-5 ± 20)
MD072		**343** ± 56 (33 ± 3)	(16 ± 6)	(7 ± 9)	(16 ± 16)

Table 6. [1,2,4]triazolo[4,3-*a*]quinoxalines with bulky R_1 substituents – 1st part



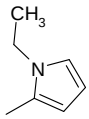
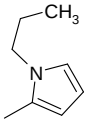
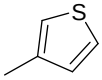
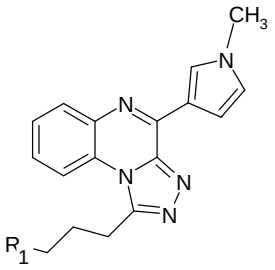
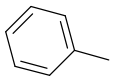
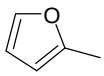
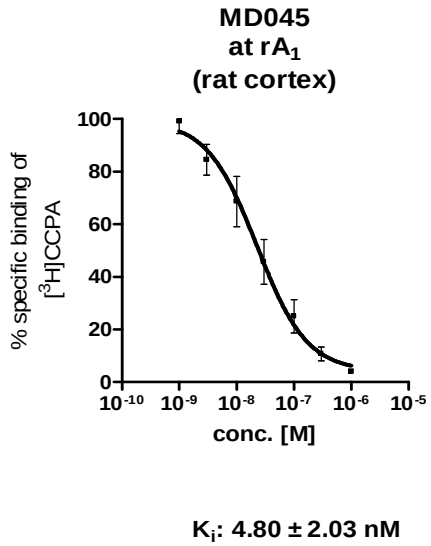
Compd.	R_4	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD045		**4.80** ± 2.03* (96 ± 1)	(13 ± 5)	(15 ± 1)	**102** ± 67 (50 ± 11)
MD032		**15.2** ± 5.8** (87 ± 6)	(16 ± 4)	(-6 ± 6)	(30 ± 0)
MD061		**0.576** ± 0.290 (104 ± 1)	**0.845** ± 0.259 (97 ± 1)	(103 ± 4)	**51.7** ± 10.4 (72 ± 4)
MD064		**4.31** ± 0.34 (101 ± 0)	**33.8** ± 14.6 (77 ± 3)	(72 ± 3)	(36 ± 7)
MD083		**5.07** ± 1.57 (98 ± 1)	**39.1** ± 5.2 (75 ± 4)	(73 ± 1)	(40 ± 2)
MD074		**0.937** ± 0.160 (102 ± 0)	**16.6** ± 3.1 (87 ± 5)	(83 ± 1)	**89.8** ± 35.7 (53 ± 9)

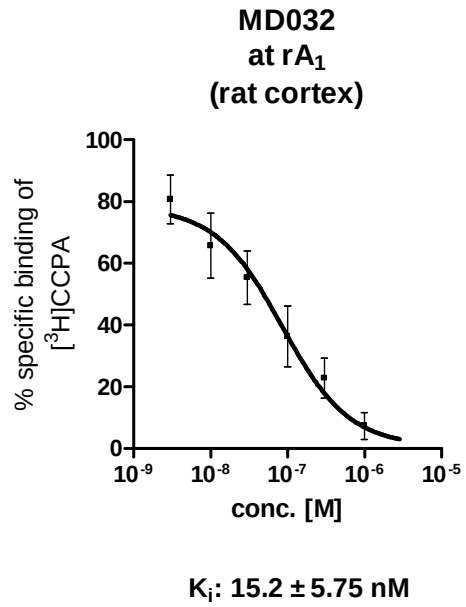
Table 6. [1,2,4]triazolo[4,3-*a*]quinoxalines with bulky R_1 substituents – 2nd part



Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
IW 18		**1.58** ± 0.23 (100 ± 1)	**35.7** ± 17.3 (83 ± 5)	(96 ± 1)	**26.9** ± 5.8 (63 ± 5)
IW 25		**3.43** ± 0.24 (100 ± 1)	**27.7** ± 8.5 (75 ± 3)	(73 ± 1)	**21.8** ± 2.01 (93 ± 5)
IW 26		**2.65** ± 0.31 (99 ± 0)	**49.4** ± 24.7 (74 ± 2)	(67 ± 7)	**25.8** ± 2.2 (85 ± 2)

