Adenosine Inhibits Tumor Cell Invasion via Receptor-independent Mechanisms

Sanna S. Virtanen\textsuperscript{1,2}, Anu Kukkonen-Macchi\textsuperscript{3}, Minna Vainio\textsuperscript{4}, Kati Elima\textsuperscript{3}, Pirkko L. Härkönen\textsuperscript{2}, Sirpa Jalkanen\textsuperscript{3}, and Gennady G. Yegutkin\textsuperscript{3}

Authors' Affiliations: \textsuperscript{1}Turku University of Applied Sciences; The Departments of \textsuperscript{2}Cell Biology and Anatomy, \textsuperscript{3}Medical Microbiology and \textsuperscript{4}Biology, University of Turku, Turku, Finland

Running title: Adenosine Inhibits Invasion via Non-Receptor Mechanisms

Note: Supplementary data for this article are available

Keywords: adenosine, breast and prostate cancer, invasion, ecto-5\'-nucleotidase/CD73, AMP-activated protein kinase

Corresponding Author: Gennady G. Yegutkin, MediCity Research Laboratory, University of Turku, Tykistökatu 6A, 20520 Turku, Finland. Phone: (358)2-3337022; E-mail: genyeg@utu.fi

Grant Support

This work was supported by grants from the Academy of Finland and the Sigrid Juselius Foundation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Word count (excluding Abstract, references and figure legends): 5192

Total number of figures and tables: 7 figures
Abstract

Extracellular adenosine mediates diverse anti-inflammatory, angiogenic and other signaling effects via binding to adenosine receptors, and also regulates cell proliferation and death via activation of the intrinsic signaling pathways. Given the emerging role of adenosine and other purines in tumor growth and metastasis, this study evaluated the effects of adenosine on the invasion of metastatic prostate and breast cancer cells. Treatment with low micromolar concentrations of adenosine, but not other nucleosides or adenosine receptor agonists, inhibited subsequent cell invasion and migration through matrigel- and laminin-coated inserts. These inhibitory effects occurred via intrinsic receptor-independent mechanisms, despite the abundant expression of A2B adenosine receptors (ADORA2B). Extracellular nucleotides and adenosine were shown to be rapidly metabolized on tumor cell surfaces via sequential ecto-5’-nucleotidase (CD73/NT5E) and adenosine deaminase (ADA) reactions with subsequent cellular uptake of nucleoside metabolites and their intracellular interconversion into ADP/ATP. This was accompanied by concurrent inhibition of AMP-activated Protein Kinase (AMPK) and other signaling pathways. No differences in the proliferation rates, cytoskeleton assembly, expression of major adhesion molecules: integrin-1β (ITGB1), CD44, focal adhesion kinase (FAK), and secretion of matrix metalloproteinases (MMP) were detected between the control and treated cells; thus, excluding the contribution of these components of invasion cascade to the inhibitory effects of adenosine. These data provide a novel insight into the ability of adenosine to dampen immune responses and prevent tumor invasion via two different, adenosine receptor-dependent and independent mechanisms.

Implications: This study suggests that the combined targeting of adenosine receptors and modulation of intracellular purine levels can impact tumor growth and metastasis phenotypes.
Introduction

Despite significant progress in cancer biology, the exact mechanisms underlying tumor growth and metastasis are not fully understood. Reasons for this, among others, include the variety of tumor types, their high plasticity and ability to adapt to environmental challenges, the redundancy of signaling pathways involved, histopathological diversity of the tumor microenvironment, and complex crosstalk between the tumor and supporting stroma as well as other adjacent host cells (1, 2). Currently, intense research efforts have focused on selective targeting of metabolic and signaling pathways that may be altered during tumorigenesis, with particular emphasis on the extracellular purinergic signalling cascade (3-5). The anticancer effects of ATP have been described 25 years ago (6) and since then, evidence is accumulating on potent tumor-suppressing or -promoting roles for ATP, adenosine and other purines (4, 7).

