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ORIGINAL ARTICLE

Putative role of the adenosine A_3 receptor in the antiproliferative action of N^6-(2-isopentenyl)adenosine

Clara C. Blad · Jacobien K. von Frijtag Drabbe Künzel · Henk de Vries · Thea Mulder-Krieger · Sara Bar-Yehuda · Pnina Fishman · Adriaan P. IJzerman

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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_i values of 159 and 649 nM, respectively). These values were comparable to the observed K_i 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 Chinese hamster ovary 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 (Cl-IB-MECA) have known antitumor effects. We demonstrated strong and highly similar antiproliferative effects of IPA and Cl-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.

Keywords Adenosine A_3 receptor · N^6-(2-isopentenyl) adenosine (IPA) · Antitumor agent · Modified nucleoside · HPLC · Zeatin riboside

Introduction

Four adenosine receptors have been cloned and pharmacologically characterized: A_1, A_{2A}, A_{2B}, and A_3. Of these subtypes, the A_3R has been identified last [1]. The hA_3R 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 A_3R an interesting target for the treatment of cancer (for a review, see [4]). A recent study even showed that the threefold A_3R upregulation in human colorectal cancer was reflected, via an unknown mechanism, in peripheral blood cells [5]. This could make the A_3R a promising biomarker for this and possibly other types of cancer. We and others have previously shown that A_3R agonists have antiproliferative effects on tumor cells in vitro and in vivo, characterized by induction of G_0/G_1 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

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C. C. Blad · J. K. von Frijtag Drabbe Künzel · H. de Vries · T. Mulder-Krieger · A. P. IJzerman (✉)
Division of Medicinal Chemistry,
Leiden/Amsterdam Center for Drug Research,
P.O. Box 9502, 2300 RA Leiden, the Netherlands
e-mail: ijzerman@lacdr.leidenuniv.nl

C. C. Blad
e-mail: bladcc@chem.leidenuniv.nl

S. Bar-Yehuda · P. Fishman
Can-Fite BioPharma,
P.O. Box 7537, Petah-Tikva 49170, Israel

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, A_3R 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 A_3R upregulation may precede asbestos-induced malignant mesothelioma formation [7]. The A_3R agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide (Cl-IB-MECA) prevented TNF-α-mediated cell survival after asbestos exposure in vitro by acting on the deregulated Akt/NF-κB pathways, showing that A_3R is a possible target for cancer prevention as well as treatment. Interestingly, A_3R 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 A_3R agonist IB-MECA has been evaluated in phase I clinical studies [11]. This agonist was tolerated very well. Related A_3R agonist Cl-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* A_3R 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 A_3R activation. However, they could not be attributed to adenosine, as the effect was not sensitive to adenosine deaminase (ADA) and could not be reproduced by administration of adenosine alone [13]. Thus, we set out to identify other endogenous agonists for the A_3R.

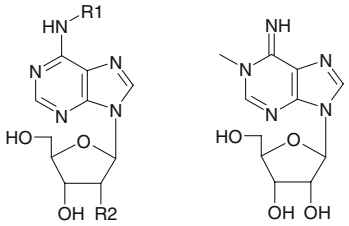
In the current work, we report the affinity of a panel of naturally occurring nucleosides for the human adenosine receptors, while focusing on the hA_3R. Then, we assessed the potency of the two higher affinity compounds, the nucleosides IPA and racemic zeatin riboside, in cAMP generation assays. The effect of IPA and the reference A_3R agonist Cl-IB-MECA on tumor cell line proliferation was also determined. We provide evidence that the antiproliferative effects of IPA are indeed linked to the A_3R as they can be partially blocked by a selective antagonist for that receptor. We also present an HPLC method to investigate the IPA content of muscle tissue. However, endogenous IPA could not be detected in our analysis.