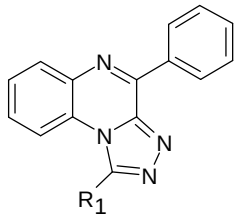


* **Figure 7:** MD045 - concentration-inhibition curve



** **Figure 8:** MD032 – concentration-inhibition curve

Phenyl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

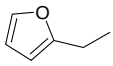
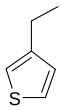
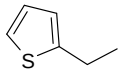
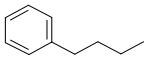


Among the phenyl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines two A_1 selective compounds were identified: MD075 (K_i = 83.8 nM) and MD092 (K_i = 23.7 nM). Both of them are furyl substituted but fur-2-yl shows higher affinity at A_1 receptors over other adenosine receptors. Methylfur-2-yl has noticeably higher affinity (MD103 - K_i = 44.4 nM) at A_3 receptors than the furyl derivatives. The methyl connection between furyl and the tricyclic system increases affinity at A_3 without effects towards selectivity.

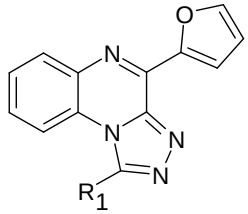
The most potent coumpound of this series is the 2-phenylpropyl derivative with K_i value = 0.937 nM at A_1 but also very high affinity at other adenosine receptors. The nonsubstituted derivative (MD072) has no or just low affinity at adenosine receptors, similarly to the chloride derivative (MD071).

Table 7. Phenyl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD072	H	**343** ± 56 (33 ± 3)	(16 ± 6)	(7 ± 9)	(16 ± 16)
MD071		**103** ± 33 (59 ± 8)	**342** ± 62 (21 ± 6)	(10 ± 3)	(39 ± 3)
MD075		**83.8** ± 10.3 (70 ± 2)	(18 ± 6)	(28 ± 4)	(15 ± 1)

MD092		**23.7** ± 5.6 (88 ± 4)	**169** ± 29 (35 ± 4)	(34 ± 8)	(39 ± 6)
MD103		**73.2** ± 45.7 (67 ± 10)	**99.0** ± 23.8 (49 ± 7)	(5 ± 5)	**44.4** ± 16.2 (80 ± 6)
MD093		**72.4** ± 12.1 (62 ± 3)	(14 ± 2)	(26 ± 1)	(37 ± 2)
MD088		**101** ± 24 (46 ± 4)	**77.5** ± 13.8 (53 ± 5)	(26 ± 5)	**27.6** ± 4.7 (78 ± 6)
MD073		**144** ± 33 (53 ± 6)	**106** ± 21 (53 ± 3)	(21 ± 4)	**25.7** ± 4.7 (80 ± 8)
MD074		**0.937** ± 0.160 (102 ± 0)	**16.6** ± 3.1 (87 ± 5)	(83 ± 1)	**89.8** ± 35.7 (53 ± 9)

Fur-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

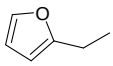
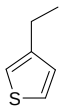
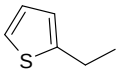


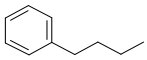
The 1-phenylpropyl-substituted derivative exhibits high affinity at all adenosine receptors (K_i = 0.576 nM at A_1, 0.845 nM at A_{2A}, 51.7 nM at A_3 and 103% inhibition at A_{2B} at 1 μM concentration). The nonsubstituted derivative (MD058) has no or just low affinity at adenosine receptors so as the chloride derivative (MD059).

Thiophenyl-substituted derivatives show comparable affinity at A_1 and A_{2A} receptors but the carbon linkage between thiophenyl and the tricyclic system results in increased affinity at A_3 receptors as shown by MD060 (K_i = 16.9 nM at A_3) and MD087 (K_i = 27.2 nM at A_3).

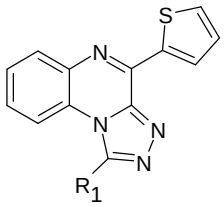
In furyl-substituted derivatives the carbon linker between furyl and the tricyclic system is again important for the affinity at A_3 receptors. The K_i value of MD090 (pyrrol-2-yl derivative) is 192 nM while the K_i value of MD102 (methylpyrrol-2-yl derivative) is 43.1 nM.

