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ORIGINAL ARTICLE

A_{2B} adenosine receptor blockade inhibits growth of prostate cancer cells

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Abstract The role of the A_{2B} adenosine receptor (AR) in prostate cell death and growth was studied. The A_{2B} AR gene expression quantified by real-time quantitative RT-PCR and Western blot analysis was the highest among four AR subtypes (A_1, A_{2A}, A_{2B}, and A_3) in all three commonly used prostate cancer cell lines, PC-3, DU145, and LNCaP. We explored the function of the A_{2B} AR using PC-3 cells as a model. The A_{2B} AR was visualized in PC-3 cells by laser confocal microscopy. The nonselective A_{2B} AR agonist NECA and the selective A_{2B} AR agonist BAY60-6583, but not the A_{2A} AR agonist CGS21680, concentration-dependently induced adenosine 3′,5′-cyclic monophosphate (cyclic AMP) accumulation. NECA diminished lactate dehydrogenase (LDH) release, TNF-α-induced increase of caspase-3 activity, and cycloheximide (CHX)-induced morphological changes typical of apoptosis in PC-3 cells, which were blocked by a selective A_{2B} AR antagonist PSB603. NECA-induced proliferation of PC-3 cells was diminished by siRNA specific for the A_{2B} AR. The selective A_{2B} AR antagonist PSB603 was shown to inhibit cell growth in all three cell lines. Thus, A_{2B} AR blockade inhibits growth of prostate cancer cells, suggesting selective A_{2B} AR antagonists as potential novel therapeutics.

Keywords Prostate cancer · Cancer · Adenosine receptor · A_{2B} · G protein-coupled receptor (GPCR) · Cell proliferation

Abbreviations

BCA	Bicinchoninic acid
Cyclic AMP	Adenosine 3′,5′-cyclic monophosphate
CGS21680	(2-[p-(2-Carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine)
CHX	Cycloheximide
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DOX	Doxorubicin
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IB-MECA	N^6-(3-Iodobenzyl)adenosine-5′-*N*-methyluronamide
MRS2365	(*N*)-Methanocarba-2′-deoxy-2-methylthio-adenosine-5′-diphosphate
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Real time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
XTT	2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt
NECA	5′-*N*-Ethylcarboxamidoadenosine
PSB603	8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine
TNF-α	Tumor necrosis factor-alpha

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Introduction

Prostate cancer is one of the most frequently diagnosed cancers in man [1], but the means of treatment of this disease is still limited. G protein-coupled receptors (GPCRs) are emerging targets for cancer [2, 3]. We recently reported that agonists of the G protein-coupled $P2Y_1$ receptor could be potential novel drugs for prostate cancer [4].

It has been reported that the A_{2B} adenosine receptor (AR) is one of those most abundantly expressed in the human prostate cancer cells [5]. However, the role of the A_{2B} AR in prostate cancer has not previously been well explored, although it has been studied in several other types of tumors [6–9]. For example, Ryzhov et al. [8], using A_{2B} AR knockout mice, demonstrated that host A_{2B} AR promoted the growth of Lewis lung carcinoma. Ma et al. [6] demonstrated that A_{2B} AR activation promoted colon carcinoma cell growth, which was significantly inhibited by a selective A_{2B} AR antagonist, MRS1754. Stagg et al. [7] demonstrated both anti-CD73 antibody and a selective A_{2B} antagonist PSB1115 slowed the growth of breast tumors. Cekic et al. [9] demonstrated that A_{2B} AR antagonists, aminophylline (nonselective) and ATL801 (selective), blocked the growth of both breast tumors and bladder tumors.

In the present study, we found that the expression level of the A_{2B} AR was the highest among the four AR subtypes in three commonly used prostate cancer cell lines. We subsequently studied the A_{2B} AR signaling in regulating cell death and growth using PC-3 cells as a model. We further examined the role of the A_{2B} AR in proliferation of the three most commonly used cell lines, PC-3 (androgen-independent), DU145 (androgen-independent), and LNCaP (androgen-dependent), using a selective A_{2B} AR antagonist PSB603.

Materials and methods

Materials

Lactate Dehydrogenase (LDH) Assay Kit was purchased from Roche Applied Science (Indianapolis, IN, USA). XTT-based Toxicology Assay Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase-3 Colorimetric Detection Kit was from Assay Designs (Ann Arbor, MI, USA). NECA (5′-*N*-ethylcarboxamidoadenosine), CGS21680 (2-[p-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine), TNF-α (tumor necrosis factor-alpha), cycloheximide (CHX), and doxorubicin (DOX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MRS2365 ([[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxy-bicyclo[3.1.0]hex-1-yl]methyl]diphosphoric acid mono ester trisodium salt) and PSB603 (8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine) were purchased from Tocris Bioscience (R&D Systems, Inc., Minneapolis, MN, USA). Predesigned small interfering RNA (siRNA) for the A_{2B} AR, negative control siRNA, and SYBR Green reagents were purchased from Applied Biosystems (Foster City, CA, USA). Annexin-FITC was from BD Biosciences (San Diego, CA, USA). LUF6210 (BAY60-6583) was synthesized at Leiden University, The Netherlands. All other reagents were from standard sources and were of analytical grade.

Cell culture

Human prostatic carcinoma cells, PC-3, DU145, and LNCaP (American Type Culture Collection (ATCC), Manassas, VA, USA), were cultured at 37 °C in a humidified incubator with 5 % CO_2 in RPMI-1640 medium supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 3 mM/l glutamine. Cells used in the present study were within 20 passages after purchasing from ATCC.

