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The anticancer activity mediated by new A_3 receptor agonists

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DECLARATION:

I hereby declare that I elaborated this bachelor thesis independently under the supervision of Mgr. Jana Kotulová, using only information sources referred in the **Literature chapter.**

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SUMMARY

This bachelor thesis is focused on summarizing the current knowledge about the role and mechanism of action of adenosine receptors with the main focus on anti-cancer activity of A_3 adenosine receptor and its new agonists.

The theoretical part of the thesis outlines basic information about the endogenous agonist of all adenosine receptors subtypes (i.e. adenosine) and explains its role in living organisms under both physiological and pathological conditions with the main focus on cancer. The thesis also provides classification of adenosine receptor family and each of its subtype, which is followed by an examination of adenosine receptor A_3 and its signaling pathways. The last section of the theoretical part of the thesis presents clinically relevant agonists, highlighting two most prospective ones - IB-MECA and CL-IB-MECA. Finally, possible resistance to A_3 adenosine receptor treatment shall be introduced.

The experimental part of the thesis aims to examine RNA and protein expression level of adenosine receptors A_3 in various cancer cell lines. The expression level is evaluated by relative quantification of the complementary DNA, immunodetection of the presence of the A_3AR protein product and by quantitative densitometry. Results could possibly help to select suitable cancer cell lines for further testing of agonists of adenosine receptor A_3. The experimental part revealed gene expression and protein expression level of adenosine receptor A_3 in different types of cancer cell lines (hematopoietic, mammary gland, liver, pancreatic, lung, colon and bone). Based on those results, several cell lines were pre-selected, which can serve as *in vitro* models for future studies of anticancer effects mediated by new adenosine receptor A_3 agonists. Following cell lines were selected: pancreatic cancer cell lines: AsPC1, BXPC3, CAPAN-2, JOPACA-1, YAPC, PACADD119, PACADD135, PATU8902, liver cancer cell lines: HEPG2, HEP3B, NCI-N87, colon cancer cell lines: HCT116 par., HCT116 p53 -/- and lung cancer cell lines: A549, HCC78.

SOUHRN

Tato bakalářská práce je zaměřená na shrnutí současných znalostí o roli a účincích adenosinových receptorů s hlavním zaměřením na protinádorovou aktivitu adenosinového receptoru A_3 a jeho nových agonistů.

Teoretická část práce uvádí základní informace o endogenním agonistovi všech subtypů adenosinových receptorů (tedy adenosinu) a popisuje jeho roli v živých organismech jak při fyziologických, tak při patologických podmínkách, s hlavním zaměřením na rakovinu. Práce se také zabývá klasifikací rodiny adenosinových receptorů a jejích jednotlivých subtypů, poté následováno rozborem adenosinového receptoru A_3 a jeho signálních drah. Poslední sekce teoretické části této práce představuje klinicky relevantní agonisty, se zaměřením především na dva nejvíce perspektivní IB-MECu a CL-IB-MECu. Nakonec je zmíněna i možná rezistence vůči léčbě A_3 adenosinového receptoru.

Experimentální část práce se zaměřuje na zkoumání úrovně exprese RNA a proteinů adenosinových receptorů A_3 v různých rakovinových buněčných liniích. Úroveň exprese je hodnocena relativní kvantifikací komplementární DNA, imunodetekcí přítomnosti proteinového produktu A_3AR a kvantitativní densitometrií. Výsledky mohou být použity při výběru vhodných linií pro další testování agonistů A_3. Experimentální část odhalila genovou expresi a úroveň proteinové exprese adenosinových receptorů A_3 v různých typech rakovinových buněčných linií (hematopoetických, prsních, jaterních, slinivky, tlustého střeva, plicních a kostních). Na základě těchto výsledků bylo vybráno několik buněčných linií, které mohou potencionálně sloužit jako *in vitro* modely pro budoucí studie protinádorových účinků zprostředkovaných novými agonisty A_3 adenosinového receptoru. Vybrány byly následující buněčné linie: AsPC1, BXPC3, CAPAN-2, JOPACA-1, YAPC, PACADD119, PACADD135, PATU8902, HEPG2, HEP3B, NCI-N87, HCT116 par., HCT116 p53-/-, A549 a HCC78.

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1 INTRODUCTION

The endeavor of finding a cure for cancer is continuous. This is not surprising, since it is the cause of death for approximately one person out of five. Cancer cells violate basic rules of cell behavior, which are vital for the existence of the multi-cellular organism and they use every opportunity to do so. Differences between the behavior of cancer and non-cancerous/ healthy cells helped to reveal the basic functioning of cellular processes and their tight regulation machineries. Therefore, the cancer research has reached a better and deeper understanding of cancer, which fuels efforts to find new approaches for fighting this fatal disease.

The current gold standard of treatment is in many cases radiation or traditional chemotherapy both extremely unspecific methods, which are in many cases ineffective and with severe side effects. On the other hand, modern treatment relies on highly selective methods targeting only specific features present on cancer cells and agonist dependent activation of adenosine receptor A_3 has potential to be one of those treatments. This type of receptor is highly expressed in cancer cell compared to non-tumor cells and therefore it was suggested as a prospective target for cancer treatment.

Adenosine receptors A_3 are known for their anti-inflammatory effects. Moreover, many *in vitro* cancer studies also confirmed that their agonist stimulation is in turn responsible for strong anti-tumor effects affecting cancer cell proliferation, invasiveness and death. In order to be able to activate specifically adenosine receptors $A_{3,}$ there is a need for highly selective and potent agonists. Many agonists fulfill those criteria, where the majority of them are adenosine derivatives. However, only two commercially available agonists stand out and those are IB-MECA and CL-IB-MECA, where *in vitro* and *in vivo* studies reported their promising results on various cancer cell lines. Furthermore, both compounds successfully completed preclinical studies, which showed that they are both well tolerated and safe in humans. Recently, IB-MECA and CL-IB-MECA were included in clinical trials for disorders including dry eye disease, psoriasis, rheumatoid arthritis and hepatocellular carcinoma.

2 THE CURRENT STATE OF THE KNOWLEDGE

2.1 Adenosine

Adenosine (Ado) is an omnipresent signaling molecule and nearly all cells produce it. It occurs in both intracellular and extracellular space of the cell in a nanomolar concentration and its level substantially rises and falls in response to different kinds of cellular distress (Fredholm *et. al.*, 2011). Ado is a purine nucleoside composed of a molecule of adenine attached to a d-ribose sugar molecule via a β-N_9-glycosidic bond **(Fig.1)** and as an endogenous organic compound with a wide occurrence in nature, Ado modulates many important physiological processes but is mainly involved in the protection of the cell (Linden, 2005). Effects of Ado are mediated via receptor-dependent and/or receptor-independent mechanisms. The first mentioned mechanism is executed by binding to different subtypes of the adenosine receptor (AR) family (A_1, A_{2A}, A_{2B} and A_3) in order to activate them as a nonselective endogenous agonist (Fredholm *et. al.* 2011). The second mechanism involves other processes, in particular regulation of the intracellular nucleoside level by specific bi-directional transporters (Antonioli *et al.*, 2013b).

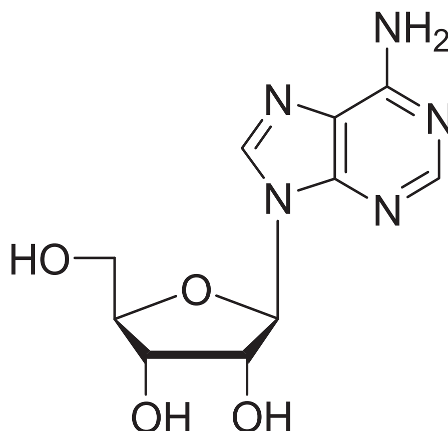


Figure 1 – The structure of Ado molecule. The structure of Ado is composed of adenine attached to d-ribose. (Fredholm *et al.*, 2011)

2.1.1 Role of adenosine in living organisms

Ado exerts pleiotropic functions throughout the whole body and it can promote its functions through receptor-independent or receptor-dependent pathway. Ado acts as a neuromodulator playing a role in suppression of arousal and as a regulator of blood flow through vasodilatation. In general, Ado exerts functions that contribute to keep homeostasis and protect an organism for example by stimulation of angiogenesis or by suppression of inflammatory response nearby an injury (Borea *et al*., 2016). It also contributes to locally equalizing energy demands with energy supply thus often being referred to as "retaliatory metabolite" (Borea *et al*., 2014). This is due to Ado metabolism, which can influence cellular concentration of adenosine triphosphate (ATP). Apart from those functions Ado is also involved in regulation of cell proliferation, cell growth and cell death (Haskó *et al*., 2008).

2.1.1.1 Receptor-independent pathway

Extracellular Ado can be transported to the inside of the cell by bi-directional nucleoside transporters (NTs), those are integral membrane proteins that regulate flux of nucleosides such as Ado, therefore NTs control its intracellular level (dos Santos-Rodrigues *et al*., 2014). This regulation is very important for ensuring the concentration equilibrium of Ado. By decreasing extracellular levels of Ado, NTs are even able to cease Ado receptor-dependent signaling pathway (dos Santos-Rodrigues *et al*., 2014). Therefore, there exist a tight regulatory interconnection between receptor-dependent and receptor-independent pathway. Apart from Ado, also other nucleosides such as guanosine or thymidine are transported via NTs, which are often being referred to as to "salvage metabolites", since they can be converted to nucleotides or they can serve as precursors of nucleic acids (Young *et al*., 2013). There were classified two distinct categories of transporters: equilibrative nucleoside transporters (ENTs), which carry Ado across the cell membrane following the concentration gradient and concentrative nucleoside transporters (CNTs) which adjust the influx of Ado caused by transmembrane sodium gradient by opposing the concentration gradient (Latini *et* Pedata, 2001, **Fig.2**). In addition, NTs facilitate an uptake of nucleoside analog drugs used in cancer chemotherapy or anti-viral therapeutic treatment, therefore their expression plays an important role in the treatment response (dos Santos-Rodrigues *et al*., 2014; Galmarini *et al*., 2001). It was demonstrated that negative regulation of ENT1 contributes to nucleoside treatment resistance in murine leukemic cells (Leisewitz *et al*.,

2011). Moreover, higher expression of some types of ENTs specifically ENT4 may constitute a survival and a growth advantage for cancer cells (Young *et al*., 2013).

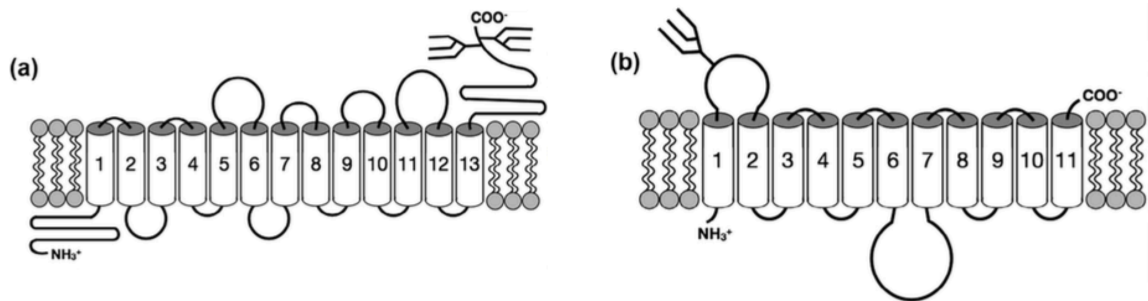


Figure 2 – Structure of NTs – CNT (a) and ENT (b). CNT is predicted to have 13 transmembrane domains whereas ECT only 11. Both types of NTs contain intracellular amino-terminus (N-terminus) and extracellular carboxy-terminus (C-terminus) and both are glycosylated but on different sites (displayed as a fork structures). (Adapted from dos Santos-Rodrigues *et al*., 2014)

2.1.1.2 Receptor-dependent pathway

Four different AR subtypes carry out receptor dependent pathway. In this case Ado acts as an endogenous ligand, which binds to ARs and influence especially regulation of adenylate cyclase (AC) activity and formation of the second messenger molecule cyclic adenosine monophosphate (cAMP). This second messenger is responsible for signal transduction and it is involved in many other intracellular downstream signaling pathways (Fredholm *et al*., 2000, **Fig.3**). Detailed information about all AR subtypes and their predominant pathways can be found in the chapter **2.2 Adenosine receptor family**.

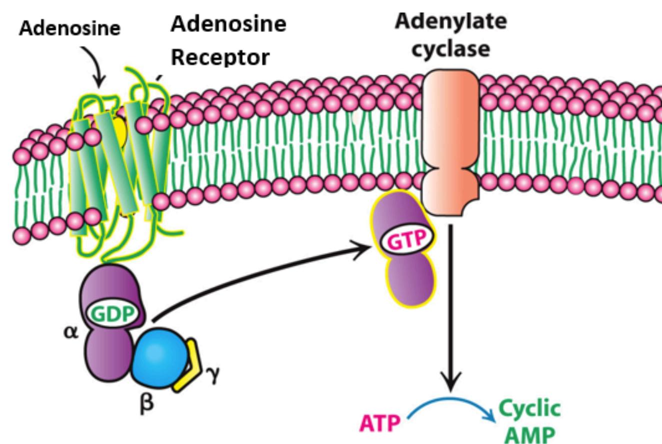


Figure 3 – AR mediated pathway. Extracellular Ado engages with surface ARs. Activation of ARs primarily triggers dissociation of subunit Gα from subunit G$\beta\gamma$. Phosphorylated GDP and subunit Gα regulate activity of enzyme AC, which is responsible for cAMP synthesis from ATP). (Tymoczko *et al*., 2015)

2.1.2 Adenosine and cancer

The extracellular concentration of Ado dramatically increases in response to unfavorable conditions such as hypoxia or necrosis caused by rapid tumor growth. In those situations, where transient or permanent damage of the cell membrane occurs, massive amount of intracellular ATP is released and is consequentially converted to Ado by different ecto-enzymes leading to increase of extracellular levels of Ado. Released ATP from damaged cells function as an alert or a signal of danger and it generates responses, which aim to restore homeostasis (Antonioli *et al.*, 2013b). In physiological conditions increased extracellular levels of Ado get back to original concentration with the help of enzymes and transporters. However, those mechanisms are disrupted in the unfavorable tumor environment and when the increased Ado level persists it can become detrimental. High expression levels of ecto-enzymes nucleoside triphosphate dephosphorylase (CD39) and ecto-5'-nucleotidase (CD73), responsible for Ado formation and low expression levels of ecto-adenosine deaminase, responsible for Ado breakdown are typical for solid tumor and are directly responsible for sustaining the extremely high extracellular Ado concentration (Eltzschig *et al.*, 2009; Synnestvedt *et al.*, 2002). In addition, under those unfavorable conditions also expression of ENTs is decreased, therefore transport of Ado to the intracellular space is significantly reduced, which even enhances massive accumulation of Ado in the extracellular space of the cell (Eltzschig, 2009). The level of Ado in body fluids and unstressed tissues is very low somewhere between 30-300 nM, thus Ado is able to activate receptors only at sites where they are highly abundant (Fredholm *et al.*, 2001). However, in tumors Ado concentrations were reported to be 10- to 20-fold higher than in healthy tissues at the same location, which leads to significant increase in AR activation and signaling (Blay *et al.*, 1997). The aberrant signaling is being associated with immunosuppression, promotion of tissue changes and deregulation of signaling pathways, which can lead to the cancer development (Antonioli *et al.*, 2013b; Chan *et* Cronstein, 2010; Karmouty-Quintana *et al.*, 2013).

