

Antiproliferative effects of selective adenosine receptor agonists and antagonists on human lymphocytes: evidence for receptor-independent mechanisms

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Received: 3 December 2012 / Accepted: 16 January 2013 / Published online: 29 January 2013
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Abstract The effects of standard adenosine receptor (AR) agonists and antagonists on the proliferation of human T lymphocytes, unstimulated and phytohemagglutinin-stimulated human peripheral blood lymphocytes (PBL), and Jurkat T cells were investigated. Real-time PCR measurements confirmed the presence of all four AR subtypes on the investigated cells, although at different expression levels. A_{2A} ARs were predominantly expressed in PBL and further upregulated upon stimulation, while malignant Jurkat T cells showed high expression levels of A₁, A_{2A}, and A_{2B} ARs. Cell proliferation was measured by [³H]-thymidine incorporation assays. Several ligands, including the subtype-selective agonists CPA (A₁), BAY60-6583 (A_{2B}), and IB-MECA (A₃), and the antagonists PSB-36 (A₁), MSX-2 (A_{2A}), and PSB-10 (A₃) significantly inhibited cell proliferation at micromolar concentrations, which were about three orders of magnitude higher than their AR affinities. In contrast, further investigated AR ligands, including the agonists NECA (nonselective) and CGS21680 (A_{2A}), and the antagonists preladenant (SCH-420814, A_{2A}), PSB-1115 (A_{2B}), and PSB-603 (A_{2B}) showed no or only minor effects on lymphocyte proliferation. The anti-proliferative effects of the AR

agonists could not be blocked by the corresponding antagonists. The non-selective AR antagonist caffeine stimulated phytohemagglutinin-activated PBL with an EC₅₀ value of 104 μM. This is the first study to compare a complete set of commonly used AR ligands for all subtypes on lymphocyte proliferation. Our results strongly suggest that these compounds induce an inhibition of lymphocyte proliferation and cell death through AR-independent mechanisms.

Keywords Adenosine receptors · Real-time PCR · Human lymphocytes · Jurkat T cells · Proliferation · [³H]Thymidine incorporation · Receptor-independent mechanisms

Abbreviations

ADA	Adenosine deaminase
AR	Adenosine receptor
BAY60-6583	2-[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide
cAMP	Cyclic AMP
CGS-21680	(2- <i>p</i> -[2-Carboxyethyl]phenethylamino)-5'- <i>N</i> -ethylcarboxamido-adenosine
CI-IB-MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-9-[5-(methyl-carbamoyl)-β-d-ribofuranosyl]adenine
CPA	<i>N</i> ⁶ -Cyclopentyladenosine
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GPCR(s)	G protein-coupled receptor(s)
IB-MECA	<i>N</i> ⁶ -(3-Iodobenzyl)-5'- <i>N</i> -methylcarboxamidoadenosine

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MSX-2	3-(3-Hydroxypropyl)-7-methyl-8-(<i>m</i> -methoxystyryl)-1-propargylxanthine
NECA	5'- <i>N</i> -Ethylcarboxamidoadenosine
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PSB-10	(<i>R</i>)-8-Ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-4,5,7,8-tetrahydro-1 <i>H</i> -imidazo[2,1- <i>i</i>]purin-5-one
PSB-36	1-Butyl-8-(3-noradamantanyl)-3-(3-hydroxypropyl)xanthine
PSB-603	8-[4-[4-(4-Chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine
PSB-1115	1-Propyl-8- <i>p</i> -sulfophenylxanthine
SCID	Severe combined immunodeficiency
SCH420814	Preladenant
TNF	Tumor necrosis factor

Introduction

The endogenous nucleoside adenosine regulates many physiological processes through the activation of four specific cell surface receptors, referred to as A_1 , A_{2A} , A_{2B} , and A_3 [1]. They belong to the family of G protein-coupled receptors characterized by seven transmembrane-spanning helical domains (seven TM receptors) [1]. While adenosine A_{2A} and A_{2B} receptors interact with G_s proteins to stimulate adenylyl cyclase activity resulting in an increase in intracellular cAMP levels, A_1 and A_3 receptors are coupled to G_i proteins inhibiting adenylyl cyclase activity and, therefore, cAMP production. Coupling to further second messenger systems has been described, e.g., stimulation of phospholipase C (A_1 , A_{2B} , A_3), activation of potassium and inhibition of calcium channels (A_1), and mobilization of intracellular calcium by an as yet unknown mechanism (A_{2B}) [1, 2]. Adenosine inhibits the release of excitatory neurotransmitters in the brain and leads to sedation, whereas in the periphery it shows negative inotropic, chronotropic, dromotropic, antihypertensive, and antidiuretic effects [3]. Adenosine receptors (ARs) are also found on cells of the immune system and have been recognized to play an important role in inflammatory conditions [4–7], in which adenosine can act as an anti-inflammatory and immunosuppressive mediator mainly via the activation of A_{2A} receptors. It inhibits TNF- α production and has been implicated as a physiological signal in the apoptotic deletion of T cells during intrathymic cell selection, a process which functions to prevent autoimmunity [8–11]. The effects of adenosine on lymphocytes are of particular interest since the accumulation of extracellular and intracellular adenosine and 2'-deoxyadenosine in the absence of adenosine deaminase (ADA) activity is lymphotoxic and causes

SCID [12, 13], which is characterized by a loss of function and depletion of T and B lymphocytes [14]. Part of this effect may be attributed to adenosine via the activation of ARs and sustained increase in cAMP levels [5]. Besides the activation of extracellular receptors [11, 15–17], direct intracellular toxicity (also by adenosine metabolites) has been described [18–21]. Furthermore, adenosine is able to stimulate cell growth and proliferation and/or to induce apoptosis in various normal and tumor cell lines. Proliferative or anti-proliferative effects appear to be dependent on the cell type, the distribution of adenosine receptor subtypes, and their expression on the cell surface [22–25]. A_3 adenosine receptors seem to play an important role [26–28], but recent evidence accumulates that A_{2B} ARs can be upregulated on malignant cells and are involved in the control of tumor growth and development [16, 24, 29–32]. In contrast to these findings, adenosine and various related AR agonists as well as antagonists can trigger apoptosis by receptor-independent mechanisms that require the entry of the compounds into the cells. A prominent representative is 2-chloro-2'-deoxyadenosine (cladribine) which is applied as a chemotherapeutic anticancer agent for the treatment of chronic lymphoid malignancies [10, 33, 34]. Further examples include 2-chloroadenosine (nonselective AR agonist) [10, 33], 1,3-dipropyl-8-cyclopentylxanthine (A_1 AR antagonist) [35], and 2-chloro- N^6 -(3-iodobenzyl)adenosine-5'- N -methylcarboxamide (A_3 AR agonist) [36].

In the present study, we investigated the effects of standard adenosine receptor agonists and antagonists on the proliferation of human T lymphocytes. Initially, we evaluated the expression of adenosine receptor subtypes in unstimulated and phytohemagglutinin (PHA)-stimulated human peripheral blood lymphocytes (PBL) and Jurkat T cells, a human lymphocytic leukemia cell line, using real-time PCR. Subsequently, the effects of various nonselective and subtype-selective AR agonists as well as antagonists on the proliferation of these cells were determined. The rate of incorporation of [6 - 3 H]-thymidine into DNA during the S-phase of the cell cycle was used as an indicator for the rate of cell proliferation. We provide evidence for an inhibitory effect of several AR agonists as well as antagonists on the proliferation of human lymphocytes through AR-independent mechanisms.

Materials and methods

Chemicals

DMSO and ADA were obtained from Fluka (St. Gallen, Switzerland), [3 H]thymidine (20 Ci/mmol) was from Hartmann Analytic (Braunschweig, Germany), EDTA was from Roth (Karlsruhe, Germany), 5'-*N*-ethylcarboxamidoadenosine (NECA) from Applichem (Darmstadt, Germany), CGS21680 from Tocris (Ellisville, USA), and Pancoll from

PANbiotech (Aidenbach, Germany). BAY60-6583 was kindly provided by Dr. Thomas Krahn, Bayer-Schering Pharma, Wuppertal, Germany. All other chemical reagents, cell culture materials, and AR ligands were obtained from Sigma (Munich, Germany) or synthesized in our laboratories.

