

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/20128> holds various files of this Leiden University dissertation.

Author: Blad, Clara Catelijne

Title: A quest for connections : ligands for the HCA2, adenosine A3 and GPR88 receptors

Date: 2012-11-15

Chapter 6



Putative role of the adenosine A₃ receptor in the anti-proliferative action of N⁶-(2-isopentenyl)adenosine

This chapter is based on: Blad CC, von Frijtag Drabbe Künzel JK, de Vries H, Mulder-Krieger T, Bar-Yehuda S, Fishman P, IJzerman AP. *Purinergic Signal* **2011**; 7(4): 453-62.

Abstract

We tested a panel of naturally occurring nucleosides for their affinity towards adenosine receptors. Both N^6 -(2-isopentenyl)adenosine (IPA) and racemic zeatin riboside were shown to be selective human adenosine A_3 receptor (hA_3R) ligands with affinities in the high nanomolar range (K_1 values of 159 and 649 nM, respectively). These values were comparable to the observed K_1 value of adenosine on hA_3R , which was 847 nM in the same radioligand binding assay. IPA also bound with micromolar affinity to the rat A_3R . In a functional assay in CHO cells transfected with hA_3R , IPA and zeatin riboside inhibited forskolin-induced cAMP formation at micromolar potencies. The effect of IPA could be blocked by the A_3R antagonist VUF5574.

Both IPA and reference A_3R agonist 2-chloro- N^6 -(3-iodobenzyl)adenosine-5'- N -methylcarboxamide (CI-IB-MECA) have known antitumor effects. We demonstrated strong and highly similar antiproliferative effects of IPA and CI-IB-MECA on human and rat tumor cell lines LNCaP and N1S1. Importantly, the antiproliferative effect of low concentrations of IPA on LNCaP cells could be fully blocked by the selective A_3R antagonist MRS1523. At higher concentrations, IPA appeared to inhibit cell growth by an A_3R -independent mechanism, as was previously reported for other A_3R agonists. We used HPLC to investigate the presence of endogenous IPA in rat muscle tissue, but we could not detect the compound.

In conclusion, the antiproliferative effects of the naturally occurring nucleoside IPA are at least in part mediated by the A_3R .

Introduction

Four adenosine receptors have been cloned and pharmacologically characterized: A₁, A_{2A}, A_{2B} and A₃. Of these subtypes, the A₃R has been identified last [1]. The hA₃R has a wide distribution in the body. High expression is observed in liver, lungs and immune cells, whereas the brain, testes, placenta and heart display moderate expression levels [2-3]. Furthermore, a very high expression level is observed in tumor cell lines and cancer tissues, making the A₃R an interesting target for the treatment of cancer (for a review, see [4]). A recent study even showed that the 3-fold A₃R upregulation in human colorectal cancer was reflected, via an unknown mechanism, in peripheral blood cells [5]. This could make the A₃R a promising biomarker for this and possibly other types of cancer. We and others have previously shown that A₃R agonists have antiproliferative effects on tumor cells in vitro and in vivo, characterized by induction of G₀/G₁ cell cycle arrest and apoptosis [4]. It has been shown that, in most cases, a deregulation of the NF-κB and Wnt signaling pathways is at the basis of these effects [4]. However, other mechanisms may be more important in specific cell types. For example, in A375 human melanoma cells, A₃R stimulation activated phosphatidylinositol 3-kinase (PI3K) which induced Akt phosphorylation, finally resulting in reduced levels of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) [6]. Another recent study suggested that A₃R upregulation may precede asbestos-induced malignant mesothelioma formation [7]. The A₃R agonist 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide (CI-IB-MECA) prevented TNF-α-mediated cell survival after asbestos exposure in vitro by acting on the de-regulated Akt/NF-κB pathways, showing that A₃R is a possible target for cancer prevention as well as treatment. Interestingly, A₃R agonists do not inhibit the growth of normal cells [7-9]. Moreover, these ligands can act as cytoprotective agents, for example by preventing the myelotoxic effects of chemotherapy [10]. In view of the promising preclinical data, the safety profile of the A₃R agonist IB-MECA has been evaluated in phase I clinical studies [11]. This agonist was tolerated very well. Related A₃R agonist CI-IB-MECA is currently in phase I/II clinical trials for hepatocellular carcinoma ([12] and trial ID: NCT00790218). On the basis of these studies and observations we hypothesized that endogenous A₃R agonists might also contribute to the body's natural defense mechanism against tumors. In particular, such compounds may be excreted by striated muscle cells, protecting the tissue from metastases [13]. We have shown that extracts from muscle cells inhibited tumor proliferation while protecting bone marrow cells in vivo. These effects were dependent on A₃R activation. However, they could not be attributed to adenosine, as the effect was not sensitive to ADA and could not be reproduced by administration of adenosine alone [13]. Thus, we set out to identify other endogenous agonists for the A₃R.

In the current work we report the affinity of a panel of naturally occurring nucleosides for the human adenosine receptors, while focusing on the hA₃R. Then, we assessed the potency of the two higher affinity compounds, the nucleosides IPA and racemic zeatin riboside, in cAMP generation assays. The effect of IPA and the reference A₃R agonist CI-IB-MECA on tumor cell line proliferation was also determined. We provide evidence that the antiproliferative effects of IPA are indeed linked to the A₃R as they can be partially

blocked by a selective antagonist for that receptor. We also present a HPLC method to investigate the IPA content of muscle tissue. However, endogenous IPA could not be detected in our analysis.

Materials & Methods

Materials

[³H]-DPCPX and [¹²⁵I]-AB-MECA were purchased from Amersham Biosciences (Roosendaal, the Netherlands). [³H]-ZM241385 and [³H]-MRS1754 were obtained from Tocris Cookson, Ltd. (Bristol, UK). All nucleosides listed in table 1 were supplied by Sigma-Aldrich (Zwijndrecht, the Netherlands). Ammonium acetate and EDTA for analysis of muscle tissue were obtained from Fluka (Zwijndrecht, the Netherlands), whereas methanol for the HPLC analysis was from Biosolve (Valkenswaard, the Netherlands).