Most models of purinergic signaling depend on functional interactions between distinct processes. These include (i) release of endogenous ATP and other agonists; (ii) triggering signaling events via a series of ligand-gated P2X and metabotropic P2Y receptors; (iii) ectoenzymatic inactivation of nucleotides; (iii) binding of generated adenosine to its own G-protein-coupled receptors; and finally, (iv) metabolism and re-uptake of adenosine and other nucleosides by the cells (8). In view of diverse, often counteracting effects of ATP and adenosine, substantial attention is given to the tumorigenic role of key ectoenzymes mediating stepwise ATP hydrolysis to adenosine: nucleoside triphosphate diphosphohydrolase-1 (NTPDase1, otherwise known as CD39) (9) and ecto-5'-nucleotidase/CD73 (4, 10-12). High activities of ecto-5’-nucleotidase/CD73 and other nucleotidases may contribute to the clearance of pro-inflammatory and pro-angiogenic ATP in the tumor environment (4, 7, 9), simultaneously leading to A2A adenosine receptor-mediated inhibition of effector immune cells, including cytotoxic T-lymphocytes, natural killer cells, dendritic cells and macrophages (3, 13, 14).
Along with "classical" receptor-mediated pathways, adenosine exerts cytotoxic and other biological effects via intrinsic mechanisms (5, 15, 16). Most of these effects occur at high micromolar and even millimolar nucleoside concentrations, which are at least two orders of magnitude higher than adenosine receptor affinities and generally imply the uptake of extracellular adenosine and/or its metabolites into the cells through adenosine transporters and their ensuing interconversion into AMP and further to ADP/ATP. As a consequence of these metabolic shifts, adenosine may induce apoptosis of different malignant cells via up-regulation of p53 (17) or AMPK (18) activities, translocation of apoptosis-inducing factor AMID from the cytosol into the nucleus (19), and other mitochondrial apoptotic pathways (15). Likewise, high concentrations of adenosine receptor agonists, antagonists and other nucleoside analogues also trigger anti-proliferative and cytotoxic effects on normal and leukemic cells via receptor-independent mechanisms (3, 20, 21), presumably acting as anti-metabolites competing with natural nucleosides and inhibiting key enzymes of purine homeostasis.

By using androgen-independent prostate carcinoma cells PC-3 and triple-negative breast cancer cells MDA-MB-231 lacking three major biomarkers of breast cancer subtypes (estrogen and progesterone receptors and the epidermal growth factor receptor 2, HER2), as appropriate in vitro models for studying tumor metastasis, this study was undertaken to identify a link between extracellular purines and invasion. The results demonstrate that pre-treatment of tumor cells with low micromolar concentrations of adenosine inhibited their invasion and migration. Strikingly, these effects mainly occurred through the metabolism and uptake of exogenous adenosine with subsequent deregulation of intracellular purine homeostasis and related signalling pathways, thus providing a novel insight into the ability of adenosine to dampen immune responses and inhibit tumor metastasis via two different, adenosine receptor-dependent and independent mechanisms.

Materials and Methods
Reagents and antibodies

The following antibodies have been used in the study: mouse mAbs against CD44 (Hermes-3), integrin β1 (ITGB1) and isotype-specific 3G6 mAb against chicken T-cells (22); rabbit polyclonal anti-phospho-AMPKα1/2 (Thr172) and mouse anti-AMPKα1/2 antibody (Santa Cruz); rabbit polyclonal anti-phospho-AMPK1 (Thr174) and mouse anti-phosphotyrosine mAb (4G10) (Millipore, Temecula, CA); mouse mAbs against focal adhesion kinase (FAK) and phospho-FAK (pY397) (Becton Dickinson Biosciences, CA); mouse monoclonal anti-β-tubulin (TUB2.1) and FITC-conjugated anti-mouse Ig (Sigma-Aldrich); horseradish peroxidase (HRP) conjugated rabbit α-mouse and swine α-rabbit Ig (DAKO A/S, Denmark); Alexa488-conjugated goat α-mouse and α-rabbit Ig and Alexa546-conjugated phalloidin (Invitrogen™, Life Technologies). Radiolabeled compounds [methyl-3H]thymidine and [3H]-cAMP were from Perkin Elmer Inc, [2-3H]AMP from Quotient Bioresearch (GE Healthcare, UK) and [2-3H]adenosine from Amersham Biosciences. Matrigel and laminin were from BD Biosciences. Compound C (also known as dorsomorphin) was from Tocris Biosciences (R&D Systems). 5'-(N-cyclopropyl)-carboxamido-adenosine (CPCA, also known as C103_SIGMA), 5'-N-ethyl-carboxamide-adenosine (NECA), lipopolysaccharide (LPS) from Escherichia coli O111:B4, adenosine and other nucleosides and chemicals were purchased from Sigma-Aldrich.