Lymphocyte isolation and stimulation

Lymphocytes were isolated from buffy coats kindly provided by the Blood Bank of the University Hospital of Bonn, prepared from the peripheral blood of healthy volunteers. A buffy coat-PBS/2 mM EDTA mixture (1:2) was centrifuged on Ficoll-Hypaque density gradients after the method of Böyum [37]. The mononuclear leukocyte fractions isolated were removed from the Ficoll gradients and washed in PBS (pH 7.2) containing 2 mM EDTA. The final pellet was resuspended in VLE-RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at a concentration of 3×10^6 cells ml⁻¹. Monocytes, which adhere to plastic dishes, were removed after an adherence time of 1 h at 37 °C, 5 % CO₂. The supernatant contained the resulting cell suspension of PBL, which was maintained in VLE-RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at 37 °C with 5 % CO₂ until use. Stimulation of the PBL was achieved by the addition of 10 µg/ml phytohemagglutinin (PHA-M).

Cell culture conditions

Human leukemia Jurkat T cells were grown in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/ml of penicillin and 0.1 mg/ml of streptomycin) at 37 °C with 5 % CO₂.

RNA isolation and real-time PCR

The total RNA from PBL or Jurkat T cells was isolated using the NucleoSpin[®]RNAII kit (Macherey-Nagel, Düren, Germany), and the cDNA was subsequently prepared with the iScript[™]cDNA Synthesis kit (BioRad, Munich, Germany), following the manufacturer's instructions, to give a final concentration of 12.5 ng/µl cDNA. Quantitative PCR was performed using the iQ[™]SYBR[®] Green Supermix (BioRad). A typical reaction contained 12.5 µl of the kit reaction mixture, 7.5 µl of molecular biology grade water, 0.5 µl each of 10 µM primer stocks, and 4 µl of cDNA. Standard curves were produced using diluted cDNA. Real-time PCR monitoring was performed using an iCycler IQ Real-Time PCR detection system (BioRad). The thermal cycler tracks fluorescence levels over 40 amplification cycles: 95 °C for 15 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 10 s (polymerization). A melt

curve was performed at the end of each run to verify that there was a single amplification product and a lack of primer dimers. All samples were normalized to the amount of β-actin mRNA present in the sample. The transcript levels and fold change in mRNA were determined by analyzing the standard curves and/or using the $\Delta\Delta C_T$ method as described previously [38]. Primer sets for the following genes (sense and anti-sense sequence, respectively) were used: A₁ AR (5'-TGC ACT GGC CTG TTC TGT AG-3', 5'-CTG CCT CTC CCA CGT ACA AT-3'); A_{2A} AR (5'-GGA GTT TGC CCC TTC CTA AG-3', 5'-CTG CTT CCT CAG AAC CCA AG-3'); A_{2B} AR (5'-ATC TCC AGG TAT CTT CTC-3', 5'-GTT GGC ATA ATC CAC ACA G-3'); A₃ AR (5'-CCT TCT CGC GTG TCC TGA CT-3', 5'-CTC TGA CTA CCG CCG TTG CT-3'); and human β-actin (5'-GGT GGC TTT TAG GAT GGC AAG-3', 5'-ACT GGA ACG GTG AAG GTG ACA G-3') in identical reactions.

Cell proliferation assay—³Hthymidine incorporation

Cell suspensions were distributed at 1×10^5 cells per vial in a vial containing a final volume of 1 ml (PBL) or 0.5 ml (Jurkat T) of the medium (PBL: VLE RPMI+FCS+antibiotics; Jurkat T: RPMI 1640+FCS+2 mM L-glutamine+antibiotics) and the test compounds (concentrations stated in the text). Cells were grown for 48 h, after which time 10 µl of [³H]thymidine (0.5 µCi per vial) in the medium was added and the cells were allowed to grow for a further 24 h. At the end of this period, cells were harvested through a GF/B glass-fiber filter using a Brandel cell harvester and washed several times with ice-cold water. The punched-out wet filters were transferred to mini-vials and incubated with 2.5 ml of Ready Safe[™] scintillation cocktail (Beckman Coulter) for 9 h before counting in a liquid scintillation counter. Data were analyzed using Graphpad Prism[®] (San Diego, CA, USA), version 4.0. IC₅₀/EC₅₀ values were determined by fitting the data to a sigmoidal curve with variable slope.

Results

Adenosine receptor transcripts

To confirm the presence of different AR subtypes in human lymphocytes, the mRNA expression levels of A₁AR, A_{2A}AR, A_{2B}AR, and A₃AR were determined. Real-time PCR analysis was performed with RNA isolated from human PBL, unstimulated as well as phytohemagglutinin (PHA)-stimulated, and Jurkat T cells. Primers were used to amplify specifically a fragment of the human AR cDNAs. As shown in Fig. 1, in PBL as well as in Jurkat T cells, the mRNA for all AR subtypes was detectable.

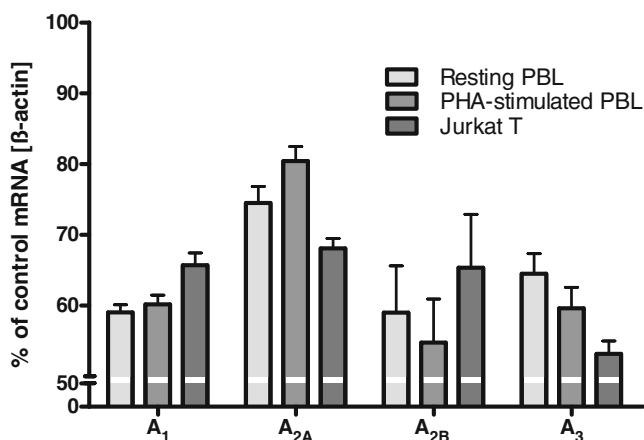


Fig. 1 Results from real-time PCR experiments determining adenosine receptor transcripts in PBL and Jurkat T cells. Data were calculated relative to the internal housekeeping gene (β -actin) and are expressed as percentage of control mRNA expression \pm SEM, $n=3$. β -Actin mRNA expression was not significantly different between the tested cells allowing a direct comparison (data not shown)

In PBL, the highest mRNA expression was found for A_{2A}ARs, which was further upregulated upon stimulation of the cells with PHA, followed by mRNA expression of A₁ARs and A₃ARs. The lowest expression levels were found for A_{2B}ARs, which—together with A₃ARs—appeared to be somewhat downregulated during stimulation. In Jurkat T cells, mRNA expression for A₁AR, A_{2A}AR, and A_{2B}AR was nearly

the same; only the expression of A₃AR was significantly lower as compared to that of the other AR subtypes.