Chinese hamster ovary (CHO) cells expressing the human adenosine A₁ receptor were kindly provided by Dr. A. Townsend-Nicholson (University College of London, UK). Human embryonic kidney (HEK) 293 cells stably expressing the human adenosine A_{2A} receptor were a gift from Dr. E. Wang (Biogen/IDEC, San Diego, CA, USA). CHO cells expressing the human adenosine A_{2B} receptor were donated by Dr. S. Rees (GSK, Stevenage, UK) and both CHO and HEK293 cells expressing the hA₃R were kindly provided by Dr. K.-N. Klotz (University of Wuerzburg, Germany). RBL-2H3 cells were a kind gift of Dr. Frank Redegeld (Utrecht University, the Netherlands). LNCaP (human prostate carcinoma) and N1S1 (rat hepatocellular carcinoma) cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA).

Fresh Wistar rat cadavers from an untreated control group of another study were kindly provided by the animal facility of the Leiden/Amsterdam Center for Drug Research (Leiden, the Netherlands).

Radioligand binding studies

Cell culture and membrane preparation

CHO cells expressing the human A₁ receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/ml), penicillin (50 IU/ml) and G418 (0.2 mg/ml) at 37 °C and 5% CO₂. HEK 293 cells stably expressing either the human A_{2A} adenosine receptor or the hA₃R were grown in DMEM containing 10% newborn calf serum, streptomycin (50 µg/ml), penicillin (50 units/ml) and G418 (0.5 mg/ml) at 37 °C and 7% CO₂. Membranes were prepared as previously described [14]. ADA was added in the final preparations at 0.8 IU/ml, except for the membranes containing the A₃R, where no ADA was included.

Human adenosine A₁ receptor

Affinity for the human A₁ receptor was determined on membranes from CHO cells expressing the human receptors, using [³H]-DPCPX as the radioligand. Membranes containing 10 µg of protein were incubated in a total volume of 200 µl of 50 mM Tris/HCl (pH 7.4) and [³H]-DPCPX (final concentration 1.6 nM) for 1 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 10 µM CPA. The incubation

was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester. Filters were washed three times with ice-cold buffer and placed in scintillation vials. Packard Emulsifier Safe (3.5 ml) was added, and after 2 h radioactivity was counted in a Perkin-Elmer Tri-Carb 2900 β -scintillation counter.

Human adenosine A_{2A} receptor

Affinity for the human A_{2A} receptor was determined on membranes from HEK293 cells stably expressing this receptor, using [³H]-ZM241385 as the radioligand. Membranes containing 30 μ g of protein were incubated in a total volume of 200 μ l of 50 mM Tris/HCl (pH 7.4) and [³H]-ZM241385 (final concentration 1.7 nM) for 2 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 10 μ M CGS21680. Filtration and counting were performed as described for the A₁ receptor.

Human adenosine A_{2B} receptor

At the human A_{2B} receptor radioligand displacement was determined on membranes from CHO cells stably transfected with this receptor, using [³H]-MRS1754 as the radioligand. Membranes containing 20 μ g of protein were incubated in a total volume of 100 μ l of 50 mM Tris/HCl (pH 7.4), 10 mM MgCl₂, 1 mM EDTA, 0.01 w/v % CHAPS (pH 8.26 at 5 °C), and [³H]-MRS1754 (final concentration 1.2 nM) for 1 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 1 mM NECA. Filtration and counting were performed as described for the A₁ receptor.

Human A₃R

The affinity at the hA₃R was measured on membranes from HEK293 cells stably expressing this receptor, using [¹²⁵I]-AB-MECA as the radioligand. Membranes containing 35 μ g of protein were incubated in a total volume of 100 μ l of 50 mM Tris/ HCl, 10 mM MgCl₂, 1 mM EDTA, 0.01% w/v CHAPS (pH 8.26 at 5 °C), and [¹²⁵I]-AB-MECA (final concentration 0.10 nM) for 1 h at 37 °C in a shaking water bath. Nonspecific binding was determined in the presence of 100 μ M R-PIA. The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester. Filters were washed three times with ice-cold buffer and placed in counting tubes. Radioactivity was counted in a Perkin-Elmer Wallac 1470 Wizard gamma-counter.

Rat A₃R

The affinity at the rat A₃R was measured on membranes from RBL-2H3 (rat basophilic leukemia) cells endogenously expressing this receptor, using [¹²⁵I]-AB-MECA as the radioligand. Membranes of these cells were prepared as described previously [15]. Membranes containing 60 μ g of protein were incubated in a total volume of 100 μ l of 50 mM Tris/ HCl, 10 mM MgCl₂ (pH 7.7 at 22 °C), and [¹²⁵I]-AB-MECA (final concentration 0.20 nM) for 1 h at 37 °C in a shaking water bath. Nonspecific binding was determined in the presence of 100 μ M R-PIA. Filtration and counting were performed as described for the hA₃R.

Second messenger studies: cAMP production in cells expressing the hA₃R

CHO cells expressing the hA₃R were grown overnight as a monolayer in 24 well tissue culture plates (400 µl/well; 2 × 10⁵ cells/well) in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/ml), penicillin (50 IU/ml) and G418 (0.2 mg/ml) at 37 °C and 5% CO₂. To determine the potencies of IPA, adenosine and zeatin riboside, cAMP generation was performed in DMEM/N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (0.60 g HEPES/50 ml DMEM pH 7.4). Each well was washed twice with HEPES/DMEM buffer (250 µl), after which the PDE inhibitors rolipram (50 µM) and cilostamide (50 µM) were added to each well. This mixture was incubated for 30 min at 37 °C followed by the introduction of either the compound of interest (10 µM), reference compound Cl-IB-MECA (10 µM) or DMEM/HEPES. After a further 10 min of incubation, forskolin was added (10 µM). After a subsequent 15 min, incubation was stopped by aspirating the assay medium and by adding 200 µl of ice-cold 0.1 M HCl. The amount of cAMP was determined by competition with [³H]-cAMP for protein kinase A (PKA). Briefly, the sample, approximately 1.8 nM [³H]-cAMP, and 100 µl of PKA solution were incubated on ice for at least 2.5 h. The incubations were stopped by rapid dilution with 2 ml of ice-cold Tris/ HCl buffer (50 mM, pH 7.4), and bound radioactive material was then recovered by filtration through Whatman GF/C filters. Filters were additionally rinsed with 2 × 2 ml of Tris/HCl buffer, Packard Emulsifier Safe (3.5 ml) was added, and after 2 h radioactivity was counted in a Perkin-Elmer Tri-Carb 2900 β-scintillation counter.