Materials and methods

Materials

[^{3}H]-DPCPX and [^{125}I]-AB-MECA were purchased from Amersham Biosciences (Roosendaal, the Netherlands). [^{3}H]-ZM241385 and [^{3}H]-MRS1754 were obtained from Tocris Cookson, Ltd. (Bristol, UK). All nucleosides listed in Table 1 were supplied by Sigma-Aldrich (Zwijndrecht,

Table 1 Chemical structures of naturally occurring modified nucleosides and their effects in radioligand binding studies at human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors



1 2

	Name	R1	R2	% displacement			K_i or % displacement
				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)	$-CH_2CHC(CH_3)_2$	-OH	14	0	0	159 ± 19 nM
1c	*Trans*-zeatin riboside	$-CH_2CHC(CH_3)CH_2OH$	-OH	2	0	6	31%
1d	Racemic zeatin riboside	$-CH_2CHC(CH_3)CH_2OH$	-OH	17	0	0	643 ± 27 nM
1e	2′-O-methyladenosine	-H	$-OCH_3$	0	0	8	0%
2	1-methyl-adenosine	-	-	2	0	4	34%

Percent displacement at 1 μM (N=2) or K_i value (N=3) is shown; data are means (±SEM) of N experiments performed in duplicate

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 A_1 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 hA_3R 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, VA, 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 A_1 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% CO_2. HEK 293 cells stably expressing either the human A_{2A} adenosine receptor or the hA_3R were grown in DMEM containing 10% newborn calf serum, streptomycin (50 μg/ml), penicillin (50 U/ml), and G418 (0.5 mg/ml) at 37°C and 7% CO_2. 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 A_3R, where no ADA was included.

Human adenosine A_1 receptor

Affinity for the human A_1 receptor was determined on membranes from CHO cells expressing the human receptors, using [^{3}H]-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 [^{3}H]-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 incubation, radioactivity was counted in a PerkinElmer 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 [^{3}H]-ZM241385 as the radioligand. Membranes containing 30 μg of protein were incubated in a total volume of 200 μl of 50 mM Tris/HCl (pH 7.4) and [^{3}H]-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 μM CGS21680. Filtration and counting were performed as described for the A_1 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 [^{3}H]-MRS1754 as the radioligand. Membranes containing 20 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 0.01 *w/v* % CHAPS (pH 8.26 at 5°C), and [^{3}H]-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 A_1 receptor.

Human A_3R

The affinity at the hA_3R was measured on membranes from HEK293 cells stably expressing this receptor, using [^{125}I]-AB-MECA as the radioligand. Membranes containing 35 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/ HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.01% *w/v* CHAPS (pH 8.26 at 5°C), and [^{125}I]-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 μM 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 PerkinElmer Wallac 1470 Wizard gamma-counter.

Rat A_3R

The affinity at the rat A_3R was measured on membranes from RBL-2H3 (rat basophilic leukemia) cells endogenously expressing this receptor, using [^{125}I]-AB-MECA as the radioligand. Membranes of these cells were prepared as

described previously [15]. Membranes containing 60 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/ HCl, 10 mM $MgCl_2$ (pH 7.7 at 22°C), and [^{125}I]-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 μM R-PIA. Filtration and counting were performed as described for the hA_3R.

Second messenger studies: cAMP production in cells expressing the hA_3R

CHO cells expressing the hA_3R were grown overnight as a monolayer in 24-well tissue culture plates (400 μl/well; 2×10^5 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% CO_2. 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 [^{3}H]-cAMP for protein kinase A (PKA). Briefly, the sample, approximately 1.8 nM [^{3}H]-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 PerkinElmer 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 hA_3-CHO cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5,000 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 3 h. 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 U/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% CO_2 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 Cl-IB-MECA and IPA (0.01, 0.1, 1, and 10 μM). A 24-h [^{3}H]-thymidine incorporation assay was used to evaluate cell growth, except for the experiments evaluating the effect of EHNA (10 μM), which lasted 48 h. For the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]-thymidine. Cells were harvested, and the [^{3}H]-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 A_3R antagonist MRS1523

The effect of IPA on the proliferation of LNCaP cells in the presence of the A_3R 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-h [^{3}H]-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 g of tissue was weighed exactly and cut into small pieces of about 5×5×5 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 four freeze–thaw cycles in liquid nitrogen and a 60°C water bath. The sample was centrifuged at 6,000×*g* at 4°C for 30 min, and the supernatant was additionally centrifuged at 200,000×*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 of 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 of 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), 125×4.6 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_1, A_{2A}, and A_{2B} receptors could not be assessed in this experimental setup 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 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.