Table 8. Fur-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD058	H	(24 ± 1)	**100** ± 21 (56 ± 2)	(41± 0)	(37 ± 2)
MD059		**179** ± 19 (37 ± 2)	**112** ± 40 (76 ± 2)	(58 ± 1)	(47 ± 5)
MD062		**72.6** ± 8.56 (71 ± 2)	**15.8** ± 1.8 (90 ± 2)	(87 ± 8)	(33 ± 1)
MD090		**23.2** ± 3.2 (89 ± 3)	**2.45** ± 0.34 (105 ± 5)	(91 ± 4)	**152** ± 41 (62 ± 4)
MD102		**69.7** ± 13.0 (68 ± 3)	**82.6** ± 10.6 (74 ± 3)	(74 ± 16)	**43.1** ± 16.2 (81 ± 6)
MD091		**81.8** ± 17.8 (68 ± 2)	**28.8** ± 5.3 (91 ± 2)	(59 ± 2)	(44 ± 4)
MD087		**178** ± 27 (53 ± 4)	**20.7** ± 6.8 (73 ± 11)	(85 ± 6)	**27.2** ± 5.4 (95 ± 8)
MD060		**64.0** ± 3.1 (74 ± 2)	26.8 ± 5.1 (77 ± 4)	(49 ± 6)	**16.9** ± 7.0 (87 ± 5)

MD061		**0.576** ± 0.290 (104 ± 1)	0.845 ± 0.259 (97 ± 1)	(103 ± 4)	**51.7** ± 10.4 (72 ± 4)

Thiophen-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines



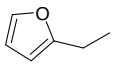
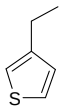
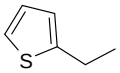
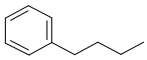
The phenylpropyl-substituted derivative (MD064) exhibits high affinity at A_1, A_{2A}, A_{2B} but not at A_3 receptors. The least active compounds from this series are the chloride-substituted (MD057) or unsubstituted (MD055) derivatives.

Furyl derivatives (MD065, MD095) have higher A_1 and A_{2A} affinity and lower affinity at A_3 receptors, while the methylfur-2-yl substituent (MD104) increases the affinity at A_3 receptors and decreases the affinity at A_1 and A_{2A}.

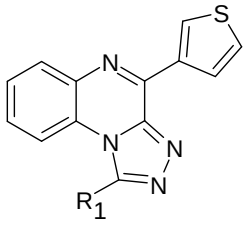
Another thiophenyl residue in the molecule induces a decrease in affinity at A_1 receptors. The carbon linker between thiophene and the tricyclic system (MD063, MD076) results in higher affinity at A_{2A}, A_{2B} and A_3 receptors.

Table 9. Thiophen-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD055	H	**363** ± 54 (34 ± 3)	**357** ± 94 (28 ± 5)	(23± 2)	(23 ± 2)
MD057		**25.4** ± 6.4 (37 ± 7)	41.9 ± 0.9 (45 ± 7)	(14 ± 11)	**36.2** ± 3.8 (62 ± 4)

MD065		**76.4** ± 12.1 (63 ± 4)	54.9 ± 7.9 (36 ± 6)	(4 ± 2)	(31 ± 5)
MD094		**51.5** ± 19.7 (75 ± 5)	**84.8** ± 18.9 (59 ± 1)	(24 ± 0)	(15 ± 9)
MD104		**137** ± 62 (42 ± 5)	**82.8** ± 32.0 (60 ± 7)	(55 ± 7)	**40.4** ± 6.0 (78 ± 6)
MD095		**58.8** ± 6.9 (49 ± 4)	(19 ± 5)	(17 ± 14)	**36.0** ± 13.7 (58 ± 6)
MD063		**248** ± 81 (38 ± 1)	**66.7** ± 30.7 (73 ± 2)	(66 ± 4)	**32.9** ± 7.0 (83 ± 5)
MD076		**194** ± 65 (53 ± 4)	**56.8** ± 18.5 (64 ± 8)	(61 ± 2)	**13.8** ± 7.2 (86 ± 4)
MD064		**4.31** ± 0.34 (101 ± 0)	**33.8** ± 14.6 (77 ± 3)	(72 ± 3)	(36 ± 7)

Thiophen-3-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

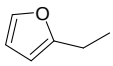
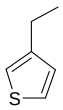
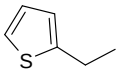


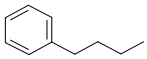
In these molecules, another thiophenyl residue results in loss of affinity for A_1 receptors. Methylthiophenyl derivatives (MD085, MD082) show higher affinity at A_{2A}, A_{2B}, and A_3 receptors than the corresponding derivatives with a thiophenyl residue (MD097). Furyl-substituted derivatives (MD084, MD096, MD105) are somewhat

weaker at all adenosine receptors, similarly as the nonsubstituted (MD080) or chloride-substituted (MD081) compounds.