Effects of adenosine deaminase on lymphocyte cell proliferation

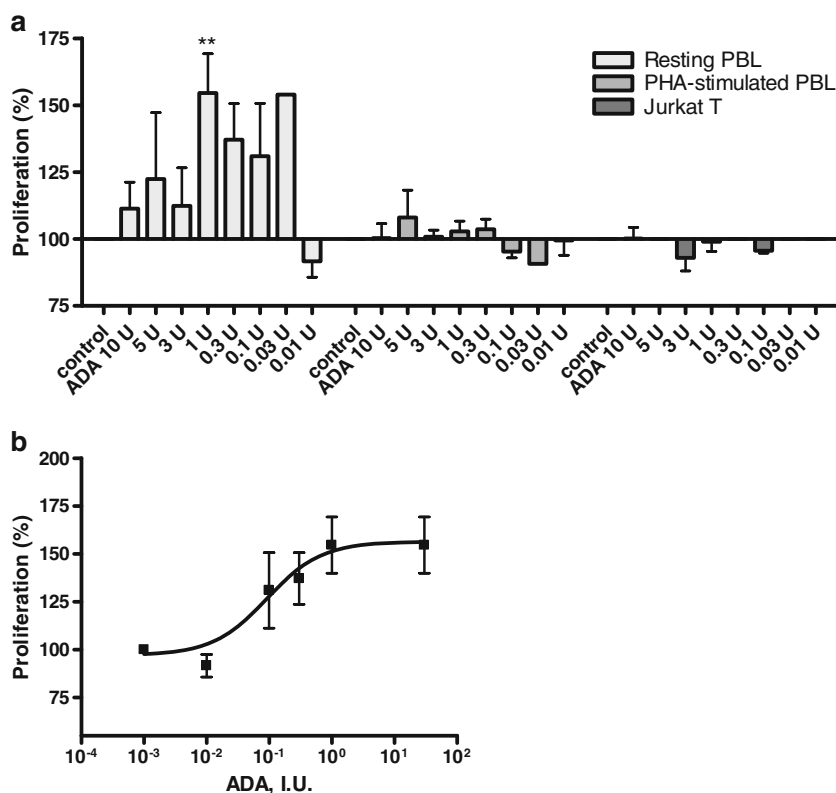
The effect of the enzyme ADA (EC 3.5.4.4), which converts adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively, on the proliferation of resting, unstimulated and PHA-stimulated PBL as well as on Jurkat T cells, was investigated. The results are shown in Fig. 2.

In the presence of ADA, resting PBL displayed higher DNA synthesis and proliferation than parallel control cultures, as measured by the incorporation of [³H]thymidine. The effect of different doses of ADA is shown in Fig. 2a. Proliferation increased up to a concentration of 1 IU/ml. An EC₅₀ value of 0.0927 IU/ml was calculated and a maximum effect of 55 \pm 15 % stimulation was observed (Fig. 2b). At higher concentrations, the proliferation of resting PBL returned to background levels. ADA had no effect on the proliferative capacity of PHA-stimulated PBL and Jurkat T cells (Fig. 2a).

Effects of adenosine receptor agonists on lymphocyte cell proliferation

As a next step, we investigated the effects of standard AR agonists on lymphocyte proliferation. The following

Fig. 2 a Effect of adenosine deaminase on [³H]thymidine incorporation in resting and PHA-stimulated PBL as well as Jurkat T cells. Cells were cultured in the presence of the indicated amounts of enzyme. Results are expressed as percentage of untreated control (=100 %) [³H]thymidine uptake. **b** Dose–response curve obtained from [³H]thymidine incorporation studies in PHA-stimulated PBL. Each bar/data point represents the mean of a minimum of three separate experiments carried out in triplicate \pm SEM



agonists were studied: NECA (non-selective), CPA (A_1), CGS21680 (A_{2A}), BAY60-6583 (A_{2B}), and N^6 -(3-iodobenzyl)-5'- N -methylcarboxamidoadenosine (IB-MECA, A_3). The structures and affinities of the compounds for the different AR subtypes are shown in Table 1.

The nonselective agonist NECA and the A_{2A} AR-selective CGS21680 showed no significant effect on the proliferation of unstimulated PBL. In contrast, the A_1 AR-selective CPA, the A_{2B} AR-selective BAY60-6583, as well as the A_3 AR-selective agonist IB-MECA appeared to show a dose-dependent inhibition of [3 H]thymidine uptake (Fig. 3a). For IB-MECA, the inhibition was statistically significant.

The effects were observed at relatively high concentrations in the micromolar range. Where possible, dose–response curves were determined and IC_{50} values were calculated: they ranged from 2.45 μ M for CPA to 17.6 μ M for IB-MECA (see Fig. 4a and Table 2). IB-MECA showed the highest maximum effect with an inhibition of the proliferation of 56 \pm 6 %. CPA and BAY60-6583 inhibited the proliferation of unstimulated PBL by 41 \pm 16 and 35 \pm 11 %, respectively (Figs. 3a and 4a and Table 2). These effects were more pronounced in PHA-

stimulated PBL: CPA, BAY60-6583, and IB-MECA showed a highly significant inhibition of the cell proliferation with a maximum effect of >95 % inhibition (CPA: 96 \pm 1 % at 250 μ M, BAY60-6583: 98 \pm 1 % at 100 μ M, IB-MECA: 96 \pm 3 % at 100 μ M; Figs. 3b and 4b). The effects were only observed at relatively high concentrations in the micromolar range (IC_{50} values: 12.7 μ M for CPA, 7.10 μ M for BAY60-6583, and 14.2 μ M for IB-MECA). NECA and CGS21680 had no significant effects on the cell proliferation of PHA-stimulated PBL at concentrations up to 100 μ M; only at a high concentration of 250 μ M were both compounds weakly inhibitory.

In Jurkat T cells, CPA and IB-MECA also showed inhibitory effects on proliferation at a concentration of 10 μ M, but not at 1 μ M (Fig. 3c).

Effects of adenosine receptor antagonists on lymphocyte cell proliferation

The following antagonists were tested in this study: caffeine (A_1 , A_{2A} , A_{2B}); PSB-36 (1-butyl-8-(3-noradamantyl)-3-(3-hydroxypropyl)xanthine, A_1); MSX-2 (3-(3-hydroxypropyl)-

Table 1 Structures of investigated adenosine receptor agonists and their affinities or potencies at human adenosine receptor subtypes (data are taken from [1])

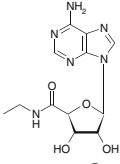
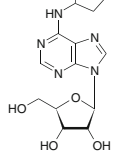
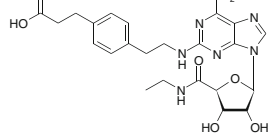
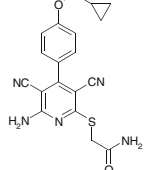
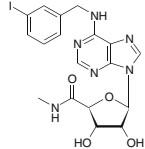
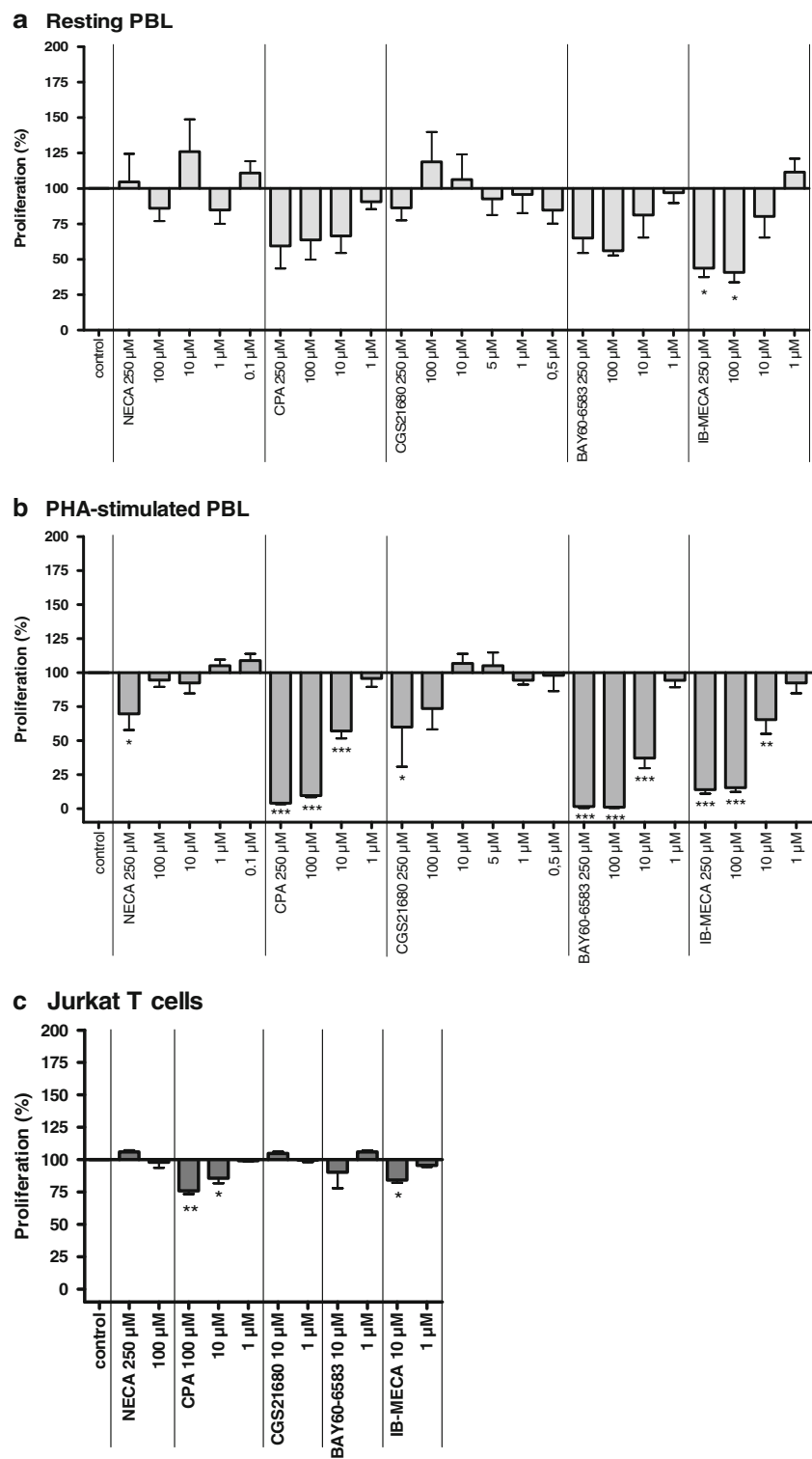
Compound	Structure	K_i (nM)			
		A_1	A_{2A}	A_{2B}	A_3
NECA		14	20	140	6.2
CPA		2.3	794	18,600	72
CGS21680		289	27	>10000	67
BAY60-6583		>10000	>10000	3-10	>10000
IB-MECA		51	2900	11000	1.8