2.2 Adenosine receptor family

Ado acts through activation of four different subtypes of AR family, namely A_1, A_{2A}, A_{2B} and A_3. ARs belong to the group of G protein-coupled receptors (GPCRs) (Ralevic *et* Burnstock, 1998). Those receptors are composed of a single polypeptide chain, which is extending from the extracellular side and transverses the membrane seven times as α-helices, where those helices are perpendicular to the cell membrane (Trincavelli *et al.*, 2010).

ARs display the extracellular N-terminus and the intracellular C-terminus and they are glycosylated on the second extracellular loop (Sheth *et al*., 2014). The third intracellular loop and the C-terminus are two intracellular sites involved in the G-protein coupling and the third intracellular loop also plays a role in desensitization and internalization of ARs (Baldwin, 1994; Schöneberg *et al*., 2002; **Fig.4**).

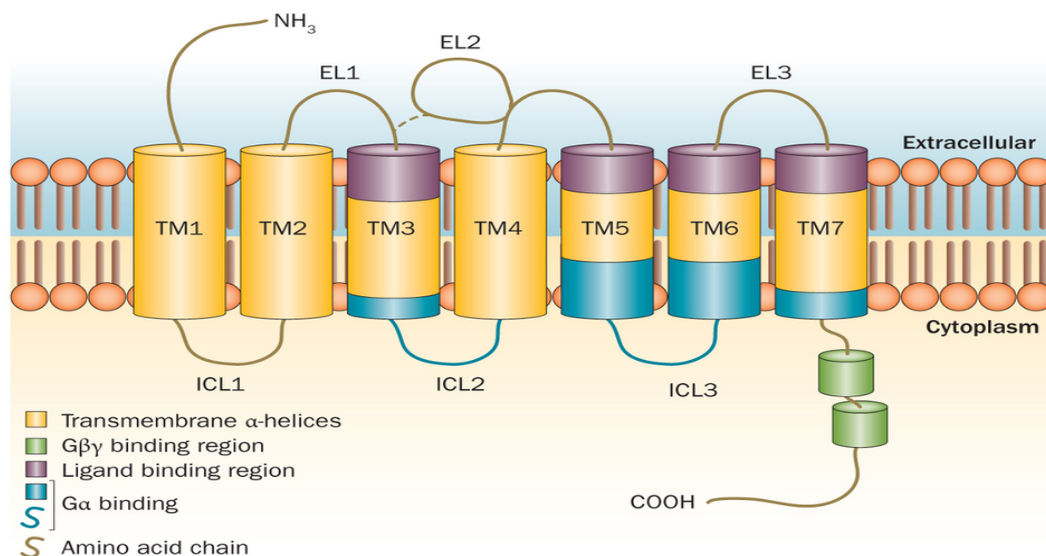


Figure 4 – The general structure of the G protein-coupled receptor.
This figure is showing how seven transmembrane-spanning domains are traversing the cellular membrane. Extracellular N-terminus and intracellular C-terminus are also displayed. For ARs extracellular sites of transmembrane helix 3 (TM3) and transmembrane helix 7 (TM7) are prior for ligand binding and intracellular TM3 and C-terminus are prior for guanine nucleotide-binding protein (G-protein) coupling. (Neumann *et al*., 2014)

Separate genes encode each AR, having following chromosomal location: A_1AR –1q32.1, A_{2B}AR – 22g11.2, A_{2B}AR – 17p11.2-12, A_3AR – 1p13.2 and also each AR exerts different physiological role (Fredholm *et al*., 2001). Generally, AR subtypes differ in tree key features; in the binding affinity, in the type of coupling G-proteins that can consequently trigger different transduction pathways and in the structure of the binding site.

First, they express different affinity for their endogenous agonist Ado. In humans the affinity for Ado of AR subtypes differs dramatically. In humans the inhibitory constant (Ki) for A_1ARs is 100 nM, for A_{2A} 310 nM, for A_{2B} 15 000 nM and for A_3 290 nM (Müller *et* Jacobson, 2011, **Tab.I**).

Second, they are preferentially coupled to different types of G-proteins, A_{2A} and A_{2B} recruit G_s, which is a stimulatory G-protein that consequentially stimulates the activity of AC, thus the level of cyclic cAMP increases. On the other hand, A_1 and A_3 recruit G_i, which is an inhibitory G-protein that consequentially inhibits the activity of AC and therefore the level of cAMP decreases. After activation of AR by ligand, multifactorial pathways are initiated with a distinct predominant signal transduction pathway for each receptor (**Fig.5**). A_1 and A_3 share 49% sequence identity and A_{2A} and A_{2B} share even higher sequence identity of 59%, which also explains that these pairs are involved in similar predominant pathways (Fredholm *et al*., 2001; Müller *et* Jacobson, 2011).

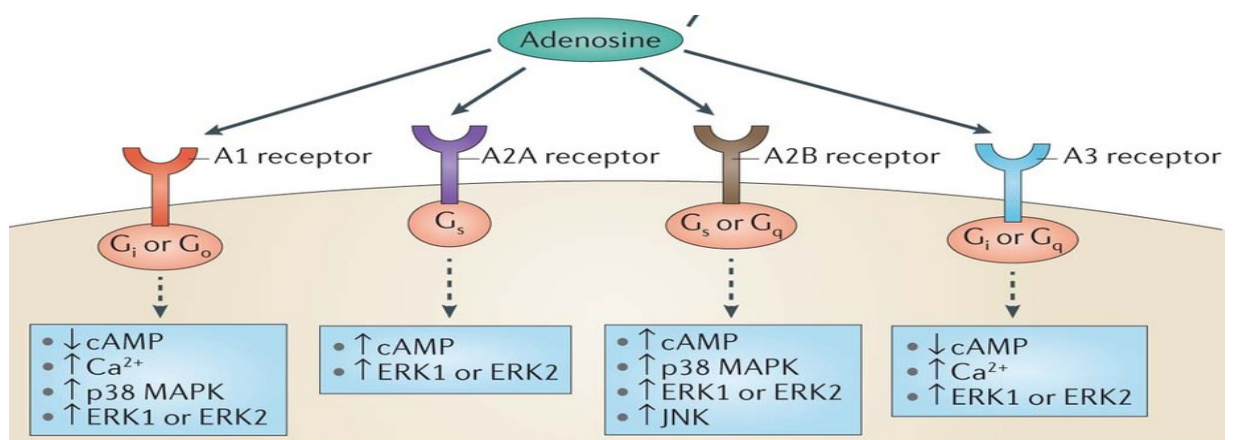


Figure 5 – Predominant downstream pathways of AR subtypes.

Extracellular Ado acting through receptor dependent pathway can bind to four different AR subtypes. ARs can either activate (A_{2A}AR and A_{2B}AR) or inhibit (A_1AR and A_3AR) AC activity. Activation of A_1AR and A_3AR can also elicit the release of calcium ions from intracellular stores via stimulation of phospholipase Cβ (PLCβ). Furthermore, AR subtypes stimulate mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK and cJun – N terminal kinase (JNK). (Adapted from Antonioli *et al*., 2013a)

Third, they differ in the structure of the ligand-binding site, which is important for receptor agonist and antagonist binding. All four receptor subtypes have been cloned from rat, mouse and human and all exert high structural similarity concerning those mammals, only exception being A_3AR, where this receptor significantly differs in ligand specificity regarding different species (Trincavelli *et al*., 2010). Two specific transmembrane-spanning domains TM3 and TM7 were identified to be responsible for receptor ligand binding. Those particular TM sites seem to be common for ligand binding of all four subtypes. Only specific aminoacids present at TM3 and TM7 are identified to differ for each AR subtype and are indicated to be of higher importance for their ligand specificity (Fredholm *et al*., 2000).

2.2.1 Adenosine receptor subtypes

ARs are unevenly distributed in various body tissues and since Ado concentration under basal physiological conditions is rather low, it only has an ability to activate receptors where they are abundant (Sheth *et al*., 2014; Fredholm *et al*., 2001). Moreover, information about AR distribution (expression) can be used for effective AR agonist targeting. Recent findings indicate promising prospects of ARs in the treatment of wide range of pathological conditions especially heart and brain ischemia, inflammation, pain, neurodegenerative diseases, rheumatoid arthritis, cancer and many others (Haskó et Cronstein, 2013; Fishman *et al*., 2004; McIntosh *et* Lasley, 2012; Ochaion *et al.,* 2009; Pugliese *et al*., 2007; Sawynok et al., 1997). The distribution, involvement in physiological processes and therapeutic potential in treatment of AR subtypes are described in the following overview.

2.2.1.1 A_1 receptor

A_1AR is particularly present in the central nervous system (CNS). High-level expression was found in the hippocampus, cerebral cortex, cerebellum and other parts of CNS (Middlekauff *et al*., 1998; Saura *et al*., 1998; Swanson *et al*., 1995). On the other hand, low-level expression was reported in the kidney, lung or colon (Fredholm *et al*., 2001). Physiological functions of A_1AR include neuroprotection, where A_1ARs modulate release of neurotransmitters in the CNS (Trincavelli *et al*., 2010). Another important physiological function of A_1AR is cardiovascular protection. A_1ARs in cardiomyocytes exert opposite effects of adrenergic sympathicus stimulation, thereby reducing heart rate (Olsson *et* Pearson, 1990). In addition, A_1ARs are involved in stimulation of glucose uptake in white adipocytes and in the inhibition of lipolysis. A_1ARs also play role in bronchoconstriction, anxiety, analgesia or sleep/arousal regulation (Trincavelli *et al*., 2010).

2.2.1.2 A_{2A} receptor

A_2AR is particularly prevalent in the striatum, olfactory tubercle, spleen, thymus, leukocytes and blood platelets. Lower level of expression was detected in the heart, lung or blood vessels (Fredholm et al., 2001; Hettinger *et al*., 2001; Koshiba *et al*., 1999; Rosin *et al*., 1998). Similarly to A_1AR, A_{2A}AR also contributes to cardio- and neuroprotection and it functions as a vasodilator of coronary arteries, which leads to improvement of nutrition supplementation of the heart and decrease of the blood pressure (Fredholm *et al*., 2011). Its primary physiological role is immunosuppression, to prevent tissue damage that can be

caused by a rogue immune response. In addition to immunosuppressive effects, A_2AR is involved in physiology of neurodegenerative diseases such as Parkinson's, Huntington's or Alzheimer's disease (Domenici *et al.*, 2007; Fredholm *et al.*, 2001; Mori *et* Shindou, 2003).

2.2.1.3 A_{2B} receptor

$A_{2B}ARs$ were reported to be expressed in the vasculature of almost all organs with higher expression in smooth muscle cells, endothelial cells and macrophages (Klotz, 2000) $A_{2B}AR$ is assigned to have a role in the mast cell activation, physiology of asthma, mediation of allergic reaction or inflammatory disorder (Feoktistov *et* Biaggioni, 1996; Trincavelli *et al.*, 2010; Yang *et al.*, 2006). Despite of those findings, $A_{2B}AR$ is still the least selective AR (**Tab.I**) and its role has not yet been fully understood mostly due to a lack of high affinity agonists and antagonists (Ham *et* Rees, 2008). However, only recently growing evidence suggests its role in cancer, renal disease and diabetes, where it can potentially serve as a target for therapeutic treatment (Sun *et* Huang, 2016).

2.2.1.4 A_3 receptor

In general, ARs display high sequence identity (80 – 95%), which means they are evolutionally well conserved with only exception being A_3AR (Chan *et* Cronstein, 2010). This receptor can vary among different species, which makes it slightly different from other ARs (Zhou *et al.*, 1992). In humans, A_3ARs are present at high-levels in the lung and liver and in lower levels in the aorta. In the CNS there are found relatively low levels of A_3AR with the thalamus and the hypothalamus being two exceptions. Other tissues or cells expressing A_3AR are mast cells, eosinophils, testis, kidney, heart, spleen, uterus, bladder or colon (Fredholm *et al.*, 2001). A_3AR plays important role in mediating allergic responses and inflammation (Gessi *et al.*, 2008). Moreover, A_3AR is also involved in neurodegeneration and autoimmune disorders including dry eye disease, psoriasis, and rheumatoid arthritis (Avni *et al.*, 2010; Baharav *et al.*, 2005; David *et al.*, 2012). In addition, this receptor plays significant role concerning cancer. A_3ARs were reported to be overexpressed in various types of cancer, for example in human colon cancer tissues or in human hepatocellular cancer tissues. Moreover, the upregulation also occurs in peripheral blood mononuclear cells (PBMC), thus A_3AR may serve as a potential biological marker to prove the presence of cancer cells but also as a potential target for anticancer treatment (Gessi *et al.*, 2004; Madi

et al., 2004). Overexpression of A_3ARs is primarily attributed to elevated Ado and cytokines, both typical for pathological conditions such as cancer or inflammation (Fishman *et al*., 2012). Therefore, it was suggested that level of A_3AR expression may also correlate with severity of cancer, thus it can potentially serve as method for estimation of the status of the tumor progression (Gessi *et al*., 2004). Mechanisms of action and anticancer effects caused by agonist dependent activation of A_3AR are described in detail in following chapters.

A_3AR signaling is very complex and the agonist activation of this receptor can stimulate series of different downstream signaling pathways (**Fig.6**), which are involved in the control of the cell cycle progression, growth, proliferation and cell apoptosis.