Affinity of IPA for the rat A_3R

The affinity of IPA for the rat A_3R was also determined in radioligand binding studies (Fig. 1). A K_i value of 4.69± 0.20 μM was found for the rat ortholog, so the affinity of IPA for the rat A_3R was lower than for the hA_3R.

Effect of IPA on the cAMP production in cells expressing the hA_3R

The potency of adenosine, IPA, and racemic zeatin riboside to modulate cAMP production was determined in intact CHO cells stably expressing the hA_3R (Table 2). Adenosine and IPA inhibited forskolin-stimulated cAMP accumulation with similar potencies in the low micromolar range and virtually identical intrinsic activities.

In a second assay, the effects of ADA and the selective hA_3R antagonist VUF5574 were investigated (Fig. 2). The selective A_3R agonist Cl-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 Cl-IB-MECA had a more potent effect. Inclusion of ADA abolished the effect of adenosine, whereas IPA and Cl-IB-MECA were insensitive to the enzyme. Modulation of the second messenger

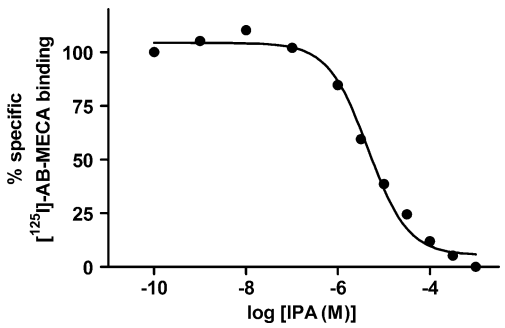


Fig. 1 Competition binding experiment on RBL-2H3 cell membranes endogenously expressing the rat A_3R. IPA displaced [^{125}I]-AB-MECA from the rat A_3R with a K_i value of 4.69±0.20 μM (N=3). Data from one representative experiment are shown. Incubation was 1 h at 37°C in 50 mM Tris/ HCl, 10 mM $MgCl_2$ (pH 7.7)

Table 2 Potency of naturally occurring nucleosides on the human adenosine A_3 receptor, as inhibitors of forskolin-stimulated cAMP production in CHO cells expressing the hA_3R

	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

EC_{50} value (N=4, except zeatin riboside N=3) is shown; data are means (±SEM) of N experiments performed in quadruplicate

pathway by each of the agonists was largely and significantly inhibited by the antagonist VUF5574.

Effect of IPA on the proliferation of tumor cell lines

In view of the reported antitumor 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 Cl-IB-MECA (Figs. 3 and 4). At 10 μM, IPA even had a significantly greater effect than Cl-IB-MECA on both cell lines (P< 0.01). An approximate EC_{50} value of 1 μM 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 μM, had only a modest effect on N1S1