Fig. 3 Effects of adenosine receptor agonists on [3 H]-thymidine incorporation in unstimulated PBL (**a**), PHA-stimulated PBL (**b**), and Jurkat T cells (**c**). Cells were cultured in the presence of the compounds. Results are expressed as percentage of [3 H]-thymidine uptake by untreated control cells (=100 %). Each *bar* represents the mean of a minimum of three separate experiments carried out in triplicate \pm SEM



7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine, A_{2A}); SCH420814 (preladenant, A_{2A}); PSB-1115 (1-propyl-8-*p*-sulphophenylxanthine, A_{2B}); PSB-603 (8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine, A_{2B}); and PSB-10 ((*R*)-8-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one, A_3). The

structures and affinities of the compounds for the different AR subtypes are shown in Table 3. Like AR agonists, several subtype-selective AR antagonists inhibited the proliferative capacity of human lymphocytes, as shown in Fig. 5.

The A_1 AR selective antagonist PSB-36, the A_{2A} AR selective antagonist MSX-2, and the A_3 AR selective antagonist

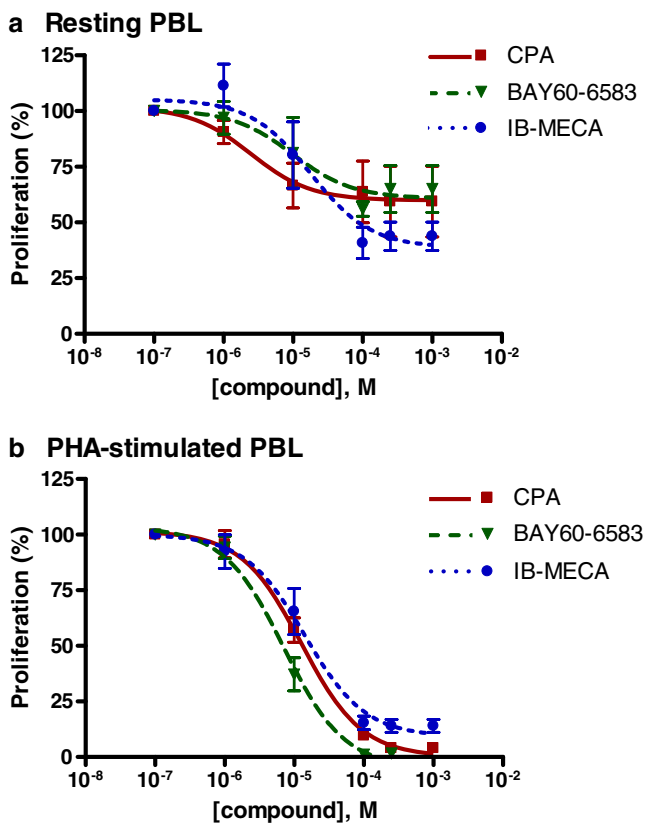


Fig. 4 Dose–response curves obtained with [^3H]thymidine incorporation studies in unstimulated PBL (**a**) and PHA-stimulated PBL (**b**). Each data point represents the mean of a minimum of three separate experiments carried out in triplicate \pm SEM (for IC_{50} values, see Table 2)

PSB-10 significantly inhibited the proliferation of unstimulated PBL in a concentration-dependent manner (Fig. 5a). Maximum effects of 80 ± 11 , 63 ± 12 , and 73 ± 11 % inhibition of cell proliferation at $250 \mu\text{M}$, respectively, were observed. The plotted dose–response curves resulted in IC_{50} values of $8.19 \mu\text{M}$ for PSB-36, $10.8 \mu\text{M}$ for MSX-2, and $4.61 \mu\text{M}$ for

PSB-10, indicating that the effects of the tested antagonists occurred at relatively high concentrations (Fig. 6a and Table 2).

Caffeine (nonselective), SCH-420814 ($\text{A}_{2\text{A}}$ -selective), and PSB-1115 and PSB-603 (both $\text{A}_{2\text{B}}$ -selective) showed no effects on the proliferation of unstimulated PBL. As observed for agonists, these results could be confirmed or were even more pronounced in PHA-stimulated PBL: PSB-36, MSX-2, and PSB-10 showed highly significant anti-proliferative properties with maximum effects and IC_{50} values of 99 ± 0 % and $7.31 \mu\text{M}$ for PSB-36, 76 ± 7 % and $5.04 \mu\text{M}$ for MSX-2, and 95 ± 1 % and $14.4 \mu\text{M}$ for PSB-10, indicating that the observed effects occurred at relatively high concentrations (Figs. 5b and 6b and Table 2). In addition, in PHA-stimulated PBL for SCH-420814 ($\text{A}_{2\text{A}}$ antagonist) and PSB-603 ($\text{A}_{2\text{B}}$ antagonist), anti-proliferative effects were observed at a concentration of $10 \mu\text{M}$. PSB-1115 had no effect either on unstimulated or on PHA-stimulated PBL. Caffeine showed an additional small but significant stimulation of already PHA-stimulated PBL, with a maximum stimulation of 26 ± 7 % and an EC_{50} value of $104 \mu\text{M}$ (Figs. 5b and 6b and Table 2).

In Jurkat T cells, the A_1 antagonist PSB-36 showed inhibitory effects on cell proliferation at $100 \mu\text{M}$ concentration and the A_3 antagonist PSB-10 at $10 \mu\text{M}$. MSX-2 exhibited the strongest inhibitory effects on the proliferative capacity of Jurkat T cells, with a maximum inhibition of 83 ± 8 % and an IC_{50} of $1.64 \mu\text{M}$ (Fig. 6c and Table 2). These results are in good agreement with the results obtained at unstimulated and PHA-stimulated PBL.