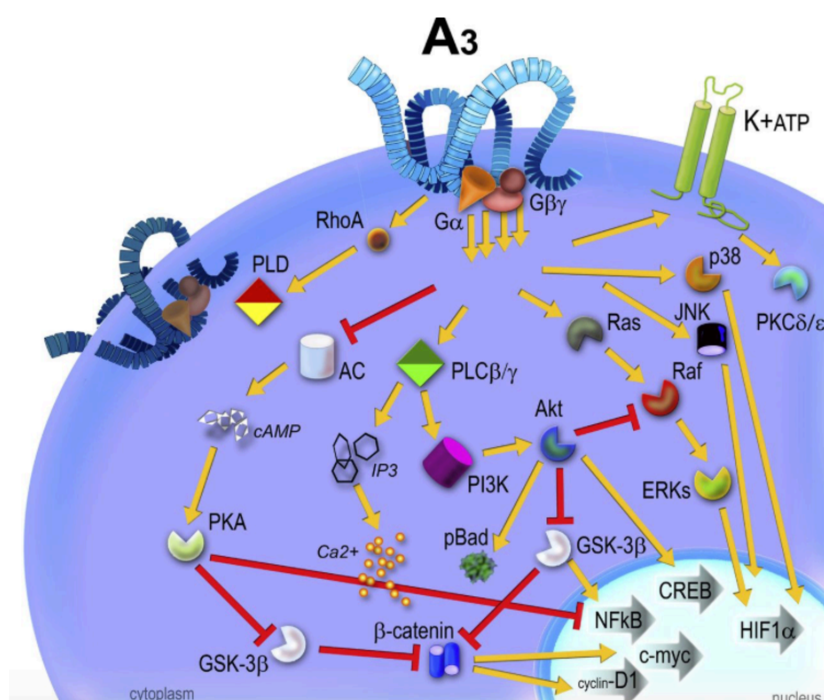


Figure 6 – Complex A_3AR signaling. This figure illustrates second messengers and downstream target proteins that are involved in A_3AR intracellular signaling pathways. It is apparent that A_3AR is able to employ multiple signaling pathways within one cell. Activation of A_3 by agonist (e.g. Ado) dissociates subunit G*α* from G*βγ* and the former inhibits AC and therefore decreases cAMP and (protein kinase A) PKA production. PKA influences activity of glycogen synthase kinase 3 beta (GSK3β), which is a key protein of Wnt downstream pathway. Consequently, inhibition of β-catenin results in decreased transcription of cyclins D1 and c-Myc, proteins necessary for the cell cycle progression. Activation of phospholipase C (PLC) leads to either phosphorylation of phosphoinositide 3-kinase (PI3K), Akt or calcium release. Another stimulated proteins are RhoA, phospholipase D (PLD) and also MAPK family (ERKs, JNK, p38) are regulated upon A_3 activation. The final series of downstream targets are situated within the cell nucleus and those are transcription factors such as nuclear factor kappa B (NF-κB), cAMP response element binding protein (CREB) or c-myc. (Adapted from Borea *et al*., 2014)

2.3 Agonists of adenosine receptor A_3

The importance of the current pursuit of new A_3ARs agonists is significant based on the fact that this type of receptor is considerably involved in the physiological regulation of several homeostatic processes as well as in the pathology of many diseases (Gessi *et al*., 2008). Neurodegenerative diseases induced by ischemia, autoimmune diseases or cancer are all pathological conditions under which A_3ARs play the major role (Baharav *et al*., 2005; Fishman *et al*., 2004; Von Lubitz *et al*., 2001). Recent aim to investigate new, selective A_3AR agonists contributed to the discovery of new promising therapeutic treatment methods arising from activation of the A_3AR. Since the beginning of the investigation several prospective agonists have been found, where some of them exhibited unusual effectiveness on animal models leading to their introduction into human clinical trials. For example results of agonist CF102 reported in several studies done in animal tumor models specifically on immunocompetent mice and rats led to its inclusion into human clinical trials on hepatocellular carcinoma (HCC) patients (Bar-Yehuda *et al*., 2008; Stemmer *et al*., 2013; Cohen *et al*., 2011). Altogether, A_3AR agonists have potential to become drugs that can improve or even save lives.

2.3.1 Clinically relevant agonists of adenosine receptor A_3

The structure-activity relationship investigation shed some light to the possible A_3AR agonists, where only derivatives of purine nucleoside Ado turned out to express high selectivity and affinity for A_3AR. Therefore, the more the chemical compound differs from Ado the more likely it will be an antagonist with only few exceptions to this generalization (Müller *et* Jacobson, 2011). Ado is synthesized in the human body and can bind to all ARs to activate them, which means it is a nonselective endogenous agonist (Fredholm *et al*., 2011). The new, selective agonists can be synthesized in several different manners. It can be through various substitutions, which primarily occurs at N^6-, C2-, and 5' positions or via changes of ribose moiety by modifications of the ribose ring (Borea *et al*., 2014).

In this section selective artificially synthesized exogenous agonists will be introduced. The first group of exogenous agonists comprise of the two most promising agonists N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (CF 101, IB-MECA, **Fig.7**) and 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (CF102, Cl-IB-MECA, **Fig.7**). Both compounds are potent and selective prototypical agonists of A_3AR and both are modified at 5'- and N^6- positions of Ado, only difference is that CF 102 has chlorine

introduced at C2- position (Jacobson *et* Gao, 2006). Recently, another selective agonist belonging to this group was synthesized; 2-chloro-N^6-(3-iodobenzyl)-5-N-methylcarbamoyl-4-thioadenosine (LJ529, **Fig.7**), which is a 4'-thio analog of CF 102 (Jeong *et al.,* 2003). There also exist other analog groups that are slightly different from the previously mentioned one but are still derivatives of Ado. The example can be 2-phenylethynyl-N^6-methyladenosine (PEMADO, **Fig.7**) or 2-hexyn-1-yl-N^6-methyladenosine (HEMADO, **Fig.7**), where both those compounds are Ado derivatives having 2-(ar)-alkynyl chains and at the N^6- position various substituents. Another group of selective A_3AR agonists is represented by 3'-amino Ado analogs modified also at the 5'- and N^6- positions and represented by N^6-[2-(3-methylisoxazol-5-ylmethoxy)-5-chloro]benzyl-3'-amino-adenosine-5'-N-methylcarboxamide (CP608,039, **Fig.7**) (Borea *et al.*, 2014). Last group that will be mentioned is more different from the previous groups because it is not defined by the substitutions at various positions of Ado but rather by modifications on the ribose ring. Agonists in this group contain (N)-methanocarba-(bicyclo[3.1.0]hexane) ring system, which is responsible for the ability of the compound, otherwise twisted in solution, to be chemically frozen in the North conformation, which is preferred conformation for binding to the A_3AR. An example can be 4-[6-(3-chlorobenzylamino)-2-chloro-9H-purin-9-yl]-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (MRS3558, CF502, **Fig.7**) or its 2-alkynyl-(N)-methanocarba derivative (MRS5151, **Fig.7**) (Müller *et* Jacobson, 2011).

Table I – Binding affinity of agonists at human AR subtypes (Adapted from Borea *et al.*, 2014).

	AR binding affinity of agonists				
		Ki [nM]			
	Agonists	**A_1AR**	**$A_{2A}AR$**	**$A_{2B}AR$**	**A_3AR**
Non-selective	Ado	100	310	15 000	290
Selective	CF101	51	2900	11 000	1.8
	CF102	220	5360	> 10 000	1.4
	LJ529	193	223	N.D	0.38
	PEMADO	32 800	41 700	> 30 000	0.44
	HEMADO	330	1200	> 30 000	1.1
	CP608,039	7300	> 50 000	N.D	5.8
Note: *N.D = no data available*	CF502	260	2330	> 10 000	0.29
	MRS5151	14 900	10 000	N.D	2.38

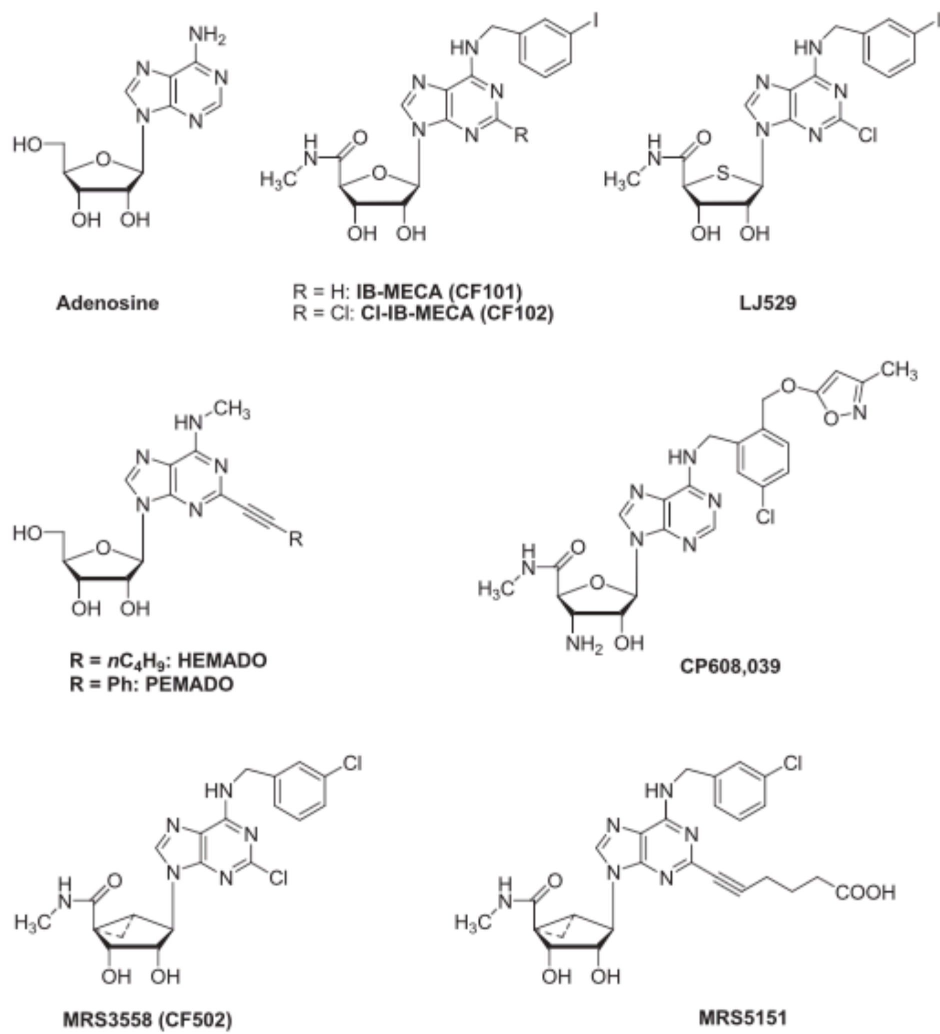


Figure 7 – A_3AR agonists. Typical structure of A_3AR agonist derived from endogenous agonist Ado. (Borea *et al*., 2014)

In this section two prototypical A_3AR agonists CF101 and CF102 and their anti-cancer potential will be introduced in detail. There exist several reasons for selecting these two very compounds. Main reason is that their behavior was tested in many studies on cancer cells both *in vitro* and *in vivo* with promising outcomes. Despite the fact that CF101 and CF102, highly selective agonists of A_3AR (Jeong *et al*., 2003, **Tab.I**), were originally synthesized to cure inflammatory rheumatoid arthritis, their therapeutic potential was revealed to be much wider. Both those small orally bioavailable molecules reached human clinical trials, where they exhibited great results in treatment of diseases such as psoriasis, rheumatoid arthritic, dry-eye syndrome, hepatitis B or hepatocellular carcinoma (Avni *et al*., 2010; Baharav *et al*., 2005; Bar-Yehuda *et al*., 2008; David *et al*., 2012). However, their full therapeutic potential is still yet to be discovered.

2.3.2 Agonist CF101 (IB-MECA)

CF101 is derived from double modification of Ado at 5'- and at N^6-positions (Gallo-Rodriguez *et. al*., 1994) (**Fig.8**) and its systematic name is N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide. CF101 is a small orally bioavailable molecule (Bar-Yehuda *et al*., 2007) and it is a selective and potent A_3AR agonist with significant affinity for A_3AR. Although CF101 is highly selective, where it is 28-, 1611- and 6111-fold less selective for A_1AR, $A_{2A}AR$ and $A_{2A}AR$, respectively, in comparison to A_3AR, (**Tab.I**) it was discovered that it is only a partial agonist of A_3AR, thus is can only trigger submaximal functional response after maximal receptor occupancy (Borea *et al*., 2010). Studies showed that response to CF101 is at the most slightly above 60 % that of Ado (Fossetta *et al*., 2003).

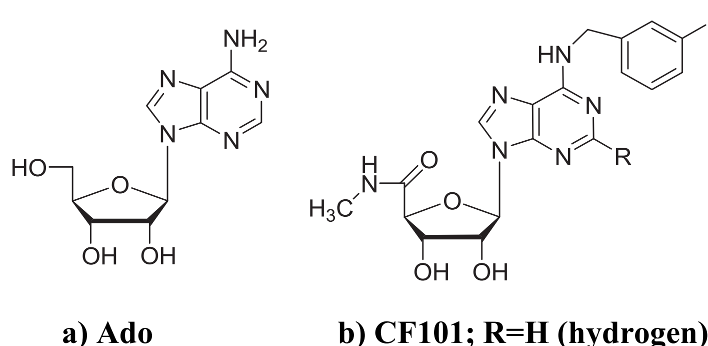


Figure 8 – Structure of Ado (a) and from it derived agonist CF101 (b). Positions 5'- and N^6- of Ado were used for CF101 creation by introduction of substituents that significantly improves its affinity. (Borea *et al*., 2014)

2.3.2.1 Effects of CF101 on cell proliferation, cell invasiveness and cell death related to cancer

It was confirmed that A_3ARs are present in various types of cancer cells for example in HL-60 human leukemia or A375 human melanoma and upregulated in other types such as in human colorectal cancer (Gessi *et al*., 2002 & 2004; Kohno *et al*., 1996; Merighi *et al*., 2001; Ohana *et al*., 2003). Since CF101 is a prototypical A_3AR agonist and is able to selectively act upon this receptor, it can directly affect cancer cell proliferation, cancer cell invasiveness and level of cell death (Fredholm *et al*., 2011).

It was demonstrated that A_3AR activation by CF101 causes deregulation of the Wnt and NF-κB signaling. Since Wnt pathway enhances tumorgenesis and NF-κB is known to regulate two key proteins necessary for the cell cycle progression D1-cyclins and c-Myc (Alberts *et al*., 2014), this deregulation resulted in an inhibition of cell proliferation and growth (Fishman *et al*., 2012). Tumor-cells growth inhibition was evident from *in vitro* experiments conducted on series of various cell lines such as PC-3 human prostate carcinoma cells, B16-F10 murine melanoma cells, OVCAR-3 human ovarian or HCT116 colon cancer cells (Abedi *et al*., 2014; Fishman *et al*., 2002 & 2003, Ohana *et al*., 2003). Simultaneously, it was demonstrated that the inhibition was receptor-dependent (Gessi *et al*., 2011, Madi *et al*., 2003, Ohana *et al*., 2003). In B16-F10 cell line treated with CF101 it was proven that the decrease in the level of c-Myc also reduce telomerase activity (Madi *et al*., 2003), which impedes cancer cell proliferation.