Detection of AR gene expression

Total mRNA in cells was isolated following the protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was completed using Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA, USA). The cDNA was then amplified by PCR with gene-specific primers for ARs and GAPDH on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol using SYBR Green PCR Master Mix. Amplification parameters were as follows: 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA) and the sequences are as follows: A_1 AR: forward 5′-CTA CCT AAT CCG CAA GCA GC-3′; reverse 5′-GTC ATC AGG CCT CTC TTC TGG-3′; A_{2A} AR: forward 5′-AAC CTG CAG AAC GTC ACC A-3′; reverse 5′-GTC ACC AAG CCA TTG TAC CG-3′; A_{2B} AR, forward 5′-GTG CCA CCA ACA ACT GCA CAG AAC-3′; reverse 5′-CTG ACC ATT CCC ACT CTT GAC ATC-3′; A_3 AR, forward 5′-CAC CAC CTT CTA TTT CAT TGT CTC T-3′; reverse 5′-GGT ACT CTG AGG TCA GTT TCA TGT T-3′; and human GAPDH, forward 5′-ATT CCA TGG CAC CGT CAA GGCT-3′; reverse 5′-TCA GGT CCA CCA CTG ACA CGT T-3′. Quantitative analysis of data was performed using the ΔΔCt method [10]. Values were normalized to GAPDH and were expressed as relative expression levels.

Measurement of cell growth

The assay method was previously described [4]. Briefly, cells were seeded in 96-well plates at a concentration of approximately 5,000 cells per well in 100 μl medium and cultured at

37 °C overnight. The A_{2B} AR agonist or antagonist was incubated with cells at 37 °C for 24, 48, and 72 h. Absorbance was measured at 450 nm using XTT Toxicology Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol and converted to cell numbers based on a standard curve (cell numbers vs. absorbance values). The A_{2B} siRNA (1 μM) was transfected at about 60–80 % cell confluency using Lipofectamine 2000 transfection reagent as instructed by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Detection of the A_{2B} AR using laser confocal microscopy

PC-3 cells were seeded on cover slips in six-well plates and grown for 48 h. Cells were then incubated with MitoTracker Red (Molecular Probes) for 45 min and washed with PBS, and fixed with cold fix solution (50 % methanol/50 % acetone) for 20 min at −20 °C. Cells were blocked with 10 % FBS for 1 h at room temperature, after washing with PBS. Anti-A_{2B} AR antibody (Alomone Labs, Ltd., Israel) was added at 1:200 dilution and incubated with cells for 1 h. Cells were then washed three times with PBS before adding Alexa fluor-488 goat antirabbit IgG (Invitrogen, Carlsbad, CA, USA) and incubated for another 1 h. This was followed by washing three times with PBS and mounting with ProLong® Gold antifade mounting reagent with DAPI (Invitrogen, Carlsbad, CA, USA). Fluorescence images were obtained with a laser scanning Zeiss LSM-510 Meta Confocal Microscope (Carl Zeiss Inc., Jena, Germany).

Western blot analysis

Cells of about 80 % confluence were lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and the cell lysates were stored at −80 °C. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Cell lysates (30 μg protein/well) were analyzed under reducing conditions by SDS-PAGE and proteins were separated on 12 % Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane by electroblotting. Membranes were blocked according to the manufacturer's instructions and probed with specific antibodies overnight at 4 °C. Subsequently, blots were probed with IRdye-conjugated secondary antibody for 1 h and then analyzed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). In case of blots probed with control antigen of the respective antibody, both antigen and antibody were premixed and incubated for 1 h with constant agitation, and this ag–ab mixture was used as control for the respective AR antibody.

Cyclic AMP accumulation assay

Cells were planted in 96-well plates in 0.1 ml medium. After overnight incubation, the medium was removed and cells were washed three times with 0.1 ml DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the test agonists in the presence of rolipram (10 μM) and adenosine deaminase (3 U/ml) for 30 min. The antagonist was added 20 min before the addition of the agonist. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 100 μl of 0.1 M ice-cold HCl. For determination of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) production, the Sigma Direct cyclic AMP Enzyme Immunoassay kit was used following the instructions provided with the kit. The absorbance was measured with a microplate reader at 405 nm.

Assay of LDH release

Cells were first cultured in 24-well plates overnight with complete medium and then replaced with 500 μl fresh medium containing 1 % serum. The A_{2B} AR agonist NECA was added 20 min before CHX (10 μg/ml) or DOX (10 μM) and the mixture was incubated at 37 °C for 24 h. If an antagonist was used, it was incubated with the cells 20 min prior to the addition of agonists. For the measurement, the culture medium was first centrifuged, and supernatant was carefully transferred to the corresponding wells of an optically clear 96-well flat bottom microplate in triplicate. LDH activity was measured using a LDH Cytotoxicity Detection kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions. The absorbance of the samples was measured at 490 nm using SpectraMax5 Microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of caspase-3 activity

PC-3 cells were plated in six-well plates at a density of 2×10^5 cells per well and cultured to 80–90 % confluence. The cells were treated with TNF-α in the absence or presence of A_{2B} AR agonist NECA for 8 h and were then lysed in RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitors, and the protein concentration was quantified. Caspase-3 activity was measured using the Caspase-3 Colorimetric Assay Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) following the manufacturer's instructions. Each sample contained 50–200 μg protein, and the absorbance in each well was measured at 405 nm using a microplate reader.