In the assays evaluating the effects of ADA and VUF5574, intracellular cAMP levels were measured using a LANCE cAMP 384 kit (PerkinElmer, The Netherlands) as described previously [16]. To each well, 5 µL of the agonist Cl-IB-MECA, adenosine or IPA (10 µM final concentration) in stimulation buffer (PBS with 5 mM Hepes, pH 7.4 supplemented with 0.1% BSA, rolipram (50 µM) and cilostamide (50 µM)) was added in the absence (control) or presence of ADA (0.8 IU/mL) or VUF5574 (1 µM). Then 4.5 µL hA₃-CHO cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5000 cells/well), which was followed by incubation for 15 min at room temperature. Subsequently, 2.5 µL forskolin (1 µM) was added and the mixture was incubated for 30 min at room temperature. Then, detection mix (6 µL) and cAMP antibody solution (6 µL) were added and incubated for 3h. Intracellular cAMP levels were measured using a TR-FRET assay on a Victor spectrometer (PerkinElmer, The Netherlands) according to instructions of the supplier. All data reflect the average of at least three independent experiments performed in duplicate.

Proliferation assays on tumor cell lines

Effect of IPA on the proliferation of tumor cell lines

Cell proliferation was studied in both N1S1, a rat hepatocellular carcinoma cell line, and LNCaP, a human prostate carcinoma cell line. The cells were grown in RPMI 1640 with penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and 10% fetal

bovine serum (FBS). The cells were maintained in T-75 flasks at 37 °C in a 5% CO₂ incubator and transferred to freshly prepared medium twice weekly. For the in vitro studies with the LNCaP cell line serum-starved cells were used. In these experiments FBS was omitted from the culture medium for 18 h and the experiment was carried out on monolayers of cells in RPMI medium supplemented with 1% FBS. For the N1S1 cells no serum starvation was used and the assay was carried out in the growth medium of the cells.

The cells (1.5×10^4 /ml) were incubated in 96-well microtiter plates in the presence of 25 μM adenosine or various concentrations of CI-IB-MECA and IPA (0.01, 0.1, 1, and 10 μM). A 24 h [³H]-thymidine incorporation assay was used to evaluate cell growth, except for the experiments evaluating the effect of EHNA (10 μM), which lasted 48 hours. For the last 18 h of incubation, each well was pulsed with 1 μCi [³H]-thymidine. Cells were harvested and the [³H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA).

Effect of IPA on the proliferation of LNCaP cells in the presence of the A₃R antagonist MRS1523

The effect of IPA on the proliferation of LNCaP cells in the presence of the A₃R antagonist MRS1523 was also examined. The cells (1.5×10^4 /ml) were incubated in 96-well microtiter plates with IPA (0.01, 0.1, 1 and 10 μM) in the absence or presence of MRS1523 (0.01, 0.1 and 1 μM). Cell growth was evaluated in a 24-hour [³H]-thymidine incorporation assay as described above.

Data analysis

All radioligand displacement curves and cAMP concentration-effect curves were analyzed with GraphPad Prism software (version 5.0). Statistical analysis of the results of the cAMP production assays was done using an unpaired Student's t-test.

HPLC analysis of the presence of IPA in rat muscle tissue

The presence of IPA in rat muscle tissue was investigated by HPLC analysis. Fresh rat muscle tissue (hind leg vastus lateralis and semimembranosus) was used. About 5 gram of tissue was weighed exactly and cut into small pieces of about 5x5x5 mm. The material was spiked with IPA and/or internal standard cyclopentyladenosine in methanol (2.5 μg). Then, 30 ml of 5 mM ammonium acetate (pH 5.2), 50 mM EDTA was added and the material was treated with a Diax 900 homogenizer (Heidolph, Schwabach, Germany) equipped with a 1.5 cm probe, for 1.5 min at speed 1. The homogenate was subjected to 4 freeze-thaw cycles in liquid nitrogen and a 60 °C water bath. The sample was centrifuged at 6000 g at 4 °C for 30 min and the supernatant was additionally centrifuged at 200000 g at 4 °C for 45 min. After the centrifugation steps, the clear supernatant was filtered over Miracloth to remove fat particles and transferred to an activated C18 solid phase extraction column (Grace, Deerfield, USA). After passing the sample, the column was washed with 25 ml 5 mM ammonium acetate and the material of interest was eluted with 5 ml methanol. The methanol was evaporated in a vacuum centrifuge and the sample was reconstituted in 0.5 ml 5 mM ammonium acetate after which HPLC analysis was performed.

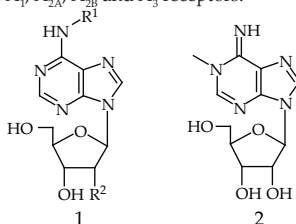
The system consisted of a gradient solvent delivery system (Gilson, Den Haag, the Netherlands), equipped with a Gilson 115 UV detector, monitoring at 270 nm. The column was a Discovery® RP 18 (Supelco, Zwijndrecht, the Netherlands), 125 x 4.6 mm, packed with 5 µm particles. As eluent a gradient between solvent A (10% methanol in 5 mM ammonium acetate) and solvent B (90% methanol in 5 mM ammonium acetate) was applied with a flow of 0.6 ml/min. The profile of the gradient was as follows; 0 min, 0% B; 10 min, 0% B; 15 min, 37.5% B; 35 min, 37.5% B; 40 min, 100% B; 50 min, 100% B; 51 min, 0% B and 60 min, 0% B. For analysis, 100 µl of sample in 5 mM ammonium acetate pH 5.2 was injected by an autoinjector. Data were recorded and processed using ADChrom software (Leiden University, the Netherlands).