Further investigations of the anti-proliferative effects of adenosine receptor agonists and antagonists on lymphocyte cell proliferation

Additional experiments were performed to investigate whether the observed anti-proliferative effects of AR

Table 2 Anti-proliferative potency of standard adenosine receptor agonists and antagonists on peripheral blood lymphocytes and Jurkat T cell determined in [^3H]thymidine uptake assays


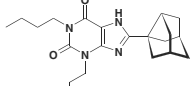
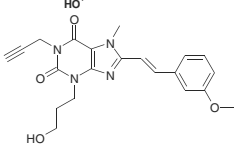
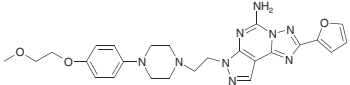
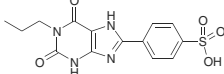
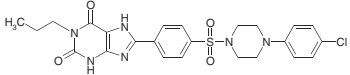
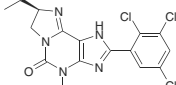
Compound	Unstimulated PBL		Stimulated PBL		Jurkat T	
	IC_{50} (μM)	% Inhibition	IC_{50} (μM)	% Inhibition ^a	IC_{50} (μM)	% Inhibition ^b
CPA	2.45 ± 2.62	41 ± 16	12.5 ± 1.2	96 ± 1	>100	25 ± 3
IB-MECA	17.6 ± 1.9	56 ± 6	14.2 ± 1.4	96 ± 3	>10	16 ± 2
BAY60-6583	9.82 ± 2.58	35 ± 11	7.10 ± 1.25	98 ± 1	>10	10 ± 12
Caffeine	>100	3 ± 7	EC_{50} : 104 ± 1	26 ± 7^c	>250	3 ± 0
MSX-2	10.8 ± 1.7	63 ± 12	5.04 ± 1.63	76 ± 7	1.64 ± 1.29	83 ± 8^a
PSB-10	4.61 ± 1.87	73 ± 11	14.4 ± 1.3	95 ± 1	>10	41 ± 11
PSB-36	8.19 ± 1.54	80 ± 11	7.31 ± 1.19	99 ± 0	~ 100	55 ± 4

^a Maximal inhibition, unless otherwise noted

^b Percent inhibition at the indicated concentration, unless otherwise noted

^c Maximal stimulation

Table 3 Structures of investigated adenosine receptor antagonists and their affinities at human adenosine receptor subtypes (data taken from [1])^a

Compound	Structure	K _i (nM)			
		A ₁	A _{2A}	A _{2B}	A ₃
Caffeine		10700	23400	38800	13300
PSB-36		0.7	980	187	2300
MSX-2		900	5.38	>10000	>10000
Preladenant (SCH420814)		>1000	0.9	>1000	>1000
PSB-1115		>10000	24000	53.4	>10000
PSB-603		>10000	>10000	0.553	>10000
PSB-10		1700	2700	>10000	0.441

^a unpublished data from our laboratory

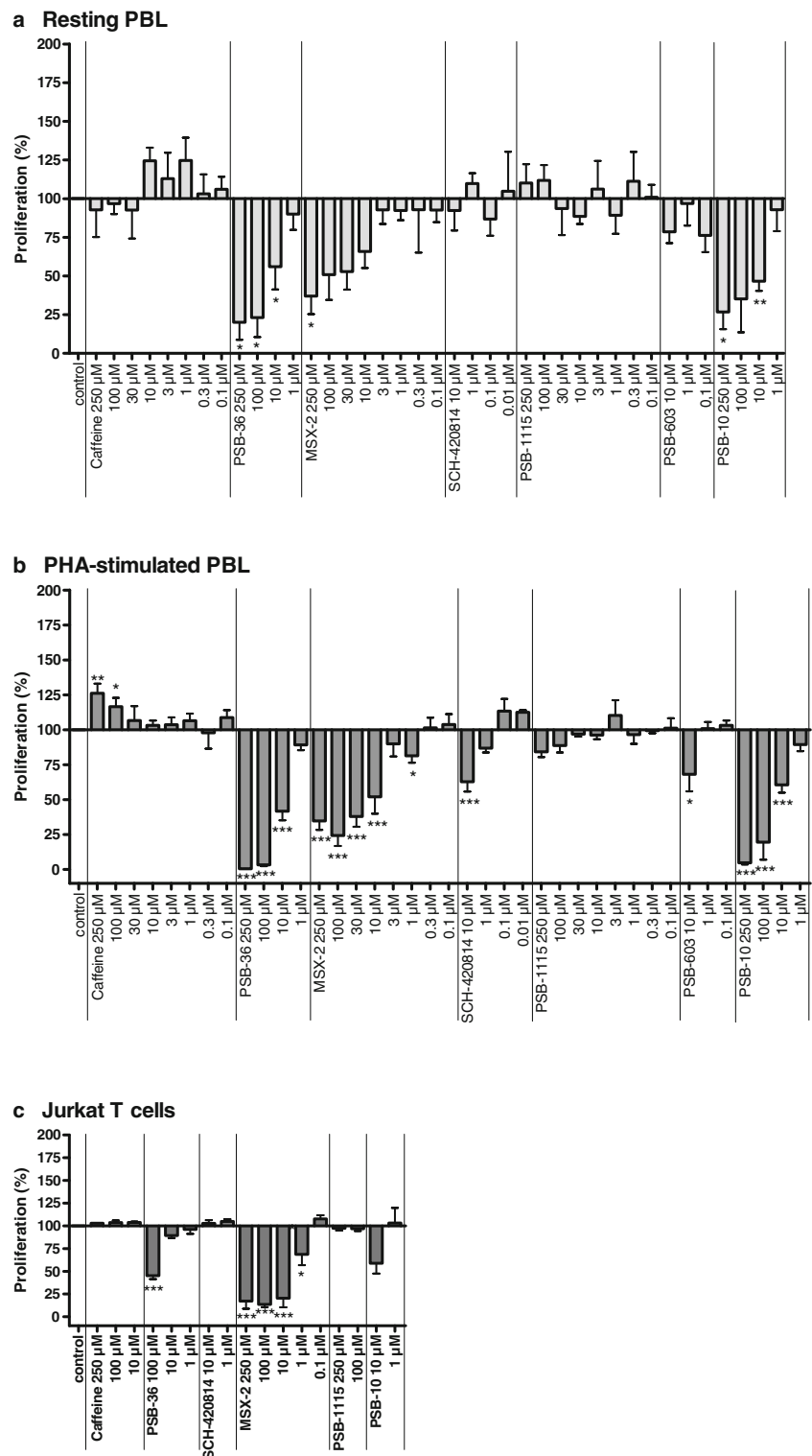
agonists and antagonists were receptor-dependent. Therefore, the effect of the co-administration of a subtype-selective AR agonist together with the respective subtype-selective AR antagonist on cell proliferation of resting and PHA-stimulated PBL was investigated. The results are shown in Fig. 7.

As observed for all AR subtypes, the co-administration of a subtype-selective agonist and the corresponding subtype-selective antagonist did not abolish the anti-proliferative effects. For example, the co-administration of PSB-36 (10 μM) together with CPA (10 μM, A₁AR) or the combination of IB-MECA (10 μM) and PSB-10 (10 μM, A₃AR), all of which had shown inhibitory effects on cell proliferation of resting as well as PHA-stimulated PBL when applied as single compounds, increased the anti-proliferative effect as compared to the effects seen upon the administration of the single compounds (Fig. 7). PSB-36 inhibited cell proliferation with a maximum effect of 79±1 % at unstimulated PBL and 78±4 % at stimulated PBL and CPA with 57±5 % (unstimulated) and 49±4 % (PHA-stimulated), while the maximum effects after co-administration of the A₁ antagonist PSB-36 and the A₁ agonist CPA amounted to 90±2 % (unstimulated) and 91±1 % (PHA-stimulated; see Fig. 7a, b). IB-MECA (A₃ agonist) inhibited cell proliferation with a maximum effect of 46±7 % (unstimulated) and 48±3 % (PHA-stimulated) and PSB-10

(A₃ antagonist) with 54±4 % (unstimulated) and 52±4 % (PHA-stimulated), while the combination of both compounds showed a maximum effect of 90±2 % (unstimulated) or 71±3 % (PHA-stimulated), respectively. The combination of MSX-2 (A_{2A} antagonist, 10 μM), exerting maximum inhibitory effects of 82±19 % (unstimulated) and 80±27 % (PHA-stimulated) when given as a single compound, in combination with CGS21680 (A_{2A} agonist, 10 μM), which alone had no effect on the proliferation of PBL, revealed maximum inhibitory effects on the cell proliferation of 79±23 % at unstimulated PBL and 82±24 % at PHA-stimulated PBL. The administration of BAY60-6583 (agonist) and PSB-1115 (antagonist), ligands of the A_{2B}AR, showed no effect on the proliferative capacity of PBL (resting and PHA-stimulated), either as single compounds or when applied in combination.