Detection of apoptosis of PC-3 cells using laser scanning confocal microscopy

The method used was as previously described by Blom et al. [11]. In brief, PC-3 cells were grown in two-chambered coverglass system (Nunc, Rochester, NY, USA) until 70–80 % confluence. The cells were washed with PBS and the medium was changed to RPMI medium with 1 % serum. Cells were incubated in the presence or absence of the A_{2B} AR antagonist PSB-603 (1 μM) for 20 min followed by treatment with 1 μM of NECA for another 20 min. CHX (100 μM) was then added to the cell medium with reduced serum, and the cells were incubated for 6 h at 37 °C. After washing twice with PBS, cells were stained with Annexin-FITC (BD Biosciences, San Diego, CA, USA) for 15 min at room temperature. After staining, cells were washed once with PBS and microscopy proceeded. Cells were analyzed using a laser scanning confocal live cell imaging system (LSM 5 Live, Carl Zeiss, Germany).

Statistical analysis

EC_{50} values were calculated with Prism 5 (GraphPad, San Diego, CA, USA). Data were analyzed by analysis of variance (ANOVA) (followed by post hoc analysis) or via Student's *t* test to check the statistical difference among groups with *P* value less than 0.05 being considered significant. Results were expressed as mean±SE.

Results

Gene expression levels of four subtypes of ARs in three prostate cancer cell lines

The gene expression levels of four AR subtypes, A_1, A_{2A}, A_{2B}, and A_3, in three prostate cancer cell lines, PC-3 (Fig. 1a), DU145 (Fig. 1b), and LNCaP (Fig. 1c), were compared using real-time quantitative RT-PCR analysis. The expression level of the A_{2B} AR was the highest among four subtypes of ARs in all three cell lines (A_{2B}>A_{2A}>A_1, A_3). However, there is a quantitative difference in the A_{2A} AR expression relative to that of the A_{2B} AR in these three cell lines. The expression of the A_{2A} AR was about 30-, five-, and onefold lower than A_{2B} AR in PC-3, DU145, and LNCaP cells, respectively. Expression of A_1 and A_3 ARs was detectable but was shown to be much lower than that of the A_{2B} AR.

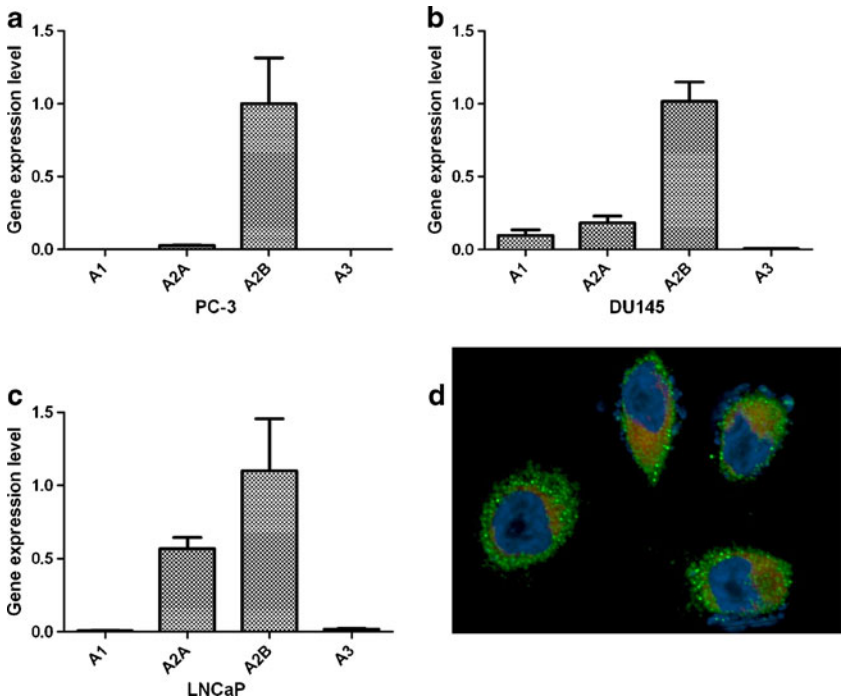

Fig. 1 **a**, **b**, and **c** Gene expression level of the A_{2B} AR in comparison to A_1, A_{2A}, and A_3 ARs in PC-3 (**a**), DU145 (**b**), and LNCaP (**c**) human prostate cancer cells. Total RNA was extracted and reverse-transcripted to cDNA and then amplified with gene-specific primers for ARs or GAPDH on a 7900HT Fast Real-Time PCR System. Results are expressed as mean±SE from three separate experiments. Values of each experiment were normalized using GAPDH as an endogenous control and were expressed as relative expression levels. Quantitative analysis of data was performed using the ΔΔCt method. The expression level of the A_{2B} AR was expressed as 1. **d** Localization of the A_{2B} AR in PC-3 cells by confocal laser scanning microscopy. *Red* MitoTracker Red (mitochondria), *green* Alexa Fluor-488 (A_{2B} AR), *blue* DAPI (nucleus)