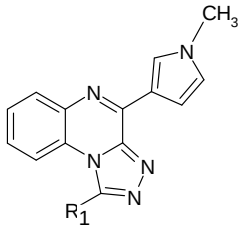
The most potent derivative from this series is MD083 with phenylpropyl substitution (K_i for A_1 = 5.07 nM) active also at other adenosine receptors.

Table 10. Thiophen-3-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD080	H	(21 ± 1)	**447** ± 116 (26 ± 9)	(28 ± 0)	(-5 ± 20)
MD081		(20 ± 4)	**224** ± 64 (37 ± 7)	(45 ± 0)	(44 ± 3)
MD084		**212** ± 18 (42 ± 4)	(28 ± 4)	(45 ± 6)	(14 ± 3)
MD096		**93.6** ± 8.7 (61 ± 5)	**171** ± 47 (49 ± 6)	(61 ± 3)	(39 ± 19)
MD105		**113** ± 70 (28 ± 2)	(31 ± 5)	(69 ± 3)	**28.6** ± 7.0 (67 ± 4)
MD097		(33 ± 0)	(14 ± 6)	(45 ± 1)	**65.7** ± 16.0 (45 ± 5)
MD082		(28 ± 5)	**68.4** ± 19.0 (49 ± 2)	(55 ± 6)	**43.7** ± 20.1 (91 ± 7)
MD085		(22 ± 2)	**54.8** ± 10.4 (56 ± 3)	(74 ± 1)	**29.2** ± 11.9 (64 ± 9)

MD083		**5.07** ± 1.57 (98 ± 1)	**39.1** ± 5.2 (75 ± 4)	(73 ± 1)	(40 ± 2)

N-methylpyrrol-3-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines



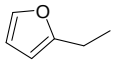
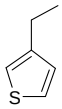
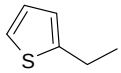
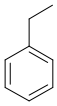
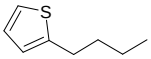
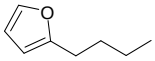
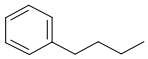
Compounds with bulky substituents on the three-carbon chain (IW18, IW25, IW26) were found to be highly potent at all adenosine receptors.

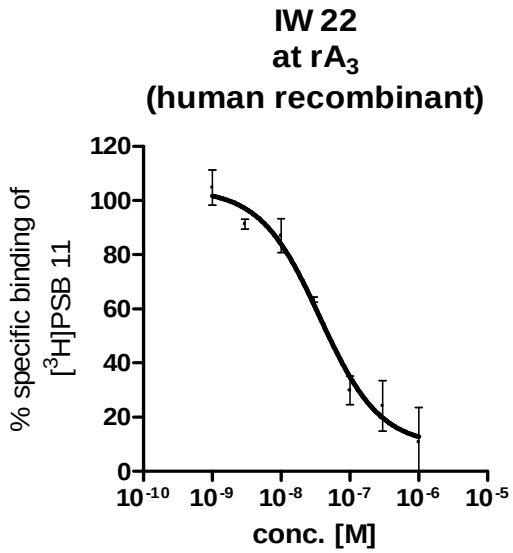
The benzyl-substituted compound IW22 shows good selectivity for A_3 receptors (K_i for A_3 = 5.91 nM). It is evident that the three-carbon chain between a bulky substituent and the tricyclic system increases affinity at all four adenosine receptor subtypes while one carbon linker results in significant selectivity towards A_3 receptors.

Nonsubstituted (IW17) or chloride-substituted (IW23) ligands have no or just low affinity at all adenosine receptors.

Methylheterocyclic substituted compounds (IW21, IW20, IW24) have comparably moderate affinity at all four adenosine receptors except for IW20, which is a very potent compound at A_3 receptors (K_i = 7.03 nM).