Cell culture and treatments

The PC-3 human prostate carcinoma cell line and the MDA-MB-231 human breast cancer cell line were from ATCC (Manassas, USA). The cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 0.03 mg/ml penicillin and 0.05 mg/ml streptomycin. For treatment experiments, the cells were harvested with trypsin/EDTA and seeded onto tissue culture flasks (25 cm²) or 6-
and 24-well plates in complete media for 24-48 hours in order to reach confluence. The medium was replaced by DMEM containing 1% BSA (DMEM-BSA). Adenosine, inosine, guanosine and uridine diluted in DMEM, as well as stock DMSO solutions of NECA, CPCA and compound C were then applied onto the cells at 1:100-1:500 dilutions. Vehicle-treated cells contained DMEM-BSA without or with 0.02% DMSO. The cells were treated for 20 hours at 37°C in a humidified 5% CO₂/95% air, detached by trypsin/EDTA and used in subsequent assays, as specified below. Cell culture plates and invasion inserts (8-μm pore size; BD Biosciences) were coated overnight at 4°C prior to the experiment with Matrigel (100 μg/cm²) or laminin (5 μg/cm²).

**Invasion and migration assays**

The invasion and migration of PC-3 and MDA-MB-231 cells were assayed as described previously (23). Briefly, the invasion was started by applying a cell suspension (5 x10³ in 300 μl DMEM-BSA) to the upper chamber of Matrigel-coated cell culture invasion inserts, followed by a 48-hour incubation. Likewise, the migration assay was performed by incubating the cells for 5 hours on the inserts coated with laminin. In both assays, 300 μl of conditioned media from the MG-63 osteosarcoma cell line was placed in the lower chamber as a bone cell-derived chemoattractant to induce invasion and migration. The insert membranes were then fixed with paraformaldehyde, stained with Mayer’s haematoxylin and cut off from the inserts. The number of cells on the lower surface was counted with a microscope (10× objective) from 10 consecutive fields representing 40% of the total area of the membrane.

**Measurement of intracellular cAMP**
PC3 cells grown on 24-well plates were pre-treated for 30 min with the inhibitor of ADA erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA, 10 μM) and the phosphodiesterase inhibitor (IBMX, 250 μM) and subsequently incubated for one hour (37 °C and 5% CO₂) in DMEM-BSA containing adenosine (0-500 μM). A₂B receptor antagonist alloxazine was added 5 min prior to adenosine treatment. Adenosine receptor agonists NECA and CPCA were also tested in the assay, but without EHNA pretreatment. The cells were then lysed with perchloric acid and neutralized by KOH. cAMP content in the supernatants was determined by a protein binding method using [³H]cAMP (~10000 cpm per tube) and MAFB Millipore MultiScreen filtration plates, as described earlier (24).

**Proliferation assay**

PC3 were cultured in 96-well plates at a density of 3000 cells per well. On the next day, cells were rinsed with PBS and incubated for 20 hours in 200 μl DMEM-BSA containing [methyl-³H]thymidine (~7x10⁵ cpm/well) and adenosine or other compounds. LPS (20 ng/ml) and NaN₃ (1 mM) were used as positive and negative assay controls, respectively. Cells were harvested using a semi-automated plate harvester (Tomtech MACH III; Fisher Scientific, Hampton, NH) and the incorporated radioactivity was determined by scintillation β-counting.

**Quantitative PCR**

Total RNA was extracted from PC3 and MDA-MB-231 cells using the Nucleo-Spin RNAII Total RNA Isolation Kit (Macherey-Nagel) and reverse-transcribed with iScript™ cDNA Synthesis kit (BioRad) according to the manufacturer’s instructions. TaqMan Gene Expression Assays for *ADORAI*, *ADORAI2A*, *ADORAI2B*, *ADORAI3* and *NT5E* were used as primer/probe sets, and the PCR reactions were carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems) in the Finnish Microarray and Sequencing Center, Turku Center of...
Biotecnology. All samples were run as triplicates and the expression values were normalized using human β-actin as an endogenous control.

**Flow cytometry**

Control and adenosine-treated PC3 cells were either used directly in the assays or additionally seeded for 2 hours onto a matrigel-coated 24-well plate. The cells were resuspended in PBS supplemented with 2% fetal calf serum (FCS) and 0.01% NaN₃ and stained with mouse monoclonal antibodies (100 μl, ~10 μg/ml each) against CD44, integrin β1, as well as isotype-specific mAb 3G6. The cells were then incubated with second-stage FITC-conjugated anti-mouse Ig and analysed using a FACScan with Cell Quest software (Becton Dickinson, CA).

**Immunofluorescence staining**

Control and adenosine-treated PC3 cells were plated for 2 hours on a 24-well plate containing matrigel-coated coverslips. The adherent cells were stained with antibodies against FAK, phospho-FAK (pY397), AMPKα1/2, phospho-AMPKα1/2 (Thr172), and tyrosine phosphorylated proteins (4G10), and subsequently incubated with appropriate second-stage Alexa₄₈₈-conjugated immunoglobulins. For actin cytoskeleton co-staining, the cells were additionally incubated with Alexa₅₄₆-Phalloidin. The slides were mounted with ProLong® Gold Antifade medium containing DAPI (Invitrogen) and examined with a fluorescent microscope (Olympus BX60). For more experimental details, see Supplementary Data.