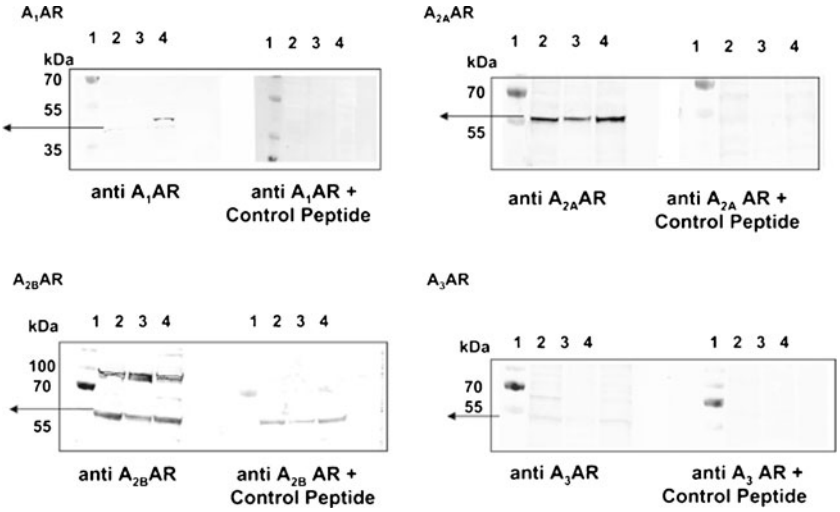

Fig. 2 Western blot analysis of the expression of all four subtypes of adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 in all three prostate cancer cell lines used in the study. In each figure, *left side blot* was probed with the specified AR antibody alone and the *right side panel* was probed with a mixture of specific antigen + antibody and used as control. *Lane 1* molecular weight marker, *lane 2* cell lysates of DU145, *lane 3* LNCaP, *lane 4* PC3. *Arrows* indicate the approximate molecular weight of monomeric ARs

Detection of the A_{2B} AR using laser confocal microscopy

The A_{2B} ARs in PC-3 cells were visualized using laser confocal microscopy. Figure 1d shows that the staining by the anti-A_{2B} AR antibody (green color) was localized mainly on the surface of the PC-3 cells.

Western blot analysis with all the four AR antibodies revealed that A_{2A} and A_{2B} antibodies were highly expressed followed by low expression of A_3 and A_1 ARs (Fig. 2). This result correlates with our findings from gene expression analysis.

Cyclic AMP accumulation induced by agonists for A_{2B} and A_{2A} ARs

Both A_{2A} and A_{2B} ARs are G_s-coupled receptors and mediate activation of adenylyl cyclase and subsequent accumulation of cyclic AMP in cells. To confirm the functional role of the A_{2B} AR in PC-3 cells, we first compared the ability of a nonselective A_{2B} agonist NECA and an A_{2A}-selective agonist CGS21680 to induce accumulation of cyclic AMP. Figure 3a shows that NECA, but not CGS21680, concentration-dependently induces accumulation of cyclic AMP, corresponding to an EC_{50} value of 469±71 nM, suggesting a functional role of the A_{2B} AR, which is consistent with its high expression in PC-3 cells as shown in Fig. 1. Figure 3b shows that the recently available A_{2B} agonist BAY60-6583 also concentration-dependently induces cyclic AMP accumulation in PC-3 cells corresponding to an EC_{50} value of 7.2±2.8 nM. Various concentrations of the selective A_{2B} antagonist PSB603 shift the agonist curve to the right in a parallel manner corresponding to a K_B value of 1.0 nM. Figure 3c shows that BAY60-6583 induces accumulation of