Table 11. N-methylpyrrol-3-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

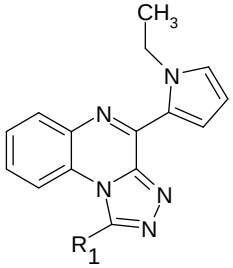
Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
IW 17	H	(21 ± 4)	(-2 ± 11)	(-11 ± 8)	**580** ± 282 (30 ± 4)
IW 23		**160** ± 31 (52 ± 4)	(-14 ± 25)	(24 ± 6)	**47.5** ± 19.9 (69 ± 4)
IW 24		**150** ± 22 (55 ± 4)	**75.9** ± 34.8 (43 ± 4)	(50 ± 0)	**25.5** ± 1.22 (84 ± 3)
IW 21		**198** ± 40 (44 ± 5)	**82.0** ± 6.1 (67 ± 5)	(60 ± 13)	**21.8** ± 12.1 (100 ± 4)
IW 20		**90.1** ± 27.6 (63 ± 5)	**79.8** ± 23.1 (50 ± 5)	(51 ± 12)	**7.03** ± 0.58 (96 ± 9)
IW 22		(28 ± 6)	(25 ± 3)	(44 ± 3)	**5.91** ± 1.20*** (96 ± 10)
IW 25		**3.43** ± 0.24 (100 ± 1)	**27.7** ± 8.5 (75 ± 3)	(73 ± 1)	**21.8** ± 2.01 (93 ± 5)
IW 26		**2.65** ± 0.31 (99 ± 0)	**49.4** ± 24.7 (74 ± 2)	(67 ± 7)	**25.8** ± 2.2 (85 ± 2)
IW 18		**1.58** ± 0.23 (100 ± 1)	**35.7** ± 17.3 (83 ± 5)	(96 ± 1)	**26.9** ± 5.8 (63 ± 5)



K_i: 5.91 ± 1.20 nM

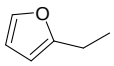
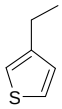
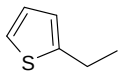
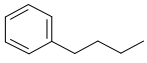
*** **Figure 9:** IW 22 – concentration-inhibition curve

N-Ethylpyrrol-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

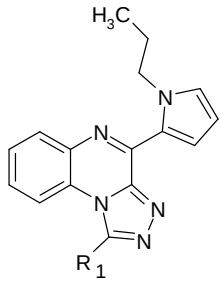


Generally, N-ethylpyrrol-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines are not active at adenosine receptors. There is only one exception - MD045. The phenylpropyl moiety increases affinity (K_i for A_1 = 4.80 nM) while the N-ethylpyrrol-2-yl residue induces selectivity towards A_1 receptor.

Table 12. N-Ethylpyrrol-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD031	H	(16 ± 1)	(-12 ± 14)	(20 ± 2)	(19 ± 7)
MD056		(12 ± 5)	(11 ± 4)	(9 ± 7)	(30 ± 1)
MD047		(18 ± 1)	(-20 ± 11)	(11 ± 5)	(6 ± 11)
MD098		**169** ± 12 (40 ± 4)	(8 ± 6)	(4 ± 12)	(45 ± 9)
MD106		(2 ± 6)	(4 ± 14)	(5 ± 2)	**73.7** ± 29.3 (66 ± 6)
MD099		(16 ± 2)	(7 ± 3)	(20 ± 6)	**120** ± 21 (40 ± 0)
MD086		(0 ± 5)	(-11 ± 14)	(-20 ± 1)	(24 ± 9)
MD042		(7 ± 1)	(6 ± 12)	(17 ± 0)	(41 ± 0)
MD045		**4.80** ± 2.03 (96 ± 1)	(13 ± 5)	(15 ± 1)	**102** ± 67 (50 ± 11)

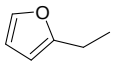
N-Propylpyrrol-2-yl-substituted [1,2,4]triazolo[4,3-*a*]quinoxalines

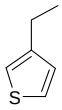
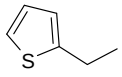
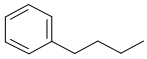


The N-propylpyrrol substituent causes dramatic loss of affinity at adenosine receptors.

The phenylpropyl moiety at MD032 increases affinity only at A_1 receptor (K_i = 15.2 nM).