A second series of experiments was carried out to exclude the possibility that the observed inhibitory effects of the antagonists (PSB-36, MSX-2, and PSB-10) on cell proliferation were due to the upregulation of the receptors and subsequent stimulation by endogenously released adenosine. To ensure high levels of antagonists during the whole experiment, the compounds (10 μM) were added daily without changing the medium, resulting in a three times administration during the 3-day assay period. In addition, ADA

Fig. 5 Effects of adenosine receptor antagonists on [^3H] thymidine incorporation in unstimulated PBL (**a**), PHA-stimulated PBL (**b**), and Jurkat T cells (**c**). Cells were cultured in the presence of the indicated compound concentrations. Results are expressed as percentage of untreated control (=100 %) [^3H]thymidine incorporation. Each bar represents the mean of a minimum of three separate experiments carried out in triplicate \pm SEM



(1 IU/ml) was added either only once, or daily, in order to remove endogenously released adenosine.

As shown in Fig. 8, the daily administration of the antagonists further increased the inhibitory effects on the proliferation of PBL, while the co-administration of ADA did not reduce the anti-proliferative effects of these compounds.

Discussion

Human lymphocytes are responsible for the adaptive cell-mediated immune response and are therefore of particular interest as immunomodulatory targets [4, 6, 9]. Inhibition of T lymphocyte proliferation and induction of apoptosis leads

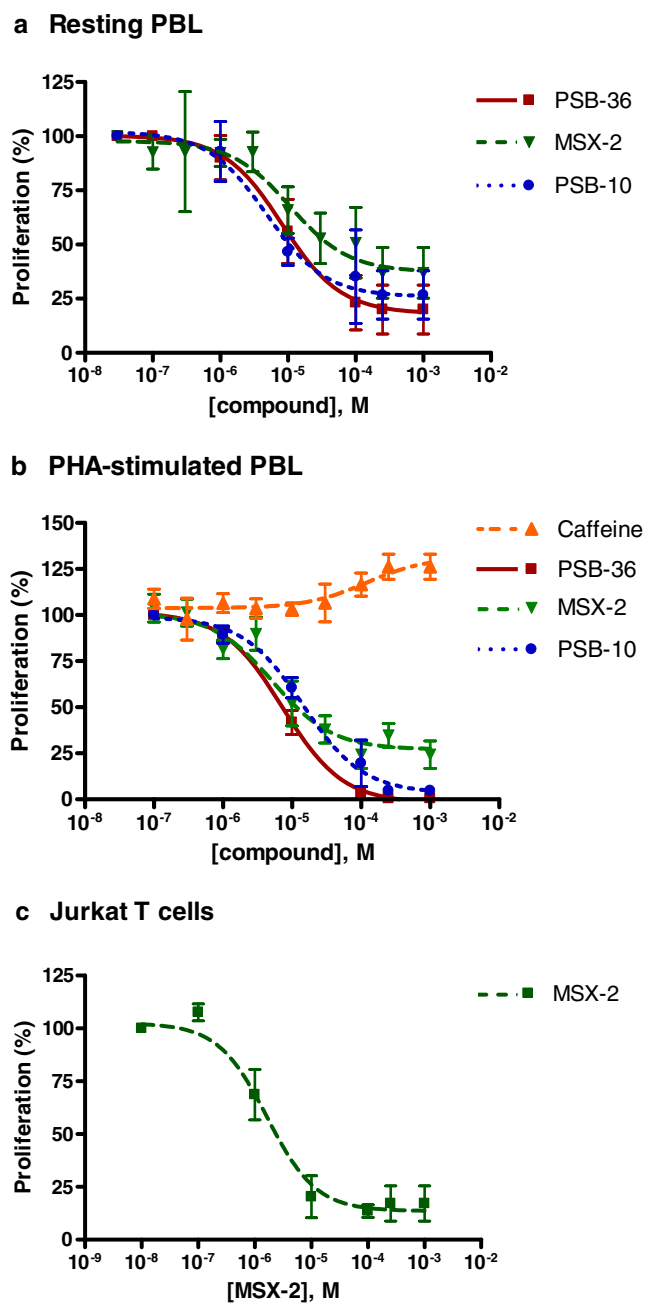
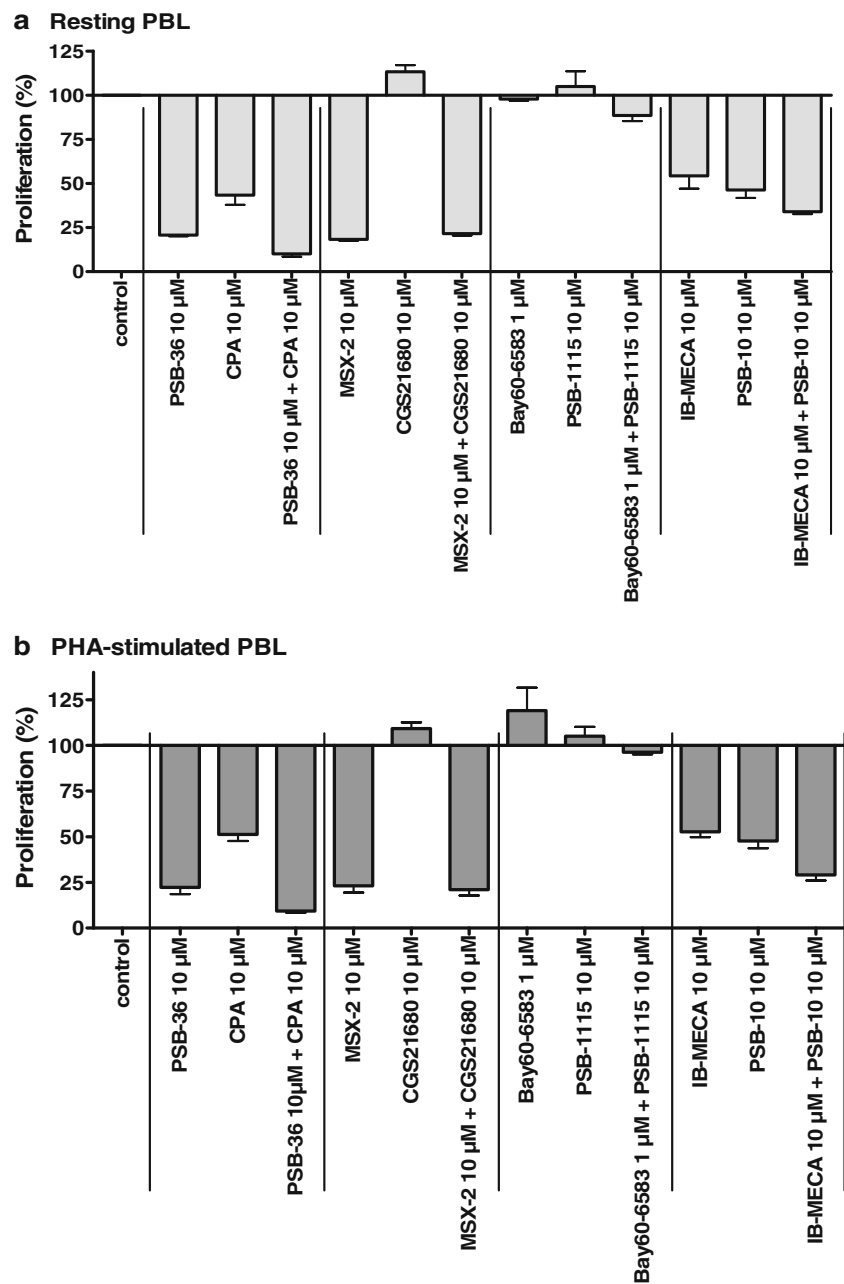