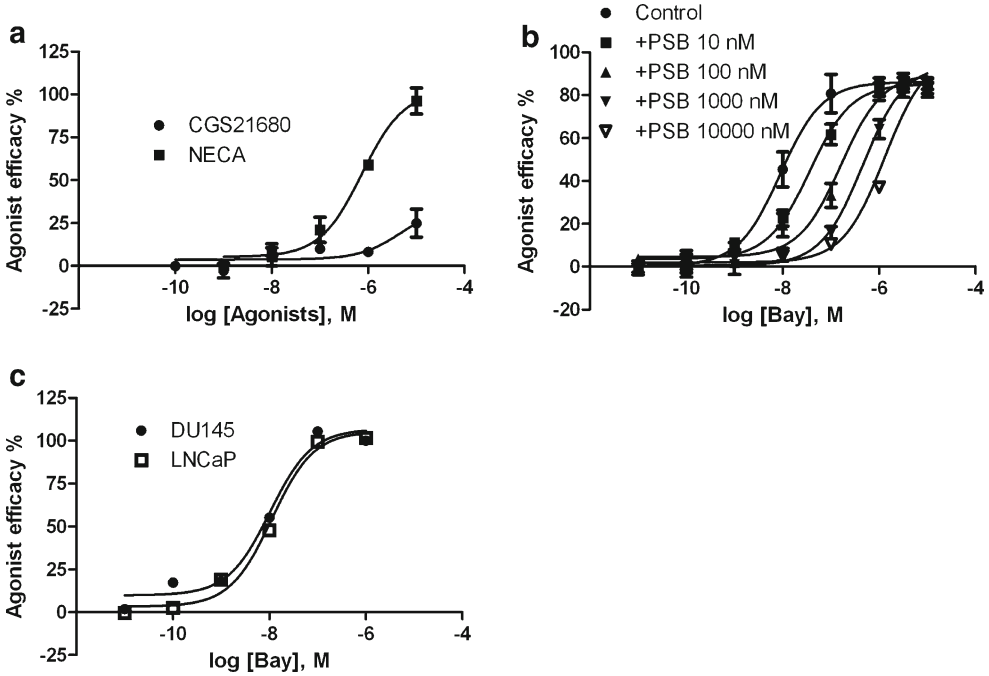

Fig. 3 **a** Cyclic AMP accumulation in PC-3 cells stimulated by the nonselective A_{2B} AR agonist NECA and the A_{2A} AR agonist CGS21680. **b** Effect of the A_{2B} AR-selective antagonist PSB603 on A_{2B} AR agonist BAY60-6583-induced cyclic AMP accumulation in PC-3 cells. **c** BAY60-6583-induced cyclic AMP accumulation in DU145 and LNCaP cells. Results are expressed as mean ± SE from three experiments performed in duplicate. The EC_{50} values of NECA, BAY60-6583, and CGS21680 were listed in the text. The K_B value of PSB603 was calculated to be 1.0 nM. *PSB* PSB603, *BAY* BAY60-6583

cyclic AMP in both DU145 and LNCaP cells corresponding to respective EC_{50} values of 9.8±1.6 and 12.2±2.7 nM.

LDH release in PC-3 prostate cancer cells

PC-3 cells, which almost exclusively express the functional A_{2B} AR, were used as a model for further exploring the role of the A_{2B} AR in prostate cancer cell death. Figure 4a shows that NECA (1 μM) decreases CHX-induced LDH release. It has been shown previously that the selective $P2Y_1$ receptor agonist MRS2365 (1 μM) enhanced the effect of CHX to promote LDH release in PC-3 cells [4]. Here, we have shown that the MRS2365-induced enhancement of CHX-induced LDH release was also diminished by NECA (Fig. 4a). Figure 4b shows that NECA significantly diminishes LDH release induced by TNF-α (10 ng/ml). Figure 4c shows that DOX, a cytotoxic drug for prostate cancer shown to induce death of PC-3 cells [12], significantly increases LDH release, which is blocked by NECA. The selective antagonist of the A_{2B} AR, PSB603, is shown to antagonize the effect of NECA (Fig. 4c). Figure 4d shows that siRNA specific for the A_{2B} AR does not affect LDH release induced by DOX but reverses the effect of the A_{2B} AR agonist NECA. The percentages of LDH release in the DOX+NECA group in the presence and absence of A_{2B} AR siRNA were 147 ±3.5 % and 116±1.9 %, respectively, which were significantly different ($P<0.05$). In control experiments, siRNA specific for an unrelated protein, GAPDH, did not reduce the effect of NECA.

Effect of NECA on TNFα-induced increase of caspase-3 activity

We next examined the role of A_{2B} AR activation in the activity of the enzyme caspase-3, an early indicator of cell apoptosis. Figure 5 shows that TNF-α causes increase of caspase-3 activity in PC-3 cells, which is significantly diminished by NECA (1 μM). The percentage of caspase-3 activity in the presence of NECA (101±1.7 %) is significantly different from that in its absence (133±6.4 %) ($P<0.05$). The A_{2B} AR antagonist PSB603 did not produce any effect alone but blocked the effect of NECA.

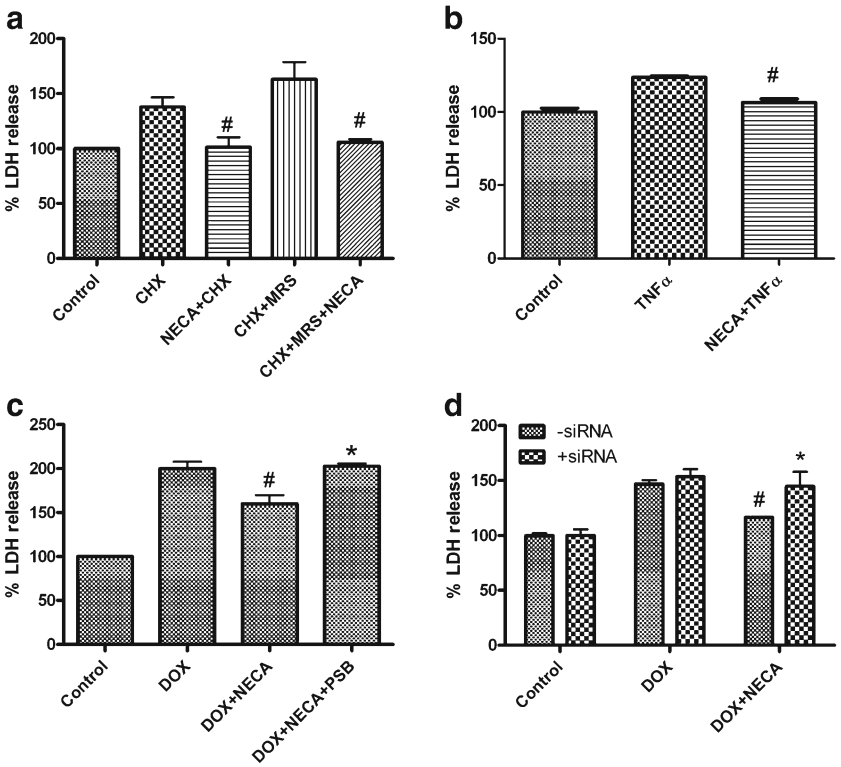

Fig. 4 Detection of cell death with an assay of LDH release in PC-3 cells. Agonists and/or CHX (10 μg/ml) (**a**) or TNF-α (10 ng/ml) (**b**) or doxorubicin (DOX, **c**) were added and incubated with the cells at 37 °C for 24 h. #Significantly different from corresponding groups in the absence of NECA (1 μM). *MRS* MRS2365 (1 μM), *PSB* PSB603 (1 μM). *Significantly different from DOX+NECA group ($P<0.05$). **d** Cells were transfected with A_{2B} AR siRNA (1 μM) at about 80 % confluency using Lipofectamine 2000 as instructed by the manufacturer. Cells were split to 24-well plates 24 h after transfection and incubated for an additional 24 h before the addition of DOX (10 μM) and/or NECA (1 μM). LDH release was measured 24 h after drug treatment. #Significantly different from the corresponding DOX group ($P<0.05$). *Significantly different from the DOX+NECA control group in the absence of siRNA ($P<0.05$)