Table 13. N-Propylpyrrol-2-yl-substituted [1,2,4]triazolo[4,3-*a]*quinoxalines

Compd.	R_1	A_1 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2A} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=3)	A_{2B} receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at 1 µM) (n=1)	A_3 receptor **K_i** ± SEM **[nM]** (% Inhib. ± SEM at1 µM) (n=3)
MD030	H	(0 ± 5)	(-17 ± 12)	(-9 ± 2)	(16 ± 4)
MD036		(-1 ± 3)	(16 ± 3)	(-7 ± 8)	(19 ± 2)
MD041		(7 ± 1)	(-4 ± 9)	(-8 ± 2)	(33 ± 6)
MD100		(16 ± 2)	(-1 ± 7)	(0 ± 6)	(27 ± 5)
MD107		(1± 5)	(-5 ± 13)	(1 ± 3)	**146** ± 99 (56 ± 2)

MD101		(11 ± 0)	(-4 ± 10)	(4 ± 2)	**120** ± 48 (48 ± 6)
MD089		(7 ± 1)	(8 ± 1)	(-13 ± 5)	**168** ± 53 (45 ± 3)
MD035		(4 ± 2)	(18 ± 1)	(-7 ± 9)	**139** ± 48 (40 ± 0)
MD032		**15.2** ± 5.8 (87 ± 6)	(16 ± 4)	(-6 ± 6)	(30 ± 0)

5. Conclusions

A novel series of [1,2,4]triazolo[4,3-*a*]quinoxalines and related compounds has been tested as potential adenosine receptor ligands. These compounds belong to the non-xanthine AR antagonists. They were designed with the expectation to have A_3 antagonistic effects.

Results were obtained using radioligand binding assays based on the principle of competitive inhibition. IC_{50} values of active compounds obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation. Structure-activity relationship between individual compounds were derived by comparing their K_i values. The main properties in view were affinity and selectivity for ARs.

Tested compounds were divided into three subgroups: quinoxalinones, tetrazoloquinoxalines and triazoloquinoxalines. The lactam group of quinoxalin-1-ones determines their selectivity towards A_3 ARs, and their affinity is rather high. Tetrazoloquinoxalines are A_3 AR selective as well, but their affinity is lower. Combining substituents in position 1 (R_1) and 4 (R_4) of the tricyclic skeleton represents the largest subgroup – the triazoloquinoxalines. It has been shown that the R_1 substituent determines affinity of a compound while the R_4 substituent confers selectivity.

There are three more promising derivatives in the series of [1,2,4]triazolo[4,3-*a*]quinoxalines: N4-ethyl-pyrrol-2-yl-1-phenylpropyl[1,2,4]triazolo[4,3-*a*]quinoxaline selective for rA_1, N4-propyl-pyrrol-2-yl-1-phenylpropyl[1,2,4]triazolo[4,3-*a*]quinoxaline selective for rA_1, and N4-methyl-pyrrol-3-yl-1-benzyl[1,2,4]triazolo[4,3-*a*]quinoxaline, selective for hA_3 ARs.

This thesis has confirmed the hypothesis of these compounds being A_3 AR ligands, but many compounds show nanomolar affinity at other AR subtypes as well.

Subsequent functional assays must be carried out to confirm that [1,2,4]triazolo[4,3-*a*]quinoxalines really have antagonistic activity at adenosine receptors.

The presented series of novel AR ligands has been tested at all four ARs. However, due to the lack of time, K_i values at A_{2B} AR have not been determined, which will have to be done in the future.

6. References

1) RIZZOLIO F., LA MONTAGNA R., TUCCINARDI T., RUSSO G., CAPUTI M., GIORDANO A.: Adenosine Receptor Lingands in Clinical Trials, Current Topics in Medicinal Chemistry, 2010, *10*, 1036-1045.

2) ALBERTS B., JOHNSON A., LEWIS J., RAFF M., ROBERTS K., WALTER P.: Molecular Biology of the cell, fourth Edition, New York: Garland Science, 2008, 904-905.

3) FORTH W., HENSCHLER D., RUMMEL W.: Allgemeine und spezielle Pharmakologie und Toxikologie, München: Elsevier GmbH, 2005, 18-21.

4) ILAŠ J., PEČAR S., HOCKEMEYER J., EULER H., KIRFEL A., MÜLLER C.E.: Development of Spin-Labeled Probes for Adenosine Receptors, Journal of Medicinal Chemistry, 2005, *48*, 2108-2114.

5) AKKARI R., BURBIEL J.C., HOCKEMEYER J., MÜLLER C.E.: Recent Progress in the Development of Adenosine Receptor Ligands as Antiinflammatory Drugs, Current Topics in Medical Chemistry, 2006, *6*, 1375-1399.

6) ABBRACCHIO M.P., BURNSTOCK G., VERKHRATSKY A., ZIMMERMANN H.: Purinergis signalling in the nervous system: an overview, Elsevier, 2009, *32*, 19-29.