Results

Radioligand binding studies on human adenosine receptors

The affinities of the naturally occurring modified nucleosides 1-methyl-adenosine, 2'-O-methyl-adenosine, IPA, trans-zeatin riboside and racemic zeatin riboside were determined in radioligand binding studies on all four subtypes of human adenosine receptors (table 1). The affinity of adenosine for the A_{1V} , A_{2A} and A_{2B} receptors could not be assessed in this experimental set-up due to the essential presence of ADA in the assays. Both IPA and the racemic mixture of cis- and trans-isomers of zeatin riboside showed affinities in the higher nanomolar range (K_i values of 159 nM and 643 nM, respectively) for the hA_3R , in the same range as the affinity of adenosine itself. The affinities of both zeatin riboside and IPA at the other adenosine receptor subtypes were negligible, given the lack of displacement at a test concentration of 1 µM.

Table 1. Chemical structures of naturally occurring modified nucleosides and their effects in radioligand binding studies at human adenosine A_{1V} , A_{2A} , A_{2B} and A_3 receptors.



Name	R ¹	R ²	% displacement			K _i or % displ.
			hA ₁	hA _{2A}	hA _{2B}	hA ₃
1a adenosine	-H	-OH	ND	ND	ND	847±60 nM
1b N ⁶ -(2-isopentenyl)-adenosine (IPA)	-CH ₂ CHC(CH ₃) ₂	-OH	14	0	0	159±19 nM
1c trans-zeatin riboside	CH ₂ CHC(CH ₃)CH ₂ OH-	-OH	2	0	6	31%
1d racemic zeatin riboside	-CH ₂ CHC(CH ₃)CH ₂ OH	-OH	17	0	0	643±27 nM
1e 2'-O-methyladenosine	-H	-OH	0	0	8	0%
2 1-methyl-adenosine	-	-OCH ₃	2	0	4	34%

% displacement at 1 µM (N=2) or K_i value (N=3) is shown; data are means (±S.E.M.) of N experiments performed in duplicate.

Affinity of IPA for the rat A₃R

The affinity of IPA for the rat A₃R was also determined in radioligand binding studies (figure 1). A K_i value of 4.69 ± 0.20 μM was found for the rat orthologue, so the affinity of IPA for the rat A₃R was lower than for the hA₃R.

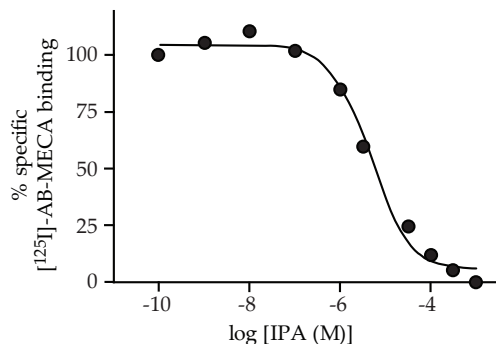


Figure 1. Competition binding experiment on RBL-2H3 cell membranes endogenously expressing the rat A₃R. IPA displaced [¹²⁵I]-AB-MECA from the rat A₃R with a K_i value of 4.69 ± 0.20 μM (N=3). Data from one representative experiment is shown. Incubation was 1 h at 37 °C in 50 mM Tris/ HCl, 10 mM MgCl₂ (pH 7.7)

Effect of IPA on the cAMP production in cells expressing the hA₃R

The potency of adenosine, IPA and racemic zeatin riboside to modulate cAMP production was determined in intact CHO cells stably expressing the hA₃R (table 2). Adenosine and IPA inhibited forskolin-stimulated cAMP accumulation with similar potencies in the low micromolar range and virtually identical intrinsic activities.

Table 2. Potency of naturally occurring nucleosides on the human adenosine A₃ receptor, as inhibitors of forskolin-stimulated cAMP production in CHO cells expressing the hA₃R.

	EC ₅₀ (μM)
adenosine	2.9 ± 1.1
N ⁶ -(2-isopentenyl)-adenosine (IPA)	2.0 ± 0.7
racemic zeatin riboside	5.8 ± 1.8

EC₅₀ value (N=4, except zeatin riboside N=3) is shown; data are means (±S.E.M.) of N experiments performed in quadruplicate.

In a second assay the effects of ADA and the selective hA₃R antagonist VUF5574 were investigated (figure 2). The selective A₃R agonist CI-IB-MECA was included for comparison next to adenosine and IPA. As expected, adenosine and IPA inhibited cAMP production to a similar extent when added at 10 μM, whereas CI-IB-MECA had a more potent effect. Inclusion of ADA abolished the effect of adenosine, whereas IPA and CI-IB-MECA were insensitive to the enzyme. Modulation of the second messenger pathway by each of the agonists was largely and significantly inhibited by the antagonist VUF5574.

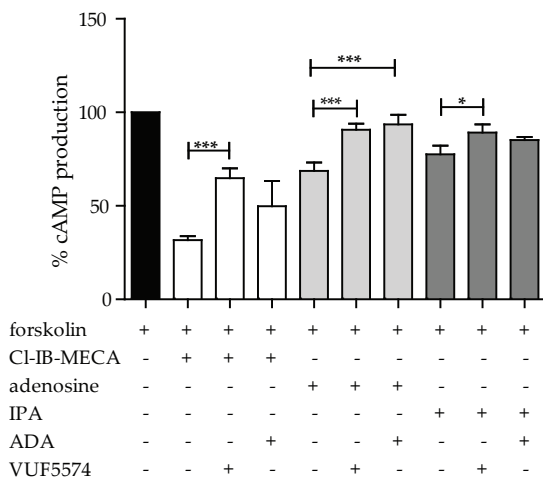


Figure 2. Effect of ADA and A_3R antagonist VUF5574 in cAMP accumulation assays. In CHO cells stably expressed with hA_3R , forskolin-stimulated cAMP production was inhibited by adenosine, IPA and CI-IB-MECA, all at 10 μ M. The effects of the concomitant presence of either the enzyme ADA (0.8 IU/ml) or the antagonist VUF5574 (10 μ M) were also assessed. The cells were incubated for 15 min at room temperature with the ligand(s) of interest \pm ADA. Then forskolin was added to the reaction mixture for a further 30 min. Data are means (\pm S.E.M.) of four experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. * $P < 0.05$, *** $P < 0.001$