In the study examining the an impact of CF101 on ALVA and PC3-MM both human prostate cancer cell lines (*in vitro)* and in nude mice with inoculated tumor cells (*in vivo)*, series of antitumor effects were demonstrated, such as reduced cell proliferation, inhibition of metastasis and suppression of cell invasiveness (Jajoo *et al*., 2009). Stimulation of A_3ARs with CF101 caused reduction of the ability of cells to migrate (*in vitro*) and metastasize (*in vivo*), those effects were assessed by wound-healing assay and by testing whether CF101 is able to block extravasation of cancer cells to the lung, respectively. Inhibition of the cell invasiveness mediated by CF101 was mainly caused by reduction of PKA stimulation of ERK1/2, which lead to decrease of NADPH oxidase activity that was responsible for generation of harmful reactive oxygen species (ROS) (Jajoo *et al*., 2009). Similar effects were observed in A375 human melanoma and NPA human thyroid cancer cells, where those two cell lines expressed high ERK1/2 activity like prostate cancer cells and A_3AR agonists

inhibited ERK1/2 in the same manner as in prostate cancer cells (Merighi *et al.*, 2005; Morello *et al.*, 2008). Since high levels of ROS are responsible for DNA and protein damage, and stimulate cellular transformation, ROS production may be a viable therapeutic target for prevention of cell cancerous differentiation (Jajoo *et al.*, 2009).

Existing studies have shown CF101 to be able to induce apoptosis but very often only at micromolar doses (Gessi *et al.*, 2011). The induction of apoptosis was observed for example in OVCAR-3 human ovarian cancer or HL-60 human leukemia cell lines (Abedi *et al.*, 2014; Kohno *et al.*, 1996). Mechanisms observed in triggering of apoptosis were various. For example, in studies examining effects of CF101 on OVCAR-3 and PC3 human prostate cancer cells, apoptosis was induced by activation of caspase-3 and caspase-9, downregulation of anti-apoptotic Bcl-2 protein expression and also by loss of mitochondrial membrane pressure, which indicated that intrinsic apoptotic signaling pathways was involved (Abedi *et al.*, 2014; Aghaei *et al.*, 2011). Another effect caused by apoptosis was observed on HL-60, where Kohno *et al.* detected DNA fragmentation, which is typical for apoptotic cells (Kohno *et al.*, 1996). In other study conducted on U937 human lymphoma cells apoptosis was induced by inhibition of TNF-α, accompanied by expression of pro-apoptotic protein Bak, which accelerated cell death (Yao *et al.*, 1997). Overall, aimed activation of A_3AR triggered apoptosis mainly by induction of intrinsic apoptotic signaling pathways. In addition, CF101 was also able to enhance effects of chemotherapeutic agent 5-Fluorouracin, used in treatment of colon carcinoma in murine model, by inhibition of NF-κB and the upstream kinase PKB/Akt, which are responsible for chemoresistance when highly expressed (Bar-Yehuda *et al.*, 2005).

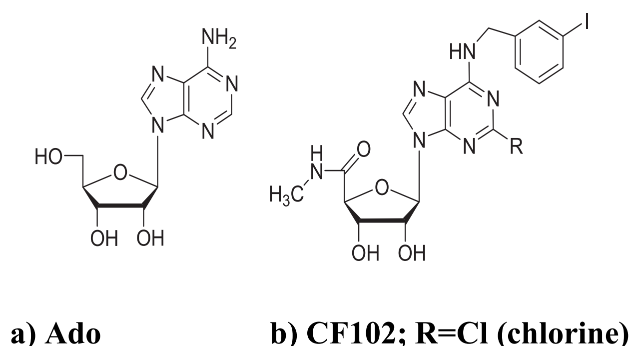
2.3.2.2 Clinical trials using CF101

Several clinical trials were held using CF101. Complete list and results of clinical trials is available at clinicaltrials.gov. Those trials investigated effects of CF101 on inflammatory autoimmune diseases particularly on rheumatoid arthritis, psoriasis and dry eye disease. Overall, in all those inflammatory pathological conditions A_3ARs were found to be upregulated, which can be attributed to the elevated level of Ado due to stress. (Ochaion *et al.*, 2009). Effects of CF101 on A_3AR observed in those clinical trials strongly suggest its therapeutic potential. Since pre-clinical and Phase I/II studies demonstrated that CF 101 is safe and well tolerated in humans it can be potentially utilize as therapeutic treatment (Bar-Yehuda *et al.*, 2007). Moreover, findings suggesting positive therapeutic effect gathered

from clinical trials and the general anti-inflammatory nature of CF101 makes it a prospective candidate for treatment of above stated pathological conditions and also cancer. Unfortunately, there has not yet been held any clinical trial using CF101 as a treatment for cancer.

2.3.3 Agonist CF102 (CL-IB-MECA)

Previously introduced agonist CF 101 was synthesized by double modification of Ado at N^6- and 5'- position. Later on, it was discovered that by introduction of small chemical groups at the C2 - position of CF 101, both selectivity and affinity for A_3AR increases. CF102 (2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide) is a typical example of this modification, where at the C2 - position chlorine is introduced (Kim *et al*., 1994) (**Fig.9**). CF102 is also a small orally bioavailable molecule (Bar-Yehuda *et al*., 2007) and it is a compound with a significant affinity and selectivity for A_3AR, primarily due to the introduction of the chlorine group. Affinity of CF102 is higher than that of CF101. It is estimated to be 157-, 3829- and 7143-fold less selective for A_1AR, A_{2A}AR and A_{2A}AR, respectively, in comparison to A_3AR (**Tab.I**). Unlike CF101, CF102 is in most literature described as full agonist of A_3AR with around 99% intrinsic activity (IA) of that of Ado (Borea *et al*., 2010).



a) Ado **b) CF102; R=Cl (chlorine)**

Figure 9 – Structure of Ado (a) and from it derived agonist CF102 (b). Positions 5'-, N^6- and C2 of Ado were used for CF102 synthesis by introduction of substituents that significantly improves its affinity and selectivity. (Borea *et al*., 2014)

2.3.3.1 *Effects of CF102 on cell proliferation and cell death related to cancer*

Unlike CF 101, CF102 has already been tested in a clinical trial as a potential drug for cancer treatment (Stemmer *et al.*, 2013). In this study, where the safety, tolerability, pharmacokinetics, and pharmacodynamics of CF102 were tested on patients suffering from advanced HCC, positive effects in treating of this condition were reported. Particularly, CF102 treatment inhibited the tumor growth of HCC via deregulation of Wnt and NF-κB signaling pathways (Bar-Yehuda *et al.*, 2008). CF102 has even higher selectivity and affinity for A_3AR than CF101 (**Tab.I**), thus CF102 can potentially be even more efficient in regulation of cell proliferation and level of induced cancer cell death (Fredholm *et. al.*, 2011). Several *in vitro* studies using CF102 as a cancer treatment in various tumor cell lines were successfully carried out.

Reduction of the cell proliferation upon CF102 treatment was reported in several cell lines including A172 human glioblastoma cells and A375 human melanoma cells (Kim *et al.*, 2012; Merighi *et al.*, 2005). The cell proliferation decrease upon A_3AR activation by CF102 is very often due to the cell cycle arrest. In the study examining effects of CF102 on A375 cell line, it was demonstrated that A_3AR stimulation by CF102 resulted in PI3K-dependent phosphorylation of Akt, which lead to reduction of ERK1/2 phosphorylation that in turn caused cell cycle arrest in G_0/G_1 transition responsible for reduction of the cell proliferation (Merighi *et al.*, 2005). In another study examining effects of CF102 on A172 cells, it was demonstrated that CF102 caused significant inhibition of ERKs and Akt, which when upregulated play prominent role in glial survival and tumorogenesis. Therefore, CF102 mediated inhibition of ERKs and Akt induced decrease in tumor cells proliferation (Kim *et al.*, 2005). Inhibitory effects of CF102 on cell proliferation were also observed on CHO cells stably transfected with human A_3AR. The final inhibition of cell proliferation was caused due to impairment of the cell cycle. In particular treatment with CF102 caused inability to neo-synthesize DNA molecules, which resulted in cell cycle arrest in G_2/M (Brambilla *et al.*, 2000). Above stated studies demonstrated that CF102 is able to influence cell cycle at multiple levels.

Induction of the cell death after CF102 treatment was reported in several studies using following cell lines: C32 and A375 human melanoma cell lines and A172 and U 87-MG human glioblastoma cell lines (Kim *et al.*, 2012; Soares *et al.*, 2014). CF102 is able to induce apoptosis using multiple mechanisms. Soares *et al.* demonstrated on human glioblastoma cell

lines that CF102 is able to trigger a caspase-dependent cell death, which is caused by suppression of ERK1/2 and Akt. In another study examining anticancer effects of CF102 in combination with paclitaxel on two human melanoma cell lines a significant cytotoxicity was reported (Soares *et al.*, 2014). Apoptotic cell death was mainly caused by CF102 mediated increase of caspases activity. Generally, the combination of those two agents not only significantly increased cytotoxicity but also contributed to inhibition of metastatic progression by inhibition of migration, invasion, adhesion and angiogenesis, where CF102 was necessary for overcoming paclitaxel chemotherapeutic resistance.

2.3.3.2 Clinical trials using CF102

To this date, two clinical trials using CF102 as potential pharmacological agent were held, where only one focused on CF102 in cancer treatment. Those trials investigated effects of CF102 on chronic hepatitis C and advanced unresectable HCC. Overall, in both pathological conditions A_3ARs were found to be significantly upregulated. Obtained data proved that CF102 is highly effective and its great advantage is its safety profile approved in those clinical trials. (Bar-Yehuda *et al.*, 2008).

Hepatocellular carcinoma

HCC is very common type of liver cancer. It exerts negative effects on life quality and it also significantly decreases live expectancy of patients. There exist various risk factors that can contribute to HCC formation, where the most relevant being viral hepatitis infection (type B or C), metabolic toxins such as alcohol or aflatoxin. (Sun *et* Sarna, 2008).

CF102 was tested in a phase I-II, open-label, dose-escalation study evaluating the safety, tolerability, pharmacokinetics, and pharmacodynamics, where the drug was orally administered in patients with advanced HCC in six 28-day cycles and the doses tested were 1, 5 and 25 mg twice daily (Stemmer *et al.*, 2013) (available at HCC-clinical-trial). In those patients, A_3AR was found to be extremely upregulated in tumor tissue and also in PBMCs, where the increase was by 78 % of A_3AR expression in PBMC compared to healthy subject. Those high expression levels strictly correlate with NF-κB upregulation, which plays an important role in HCC pathogenesis. Overall, this trial revealed good tolerance of the drug at all tested doses with no adverse effects observed and also deceleration of tumor progression. In addition, complete regression of metastatic nodules in one of the patients was reported. In an *in vitro* study it was proven that treatment of rat derived N1S1 HCC cells with CF 102 induced apoptosis via deregulation of the Wnt and NF-κB signaling pathways, which

resulted in an inhibition of tumor growth (Bar-Yehuda *et al.*, 2008). The apoptosis was primarily triggered by an increase in expression levels of pro-apoptotic proteins such as Bad or Bak and also an increase in expression of caspase-3. Overall, this study proved favorable pharmacokinetic characteristic and showed improvement of this pathological condition, which makes it a good candidate for further clinical tests.

In conclusion, data obtained from studies using CF102 as a treatment of pathological conditions, particularly HCC, suggest its prospective therapeutic potential. Great advantage of CF102 is its high selectivity and affinity for A_3AR (**Tab.I**) and also its safety profile and good tolerability. CF102 exerted strong anti-cancer effects; it was able to induce apoptosis of HCC tumor cells preventing further tumor growth and progression (Bar-Yehuda *et al.*, 2008; Cohen *et al.*, 2011; Stemmer *et al.*, 2013).

2.4 Sensitivity and resistance to adenosine receptor A_3 agonist treatment

Although many studies reported promising results of A_3AR agonists in cancer treatment, their effects on cancer are still not well understood, since in some types of cancer it has strong anti-tumor effects, while in others it fuels mechanisms for cancer progression (Borea *et al.*, 2014; Sakowicz-Burkiewicz *et al.,* 2013). Following factors could be an explanation why A_3AR stimulation provides such differential results.

Efficiency of A_3AR treatment is strongly dependent on used agonist dosage. Experiments confirmed its positive effects on suppressing cancer and provoking cell death very often only at micromolar doses (Morello *et al.,* 2008; Kim *et al.*, 2002). Receptor desensitization can be a reason for this striking difference between effects on cancer caused by high (micromolar) or low (nanomolar) doses of agonists. When the receptor is exposed to higher agonist doses after certain time it can lead to its desensitization and then the agonist can act via A_3 receptor-independent pathway (Gessi *et al.*, 2011, Morello *et al.*, 2008). It can either enter the cell via bi-directional transporters or it can follow arrestin-dependent signaling pathway as in the case of CL-IB-MECA tested on human astrocytoma cells. (Klaasse *et al.*, 2007; Trincavelli *et al.*, 2002). This suggests that high levels of A_3AR agonist can pose anti-cancer effects independently of A_3AR indicating that those effects are highly unspecific and may also adversely affect non-tumor cells. Given the fact that A_3AR are present in nearly all cells sufficient amount of agonist would active them in most tissues leading to nonspecific diverse response (Chen *et al.*, 2013). Therefore, it is necessary to use nanomolar concentrations of highly selective and potent agonists to be able to only activate A_3AR

in cells that have high overexpression of this receptor. This approach would lead to specific targeting of only cancer cells, since cancer cell lines appear to overexpress this particular subtype (Gessi *et al*., 2004).

Another confounding factor bound to agonist dosage is general potency of AR agonist, which is substantially influenced by the number of receptors. They exhibit behavior described as “spare receptors” since the change in their overall number is manifested as shifts in the dose-response curve, not in the maximal response curve (Fredholm, 2010). Meaning that when the number of receptors available for agonist activation deceases, then it is still possible to achieve maximal receptor response, just this time with higher dose of agonist.

Furthermore, the time period of exposure to agonists is another important factor. Since A_3ARs are subjected to downregulation upon agonist exposure, their number is strictly regulated. This regulation is manifested by desensitization often followed by internalization, which after longer period of exposure can possibly result in the degradation of A_3AR (Klaasse *et al*., 2007; Trincavelli *et al*., 2002).