**Western blotting**

Control and adenosine-treated PC-3 cells were seeded for 2 hours onto Matrigel-coated 6-well plates. The cells were lysed and subjected to 8% SDS-PAGE electrophoresis (~30 μg
protein/lane), followed by transfer of separated proteins to nitrocellulose membranes. The amounts of total and phospho-AMPK1 (Thr174), total and phospho-FAK (pY397), integrin β1, CD44, β-tubulin and phosphotyrosine kinases were determined by using appropriate primary and secondary HRP-conjugated antibodies as described in Supplementary Data.

**Thin-layer chromatographic (TLC) analysis of purine-converting pathways**

For ecto-5'-nucleotidase and adenosine deaminase (ADA) assays, PC3 and MDA-MB-231 cells were seeded overnight onto 96-well clear plates (8000 cells per well), and incubated at 37°C in a final volume of 100 μl DMEM containing 4 mM β-glycerophosphate and 10 μM of [3H]AMP or [3H]adenosine. Aliquots of the mixture were applied onto Alugram SIL G/UV<sub>254</sub> TLC sheets (Macherey-Nagel, Duren, Germany). 3H-labeled AMP and nucleosides were separated using appropriate solvent systems and quantified by scintillation β-counting as described earlier (25). Intracellular purine-converting pathways were was evaluated by incubation of PC-3 cells grown on 24-well plates for 20 hours in 500 μl DMEM containing 0,2% BSA, 4 mM β-glycerophosphate and 10 μM [3H]adenosine. The cells were washed to remove non-bound radioactivity and lysed in mammalian lysis buffer (Perkin Elmer) with subsequent TLC analysis of the relative amounts of cell-incorporated [3H]adenosine and its metabolites.

**Quantification of ATP and other purines and bases**

PC-3 cells were incubated in 6-well plates in a starting volume of 1.2 ml DMEM-BSA without (control) and with 10 μM adenosine. Aliquots of the media (100 μl) were collected at the beginning (zero point) and after 2 and 20 hours of incubation. Extracellular nucleotides/nucleosides were extracted by adding perchloric acid, adjusted to neutral pH by KOH, and stored at -70°C. At the end of experiment, control and adenosine-treated cells were
washed and lysed for assaying intracellular content. Nucleotides and nucleosides were separated by reverse-phase HPLC, monitored by absorption at 258 nm and quantified from the areas under the corresponding peaks, as described previously (25). For more experimental details, see Supplementary Data. Intracellular ATP was additionally determined in the obtained cell lysates by using luciferin-luciferase kit ATPLite (Perkin Elmer).

**Human Phospho-Kinase Array**

Control and adenosine-treated PC3 and MDA-MB-231 cells were plated for 2 hours onto 6-well plates coated with Matrigel. The phosphorylation profile of major kinases was determined by using the human Phospho-Kinase Array Kit with a cocktail of biotinylated antibodies (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The protein concentration in cell lysates was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). After exposing the membranes to a streptavidin-HRP reagent, the obtained images were acquired to the Gel Doc-2000 (Bio-Red Laboratories) and the intensities of the spots were quantified using the accompanying Quantity One software after subtracting the background signal.

**Statistical analyses**

The experiments were repeated at least three times and each treatment was done as duplicate or triplicate. Data from kinetic experiments were subjected to computer analysis by non-linear least-squares curve fitting (GraphPad Prism, San Diego, CA), and qPCR results were analyzed with the SDS 2.3 software (SABiosciences, Qiagen). Statistical comparisons were made using Student's $t$ test, and $P$ values $< 0.05$ were taken as significant.
Results

Treatment of PC3 cells with adenosine, but not with other nucleosides and purinergic agonists, diminishes their invasion and migration

To evaluate the putative in vitro effects of adenosine, other nucleosides and purinergic agonists on tumor invasion and metastasis, cultured PC3 cells were pre-treated for 20 hours with different concentrations of test compounds followed by incubation in invasion assays for a subsequent 48 hours in the absence of treatments. As shown in Figure 1A, treatment of PC-3 cells with increasing concentrations of adenosine, but not inosine, guanosine and uridine, progressively diminished their invasion through matrigel-coated inserts with IC50 of 0.19±0.04 μM. Pre-treatment of PC-3 cells with a non-selective agonist of adenosine receptors NECA (21, 26) diminished their invasion by ~40%, whereas a general A2 receptor agonist CPCA (capable of stimulating both A2A and A2B adenosine receptors (27)) did not affect the numbers of invaded cells (Fig. 1B). Adenosine, but not other adenosinergic agonists, also diminished the migration of PC-3 cells through laminin, although these inhibitory effects occurred only at relatively high (50 μM) concentrations of the nucleoside (Fig. 1B).