Effect of IPA on the proliferation of tumor cell lines

In view of the reported anti-tumor effects of agonists for the A_3R we investigated the effect of IPA on the proliferation of human prostate carcinoma cells (LNCaP) and rat hepatocellular carcinoma cells (N1S1). In both tumor cell lines IPA inhibited the incorporation of [3 H]-thymidine in a similar fashion as the reference A_3R agonist CI-IB-MECA (figures 3 and 4). At 10 μ M, IPA even had a significantly greater effect than CI-IB-MECA on both cell lines ($P < 0.01$). An approximate EC_{50} value of 1 μ M for IPA was established in the LNCaP cell line, whereas the potency on the N1S1 cell line seemed somewhat lower. Adenosine, at a concentration of 25 μ M, had only a modest effect on N1S1 proliferation and no effect on the LNCaP cell line. Addition of ADA inhibitor EHNA (10 μ M) did not increase the adenosine effect in the LNCaP cell line (data not shown), although it significantly increased the effect of adenosine on the N1S1 cells, yielding almost full growth inhibition (data not shown; available with the published article as Online Resource 1). Addition of EHNA but not adenosine to N1S1 cells also revealed a 22% basal growth inhibition, probably mediated by adenosine excreted by the tumor cells.

Effect of IPA on the proliferation of LNCaP cells in the presence of the A_3R antagonist MRS1523

In a final proliferation assay we investigated whether MRS1523, an antagonist with appreciable affinity for both human and rat A_3R , was capable of blocking the effect of IPA on LNCaP cell proliferation (figure 5). The antagonist (at 0.1 and 1 μ M) prevented the anti-proliferative effect of 0.01 and 0.1 μ M IPA. When IPA was added at 1 μ M, the inhibition by MRS1523 was small but still significant at higher concentrations. At 10 μ M IPA, the antagonist had no effect.

Determination of IPA in muscle tissue by HPLC

Since IPA is a naturally occurring nucleoside we investigated its occurrence in muscle

PUTATIVE ROLE OF A₃R IN THE ANTICANCER ACTION OF IPA

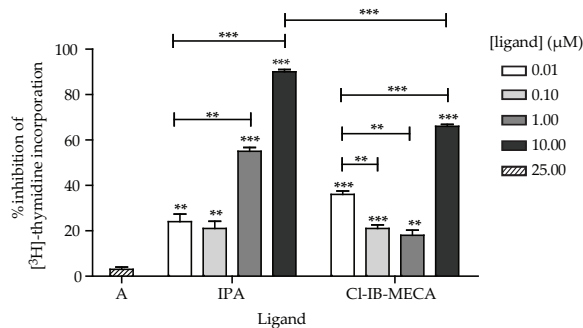


Figure 3. IPA and CI-IB-MECA have similar antiproliferative effects on LNCaP tumor cells. IPA and CI-IB-MECA (0.01-10 μM) both inhibited proliferation of the human prostate carcinoma cell line LNCaP. Adenosine (A) (25 μM) had virtually no effect. IPA and CI-IB-MECA had significantly larger antiproliferative effects than adenosine at all concentrations ($P < 0.01$). The effect of IPA increased significantly at 1 μM compared to the lower concentrations ($P < 0.01$), and the effect increased further at 10 μM . The effect of CI-IB-MECA showed a significant decrease from 0.01 μM to 1 μM ($P < 0.01$), and then significantly increased again at 10 μM ($P < 0.001$). At 10 μM , IPA had a significantly larger effect than CI-IB-MECA ($P < 0.001$). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μCi of [^3H]-thymidine was included in the buffer. Data are means (\pm S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. ** $P < 0.01$; *** $P < 0.001$. Stars without brackets indicate the significance of the difference to adenosine treatment.

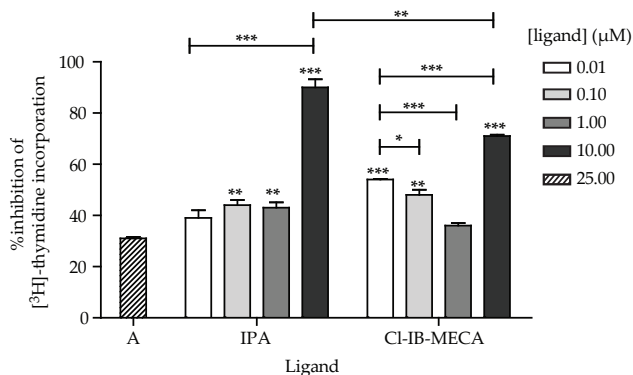


Figure 4. IPA and CI-IB-MECA have similar antiproliferative effects on N1S1 tumor cells. IPA and CI-IB-MECA (0.01-10 μM) both inhibited proliferation of the rat hepatocellular carcinoma cell line N1S1. Adenosine (A) (25 μM) had only a modest effect. IPA and CI-IB-MECA had significantly larger antiproliferative effects than adenosine ($P < 0.01$), with the exception of 0.01 μM IPA and 1 μM CI-IB-MECA. The effect of IPA increased significantly at 10 μM compared to the other concentrations ($P < 0.001$). The effect of CI-IB-MECA showed a significant decrease from 0.01 μM to 1 μM , and then significantly increased again at 10 μM (both $P < 0.001$). At 10 μM , IPA had a significantly larger effect than CI-IB-MECA ($P < 0.01$). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μCi of [^3H]-thymidine was included in the buffer. Data are means (\pm S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

tissue. We therefore analyzed extracts from Wistar rat muscle. The spectra of spiked and unspiked tissue extracts are shown in figures 6A and 6B, respectively. From the spiked tissue, 23% of the added IPA was recovered in the extract. In the unspiked spectrum no IPA peak was observed.