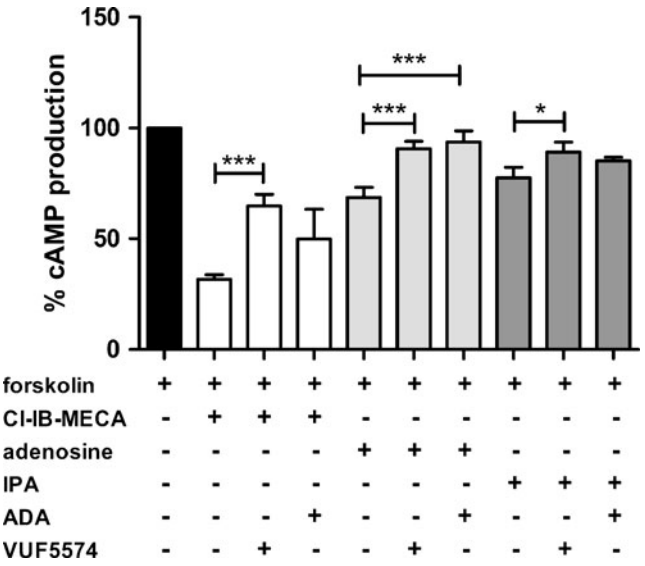


Fig. 2 Effect of ADA and A_3R antagonist VUF5574 in cAMP accumulation assays. In CHO cells stably expressed with hA_3R, forskolin-stimulated cAMP production was inhibited by adenosine, IPA, and Cl-IB-MECA, all at 10 μM. The effects of the concomitant presence of either the enzyme ADA (0.8 IU/ml) or the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

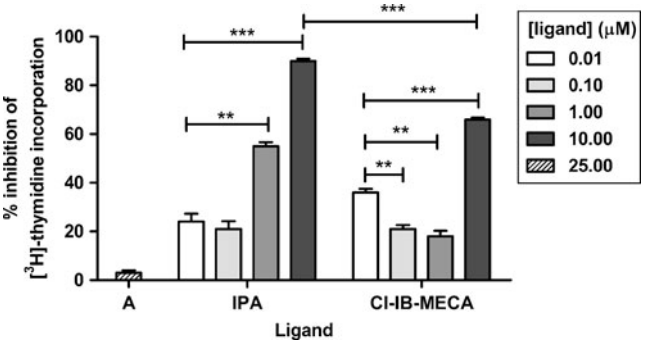


Fig. 3 IPA and Cl-IB-MECA have similar antiproliferative effects on LNCaP tumor cells. IPA and Cl-IB-MECA (0.01–10 μM) both inhibited proliferation of the human prostate carcinoma cell line LNCaP. Adenosine (**A**) (25 μM) had virtually no effect. IPA and Cl-IB-MECA had significantly larger antiproliferative 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 Cl-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 Cl-IB-MECA (P<0.001). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24 h with the compound of interest. For the last 18 h, 1 μCi of [^{3}H]-thymidine was included in the buffer. Data are means (±SEM) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t test. **P<0.01; ***P<0.001

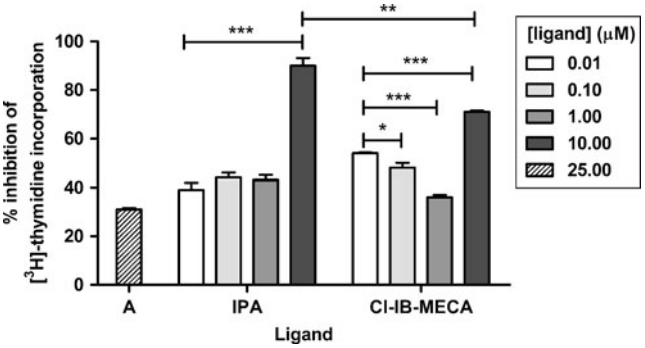


Fig. 4 IPA and Cl-IB-MECA have similar antiproliferative effects on N1S1 tumor cells. IPA and Cl-IB-MECA (0.01–10 μM) both inhibited proliferation of the rat hepatocellular carcinoma cell line N1S1. Adenosine (**A**) (25 μM) had only a modest effect. IPA and Cl-IB-MECA had significantly larger antiproliferative effects than adenosine (P<0.01), with the exception of 0.01 μM IPA and 1 μM Cl-IB-MECA. The effect of IPA increased significantly at 10 μM compared to the other concentrations (P<0.001). The effect of Cl-IB-MECA showed a significant decrease from 0.01 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 Cl-IB-MECA (P<0.01). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24 h with the compound of interest. For the last 18 h, 1 μCi of [^{3}H]-thymidine was included in the buffer. Data are means (±SEM) 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

proliferation and no effect on the LNCaP cell line. Addition of ADA inhibitor EHNA (10 μM) 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 (see Online resource 1). Addition of EHNA alone, without 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 (Fig. 5). The antagonist (at 0.1 and 1 μM) prevented the antiproliferative effect of 0.01 and 0.1 μM IPA. When IPA was added at 1 μM, the inhibition by MRS1523 was small, although still significant at higher concentrations. At 10 μM 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 tissue. We therefore analyzed extracts from Wistar rat muscle. The spectra of native and spiked tissue extracts are shown in Figs. 6a, b, respectively. From the spiked tissue, 23% of the added IPA was recovered in the extract. In the unspiked spectrum, no IPA peak was observed.