7) http://thebrain.mcgill.ca/flash/a/a_11/a_11_m/a_11_m_cyc/a_11_m_cyc.html, downloaded 2.4.2010.

8) RALEVIC V., BURNSTOCK G.: Receptors for Purines and Pyrimidines, Pharmacological Reviews, 1998, *50*, 418-492.

9) YUZLENKO O., KIEĆ-KONONOWICZ K.: Potent Adenosine A_1 and A_{2A} Receptors Antagonists: Recent Developments, Current Medicinal Chemistry, 2006, *13*, 3609-3625.

10) HALLDNER L., LOZZA G., LINDSTRÖM K., FREDHOLM B.B.: Lack of tolerance to motor stimulant effects of selective adenosine A_{2A} receptor antagonist, European Journal of Pharmacology, 2000, *406*, 345-354.

11) FARRAR A.M., PEREIRA M., VELASCO F., HOCKEMEYER J., MÜLLER C.E., SALAMONE J.D.: Adenosine A_{2A} receptor antagonism reverses the effects of dopamine receptor antagonism on instrumental output and effort-related choice in the rat: implications for studies of psychomotor slowing, Psychopharmacology, 2007, *191*, 579-586.

12) WORDEN L.T., SHAHRIARI M., FARRAR A.M., SINK K.S., HOCKEMEYER J., MÜLLER C.E., SALAMONE J.D.: The adenosine A_{2A} antagonist MSX-3 reverses the effort-related effects of dopamine blockade: differential interaction with D1 and D2 family antagonists, Psychopharmacology, 2009, *203*, 489-499.

13) MÜLLER C.E., FERRÉ S.: Blocking Striatal Adenosine A_{2A} Receptors: A New Strategy for Basal Ganglia Disorders, Recent Patents on CNS Drug Discovery, 2007, *2*, 1-21.

14) CRISTALLI G., MÜLLER C.E., VOLPINI R.: Recent Developments in Adenosine A_{2A} Receptor Ligands, Handbook of Experimental Pharmacology, 2009, *193*, 59-98.

15) ORTORE G., MARTINELLI A.: A_{2B} Receptor Ligands: Past, Present and Future Trends, Current Topics in Medicinal Chemistry, 2010, *10*, 923-940.

16) JACOBSON K.A., KLUTZ A.M., TOSH D.K., IVANOV A.A., PRETI D., BARALDI P.G.: Medicinal Chemistry of the A_3 Adenosine Receptor: Agonists, Antagonists, and Receptor Engineering, Handbook of Experimental Pharmacology 2009, *193*, 123-159.

17) DUPREE J.D., BYLUNDD.B.: Basic principles and techniques for receptor binding, 2002, Tocris Reviews No.18, 1-7.

18) MOTULSKY H: The GraphPad Guide to Analyzing Radioligand Binding Data, GraphPad, SanDiego, CA, 2001.

19) http://www.graphpad.com/www/radiolig/radiolig.htm, downloaded 18.4.2010.

20) DAVENPORT A.P., RUSSELL F.D.: Radioligand binding assays: theory and practise, Current Directions in Radiopharmaceutical Research and Development, Netherlands: Kluwer Academic Publishers, 1996, 169-179.

21) BYLUND D.B., TOEWS M.L.: Radioligand binding methods: practical guide and tips, American Journal of Physiology, 1993, *265*, 421-429.

22) A glass fiber filter paper, Journal of the Franklin Institute, 1951, *251*, 358.

23) Principles and applications of liquid scintillation counting, National Diagnostics, 2004, 1-15.

24) BIGOTT-HENNKENS H.M., DANNOON S., LEWIS M.R, JURISSON S.S.: In vitro receptor binding assays: general methods and considerations, The Quarterly Journal of Nuclear Medicine and Molecular Imaging, 2008, *52*, 245-253.

25) LOWRY O.H,, ROSEBROUGH N.J., FARR A.L., RANDALL R.J.: Protein Measurement with the Folin Phenol Reagent, The Journal of Biological Chemistry, 1951, *193*, 265-275.

26) AKKARI R., BURBIEL J.B., HOCKEMEYER J., MÜLLER C.E.: Recent Progress in the Development of Adenosine Receptor Ligands as Antiinflammatory Drugs, Current Topics in Medicinal Chemistry, 2006, *6*, 1375-1399.