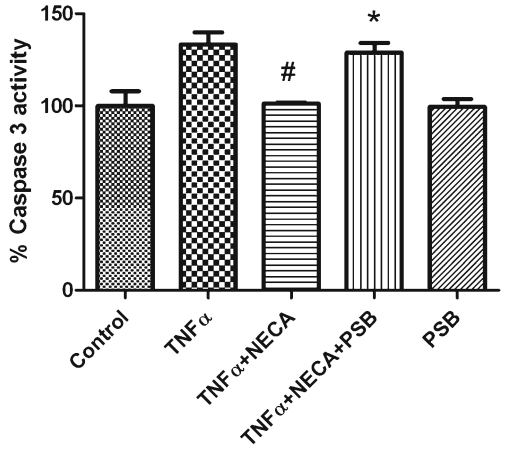

Fig. 5 Detection of apoptosis by measuring caspase-3 activity in PC-3 cells. Cells were plated in six-well plates at a density of 2×10^5 cells per well and cultured to approximately 80–90 % confluence for the experiments. The complete medium was replaced with fresh serum-free medium after washing with PBS for three times. Cells were then treated with the antagonist PSB603 (1 μM) for 20 min before the addition of the agonist NECA (1 μM) and incubated for another 20 min, followed by addition of TNF-α (10 ng/ml) for 8 h. Caspase-3 activity was measured using the Caspase-3 Colorimetric Assay Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). The protein concentration of each sample was quantified. Each sample contains 50–200 μg protein that was used in the assay. The absorbance in each well was measured at 405 nm with a microplate reader. Results are from three separate experiments performed in triplicate. #Significantly different from the TNF-α group ($P<0.05$). *Significantly different from the TNF-α+NECA group ($P<0.05$). *PSB* PSB603

Detection of morphological changes of apoptotic PC-3 cells with confocal laser scanning microscopy

CHX has been shown in many studies to induce apoptosis with characteristics such as formation of blebs, chromatin condensation, and exposition of phosphatidyl serines at the plasma membrane. Therefore, we evaluated the protective effect of the A_{2B} AR agonist NECA on CHX-induced apoptosis in PC-3 cells. In control cells (Fig. 6a), the nucleus was intact, and the morphology of PC-3 cells was not altered. As shown in Fig. 6b, cells treated with CHX exhibited formation of blebs and altered morphology, and the nucleus was fragmented. Annexin V binds to the extracellularly exposed phosphatidyl serine residues in cells that are undergoing apoptosis caused by CHX treatment. Pretreatment with NECA protected the cells against CHX-induced apoptosis, which was evident from Fig. 6c, in which the cells displayed decreased chromatin condensation and intact morphology. To confirm that the protective effect of NECA on CHX-induced apoptosis in PC-3 cells occurred through the A_{2B} AR, a selective A_{2B} antagonist PSB603 was used. Figure 6d shows that PC-3 cells pretreated with PSB603 plus NECA and CHX have characteristics similar to those of cells treated with CHX alone, which confirms that the protective effect of NECA was via the A_{2B} AR.

Activation of the A_{2B} AR-induced proliferation of prostate cancer cells

The activation the A_{2B} AR has been shown to induce proliferation of colon carcinoma cells [6] and growth of breast tumors [7]. In the present study, the potential proliferative effect of A_{2B} AR activation in PC-3 prostate cancer cells was examined using an XTT assay. Figure 7a shows that the nonselective A_{2B} AR agonist NECA concentration-dependently promotes growth of PC-3 cells (measured 72 h after addition of NECA), corresponding to an EC_{50} value of 266±78 nM. At 1 μM, NECA produced an effect close to maximum; thus, we selected this concentration in the following experiment. Figure 7b shows that NECA (1 μM) significantly enhanced growth of PC-3 cells, which was diminished by specific siRNA for the A_{2B} AR (Fig. 7b). The selective A_{2A} AR agonist CGS21680 (1 μM) did not promote cell growth (data not shown).

We further tested the role of A_{2B} AR blockade in the proliferation of all three cell lines using the selective A_{2B} AR antagonist PSB603. Figure 8 shows that PSB603 inhibits its growth of all three types of commonly used prostate cancer cells.