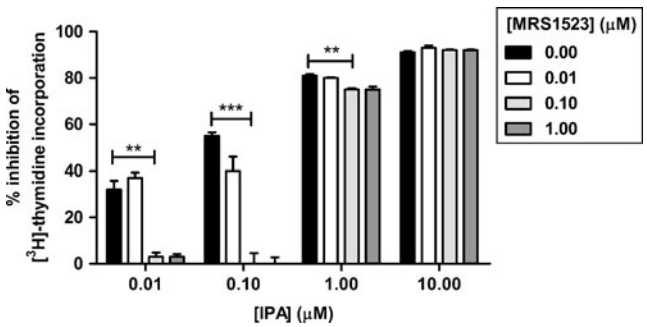


Fig. 5 The antiproliferative effect of IPA is partly blocked by A_3R antagonist MRS1523. The selective A_3R antagonist MRS1523 (0.01–1 μM) blocked the effect of low concentrations (0.01–0.1 μM) of IPA on human prostate carcinoma (LNCaP) cells. MRS1523 did not inhibit the effect of high concentrations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

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 ortholog 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 homologs 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 two- or threefold 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 A_1 and A_{2A} receptors [18]. In contrast, several more recent publications report similar potencies of adenosine towards the A_1, A_{2B}, and A_3 subtypes, in the submicromolar range [19–21]. The affinity and potency that we observed for A_3R 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 EC_{50} values of tested agonists.

Antiproliferative 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 20 [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 antitumor activity of IPA has not been elucidated so far.

In view of the well-known role of the A_3R 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 A_3R reference agonist Cl-IB-MECA, which has nanomolar affinity at the receptor. These effects may be mediated by the A_3R 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