Factor also influencing efficiency of agonists, which is important to be taken into account, is tumor environment, where agonists exert less persuasive anti-tumor effects, when used in hypoxic tissues (Trincavelli *et al*., 2010).

Several studies also revealed that mutations present at specific amino-acid residues at transmembrane-spanning domain play a significant role, affecting not only agonist binding but also signal transduction, thus potentially leading to resistance to A_3AR treatment. This was clearly demonstrated by a study, which discovered that mutation in Asp250 significantly decreased binding affinity and functional potency, which have been caused by changes in receptor-ligand recognition or receptor structure. On the other hand, mutation in Trp243 caused A_3AR functional inactivation, which indicated that this amino-acid residue plays critical role in receptor signal transduction (Gao *et al*., 2002).

Literature concerning A_3AR treatment is in many cases fragmented. Therefore, current contradictory findings raise the need to find under what circumstances agonist activation of A_3AR contributes to fight cancer.

3 AIMS OF THE THESIS

Theoretical part shall provide comprehensive review on the current knowledge of adenosine receptors with focus on the role of A_3 adenosine receptor in cancer and the anticancer activity of new clinically relevant A_3 agonists.

Experimental part shall provide assessment of RNA and protein expression level of A_3 adenosine receptor in selected cell lines and pre-selection of candidates for *in vitro* model of A_3 mediated anticancer effects based on the A_3 expression level.

4 MATERIAL AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

- Acetone 100% (20001-ATO, LECH-NER)
- Acrylamide/Bis Solution (30%) 29:1 (161-0156, BIO-RAD)
- Amonium persulphate (APS) (A3678, SIGMA ALDRICH)
- Benzamidine (12072, SIGMA ALDRICH)
- β-mercaptoethanol (M3148, SIGMA ALDRICH)
- Bovine serum albumin (BSA) (A 7906, SIGMA ALDRICH)
- CHAPS (226947, SIGMA ALDRICH)
- Chloroform (39553, SERVA)
- Deionized water (dH_2O)
- Diethyl pyrocarbonate treated water (DEPC-H_2O) (AM9922, AMBION)
- Dimethyl sulfoxide (DMSO) (D8418, SIGMA ALDRICH)
- Disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$) (30388APO, LECH-NER)
- Dithiotreitol (DTT) (DTT-ROCHE, SIGMA ALDRICH)
- Sodium pyrophosphate tetrabasic decahydrate ($Na_4P_2O_7 \cdot 10H_2O$) (S6422, SIGMA ALDRICH)
- Dulbecco's Modified Eagle's Medium (DMEM) (D5796, SIGMA ALDRICH)
- Eagle's Minimum Essential Medium (EMEM) (M4655, SIGMA ALDRICH)
- Ethanol (437433T, VWR)
- Ethylenediamine tetraacetic acid (EDTA) (11280.02, SERVA)
- Eva Green Dye 20x in water (31000, BIOTIUM)
- F12 Ham’s (SH30026.02, HYCLONE)
- FCSi (10270, GIBCO)
- Glycerol (G5516, SIGMA ALDRICH)
- Glycine (23390.03, SERVA)
- HEPES (H4034, SIGMA ALDRICH)
- Horse serum (P30-0702, PAN BIOTECH)
- Iscove’s (BE12-722F, LONZA)

- Inzulin Humulin (968SPC400, ELI LILLY)
- Isopropanol (I9516, SIGMA ALDRICH)
- Keratinocyte serum free medium (SFM) (10724-011, GIBCO)
- L – glutamine (G6392, SIGMA ALDRICH)
- Loading dye 6x (bromphenol blue) (R0611, THERMO FISHER SCIENTIFIC)
- Luminata Forte Western HRP Substrate (WBLUF0100, SIGMA ALDRICH)
- Magnesium chloride ($MgCl_2$) (25 mM) (N/A, THERMO FISHER SCIENTIFIC)
- McCOY (M8403, SIGMA ALDRICH)
- Methanol (10202LP20-10000, MIKROCHEM)
- Mili-Q water (mili-Q H_2O)
- Monopotassium phosphate (KH_2PO_4) (APO-30016, LECH-NER)
- Nonyl phenoxypolyethoxylethanol (NP-40) (74385, SIGMA ALDRICH)
- Nucleosides triphosphates (dNTPs) (U1330, PROMEGA) – diluted in DEPC water to concentration of 10mM
- Pharmalytes (pH range 8 – 10) (P2147, SIGMA ALDRICH)
- Phenylmethylsulfonyl fluoride (PMSF) (20203, USB)
- Phosphatase Inhibitor Mix II (PHIM) (100x) (39055.01, SERVA)
- Pierce Bovine Gamma Globulin Standard Pre-Diluted Set (concentrations of 125, 250, 500, 750, 1000, 1500, 2000 μg/ml) (23213, THERMO FISCHER SCIENTIFIC)
- Potassium chloride (KCl) (APO-30076, SIGMA ALDRICH)
- Primary antibody Anti-A_3AR (polyclonal – rabbit) (52 kDa) (ab203298, ABCAM)
- Primary antibody Anti-β-actin (monoclonal – mouse) (42 kDa) (sc47778, SANTA CRUZ)
- Primers **(Table VI.)** (GENERIBIOTECH)
- Protease inhibitor mix (PIM) (1 tablet dissolved in 200 ml dH_2O) (05892970001, ROCHE)
- Random Primers 20 μg (C118A, PROMEGA)
- Reverse Transcriptase RevertAid H Minus 200 U/μl (EP0451, THERMO FISCHER SCIENTIFIC)
- Ribonuclease inhibitor (RNAsin) 20-40 U/μl (N2518, PROMEGA)
- RPMI (R8758, SIGMA ALDRICH)
- Secondary antibody Anti-Mouse IgG (A9917, SIGMA ALDRICH)

- Secondary antibody Anti-Rabbit IgG (A0545, SIGMA ALDRICH)
- Skim milk (LAKTINO)
- Sodium chloride (NaCl) (A131428, MIKROCHEM)
- Sodium dodecyl sulfate (SDS) (71729, SIGMA ALDRICH)
- Sodium fluoride (NaF) (1504, SIGMA ALDRICH)
- Sodium orthovanadate (Na_3VO_4) (S6508, SIGMA ALDRICH)
- Sodium pyrophosphate tetrabasic decahydrate (S6422, SIGMA ALDRICH)
- Sodium pyruvate (PYNA) (8636, SIGMA ALDRICH)
- Spectra Multicolor Broad Range Protein Ladder (26634, THERMO FISCHER SCIENTIFIC)
- Tetramethylethylendiamine (TEMED) (15524-010, INVITROGEN)
- Thermo Start Taq DNA Polymerase 5 U/µl (AB-0908, THERMO FISCHER SCIENTIFIC)
- Trichloracetic acid (TCA) (BP80, CHEMAPOL)
- TRI Reagent (TR118200, GENBIOTECH)
- Tris (37180.04, SERVA)
- Tris-hydrochloride (Tris-HCl) (T3253, SIGMA ALDRICH)
- TRYPLE Tryple express (GIBCO, 12604)
- Tween – 20 (P2287, SIGMA ALDRICH)
- Urea (208884, SIGMA ALDRICH)

4.1.2 Buffers

- 1.5M Tris-HCl separating gel buffer, pH=8.8 (161-0798, BIORAD)
- 0.5M Tris-HCl stacking gel buffer, pH=6.8 (161-0799, BIORAD)
- 10x Tris-glycine-SDS (10x TGS) electrode buffer (1610772, BIO-RAD)
- 3x Laemmli buffer (4.8 ml Tris-HCl 0.5 M, 0.3 g SDS, 3 ml glycerol, 1.6 ml β-mercaptoethanol, 600 ml bromphenol blue, 10 ml dH_2O, pH = 6.8)
- Anode buffer I (18.15 g Tris, 50 ml methanol, 450 ml dH_2O, pH = 10.4)
- Anode buffer II (1.5 g Tris, 50 ml methanol, 450 ml dH_2O, pH = 9.4)
- Blocking buffer (5% skim milk in TBS-T buffer, pH = 7.4)
- Cathode buffer (1,5 g Tris base, 7.1 g glycine, 50 ml methanol, 450 ml dH_2O, pH = 9.4)

- PBS with inhibitors (2.23 g $Na_4P_2O_7 \cdot 10H_2O$, 183.9 mg Na_3VO_4, 210 mg NaF, (174.19 mg PMSF dissolved in 1 ml 100% methanol) and 1000 ml 1xPBS, pH = 7.4)
- PBS/EDTA (166.5 mg EDTA, 1 l 1x PBS, pH = 7.4)
- Phosphatase buffed saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO_4, 1000 ml dH_20, pH = 7.4)
- RT buffer 5x (EP0451, THERMO FISCHER SCIENTIFIC)
- TGS (100 ml 10x TGS, 900 ml dH_2O, pH = 8.3)
- Thermo-Start PCR Buffer (concentration 10x) (AB-1270, THERMO FISHER SCIENTIFIC)
- Tris buffered saline – Tween (TBS-T) (2.42 g Tris base, 8 g NaCl, 1 ml Tween-20, 1000 ml dH_2O, pH = 7.4)
- Urea lysis buffer (13.51 g urea, 0.75 g CHAPS, 500 μl NP-40, 0,27 g DTT, 500 μl pharmalytes, 3.22 g benzamidine, 27.7 g $Na_4P_2O_7 \cdot 10H_2O$, 5.249 g NaF, PIM to buffer 1:50, PHIM to buffer 1:100, + add dH_20 to final volume 25 ml)

4.1.3 Instrumental equipment

- Biological safety cabinet MSC Advantage (THERMO FISHER SCIENTIFIC)
- Biological safety cabinet Mars Safety Class 2 SCANLAF (LABOGENE)
- Biological safety cabinet Aura 2000 M.A.C (BIOAIR)
- Cell counter and viability analyzer Vi-CELL XR (BECKMAN COULTER)
- Cell culture CO_2 incubator Heracell 150i (THERMO FISCHER SCIENTIFIC)
- Centrifuge 5810 R (EPPENDORF)
- Centrifuge Mini Spin (EPPENDORF)
- Centrifuge 5430 (EPPENDORF)
- Centrifuge Micro Smart 15 (HANIL)
- Centrifuge Hermle Z323 K (DJB LABCARE)
- Dry block thermostat Bio TDB-100 (BIOSAN)
- Fume closet (MERCI)
- Imaging system Odyssey FC (LI-COR)
- Inverted Microscope 1x51 (OLYMPUS)
- Light-cycler 480 Instrument II (ROCHE)
- Spectrophotometer ND 1000 (NANODROP)

- Thermo-cycler PTC 100 (MJ RESEARCH)
- Vortex mixer (LABNET)
- Western blotting transfer system TransBlot Turbo (BIO-RAD)

4.1.4 Other equipment

- Polyvinylidene difluoride membrane Hybond P 0.45μm (PVDF) (10600023, AMERSHAM)
- Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, 4-gel, for 1.5 mm thick handcast gels (buffer tank, lid power cables, electrode assembly, casting frames, casting stand, cell buffer dam, 1.5 mm gel casting module, comb, gel releaser + basic power supply PowerPac Hc) (1658006FC, BIO-RAD)
- TPP tissue culture flasks (TPP TRASADIGEN)

4.1.5 Software

- Microsoft Excel 2010
- Microsoft Word 2010
- GrafPad Prism 7
- Li-COR Softtware
- LightCycler® 480 Software

4.1.6 Biological material

Cell lines were cultured according to specific datasheets provided by manufacturer on TPP tissue culture flasks in incubator at 37 °C in 5% CO_2 and 95% air (**Tab.II**). Cells were passaged 2 – 3 times a week. Detachment of cells during subculturing was achieved using tryple. All cell lines were maintained in antibiotics free media.

Table II – Characteristics and basic information about cell lines used in the experimental part.

Cell line	Origin	Cancer type	Cultivation medium
Hematopoietic cancer			
CCRF-CEM	ATCC 2015	human, acute lymphoblastic leukemia	RPMI + 10% FCSI
CEM-DNR	LEM	human, acute lymphoblastic leukemia	RPMI + 10% FCSI
HL-60	ATCC 2014	human, promyeloblast, peripheral blood, acute promyelocytic leukemia	RPMI + 10% FCSI

Cell line	Origin	Cancer type	Cultivation medium
JURKAT	DSMZ 2014	human, acute T cell leukemia	RPMI + 10% FCSI
JURKAT NSS09	ATCC 2015	human, acute T cell leukemia	RPMI + 10% FCSI
JURKAT F9 NSS09	ATCC 2015	human, acute T cell leukemia	RPMI + 10% FCSI
K562	ATCC 2015	human, Bone Marrow, chronic myelogenous meukemia (CML)	Iscove´s + 10% FCSI
K562-TAXOL bulk	LEM	human, Bone Marrow, chronic myelogenous leukemia (CML)	RPMI + 10% FCSI
MOLT-4	NCI 2009	human, peripheral blood, acute lymphoblastic leukemia	RPMI + 10% FCSI
RAMOS	ATCC 2014	human, B Lymphocyte, Burkitt's lymphoma (American)	RPMI + 10% FCSI
RPMI 8226	ATCC 2013	human, B lymphocyte, peripheral blood, plasmacytoma; myeloma	RPMI + 10% FCSI
SR	ATCC 2014	human, lymphoblast, pleural effusion, large cell immunoblastic lymphoma	RPMI + 10% FCSI
U-937	DSMZ 2014	human, histiotic lymphoma	RPMI + 10% FCSI
Mammary gland cancer			
BT-549	NCI 2010	human, mammary gland, infil. ductal carcinoma	RPMI + 10% FCSI + 2mM l-glutamine
MDA-MB-231	ATCC 2013	human, mammary gland/breast; derived From metastatic site: pleural effusion, adenocarcinoma	RPMI + 10% FCSI
MCF-7	NCI 2010	human, pleural effusion adenocarcinoma	RPMI + 10% FCSI + 2mM l-glutamine
T-47D	ATCC 2015	human, mammary gland, derived from metastatic side, pleural effusion	RPMI+10% FCSI+2U/ml insulin
Pancreatic cancer			
AsPC-1	ATCC 2003	human, pancreas; derived from metastatic site, ascites, adenocarcinoma	RPMI + 10mM HEPES + 1mM PYNA + 10% FCSI
BxPC-3	ATCC 2007	human, pancreas , adenocarcinoma	RPMI + 10mM HEPES + 1mM PYNA+ 10% FCSI
CAPAN-2	DSMZ 2015	human pancreas adenocarcinoma	85% RPMI 1640 + 15% FCSI
JOPACA-1	DSMZ 2015	human, pancreas carcinoma	80% Iscove's MDM + 20% FCSI
YAPC	DSMZ 2015	human, pancreas carcinoma	90% RPMI 1640 + 10% FCSI
PACADD-119	DSMZ 2015	human, pancreas ductal adenocarcinoma	80% mixture of DMEM and Keratinocyte SFM (at 1:1)
PACADD-135	DSMZ 2015	human, pancreas ductal mucinous adenocarcinoma	90% DMEM + 10% FCSI