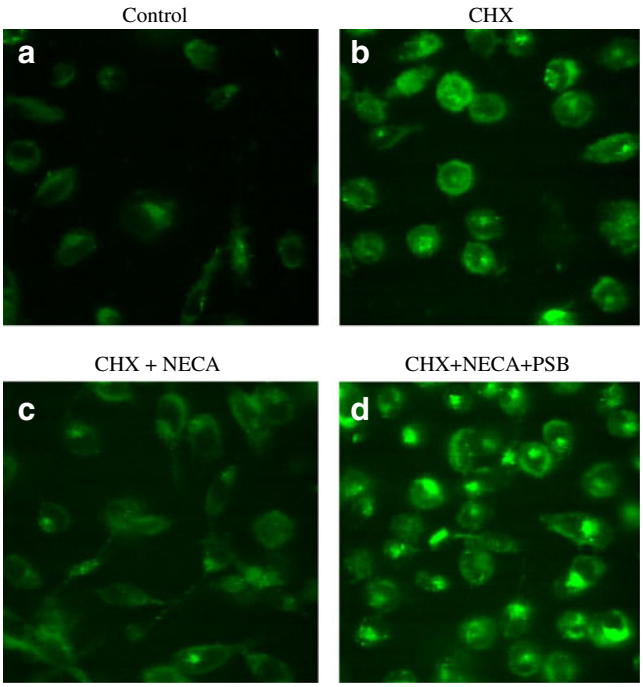

Fig. 6 Detection of morphological changes in CHX-induced apoptotic PC-3 cells using confocal microscopy. PC-3 cells grown to 70–80 % confluency were treated with 1 μM PSB603 (PSB) for 20 min before treatment with 1 μM NECA and/or 100 μM cycloheximide and were incubated for 6 h. Cells were then stained with FITC-Annexin-V and images were captured with confocal microscope (LSM 5 Live, Carl Zeiss, Germany). **a** Control. **b** CHX. **c** CHX+NECA. **d** CHX+NECA+PSB

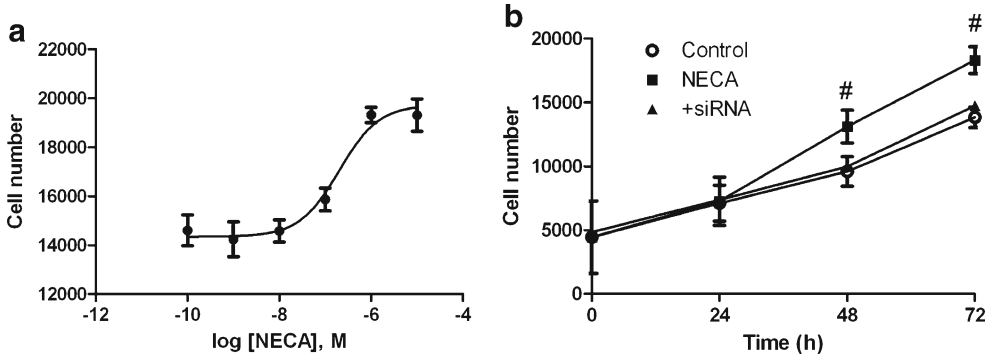

Fig. 7 Measurement of A_{2B} AR-promoted cell growth using an XTT assay. Cells were seeded in 96-well plates about 5,000 cells per well in a volume of 100 μl medium containing 10 % serum and cultured at 37 °C overnight. Cells were then treated with and incubated at 37 °C for 24, 48, or 72 h. **a** Concentration dependence of NECA-promoted PC-3 cell growth measured at 72 h after drug treatment. The EC_{50} value of NECA was calculated to be 266±78 nM. **b** The effects of A_{2B} AR siRNA (1 μM) on NECA (1 μM)-promoted growth of PC-3 cells. #Significantly different from control and siRNA groups ($P<0.05$)

Discussion

In the present study, we explored the role of the A_{2B} AR in the death and growth of prostate cancer cells. We found that A_{2B} AR activation blocked cell death and promoted cell growth. Both the A_{2B} AR antagonist PSB603 and siRNA specific for the A_{2B} AR slowed cell growth, suggesting that selective A_{2B} AR blockade could be useful for the treatment of prostate cancer.

The A_{2B} AR has been identified as one of several GPCRs expressed at a considerably high level in some cancer tissues including prostate cancer [5]. However, the role of the A_{2B} AR in prostate cancer has not been systematically investigated and reliably identified [13, 14]. This was, at least, in part, because many previous studies utilized adenosine and analogs that acted nonselectively on the ARs [13, 15] rather than selective A_{2B} AR agonists or antagonists or siRNA specific for the A_{2B} AR.

Regarding the expression profile of the four subtypes of ARs in prostate cancer cells, the results from the present study are in line with those from several previous reports [5, 15, 16]. Minelli et al. [15] suggested that the A_{2B} AR was the highest expressed of the four ARs in PC-3 cells. Panjehpour et al. [16] demonstrated that in DU145 prostate cancer cells, the A_{2B} AR was the highest expressed and the A_3 AR was the lowest expressed (almost undetectable) among the four subtypes of ARs. The results from the present study are different from those of Aghaei et al. [14], who reported a different AR expression pattern with the A_3 AR being highly expressed. Fishman et al. [17] reported that the A_3 AR agonist IB-MECA at concentrations from 0.01 to