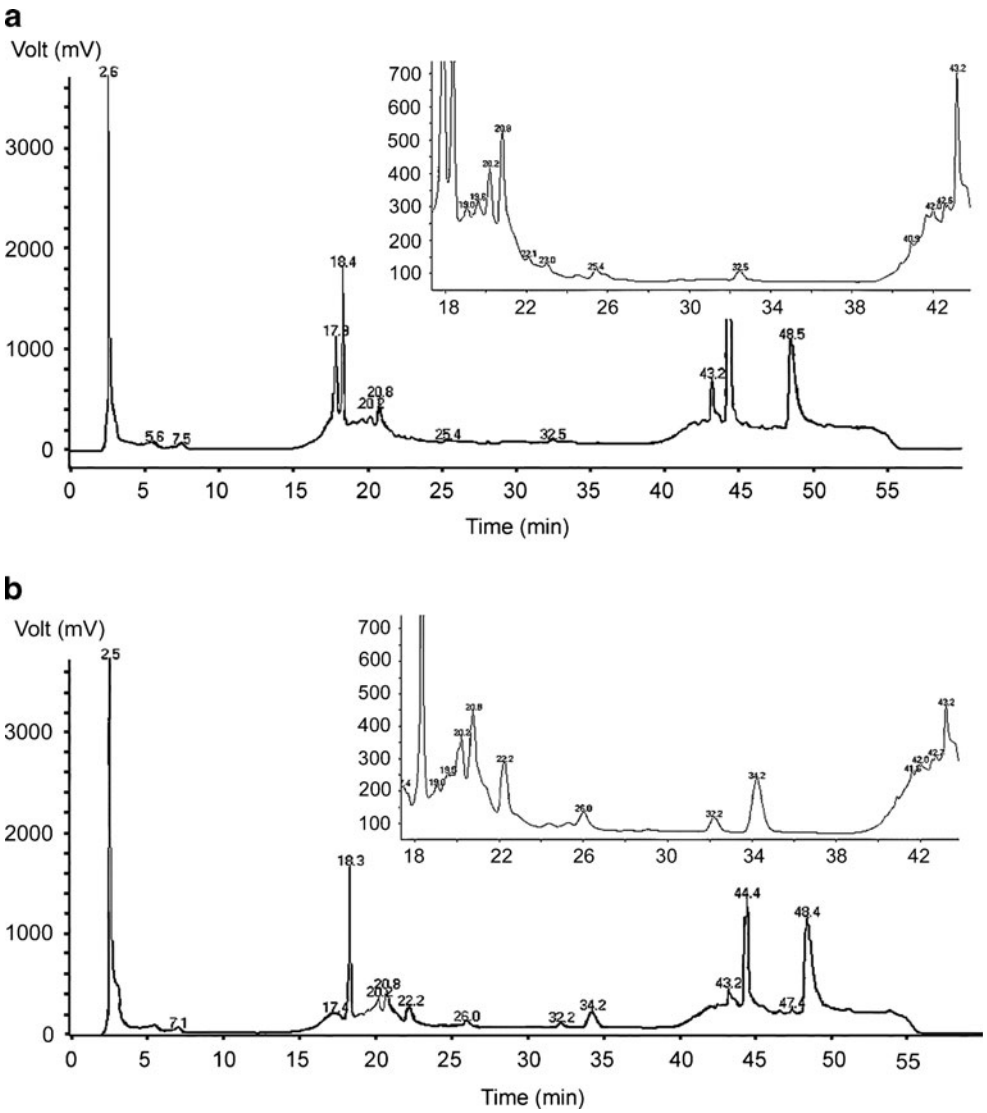


Fig. 6 HPLC analysis of rat muscle extract detects no IPA. Comparison of rat muscle tissue spiked with IPA (**b**) and unspiked muscle extract (**a**) tissue shows that IPA, which is eluted at 34.2 min in the spiked tissue, cannot be detected in the blank tissue

human A_3R. At 10 μM, IPA is significantly more active than Cl-IB-MECA in both cell lines.

The A_3R 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 hA_3R is approximately sixfold higher compared to IPA [27]. Therefore, it may be that the antiproliferative effect of higher concentrations of IPA is not mediated by the A_3R but by a different mechanism. A_3R-independent antiproliferative effects of high concentrations of A_3R agonists, including Cl-IB-MECA and IB-MECA, have previously been observed [28–30]. For example, 30 μM Cl-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, Cl-IB-MECA and IB-MECA, but also adenosine, 2-chloroadenosine, and 3′-deoxyadenosine, had antiproliferative effects on breast cancer cell lines lacking A_3R 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 A_3R-independent effect of IPA seems to occur even at 1 μM, so it may be a significantly more potent anticancer 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 A_3R 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 h, mostly in metabolized form.

Metabolites include N^6-(3-methyl-hydroxybutylamino)purine, hypoxanthine, adenine, and several N^6-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 N^6-(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. Tuberous 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 A_3R, 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 A_3R may occur at the cellular membrane or, keeping in mind that A_3R has a nuclear localization signal, in or at the nucleus [39]. A_3R 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 A_3R agonist with antitumor properties is excreted by muscle cells [13]. Since IPA activates A_3R and has antiproliferative 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 A_3R agonist we observed previously [13].

Conclusion

In conclusion, we have shown that both IPA and zeatin riboside bind selectively to the A_3R. Moreover, we provide evidence that the antiproliferative effect of low concentrations of IPA is mediated by the A_3R. Higher concentrations of the compound seem to have a potent A_3R-independent antitumor effect. It is, however, unlikely that IPA is the previously reported muscle-derived antiproliferative A_3R 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 A_3R for the antitumor action of IPA.

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