Cell line	Origin	Cancer type	Cultivation medium
PA-TU-8902	DSMZ 2015	human, pancreas adenocarcinoma	90% DMEM (4.5 g/l glucose) + 10% FCSI + 2 mM l-glutamine
MIA-PACA-2	DSMZ 2015	human, pancreas carcinoma	77.5% DMEM + 20% FCSI + 2.5% horse serum
Liver cancer			
HEP G2	ATCC 2013	human, hepatocellular carcinoma	EMEM + 10% FCSI
HEP-3B	DSMZ 2015	human hepatocellular carcinoma	90% EMEM + 10% FCSI + 2 mM l-glutamine
NCI-N87	ATCC 2013	human, stomach; derived from metastatic site, liver, gastric carcinoma	RPMI + 10% FCSI
Other types of cancer + non-tumor cell lines			
BJ	ATCC 2014	human, fibroblast , skin; foreskin , **non-tumor**	EMEM + 10% FCSI
MRC-5	ATCC 2014	human, fetal, lung, **non-tumor**	EMEM + 10% FCSI
A549	ATCC 2013	human, epithelial cell, lung, carcinoma	F-12 Ham's + 10% FCSI
HCT 116 parental	Horizon 2011	human, colon carcinoma	McCOY + 1,5mM l-glutamine + 10% FCSI
HCT 116p53(-/-)	Horizon 2011	human, colon carcinoma	McCOY + 1,5mM l-glutamine + 10% FCSI
U2OS	ATTC 2014	human, osteosarcoma	McCOY + 10% FCSI
HCC-78	DSMZ 2013	human, non-small cell lung carcinoma	RPMI + 10% FCSI

***Note:** LEM cell lines were prepared in the Laboratory of Experimental medicine by Children's Clinic of Faculty of Medicine and dentistry, Palacký University Olomouc*

4.2 Methods

4.2.1 Gene expression analysis

RNA lysates:

After the medium removal cells were washed 2x in the PBS and then incubated with PBS/EDTA at 37 °C for 10 min. Detached cells in the amount of $8x10^6$ were harvested and resuspended in the PBS and centrifuged at 180 g for 5 min. Supernatant was removed and the pellet was resuspended in the ice cold PBS. Another centrifugation, this time at 12 000 g for 5 min at 4 °C, was performed with the cell suspension in order to acquire dry cell pellet by subsequent supernatant removal. Dry cell pellet was thoroughly resuspended in 1 ml of TRI Reagent. Samples were left to stand 5 min at room temperature to ensure complete dissociation of nucleoproteins. All samples were stored in -20 °C freezer.

Isolation of the total RNA:

Samples were resuspended in 200 μl of chloroform and properly vortexed approximately for 30 s and after 10 min of standing at room temperature centrifuged at 12 000 g for 15 min at 4 °C. Upper layer of supernatant, which contained RNA, was transferred to new 1.5 ml micro test tube. Afterwards isopropanol was added to the sample in approximately same volume as the sample itself (400 – 500 μl). After 5 min of incubation at room temperature samples were centrifuged at 12 000 g for 10 min at 4 °C, which separated proteins in the form of a pellet at the bottom. Supernatant was removed and remaining pellet was washed in 75% ethanol and another centrifugation was performed at 12 000 g for 5 min at 4 °C. After ethanol removal, pellet was briefly air-dried until it became almost transparent and an appropriate volume (40 – 80 μl according to the pellet size) of DEPC-treated water was added. Dissolution was facilitated by repeated pipetting and then by using thermo-block heater set at 60 °C. RNA concentration and purity were measured using the spectrophotometer Nanodrop ND 1000.

RT (cDNA):

According to the RNA concentration appropriate volume of the total RNA sample and DEPC-treated water were mixed. Final concentration of RNA samples of 1 μg/μl per sample was incubated in thermo-cycler at 70 °C for 5 min with random primers. Properly mixed samples were incubated in thermo-cycler with heated lid at 70 °C for 5 min and immediately afterwards cooled down for 1 min in a cooler. Subsequently, 9.75 μl of the MM_{RT} (**Tab.III**) and 0.75 μl of reverse transcriptase were added, respectively, having 5 min of incubation in between. Sample was gently mixed and incubated 10 min at room temperature. RT was performed using thermo-cycler with heated lid at 42 °C for 60 min and 70 °C for 10 min followed by cooling to 4 °C. Samples were stored in -20 °C freezer for further analysis.

Table III – Chemicals used for the preparation of the master mix for reverse transcription (MM_{RT}).

MM_{RT}	
	Amount of reagents for 1 reaction [μl]
RT buffer	6
dNTPs	3
RNAsin	0,75

RT-PCR:

RT-PCR of A_3AR gene (**Tab.VI**) was performed according to conditions (**Tab.V**) with $MM_{RT\text{-}PCR}$ (**Tab.IV**) using light-cycler. As reference genes for relative quantification were used ATBC and POL2A (**Tab.VI**). Fluorescent signals were measured at set channel 465 – 510 nm and Ct values were automatically calculated by LightCycler® 480 Software. For statistical analysis of RT-PCR results, basic relative quantification was performed using $2^{-\Delta\Delta Ct}$ method (Livak *et* Schmittgen, 2001), assuming amplification efficiency E=2. The calibrator was derived from the expression (Ct values) of two non-tumor cell lines (BJ, MRC-5) by averaging.

Table IV – Chemicals used for preparation of the master mix for the real-time polymerase chain reaction ($MM_{RT\text{-}PCR}$).

$MM_{RT\text{-}PCR}$	
	Amount of reagents for 1 reaction [µl]
PCR buffer	2
$MgCl_2$	1.6
dNTPs	0.4
Taq polymerase	0.4
DMSO	1
DEPC-H_2O	10.6
Primer pair	2
dye Eva Green	1
cDNA	1

Table V – Conditions used for RT-PCR.

Program	Number of cycles	Temperature [°C]	Time [hh:mm:ss]
Denaturation	1	95	00:05:00
Cycling:	35	-	
• ***denaturation***		95	00:00:30
• ***annealing***		60	00:00:30
• ***extension***		72	00:01:00
Melting	1	60/95	00:01:00/continuous
Cooling	1	37	00:00:01

Table VI – Primers used for RT-PCR.

Gene	Sequence	Product length
A_3AR_h	**FP** 5'-TGTTTGGCTGGAACATGAAA-3'	155
	RP 5'-ATAGATGGCGCACATGACAA-3'	
$POLR2A_h$	**FP** 5'-GCACCACGTCCAATGACAT-3'	267
	RP 5'-GTGCGGCTGCTTCCATAA-3'	
$ACTB_h$	**FP** 5'-GGACTTCGAGCAAGAGATGG-3'	234
	RP 5'-AGCACTGTGTTGGCGTACAG-3'	

Note: *POL2A = gene for RNA polymerase II subunit A***,** *ACTB = gene beta actin, h = human, FR = forward primer, RP = reverse primer*

4.2.2 Protein analysis

Cell pellets:

After the medium removal cells were washed 2x in the PBS and then detached using the scraper. Detached cells in the amount of $2x10^6$ were harvested and resuspended in the PBS and centrifuged at 180 g for 5 min. The supernatant was removed and the cell pellet was resuspended in the ice cold PBS. Another centrifugation, at 12 000 g for 5 min at 4 °C, was performed with the cell suspension. After the supernatant was removed, 1 ml of the PBS containing inhibitors was added and the last round of the centrifugation was performed in the same manner as the previous one in order to acquire dry cell pellet by the subsequent supernatant removal. Dry cell pellets of all samples were stored in a -80 °C freezer for further utilization.

Protein isolation:

To cell lysates, in 100 µl of PBS with inhibitors, was added 800 µl of ice-cold acetone and 100 µl TCA. After 1 hour of precipitation at -20 °C samples were centrifuged at 18 000 g for 15 min at 4 °C. Supernatant was removed and samples were completely resuspended in ice-cold acetone and centrifuged again at 18 000 g for 15 min at 4 °C, this was repeated twice. After supernatant removal cell pellets were left to dry at room temperature. Dry cell pellets were thoroughly resuspended in urea lysis buffer and incubated at room temperature. After 30 min incubation samples were passed through 21-gauge needle to shear the DNA. Samples were incubated for 30 min at 37 °C with Laemmli buffer and stored at -80 °C

for further utilization. Protein concentration was not quantified, since urea lysis buffer interfered with all protein assay kits used in our laboratory.

SDS-PAGE electrophoresis:

Samples containing approximate protein concentration (20 μg) together with 4 μl of Spectra Multicolor Broad Range Protein Ladder were loaded on 10% SDS-polyacrylamide gel (**Tab.VII**). Protein samples were then separated at 80 V until they settled down in wells, at 100 V until they passed through the stacking gel and at 120 V until they reached the bottom of the separating gel.

Table VII – Chemicals used for the preparation of the separating and the stacking gel for the SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

	Separating gel (10%) [μl]	Stacking gel (4%) [μl]
30% acrylamide	2490	510
Mili-Q H_2O	2970	2040
1.5 M Tris-HCl (pH = 8.8)	1875	-
1 M Tris-HCl (pH = 6.8)	-	375
10% SDS	75	30
10% APS	75	30
TEMED	7,5	3

Note*: amount used for one 1.5mm gel*

Semi-dry western blotting (WB):

Resolved proteins were electro-blotted onto the PVDF membrane using the semi-dry western-blot transfer system with the set program Mix MW Turbo (7 min, up to 1.3 A; 25 V). The electro-blotting typical "sandwich" composition was assembled in the following order - filter papers, the PVDF membrane, the polyacrylamide gel and filter papers. All parts were beforehand immersed in special electrode buffers (anode buffer I, anode buffer II and cathode buffer). Moreover, the PVDF membrane was firstly wetted for 15 s in methanol and subsequently rinsed in dH_2O for 2 min. The first 3 filter papers were immersed in the anode buffer I, next 2 filter papers and the membrane in the anode buffer II and lastly the gel and 3 filter papers in the cathode buffer.

Immunodetection:

After the electro-blotting, the PVDF membrane was incubated with the blocking buffer. After one-hour incubation the membrane was probed with the anti-A_3AR primary antibody in 5% BSA in the TBS-T buffer (dilution 1:400) overnight at 4 °C. After the incubation, the membrane was washed 3x for 10 min in the TBS-T buffer and probed with the secondary antibody goat anti-rabbit IgG conjugated with horseradish peroxidase in 5% skim milk powder in the TBS-T buffer (dilution 1:5000) for 1 hour at room temperature. The membrane was then washed again in the TBS-T buffer 4x for 10 min.

Visualization:

The membrane was incubated with Luminata Forte Western HRP substrate for 5 min in the dark. Chemiluminescent signals of proteins were visualized using the imaging system Li-COR Oddysey Fc (program: chemi/600/700/ - 2/2/2 min). Molecular size of proteins was determined using the molecular-weight size marker Spectra Multicolor Broad Range Protein Ladder.

Membrane reprobing:

After the visualization, the membrane was thoroughly washed in the TBS-T buffer 2x for 10 min and then incubated with anti-β-actin primary antibody in 5% BSA in the TBS-T buffer (dilution 1:3000) overnight at 4 °C. Process of incubation, this time with the secondary antibody goat anti-mouse IgG conjugated with horseradish peroxidase (dilution 1:5000), and visualization were the same as with the detection of A_3AR protein.

Protein expression quantification:

Densities of individual bands were measured using Li-COR software. Densitometry was used to relatively quantify protein expression of individual cancer cell lines. The intensity of each A_3AR band was normalized to the intensity of corresponding β-actin band by calculating the density ratio.

DENSITY RATIO = *density A_3AR / density β-actin*

5 RESULTS

5.1 RT-PCR

In order to determine level of A_3AR gene expression, RT-PCR (**see chapter 4.2.1 Gene expression analysis)** was performed and two different reference genes ATBC and POL2A were used for quantification (**Tab.VI**). All reactions were conducted in doublets and for further calculations average values were used. Data are presented as the fold change in A_3AR gene expression of sample cell lines normalized to two endogenous reference genes ATBC and POL2A and relative to the calibrator (non-tumor) and has the fold change value 1 (**Tab.IX**). A total of 35 PCR cycles were carried out and therefore cell lines with Ct > 35 for A_3AR gene (*marked as "-"* **in Tab.IX**) were automatically classified as non-expressing, since due to their low expression it is impossible to calculate their relative expression and therefore they were excluded from further calculations.

Majority of cancer cell lines showed significant A_3AR gene overexpression. However, some cancer cell lines exerted only slightly higher expression in comparison to the calibrator. Furthermore, several cell lines had even lower expression compared to the calibrator. Gene expression was classified according to the **Tab.VIII.**

Table VIII – Criteria for classification of A_3AR gene expression of cancer cell lines.

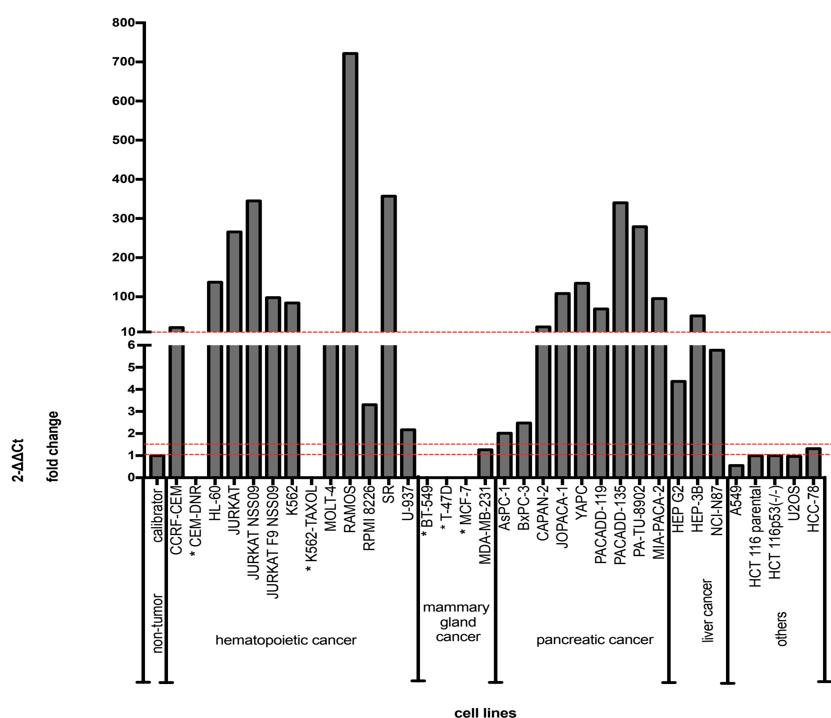
Classification	Fold change
Low expression	< 1
Normal expression	> 1; < 1,5
Overexpression	> 1,5; < 10
Significant overexpression	< 10

Table IX – Fold change of A_3AR gene expression normalized to ATBC and POL2A and relative to the calibrator.