Fig. 6 Dose–response curves obtained from [3 H]thymidine incorporation studies in unstimulated PBL (**a**), PHA-stimulated PBL (**b**), and Jurkat T cells (**c**). Each data point represents the mean of a minimum of three separate experiments carried out in triplicate \pm SEM (for IC_{50} values, see Table 2)

to immunosuppressive and anti-inflammatory effects. In the present study, we investigated the expression and function of AR subtypes in native human peripheral blood lymphocytes as well as in the human lymphoma cell line Jurkat T using real-time PCR. Moreover, we studied the effects of standard AR agonists and antagonists on lymphocyte proliferation. The results obtained from real-time PCR analysis showed expression for all four AR subtypes in the investigated

cells, consistent with previous reports [22–24, 39]. The adenosine A_{2A} receptor mRNA expression level was higher in PBL than in Jurkat T cells and was further upregulated after stimulation of the cells with PHA, as previously reported [40, 41]. The levels for A_1 AR mRNA did not change upon cell stimulation, while mRNA for the A_{2B} and A_3 ARs appeared to be somewhat downregulated upon PHA stimulation. This result is in contrast to the results by Gessi et al. [42] who had observed an upregulation of A_3 ARs upon the activation of human lymphocytes. In comparison to the non-malignant peripheral blood lymphocytes, Jurkat T cells showed a much higher expression of A_1 AR and especially A_{2B} AR, whereas the expression of A_{2A} AR and A_3 AR was somewhat lower. This indicates that A_{2B} AR may play a more important role than A_3 AR in this cancer cell line. Both receptor subtypes have previously been postulated to be associated with cancer cell proliferation [27, 29, 32, 42]. Our results indicate that all four AR subtypes are present on human lymphocytic cells, however at different expression levels, resulting in different receptor profiles. It should, however, be kept in mind that mRNA expression levels are not always well correlated with the protein levels expressed on the cell surface. The high expression levels of adenosine A_{2A} receptors in native PBL and their upregulation after stimulation of the cells have previously been reported [40, 41]. These results underscore the importance of this receptor subtype as a mediator of anti-inflammatory effects triggering the emergency downregulation of overactive immune cells [43]. On the other hand, the relatively high expression levels of adenosine A_{2B} receptors in cells of the malignant Jurkat T cell line compared to non-malignant PBL cells further indicate an important role of this subtype in malignant cell lines, as previously described [29, 32]. The adenosine A_{2B} receptor may therefore serve as a target to control cell growth and proliferation in these cells. Previous investigations of the role of adenosine in the control of cell proliferation in a number of cell types had yielded conflicting results [24, 30, 44]. The next step of this study was therefore aimed at investigating the effects of several nonselective and subtype-selective AR agonists as well as antagonists on the proliferative capacity of these cells, as measured by [3 H]thymidine uptake during DNA synthesis. In agreement with the reports showing that adenosine could impair cell proliferation [22, 24, 26], the present observations demonstrated an increased proliferation of human lymphocytes upon removal of endogenously released adenosine. The addition of ADA, which degrades adenosine to inosine, resulted in a significant stimulation of unstimulated PBL. No effect was seen upon treatment of cells, which were already

Fig. 7 Effects of the combination of adenosine receptor agonists and antagonists on [3 H]thymidine incorporation in resting PBL (a) and PHA-stimulated PBL (b). Cells were cultured in the presence of the indicated compound concentrations. Results are expressed as percentage of untreated control (=100 %) [3 H]thymidine uptake. Each bar represents the mean of a minimum of two separate experiments carried out in triplicate \pm SEM



strongly activated to grow, namely, PHA-stimulated PBL or Jurkat T cells, respectively. On the other hand, AR agonists, namely, the A_1 AR-selective CPA, the A_{2B} AR-selective BAY60-6583, and the A_3 AR-selective IB-MECA, showed clear anti-proliferative properties in all investigated cells, stimulated and unstimulated PBL, and Jurkat T cells. However, for the observed effects, relatively high concentrations were required. All three agonists inhibited cell proliferation with IC_{50} values in the micromolar range despite low nanomolar affinities for these compounds, as previously demonstrated in radioligand binding and/or functional studies (see Table 1). However, there were some differences between resting

and PHA-stimulated PBL concerning the maximum inhibitory effects: proliferation of unstimulated cells was inhibited to a lesser extent; maximum effects for the three compounds range from 35 to 56 % compared to proliferation inhibition rates of >95 % in stimulated PBL. The non-selective agonist NECA and the structurally related A_{2A} -selective agonist CGS21680 showed no significant effect on T lymphocyte proliferation in our experiments. A very small A_{2A} AR-dependent inhibition of thymocytes (7–15 % cell death) had been reported for CGS21680 [12].

Surprisingly, several AR antagonists, namely, the A_1 AR-selective PSB-36, the A_{2A} AR-selective MSX-2,

and the A₃AR-selective PSB-10 also showed significant anti-proliferative properties on human lymphocytes. For these compounds, the same phenomena could be observed, as seen for AR agonists: the IC₅₀ values determined for anti-proliferative activity were in the high micromolar range, while the previously determined AR affinities were about 1,000-fold lower (K_i values, see Table 3). The maximum inhibitory effects for the three compounds at resting PBL with 63–80 % inhibition of the proliferation were lower as compared to inhibitory rates of 76–99 % inhibition of cell proliferation of stimulated PBL. These results indicated that the observed inhibitory effects on cell proliferation by the agonists CPA, BAY60-6583, and IB-MECA and by the antagonists PSB-36, MSX-2, and PSB-10 may be caused by receptor-independent mechanisms. All of these anti-proliferative AR antagonists are xanthine derivatives related to caffeine. However, caffeine, and the A_{2B}-selective xanthine derivative PSB-1115, did not show any anti-proliferative effect. Caffeine even showed a small stimulatory effect on the proliferation of PBL. Two other AR antagonists, the A_{2B}-selective xanthine PSB-603 and the A_{2A}-selective non-xanthine AR antagonist SCH420814, only showed a very small anti-proliferative effect on PHA-stimulated PBL at a high concentration of 10 μ M. Thus, the xanthine structure does not appear to be responsible for the anti-proliferative effect of some of the AR antagonists.