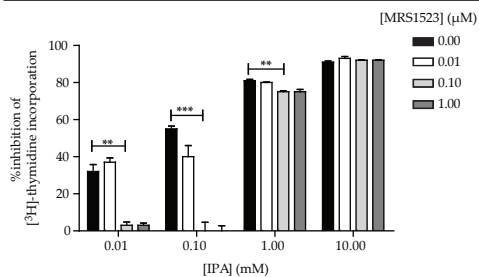


Figure 5. The antiproliferative effect of IPA is partly blocked by A_3R antagonist MRS1523. The selective A_3R antagonist MRS1523 (0.01-1 μ M) blocked the effect of low concentrations (0.01-0.1 μ M) of IPA on human prostate carcinoma (LNCaP) cells. MRS1523 did not inhibit the effect of high concentrations of IPA (1-10 μ M). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μ Ci of [3 H]-thymidine was included in the buffer. Data are means (\pm S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. ** $P < 0.01$, *** $P < 0.001$

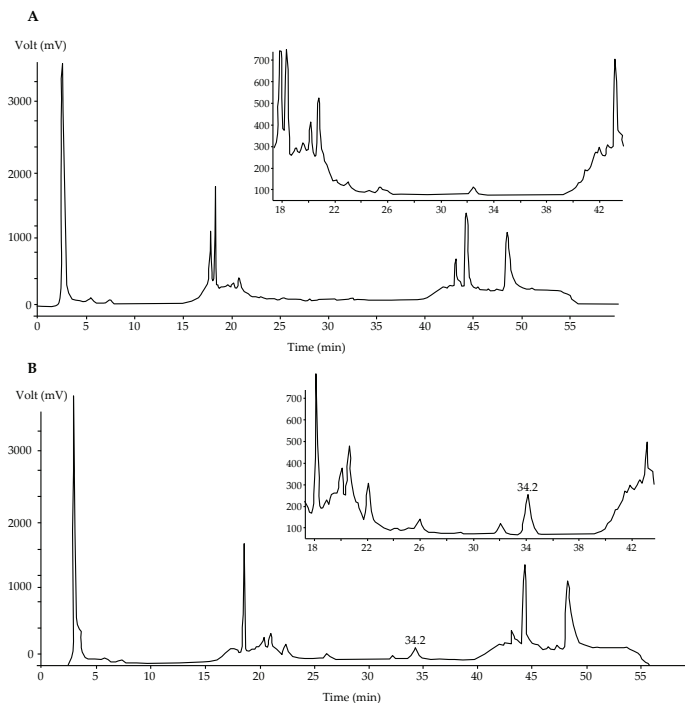


Figure 6. HPLC analysis of rat muscle extract detects no IPA. Comparison of rat muscle tissue spiked with IPA (B) and unspiked muscle extract (A) tissue shows that IPA, which is eluted at 34.2 min in the spiked tissue, cannot be detected in the blank tissue.

Discussion

In vitro evaluation of IPA activity

We have shown that zeatin riboside and particularly IPA are ligands for the A_3R . These naturally occurring modified nucleosides bind with submicromolar affinity to the hA_3R . Moreover, the affinity of IPA for A_3R is not restricted to the human orthologue, but extends to the rat A_3R . In line with previous findings on N^6 -substituted adenosine derivatives with small alkyl substituents, IPA discriminates between the two A_3R homologues and has a 30-fold higher affinity for the human A_3R [17]. Our next step was the evaluation of receptor activation by IPA, zeatin riboside and adenosine in cAMP generation assays. The compounds had similar potencies in the low micromolar range, with zeatin riboside 2- or 3-fold less potent than IPA and adenosine.

The potency of adenosine towards the A_3R was classically considered to be in the

micromolar range and rather lower than the potency towards the adenosine A₁ and A_{2A} receptors [18]. In contrast, several more recent publications report similar potencies of adenosine towards the A₁, A_{2B} and A₃ subtypes, in the submicromolar range [19-21]. The affinity and potency that we observed for A₃R activation by adenosine are clearly in better agreement with the more recent reports. Some care should be taken in the interpretation of cAMP assay results, however, as receptor expression levels may have a strong impact on the EC₅₀ values of tested agonists.

Anti-proliferative effects of IPA

Interestingly, IPA has antitumor activity both in vitro and in vivo [22-23]. In 1975 this compound has been tested in a pilot clinical trial for leukemia, where it caused remission in three patients out of twenty [24]. Currently, there is a renewed interest in the compound; in a recent review IPA was defined as an emerging anticancer drug [25]. The molecular mechanism of the anti-tumor activity of IPA has not been elucidated so far.

In view of the well-known role of the A₃R in cancer, we decided to investigate whether the antiproliferative effects of IPA are mediated by this receptor. IPA inhibits the proliferation of human prostate cancer cells (LNCaP) and rat hepatocellular cancer cells (N1S1) to a similar extent as the A₃R reference agonist CI-IB-MECA, which has nanomolar affinity at the receptor. These effects may be mediated by the A₃R which is highly expressed in N1S1 cells and probably also in LNCaP cells [12, 26]. The potency of IPA in this assay seems somewhat lower in the rat cell line than in the human cell line; this would be in line with the difference in affinity of IPA towards the rat and human A₃R. At 10 μM, IPA is significantly more active than CI-IB-MECA in both cell lines.

The A₃R antagonist MRS1523 was able to completely inhibit the effect of low concentrations of IPA (10 and 100 nM) but not higher concentrations (1 and 10 μM). The affinity of MRS1523 for the hA₃R is approximately 6-fold higher compared to IPA [27]. Therefore, it may be that the antiproliferative effect of higher concentrations of IPA is not mediated by the A₃R, but by a different mechanism. A₃R-independent antiproliferative effects of high concentrations of A₃R agonists, including CI-IB-MECA and IB-MECA, have previously been observed [28-30]. For example, 30 μM CI-IB-MECA inhibited growth of leukemia cell lines HL-60 and MOLT-4 in the presence of antagonists MRS1523 (10 μM) or MRS1220 (5 μM) [28]. Furthermore, CI-IB-MECA and IB-MECA, but also adenosine, 2-chloro-adenosine and 3'-deoxyadenosine had antiproliferative effects on breast cancer cell lines lacking A₃R mRNA [29]. Involvement of other adenosine receptor subtypes was excluded. Suggested pathways for these effects include downregulation of the estrogen receptor α [29], upregulation of death receptor Fas [28] and downregulation of cyclins D1 and E2 together with dephosphorylation of ERK1/2 [30]. It should be noted that the concentrations used in these reports are between 10 and 100 μM (even 500 μM for adenosine). The A₃R-independent effect of IPA seems to occur even at 1 μM, so it may be a significantly more potent anti-cancer agent than the other agonists that have been assessed. At 10 and 100 nM IPA, the antiproliferative effect was entirely blocked by antagonist MRS1523, so at these low IPA concentrations the effect seems entirely A₃R-dependent. Plasma concentrations of IPA were not reported in the pilot clinical trial on leukemia patients [24, 31]. However, they