Cell line	Fold change (ATBC)	Fold change (POL2A)
Calibrator	**1.00**	**1.00**
A549	0.55	0.26
AsPC-1	2.01	1.91
BT-549	0.89	0.20
BxPC-3	-	-
CAPAN-2	22.94	13.78
CCRF-CEM	21.41	14.32
CEM-DNR	-	-
HCC-78	1.31	3.58
HCT 116 parental	1.00	0.10
HCT 116p53(-/-)	1.00	0.13
HEP G2	4.36	1.00
HEP-3B	51.27	24.00
HL-60	**137.19**	**346.09**
JOPACA-1	**108.38**	**81.01**
JURKAT	**265.95**	**177.91**
JURKAT F9 NSS09	98.02	30.38
JURKAT NSS09	**344.89**	**61.61**
K562	84.45	31.02
K562-TAXOL	-	-
MCF-7	-	-
MDA-MB-231	1.27	0.71
MIA-PACA-2	**95.34**	**158.68**
MOLT-4	6.23	5.50
NCI-N87	5.78	1.19
PA-TU-8902	**279.17**	**202.95**
PACADD-119	69.07	54.76
PACADD-135	**340.14**	**198.78**
RAMOS	**721.57**	**436.55**
RPMI 8226	3.31	1.71
SR	**357.05**	**170.07**
T-47D	-	-
U-937	2.17	0.84
U2OS	0.97	0.60
YAPC	**134.36**	**88.34**

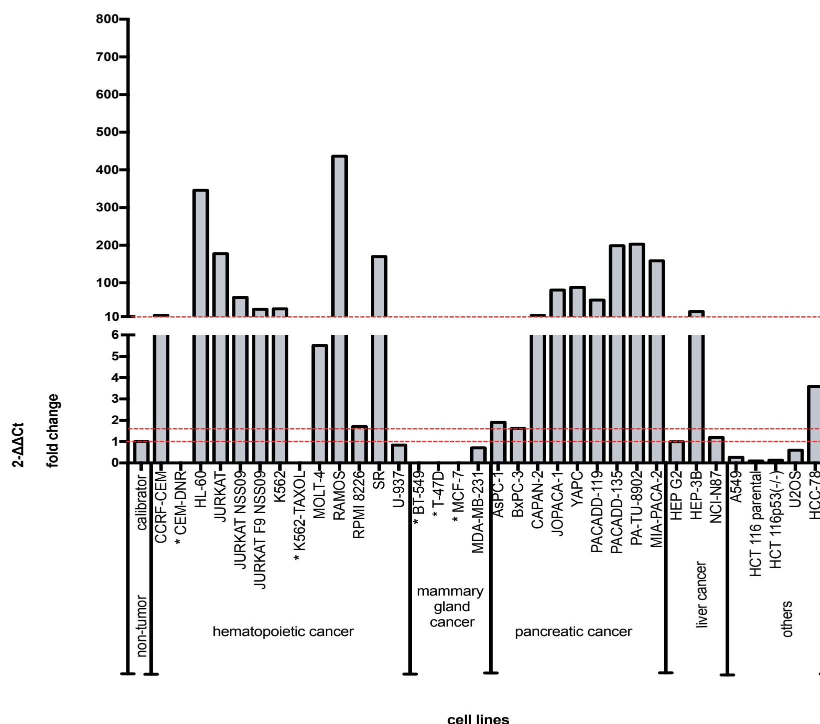
Notes: *all values are rounded up to two decimal digits, cancer cell lines with fold change values higher than 100 for one of the normalization genes are written in* ***bold,*** *- = cell lines with Ct > 35 for A_3AR gene (classified as non-expressing)*

Relatively consistent A_3AR gene expression was observed in the pancreatic cancer group of cell lines, where all cell lines except AsPC1 and BxPC-3 displayed significant overexpression of this receptor. On the other hand, mammary gland cancer cell lines, except MDA-MB-231, had Ct values for A_3AR gene > 35, which suggests low expression. In addition, expression levels of hematopoietic cancer cell lines fluctuate between extreme overexpression (JURKAT, JURKAT NSS09, JURKAR NSS09 F9, SR and especially RAMOS) and practically no expression (CEM-DNR, K562 TAXOL). Furthermore, for all liver cancer cell lines significant overexpression was detected. Last group marked as "others" had A_3AR gene expression comparable to the calibrator and therefore similar to the normal gene expression (expression in non-tumor cells). – (**Gra.1**)



Graph 1 – Graphical display of the fold change of A_3AR gene expression normalized to the reference gene ATBC. The graph illustrates results of relative quantification of A_3AR cDNA reversely transcribed from RNA of each cell line. Fold change values were calculated using $2^{\wedge-\Delta\Delta Ct}$ method (exact figures in **Tab.IX**). Red dashed lines separate columns according to gene expression classification criteria (**Tab.VIII**). Cell lines marked with "*" have Ct values > 35 and were omitted from any calculations.

Overall, the A_3AR gene expression of individual cancer cell lines normalized to POL2A was in a very similar mutual ratio to those normalized to ATBC, only numerical values were lower. Similarly to ATBC, consistently high A_3AR gene expression was detected in the pancreatic cancer group of cell lines again with two exceptions (AsPC1 and BxPC-3). In the mammary gland cancer group, only cell line MDA-MB-231 displayed some A_3AR gene expression, although lower than that of calibrator, other cell lines in this group were marked as non-expressing, since their previously measured A_3AR Ct values were > 35. The most significant fluctuations occurred in the hematopoietic cancer group, where JURKAT, JURKATNSS09, JURKATNSS09, SR and especially RAMOS showed extreme A_3AR gene overexpression compared to virtually no expression of CEM-DNR and K562 TAXOL cancer cell lines. Liver cancer group is the only group that differs slightly more from ATBC normalization then other, where only cell line HEP-3B overexpressed A_3AR gene. Last group marked as "others" had small but detectable gene expression, which was lower than the expression in non-tumor cell lines. One exception in this group was cell line HCC-78, which exerted moderate overexpression of A_3AR gene. – (**Gra.2**)



Graph 2 – Graphical display of fold change of A_3AR gene expression normalized to the reference gene POL2A. The graph illustrates results of relative quantification of A_3AR cDNA reversely transcribed from RNA of each cell line. Fold change values were calculated using $2^{\wedge-\Delta\Delta Ct}$ method (exact figures shown in **Tab.IX**). Red dashed lines separate columns according to gene expression classification criteria (**Tab.VIII**). Cell lines marked with "*" have Ct values > 35 and were omitted from any calculations.

5.2 WB

In order to detect the level of protein expression WB analysis was performed (**see chapter 4.2.2 Protein analysis**). Results are displayed in **Figure 10**. Detection of protein β-actin served as a loading control. Quantitative densitometry was used to compare the level of protein expression of individual cancer cell lines. Density of each band was measured and the relative protein quantification was calculated according to the formula in the chapter **4.2.2 Protein analysis.**

All tested cancer cell lines expressed endogenous reference protein β-actin, for which corresponding band was detected at 42 kDa for each cell line. In the group of hematopoietic cancer cell lines (CCFR CEM, CEM DNR, HL-60, JURKAT, JURKAT NSS09, JURKAT NSS09 F9, K562, K562 TAX, MOLT-4, RAMOS, RPMI8226, SR, U-937) no expression of A_3AR protein was detected. Similarly, no expression of A_3AR protein was detected in the mammary gland cancer group (BT549, T47D, MCF-7), with only one exception, cell line MDA-MB-231 expressed A_3AR. For all pancreatic (AsPC1, BXPC3, CAPAN-2, JOPACA-1, YAPC, PACADD119, PACADD135, PATU8902, MIA-PACA-2), liver (HEPG2, HEP3B, NCI-NC87), colon (HCT116 PARENTAL, HCT116 p53 -/-), lung (A549, HCC78) and bone (U2OS) cancer cell lines A_3AR protein expression was detected, although for the cell line U2OS only weak band was observed. Non-tumor cell lines had no A_3AR protein expression.

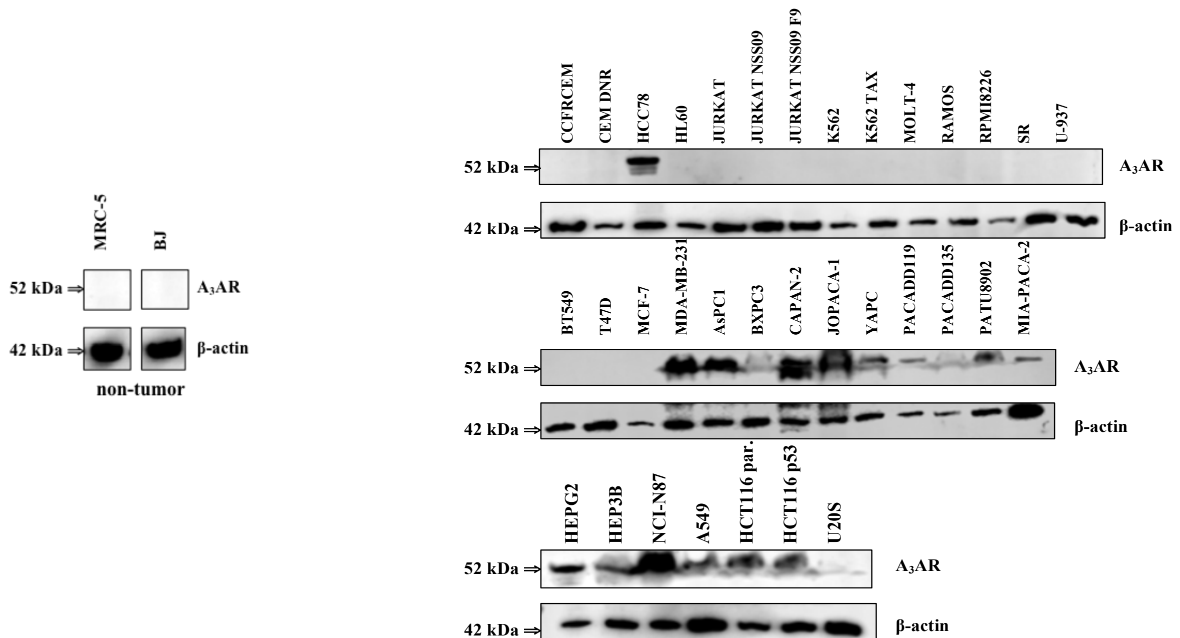
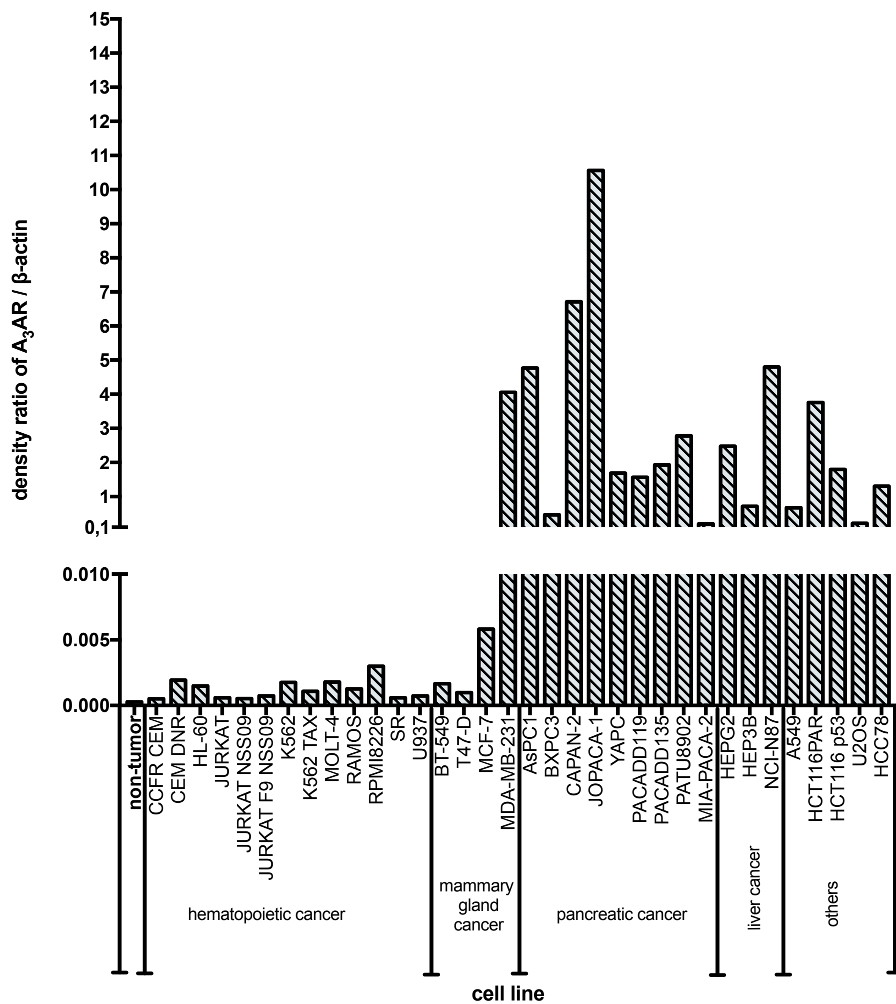


Figure 10 – Comparison of A_3AR protein expression in individual cell lines using WB analysis. A comparison with β-actin expression was used to quantitate the amount of A3 protein. WB analysis was done in a single repeat.

Density ratio values correspond to the intensity of bands displayed on membranes and are directly proportional to the protein expression of A_3AR normalized to β-actin. Density ratios of hematopoietic and mammary gland cancer cell lines were very small corresponding to nearly no expression of A_3AR protein. Only exception was MDA-MB-231, which displayed moderate density ratio. Highest density ratios were observed in the group of pancreatic and liver cancer cell lines, where the pancreatic cancer cell line JOPACA-1 had the highest density ratio of all samples. Also group marked as "others" showed relatively high density ratio values, suggesting their high A_3AR protein expression. – (**Gra.3**)



Graph 3 – Graphical display of densitometric analysis of blot density ratio of A_3AR to β-actin. Density values were acquired by measuring the intensity of bands, which were detected after the immunodetection. Density ratio ensured normalization of the protein expression of individual cell lines, since the protein concentration could differ among the samples. Density ratio "non-tumor" is average of density ratios of cell lines BJ and MRC-5.