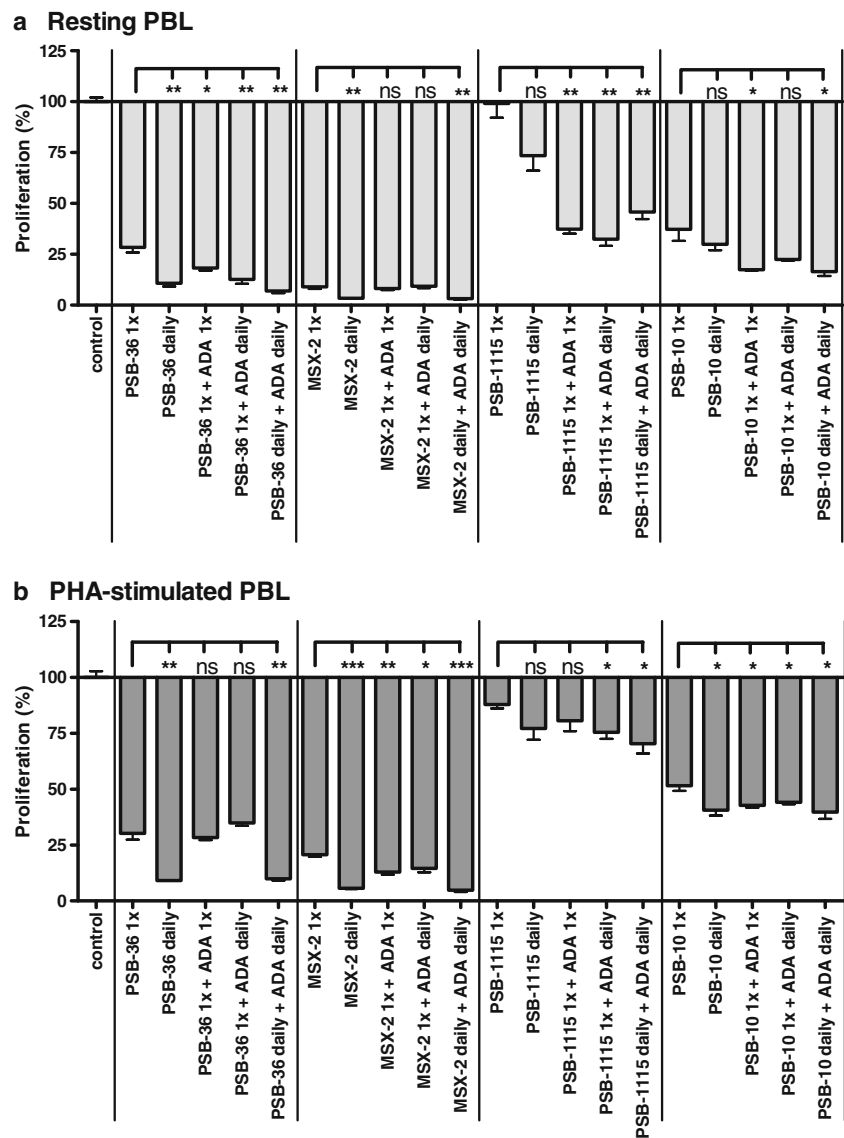
To further explore a possible AR-independent mechanism of the active AR antagonists, we tried to inhibit the agonist-induced inhibitory effects on lymphocyte proliferation by blocking the subtype-selective agonist effects with subtype-selective antagonists. As can be seen in Fig. 7, for none of the investigated AR agonists could the inhibition of cell proliferation be blocked by the addition of the corresponding AR antagonist, either in resting or in PHA-stimulated PBL. On the contrary, the inhibitory effects were retained or even increased for PSB-36+CPA and IB-MECA+PSB-10, respectively. These results provide further evidence that the mechanism of the anti-proliferative action of AR agonist is independent of ARs. Another possible explanation for the fact that agonists as well as antagonists at ARs can cause a strong inhibition of the proliferation of resting and PHA-stimulated PBL and of Jurkat T cells could be an upregulation of ARs caused by the addition of antagonists, resulting in a subsequent increased stimulation by endogenous adenosine. To exclude this possibility, we chose an assay design in which it is guaranteed that the concentration of antagonist present is always high enough to block the receptors. This was achieved by a repeated administration of the antagonist during the incubation time. Additionally, we tested the effect of the

presence of ADA in addition to the antagonist in order to remove endogenous adenosine. The results shown in Fig. 8 disproved the above hypothesis by showing that the observed inhibitory effects on cell proliferation could neither be diminished nor abolished by the repeated administration of the antagonist or by the presence of ADA. These findings exclude the possibility that AR antagonists inhibit lymphocyte cell proliferation by an upregulation of ARs.

Taken together, our results strongly indicate that the observed inhibitory effects of the subtype-selective AR agonists CPA, BAY60-6583, and IB-MECA, as well as those of the subtype-selective antagonists PSB-36, MSX-2, and PSB-10, on the proliferation of human lymphocytes are not mediated by AR-dependent mechanisms.

As can be seen in Fig. 6, caffeine was the only AR antagonist in this study which was able to stimulate the proliferation of already PHA-stimulated PBL. On the proliferation of resting PBL as well as on Jurkat T cells, caffeine had no measurable effect. The fact that the stimulating effect of caffeine only occurred in already stimulated PBL is indicative of an AR-independent mechanism. If it were a receptor-dependent mechanism, the effect should be observable also in resting PBL. For Jurkat T cells, one can argue that these cells—due to their malignancy—already proliferate at a maximum degree and cannot be further stimulated, a fact we also observed for stimulation with PHA, a strong T cell mitogen which was nevertheless unable to stimulate further proliferation in Jurkat T cells (data not shown). However, the stimulating effect of caffeine on the proliferation of already stimulated PBL can be explained by the reported ability of the methyl-xanthine to abolish cell cycle checkpoints [45]. As already mentioned, unstimulated PBL are resting in the G₀ phase of the cell cycle after isolation and therefore show per se no mitotic and cell cycle activity. For these cells, a manipulation of the cell cycle exerts no effects, quite contrary to cells running through the cycle like PHA-stimulated PBL. In these cells, as observed in the present study, the abolishment of cell cycle checkpoints (e.g., through caffeine) results in an increased proliferation rate. The results of the present study indicate that the compounds probably do not only induce cell cycle arrest, as previously shown for certain AR ligands in some cancer cell lines [22, 36], but inhibit proliferation by additional mechanisms. An indication for this assumption is that unstimulated PBL resting in the G₀ phase were significantly affected and [³H]thymidine uptake was potentially reduced after the administration of CPA, BAY60-6583, and IB-MECA as well as PSB-36, MSX-2, and PSB-10.

Fig. 8 Effect of the daily administration of adenosine receptor antagonists (10 μ M), ADA (1 IU/ml), or both on [3 H]-thymidine incorporation in unstimulated PBL (**a**) and PHA-stimulated PBL (**b**). Cells were cultured in the presence of the indicated compound concentrations. Results are expressed as percentage of untreated control (=100 %) [3 H]-thymidine uptake. Each bar represents the mean of a minimum of two separate experiments carried out in triplicate \pm SEM



The investigated AR agonists and antagonists can be structurally subdivided into several groups: (1) adenosine derivatives, (2) xanthine derivatives, and (3) non-nucleosidic adenine-like structures [46, 47]. Among the compounds that showed anti-proliferative activity, members of all three structural classes were represented. The mechanism of their anti-proliferative activity on lymphocytes is currently unknown. It might be speculated that adenosine derivatives, such as CPA, could serve as anti-metabolites of nucleic acid metabolism and thereby disrupt DNA or RNA synthesis. Another conceivable mechanism of the action of adenosine derivatives (CPA, IB-MECA), but also of the adenine-like compounds (BAY60-6583), and even of xanthine derivatives (PSB-36, MSX-2, PSB-10) might be due to kinase inhibition targeting the ATP co-substrate binding site [48–50]. However, further studies would be required to

elucidate the mechanism of action for each of the compounds.

Conclusions

The first comprehensive study investigating the effects of standard agonists and antagonists for all four AR subtypes on the proliferation of unstimulated and PHA-stimulated PBL and on Jurkat T cells was performed. For several AR agonists and antagonists, anti-proliferative effects were determined. Several lines of evidence indicated that the effects found for the structurally diverse ligands—the agonists CPA, BAY60-6583 and IB-MECA and the antagonists PSB-36, MSX-2, and PSB-10—were AR-independent: (1) agonists as well as antagonists for the same AR subtype had the same effect (CPA and PSB-36; IB-MECA and PSB-10);

(2) ligands for all four AR subtypes, some with opposing signaling pathways, were active; (3) subtype-selective antagonists did not block the effects of the agonists; and (4) the required concentrations for the anti-proliferative effects were about three orders of magnitude higher than those for binding to, blocking, or activating the ARs. The mechanisms of the anti-proliferative action on lymphocytes remain to be elucidated and can be expected to be different for each of the compounds.

Acknowledgments This study was supported by the Deutsche Forschungsgemeinschaft (DFG) within the Graduiertenkolleg GRK 677 (S.K.L., C.E.M.) and the NRW-Forschungsschule Biotech Pharma (A.C.S., C.E.M.). We thank H. Eltzschig and S. Zug (University of Tübingen, Germany) and A. Bill (University of Bonn, Germany) for their support in real-time PCR experiments. The technical assistance of N. Florin is gratefully acknowledged.

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