are expected to be very low since IPA is metabolized very fast [31]. After IPA enters the circulation, more than 50% is excreted in urine in the first 4 hours, mostly in metabolized form. Metabolites include N⁶-(3-methyl-hydroxybutylamino)purine, hypoxanthine, adenine and several N⁶-alkylated adenines and N-alkylated xanthines [31].

Origin and levels of endogenously occurring IPA

IPA and zeatin riboside are widely studied plant cytokinins controlling various processes in plant growth and development [32-33]. The nucleosides are also components of tRNA, but only IPA has been found in mammalian tRNA [33-34]. Free IPA is probably present in the mammalian cytoplasm, since its precursor N⁶-(2-isopentenyl)adenosine-3-monophosphate has been identified in several cell lines [35]. Unchanged IPA has been detected in human urine at an average amount of 50 µg per day [36]. Although levels of IPA and zeatin riboside in grains, vegetables and fruits at the moment of consumption could not readily be extracted from the literature, these foods might be exogenous sources of these compounds. Tuberos roots, such as (sweet) potatoes, seem especially likely sources [37-38]. Furthermore, ingested or residential bacteria in the gut may provide additional cytokinins [33]. In plants, free zeatin riboside is in the trans conformation, but tRNA is a source of cis-zeatin riboside. Most likely, the cis-isomer has the higher affinity for the A₃R, since the trans-isomer is less active than the racemate (table 1). However, cis-zeatin riboside is not commercially available and was therefore not tested in our study. Colocalization and thus interaction of the proposed nucleoside ligands and the A₃R may occur at the cellular membrane or, keeping in mind that A₃R has a nuclear localization signal, in or at the nucleus [39]. A₃R localized in colon could interact with IPA and zeatin riboside from exogenous sources [40].

Detection of endogenous IPA

We have previously reported that an unknown A₃R agonist with anti-tumor properties is excreted by muscle cells [13]. Since IPA activates A₃R and has anti-proliferative properties, the compound might qualify as this unknown factor. We used HPLC to investigate its presence in commercial cow, pork and chicken meat, but detected no IPA. Since the freshness and storage conditions of commercial meat may not be ideal, we then switched to fresh rat muscle tissue. Again, IPA could not be detected, nor did we detect peaks of IPA metabolites. It is therefore unlikely that IPA is the muscle-derived A₃R agonist we observed previously [13].

Conclusion

In conclusion, we have shown that both IPA and zeatin riboside bind selectively to the A₃R. Moreover, we provide evidence that the antiproliferative effect of low concentrations of IPA is mediated by the A₃R. Higher concentrations of the compound seem to have a potent A₃R-independent antitumor effect. It is however unlikely that IPA is the previously reported muscle-derived antiproliferative A₃R agonist, since it could not be detected in rat muscle. More research is needed to elucidate the putative physiological roles of IPA and the importance of the A₃R for the antitumor action of IPA.

Acknowledgement

Financial support by the Dutch Top Institute Pharma (project D1-105) is gratefully acknowledged.

References

1. Zhou, Q.Y., et al., *Molecular cloning and characterization of an adenosine receptor - the A₃ adenosine receptor*. Proc Natl Acad Sci U S A, 1992. **89**(16): p. 7432-7436.
2. Sajjadi, F.G. and G.S. Firestein, *cDNA cloning and sequence-analysis of the human A₃ adenosine receptor*. Biochim Biophys Acta, 1993. **1179**(1): p. 105-107.
3. Salvatore, C.A., et al., *Molecular cloning and characterization of the human A₃ adenosine receptor*. Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10365-10369.
4. Fishman, P., et al., *Adenosine receptors and cancer*. Handb Exp Pharmacol, 2009(193): p. 399-441.
5. Gessi, S., et al., *Elevated expression of A3 adenosine receptors in human colorectal cancer is reflected in peripheral blood cells*. Clin Cancer Res, 2004. **10**(17): p. 5895-901.
6. Merighi, S., et al., *A3 adenosine receptor activation inhibits cell proliferation via phosphatidylinositol 3-kinase/Akt-dependent inhibition of the extracellular signal-regulated kinase 1/2 phosphorylation in A375 human melanoma cells*. J Biol Chem, 2005. **280**(20): p. 19516-26.
7. Varani, K., et al., *A receptors are overexpressed in pleura from patients with mesothelioma and reduce cell growth via Akt/nuclear factor-kappaB pathway*. Am J Respir Crit Care Med. **183**(4): p. 522-30.
8. Fishman, P., S. Bar-Yehuda, and L. Vagman, *Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth*. Cancer Res, 1998. **58**(14): p. 3181-7.
9. Fishman, P., et al., *Adenosine acts as a chemoprotective agent by stimulating G-CSF production: A role for A₁ and A₃ adenosine receptors*. J Cell Physiol, 2000. **183**(3): p. 393-398.
10. Fishman, P., et al., *The A3 adenosine receptor as a new target for cancer therapy and chemoprotection*. Exp Cell Res, 2001. **269**(2): p. 230-6.
11. van Troostenburg, A.R., et al., *Tolerability, pharmacokinetics and concentration-dependent hemodynamic effects of oral CF101, an A₃ adenosine receptor agonist, in healthy young men*. Int J Clin Pharmacol Ther, 2004. **42**(10): p. 534-542.
12. Bar-Yehuda, S., et al., *The A3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappa B signal transduction pathways*. Int J Oncol, 2008. **33**(2): p. 287-295.
13. Bar-Yehuda, S., et al., *Resistance of muscle to tumor metastases: A role for A3 adenosine receptor agonists*. Neoplasia, 2001. **3**(2): p. 125-131.
14. Lane, J.R., et al., *The endocannabinoid 2-arachidonylglycerol is a negative allosteric modulator of the human A₃ adenosine receptor*. Biochem Pharmacol, 2010. **79**(1): p. 48-56.
15. Gao, Z.-G., et al., *Differential allosteric modulation by amiloride analogues of agonist*