6 DISCUSSION

The knowledge of the structure and function of A_3AR has recently evolved significantly. However, the specific role of A_3AR under various conditions still remains mysterious. Therefore, there is a need to characterize the mechanism of action and effects of agonist dependent activation of this receptor in different *in vitro* models and then correctly address those outcomes.

The study of A_3ARs modulation by various chemical compounds is a new topic studied at the Institute of Molecular and Translational Medicine, so it was first necessary to determine the level of expression of this receptor in cancer cell lines commonly used here, in order to correctly select cell lines for further experiments. The experimental part of the thesis focused on the determination of expression level of A_3AR gene and protein.

The selection of cell lines tested in the experimental part was done based on several criteria. All tested cancer cell types (i.e. liver cancer) were previously included in studies, which examined effects of A_3AR agonists (Bar-Yehuda *et al*., 2008; Cohen *et al*., 2011; 2008; Kamiya *et al*., 2012; Kim *et al*., 2002; Kohno *et al*., 1996; Lu *et al*., 2003; Madi *et al*., 2004; Ohana et al., 2003; Otsuki *et al*., 2012; Stemmer *et al*., 2013). Moreover, patient samples of mammary gland, liver and pancreatic cancer exhibited elevated expression of A_3AR. Furthermore, A_3AR receptor agonist CL-IB-MECA was tested in human clinical trial on HCC (Bar-Yehuda *et al*., 2008), with promising results. The group marked as "others" comprise of cell lines that belong to the basic panel of cell lines, which are regularly screened for anti-proliferative activity of commercially available substances and also substances prepared by the Department of Organic Chemistry at Palacký University Olomouc or by other cooperating groups.

The most significant differences in A_3AR gene expression occurred in the group of hematopoietic cancer cell lines. Expression fluctuated between extreme overexpression in JURKAT or RAMOS cell lines and almost no expression in CEM-DNR and K562 TAXOL resistant cell lines to daunorubicin and paclitaxel (taxol), respectively (**Gra.1 & 2**). Interestingly, after immunodetection with specific anti-A_3AR antibody no band for any hematopoietic cancer cell line was detected (**Fig.10**). Therefore, although they had in several cases extreme A_3AR gene overexpression there was practically no A_3AR protein product to be detected (**Gra.3**). Those results are in compliance with previous studies, such as the one by Kohno *et al*., 1996 that showed high A_3AR gene expression in HL-60. On the other hand,

different study using HL-60 and MOLT-4 cell lines (Kim *et al.*, 2002) proved that anticancer effects induced by A_3AR agonist were receptor independent, since their appearance required prolonged incubation, high agonist doses and apoptotic effects were not suspended by addition of receptor antagonist, those facts correspond to low or none A_3AR protein expression.

Mammary gland cancer cell lines (BT-549, MCF-7, T47-D) had Ct values higher than 35, which suggested a very low expression of A_3AR gene; this low expression was also confirmed by the protein expression analysis, where practically no band appeared after the immudetection with anti-A_3AR antibody (**Fig.10**). Similar outcomes were presented by Chung *et al.*, 2006. In contradiction to my findings, Panjehpour *et* Kamari-Tehrani, 2007 detected low levels of A_3AR gene expression in the cell line MCF-7. The possibility that mammary gland cancer cell lines express A_3AR gene and protein only minimally was suggested by studies, which proved that A_3 agonists are able to promote anticancer effect only at high doses (micromolar) (Kim *et al.*, 2002; Lu *et al.*, 2003). This indicated that anticancer effects of A_3 agonists are most likely A_3AR independent. Only exception in this group was the cell line MDA-MB-231, which displayed moderate expression of A_3AR gene (**Gra.1 & 2**) and higher expression of A_3AR protein (**Gra.3**).

Pancreatic group of cell lines (CAPAN-2, JOPACA-1, YAPC, PACADD119, PACADD135, PATU8902, MIA-PACA-2) exhibited significant A_3AR gene overexpression (**Gra.1 & 2**). High A_3AR gene expression was also reported by a study, which tested other pancreatic cancer cell lines (Madi *et al.*, 2004). After the immunodetection, each pancreatic cancer cell line had its corresponding band at 52 kDa. High density ratio indicated high A_3AR protein expression (**Gra.3**). Exceptions were two cell lines AsPC1 and BXPC3, which showed only slight A_3AR gene overexpression (**Gra.1 & 2**), but the level of A_3AR protein expression was similar to other cell lines in this group (**Gra.3**).

Relative quantification results of liver cancer cell lines (HEPG2, HEP3B, NCI-NC87) showed A_3AR gene overexpression in each cell line in this group (**Gra.1 & 2**). Staining liver cancer cell lines samples with A_3AR-specific antibody gave a rise to specific bands at 52 kDa, suggesting A_3AR expression (**Fig.10**). Quantitative densitometry confirmed high protein expression of A_3AR in all liver cancer cell lines (**Gra.3**). The A_3AR protein overexpression was previously described by Cohen *et al.*, 2011 for HEP3B and for another liver cancer cell line by Bar Yehuda *et al.*, 2008. Several studies proved that agonists of A_3AR have positive

anticancer effect on liver cancer *in vitro* and *in vivo*, those effects were confirmed to be A_3AR dependent (Bar-Yehuda *et al*., 2008, Stemmer *et al*., 2013). Moreover, agonist of A_3AR, particularly CL-IB-MECA, has already been successfully tested in human clinical trial (Bar-Yehuda *et al*., 2008).

The group of cancer cell lines marked as "others" was represented by three different cancer cell line types: colon (HCT116 par., HCT116 p53 -/-), lung (A549, HCC78) and bone (U2OS). Although relative quantification analysis detected low A_3AR gene expression, comparable to non-tumor cell lines (**Gra.1 & 2**), their A_3AR protein expression was relatively high, except for the cell line U2OS, which exhibited low A_3AR expression of both gene and protein (**Gra.1, 2 & 3**). Ohana et al., 2003, also previously reported high A_3AR protein expression in HCT116 colon cancer cell line. In addition, several studies showed that a high expression of A_3AR gene and A_3AR protein was also found in lung cancer cell lines (Kamiya *et al*., 2012; Madi *et al*., 2004; Otsuki *et al*., 2012).

I suggest, that in order to acquire representative characteristic of anticancer effects caused by A_3AR activation, also other parameters apart from RNA and protein expression need to be taken into account, such as examining whether the anti-cancer effects are receptor-dependent. It is also vital to study which cell signaling pathways are triggered by A_3AR activation and assess the interconnection between those pathways. Then is possible to specifically determine, which pathways are responsible for observed effects. In addition, it is important to use highly specific and potent agonists to prevent unspecific binding.

The experimental part revealed gene expression and protein expression level of A_3AR in different types of cancer. Cell lines of the same cancer type usually exhibited very similar A_3AR expression (**Gra.1, 2 & 3**). The experimental part also proved that there is not always correlation between gene expression and protein expression. These finings can serve as a tool for selection of cancer cell lines suitable for further studies of A_3AR modulation.

7 CONCLUSION

Investigation of the role of A_3AR in cancer is currently rapidly growing, thanks to discoveries of new highly selective and potent agonists, such as IB-MECA and CL-IB-MECA. This thesis presented comprehensive review on the current knowledge of AR, specifically on A_3AR and its clinically relevant agonists. Moreover, numerous studies were introduced, which provided extensive evidence that new A_3 receptor agonists are able to effectively bind to this receptor and through its activation promote anticancer effects. Therefore, A_3AR is considered as a prospective target for cancer treatment.

The experimental part of the thesis assessed selected cancer cell lines by their RNA and protein expression level of A_3AR. It is now possible to make a pre-selection of candidates for future *in vitro* models based on current knowledge about this receptor, its agonists and results of experiments conducted in the experimental part.

In order to reasonably choose an *in vitro* model one shall examine both RNA expression and protein expression. It is desirable to monitor both those values, since high RNA expression does not necessarily mean high receptor protein product, as shown also in my results. In many cases studies, which did not deliver promising results, meaning that anticancer effects were weak or appeared only after prolonged exposure of high drug concentration, were conducted on cell lines that had relatively high RNA expression but protein product was either very low or the study did not provided analysis of the protein expression level. Moreover, even those weak effects might have been receptor-independent. On the other hand, majority of successful studies used cancer cell lines, which had both high RNA expression and high protein expression; those are prerequisites for highly specific agonist-receptor interaction. For successful selection of suitable cancer cell line one should preferentially seek for high expression of A_3AR protein product.

Agonists of A_3AR are highly specific. However, when used at low concentration (nanomolar), they are able activate receptors only at sites where they are highly abundant, which is exactly a feature of certain cancer cell lines. Therefore, when the cell line is carefully chosen, agonists of A_3AR can potentially act as extremely specific targeted therapy, which only affects cancer cells.

Overall, it can be stated that suitable cancer cell lines are those, which have both high RNA and protein expression. Several candidates for *in vitro* model were preselected based on results of the experimental. The pre-selected cancer cell lines fulfill previously mentioned criteria and are therefore suitable for future studies of anticancer effects mediated by new A_3 receptor agonists.

Cell lines with high gene and protein expression of A_3AR:

- **Pancreatic cancer cell lines** - AsPC1, BXPC3, CAPAN-2, JOPACA-1, YAPC, PACADD119, PACADD135, PATU8902
- **Liver cancer cell lines** - HEPG2, HEP3B, NCI-N87
- **Colon cancer cell lines** - HTC116 par., HCT116 p53 -/-
- **Lung cancer cell lines** - A549, HCC78

For the experimental purposes also **mammary gland cancer group of cell lines** could represent an interesting *in vitro* model, since it include cell lines with very different A_3AR expression, which could potentially be suitable for comparing the effects of A_3AR agonist on cell lines that has high expression to the cell line that has low expression.

In conclusion, the results indicate that above listed cancer cell lines could be suitable *in vitro* models for the examination of A_3AR modulation.

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9 LISTS

9.1 Abbreviations

AC	adenylate cyclase
Ado	adenosine
APS	amonium persulphate
AR	adenosine receptor
Asp250	aspartic acid residue 250
ATCB	beta actin
ATCC	American type culture collection
ATP	adenosine triphosphate
Bad	pro-apoptotic protein
Bak	pro-apoptotic protein
BSA	bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate]
c-Myc	transcription factor
C-terminus	carboxy terminus
cAMP	cyclic adenosine monophosphate
CD39	nucleoside triphosphate dephosphorylase
CD73	ecto-5'-nucleotidase
cDNA	complementary deoxyribonucleic acid
CML	chronic myeloid leukemia
CNS	central nervous system
CNTs	concentrative nucleoside transporters
CREB	cAMP response element binding protein
DEPT H_2O	diethyl pyrocarbonate treated water
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
dNTPs	nucleosides triphosphates
DSMZ	German collection of microorganisms and cell cultures

DTT	dithiothreitol
E	efficiency
EDTA	ethylenediamine tetraacetic acid
EMEM	Eagle's Minimum Essential Medium
ENTs	equilibrative nucleoside transporters
ERK1/ERK2	extracellular signal-regulated kinase 1/2
F12K	Ham's F-12K (Kaighn's) Medium
FCSi	fetal calf serum inactivated
FP	forward primer
G-protein	guanine nucleotide-binding proteins
Gi	inhibitory G-protein
GPCR	g-protein coupled receptor
Gs	stimulatory G-protein
GSK3β	glycogen synthase kinase
h	human
HCC	hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HSP90	heat shock protein 90
IA	intrinsic activity
JNK	cJun – N terminal kinase
Ki	inhibitory constant
LEM	laboratory of experimental medicine
MAPK	mitogen activated protein kinase
Mili-Q H_2O	mili-Q water
MM	master mix
MM_{RT}	master mix for reverse transcription
$MM_{RT\text{-}PCR}$	master mix for reverse transcription for real time polymerase chain reaction
N-terminus	amino terminus
NF-κB	nuclear factor kappa B
NP-40	nonyl phenoxypolyethoxylethanol

NTs	nucleoside transporters
PBMC	peripheral blood mononuclear cells
PBS	phosphatase buffed saline
PHIM	phosphatase inhibitor mix
PI3K	phosphoinositide 3-kinase
PIM	protease inhibitor mix
PKA	protein kinase A
PLC	protein lipase C
PLCβ	protein lipase C beta
PLD	protein lipase D
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
PYNA	sodium pyruvate
RIPA buffer	radioimmunoprecipitation assay buffer
RNAsin	ribonuclease inhibitor
ROS	reactive oxygen species
RP	reverse primer
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
RT-PCR	real time polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	serum free medium
TBS-T	tris buffered saline – tween
TCA	trichloracetic acid
TEMED	tetramethylethylendiamine
TGS	tris-glycine- sodium dodecyl sulfate
TM3	transmembrane helix 3
TM7	transmembrane helix 7
Trp243	tryptophan residue 243
WB	western blotting

9.2 Figures

Figure 1 The structure of Ado molecule

Figure 2 Structure of NTs – CNT (a) and ENT (b)

Figure 3 AR mediated pathway

Figure 4 The general structure of the G protein-coupled receptor

Figure 5 Predominant downstream pathways of AR subtypes

Figure 6 Complex A_3AR signaling

Figure 7 A_3AR agonists

Figure 8 Structure of Ado (a) and from it derived agonist CF101 (b)

Figure 9 Structure of Ado (a) and from it derived agonist CF102 (b)

Figure 10 Comparison of A_3AR protein expression in individual cell lines using WB analysis

9.3 Tables

Table I Binding affinity of agonists at human AR subtypes

Table II Characteristics and basic information about cell lines used in the experimental part

Table III Chemicals used for the preparation of the master mix for reverse transcription (MM_{RT})

Table IV Chemicals used for preparation of the master mix for the real-time polymerase chain reaction ($MM_{RT\text{-}PCR}$)

Table V Conditions used for RT-PCR

Table VI Primers used for RT-PCR

Table VII Chemicals used for preparation of the separating and the stacking gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Table VIII Criteria for classification of A_3AR gene expression of cancer cell lines

Table IX Fold change of A_3AR gene expression normalized to ATBC and POL2A and relative to the calibrator

9.4 Graphs

Graph 1 Graphical display of fold change of A_3AR gene expression normalized to the reference gene ATBC

Graph 2 Graphical display of fold change of A_3AR gene expression normalized to the reference gene POL2A

Graph 3 Graphical display of densitometric analysis of blot density ratio of A_3AR to β-actin