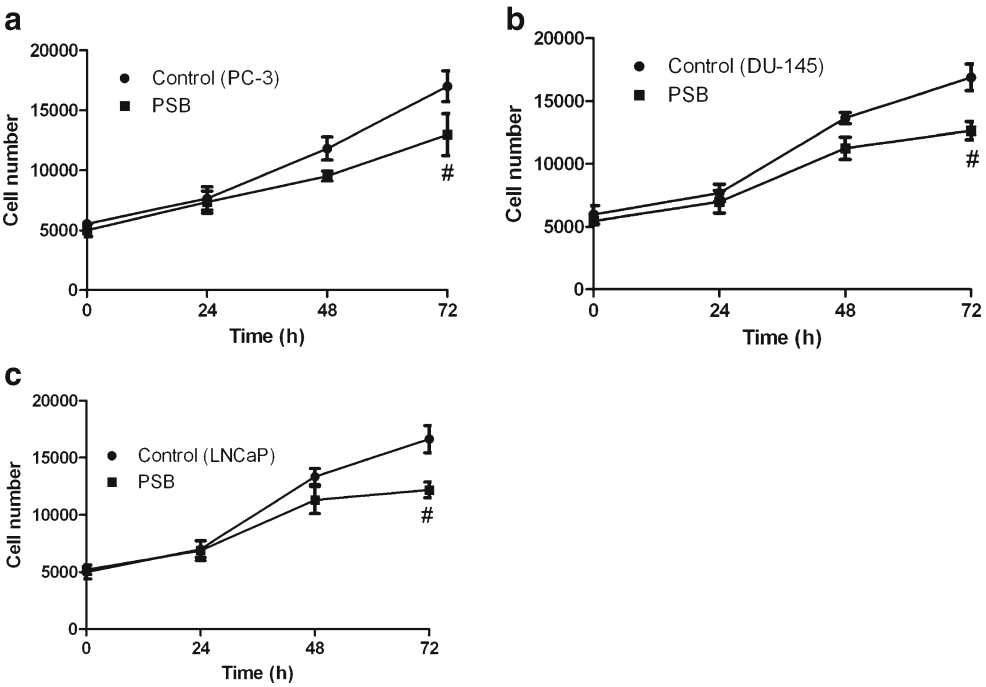

Fig. 8 Effect of the A_{2B} AR antagonist PSB603 on the growth of PC-3 (**a**), DU145 (**b**), and LNCaP (**c**) cells. Cells were seeded in 96-well plates about 5,000 cells per well in a volume of 100 μl cell culture medium with 10 % serum and cultured at 37 °C overnight. Cell viability was measured using an XTT Assay Kit following the manufacturer's protocol. Absorbance values were transformed into cell numbers based on a standard curve measured with various known numbers of cells. *PSB* PSB603. Results are expressed as mean ± SE, from at least three separate experiments. #Significantly different from the control group ($P<0.05$)

10 μM inhibited growth of PC-3 cells, but the expression profile of four ARs was not examined. The reason for this discrepancy is unclear. However, it should be noted that the A_3 AR was not found to have a high expression level in primary prostate cancer tissue [5].

The XTT assay was used in the present study to measure cell growth and a stimulatory effect of NECA was detected. The direct interpretation for this is that A_{2B} ARs mediated a proliferative effect. However, an antiapoptotic effect of NECA was also demonstrated in the same cells. Therefore, it is unclear if the effect of NECA is due to increased proliferation or decreased death or a combination of both, which could be addressed directly using a proliferation assay, such as the BrdU incorporation test.

Administration of the naturally occurring AR antagonists, i.e., caffeine and theophylline, has long been suspected to counteract the growth of cancer. Both caffeine and theophylline are weak and nonselective A_{2B} antagonists, which might have a role in both cancer promotion and prevention depending on specific situations involved. In this study, a more potent and selective A_{2B} AR antagonist, PSB603, has been clearly shown to inhibit the growth of prostate cancer cells. It remains to be seen how general is the antiproliferative effect of A_{2B} AR antagonists in primary prostate cancer cells and other types of cancer cells.

The findings from the present study that the A_{2A} AR is also relatively highly expressed in DU145 and LNCap cells, suggest that under some specific conditions, selective antagonists of the A_{2A} AR may also have a therapeutic role in prostate cancer management. Consistent with this finding, a recent report from Kalhan et al. [18] also suggested that blockade of both A_{2A} and A_{2B} ARs may have a therapeutic role in endocrine tumors.

In addition to the high expression level of the A_{2B} AR, $P2Y_1$ receptors [4], which are activated by endogenous adenine nucleotides, and ectonucleotidases CD39 and CD73 [19], which together degrade adenine nucleotides to produce adenosine, are also abundantly expressed in prostate cancer cells. Stagg et al. [20] recently reported that CD73 deficiency suppressed prostate tumorigenesis in a mouse transgenic model of prostate cancer. In addition to their early findings that A_{2B} AR antagonism blocked breast cell tumor growth [7], the authors show that the inhibitory effect of an anti-CD73 antibody on tumorigenesis depends on CD8+ T cells. Therefore, adenosine may act through its tolerogenic action leading to immune escape, in addition to a direct effect on tumor cell proliferation. On the other hand, CD39 might promote tumor cell growth by scavenging ATP rather than by generating adenosine. Indeed, ATP inhibits the growth of prostate tumor cells through the activation of P2X receptors [13]. Vascular CD39 directly promotes tumor cell growth [21]. It has been suggested that inhibition of either enzyme may find utility in cancer management. The present study demonstrated that A_{2B} AR activation may have tumor-promoting effects, thus indicating that the tumorigenic effects of ectonucleotidases might be, at least in part, via the activation of the A_{2B} AR, due to their ability to cleave ATP to produce adenosine.

In summary, the present study demonstrated that activation of the A_{2B} AR promotes, and blockade of the A_{2B} AR inhibits, the growth of prostate cancer cells. Thus, A_{2B} AR blockade might be a novel therapy for prostate cancer patients.

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Conflicts of interest None.

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