- and antagonist binding at A_1 and A_3 adenosine receptors. *Biochem Pharmacol*, 2003. **65**(4): p. 525-534.
16. van Veldhoven, J.P.D., et al., *A new generation of adenosine receptor antagonists: From di- to trisubstituted aminopyrimidines*. *Bioorganic & Medicinal Chemistry*, 2008. **16**(6): p. 2741-2752.
 17. Gao, Z.G., et al., *N^6 -substituted adenosine derivatives: selectivity, efficacy, and species differences at A_3 adenosine receptors*. *Biochem Pharmacol*, 2003. **65**(10): p. 1675-1684.
 18. Fredholm, B.B., et al., *Nomenclature and classification of purinoceptors*. *Pharmacol Rev*, 1994. **46**(2): p. 143-56.
 19. Yan, L., et al., *Adenosine receptor agonists: from basic medicinal chemistry to clinical development*. *Expert Opin Emerg Drugs*, 2003. **8**(2): p. 537-76.
 20. Fredholm, B.B., et al., *Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells*. *Biochemical Pharmacology*, 2001. **61**(4): p. 443-448.
 21. Schulte, G. and B.B. Fredholm, *Human adenosine $A_{1'}$, $A_{2A'}$, $A_{2B'}$ and A_3 receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2*. *Mol Pharmacol*, 2000. **58**(3): p. 477-82.
 22. Spinola, M., et al., *N^6 -isopentenyladenosine: A potential therapeutic agent for a variety of epithelial cancers*. *Int J Cancer*, 2007. **120**(12): p. 2744-2748.
 23. Suk, D., C.L. Simpson, and E. Mihich, *Toxicological and antiproliferative effects of N^6 -(delta2-isopentenyl)adenosine, a natural component of mammalian transfer RNA*. *Cancer Res*, 1970. **30**(5): p. 1429-36.
 24. Mittelman, A., J.T. Evans, and G.B. Chheda, *Cytokinins as chemotherapeutic agents*. *Ann N Y Acad Sci*, 1975. **255**(AUG8): p. 225-234.
 25. Bifulco, M., et al., *Biological and pharmacological roles of N^6 -isopentenyladenosine: an emerging anticancer drug*. *Anticancer Agents Med Chem*, 2008. **8**(2): p. 200-4.
 26. Ohana, G., et al., *Differential effect of adenosine on tumor and normal cell growth: Focus on the A_3 adenosine receptor*. *J Cell Physiol*, 2001. **186**(1): p. 19-23.
 27. Li, A.-H., et al., *Structure-activity relationships and molecular modeling of 3,5-diacyl-2,4-dialkylpyridine derivatives as selective A_3 adenosine receptor antagonists*. *J Med Chem*, 1998. **41**(17): p. 3186-3201.
 28. Kim, S.G., et al., *p53-Independent induction of Fas and apoptosis in leukemic cells by an adenosine derivative, CI-IB-MECA*. *Biochemical Pharmacology*, 2002. **63**(5): p. 871-880.
 29. Lu, J., A. Pierron, and K. Ravid, *An Adenosine Analogue, IB-MECA, Down-Regulates Estrogen Receptor and Suppresses Human Breast Cancer Cell Proliferation*. *Cancer Research*, 2003. **63**(19): p. 6413-6423.
 30. Morello, S., et al., *CI-IB-MECA inhibits human thyroid cancer cell proliferation independently of A_3 adenosine receptor activation*. *Cancer Biol Ther*, 2008. **7**(2): p. 278-84.
 31. Chheda, G.B. and A. Mittelman, *N^6 -(2-isopentenyl)adenosine metabolism in man*. *Biochem Pharmacol*, 1972. **21**(1): p. 27-37.
 32. Sakakibara, H., *Cytokinins: activity, biosynthesis, and translocation*. *Annu Rev Plant*

- Biol, 2006. **57**: p. 431-449.
33. Skoog, F. and D.J. Armstrong, *Cytokinins*. Annu Rev Plant Physiol, 1970. **21**: p. 359-384.
 34. Robins, M.J., R.H. Hall, and R. Thedford, *N⁶-(delta2-isopentenyl) adenosine. A component of transfer ribonucleic acid of yeast and of mammalian tissue methods of isolation and characterization*. Biochemistry, 1967. **6**(6): p. 1837-48.
 35. Burns, D.M., C.P. Rodi, and P.F. Agris, *Natural occurrence of an inhibitor of mammalian cell growth in human and mouse cell lines of normal and tumor origin*. Cancer Biochem Biophys, 1976. **1**(6): p. 269-280.
 36. Vold, B.S., D.E. Keith, and M. Slavik, *Urine levels of N-[9-(beta-D-ribofuranosyl) purin-6-ylcarbamoyl]-L-threonine, N⁶-(delta-2-isopentenyl)adenosine, and 2'-O-methylguanosine as determined by radioimmunoassay for normal subjects and cancer-patients*. Cancer Res, 1982. **42**(12): p. 5265-5269.
 37. Ravi, V., et al., *Molecular Physiology of Storage Root Formation and Development in Sweet Potato (Ipomoea batatas (L.) Lam.)*. J Root Crops, 2009. **35**(1): p. 1-27.
 38. Suttle, J.C., *Postharvest changes in endogenous cytokinins and cytokinin efficacy in potato tubers in relation to bud endodormancy*. Physiologia Plantarum, 1998. **103**(1): p. 59-69.
 39. Klaasse, E.C., et al., *Internalization and desensitization of adenosine receptors*. Purinergic Signalling, 2008. **4**(1): p. 21-37.
 40. Madi, L., et al., *The A₃ adenosine receptor is highly expressed in tumor versus normal cells*. Clin Cancer Res, 2004. **10**(13): p. 4472-4479.