Inhibitory effects of adenosine are mediated via non-cytotoxic and presumably receptor-independent mechanisms, despite expression of A2B receptors on PC-3 cells

The most common pathway implicated in adenosine effects includes the activation of certain adenosine receptor(s). In fact, the incubation of PC-3 cells with increasing concentrations of adenosine triggered a concentration-dependent increase in the cAMP level with EC50 values of ~100 μmol/L (Fig. 2A), which could be markedly attenuated in the presence of the A2B-selective antagonist alloxazine (Fig. 2B). No changes in cAMP levels were observed in the presence of other nucleosides, uridine, guanosine or inosine. In comparison with adenosine, adenosine
receptor agonists served as more potent activators of adenylyl cyclase (Fig. 2B). These data are consistent with previous reports showing the ability of sub-micromolar and low micromolar concentrations of NECA (26, 28) and CPCA (27) to trigger cAMP accumulation via activation of low-affinity A2B receptors on PC-3 and other cells. The presence of A2B receptors is further supported by qPCR data showing the abundant mRNA expression for A2B (ADORA2B), but not for other adenosine receptor subtypes in PC-3 cells (Fig. 2C). Nevertheless, relatively moderate (in comparison with adenosine) inhibition of tumor invasion by NECA, and the inability of another adenosine A2 receptor agonist CPCA to impair PC-3 cell invasion (see Fig. 1B) implies the potential involvement of non-receptor-mediated mechanisms in the revealed inhibitory effects. To rule out direct cytotoxic effects of adenosine and other compounds, the proliferation rate of the treated cells was evaluated by measuring [methyl-3H]thymidine incorporation. NECA, adenosine and other nucleosides had no effects on PC-3 cell growth, whereas a known stimulator (LPS) or inhibitor (NaN3) of cell proliferation caused the expected increase or decrease in [3H]thymidine incorporation, respectively (Fig. 2D). Furthermore, staining of control and adenosine-treated PC-3 cells with a caspase-3 substrate NucView 488 and continuous imaging of the cells during the subsequent 48 hours did not reveal any effect of adenosine (10 µM) on the percentage of fluorescent apoptotic cells (data not shown).

Adenosine does not affect the expression of adhesion molecules, pattern of cell-matrix interaction and MMP secretion by PC3 cells

To further evaluate the effects of adenosine on the initial binding steps, the expression levels of major adhesion molecules potentially implicated in tumor cell adhesion and metastasis were determined. Flow cytometric and immunoblotting stainings with antibodies against integrin-β1 and CD44 revealed an abundant cell surface expression of these molecules, with no differences in expression levels being detected between the control and adenosine-treated PC-3 cells (Fig. 3).
Adenosine also did not affect the expression (Fig. 3C) and staining patterns (Supplementary Fig. S1) of neither total nor phosphorylated (Y397) forms of yet another important adhesion-associated kinase FAK. Consistent with these staining results, direct kinetic analysis of binding of fluorescently labeled PC-3 cells to matrigel-coated wells showed that adenosine treatment does not affect the maximal binding capacity nor time-course of cell adhesion to extracellular matrix (Supplementary Fig. S2A). Furthermore, pretreatment of PC-3 cells with adenosine had no effects on the levels of MMP-2 or MMP-9 accumulated in the inserts during the invasion assay (Supplementary Fig. S2B). Taken together, these results exclude the possibility that adenosine-dependent inhibition of PC-3 invasion is mediated via the down-regulation of adhesion molecules, impaired cell-matrix interaction or changes in gelatinase secretion.

**Evidence for rapid metabolism of adenosine and other purines by PC-3 cells, followed by uptake of nucleosides and their re-synthesis into intracellular ATP**

The following studies were aimed to characterize major pathways involved in adenosine homeostasis. Ecto-5'-nucleotidase/CD73 is known to be a key enzyme responsible for phosphohydrolysis of ATP/ADP-derived extracellular AMP to adenosine (8). Our qPCR data (Fig. 2C) together with flow-cytometric staining with anti-CD73 mAb 4G4 (data not shown) confirm the abundant expression of this enzyme on the PC-3 cell surface both at mRNA and protein levels. The ability of PC-3 cells to dephosphorylate [3H]AMP into [3H]adenosine has been further ascertained by using TLC assays (Fig. 4A; upper panel). Furthermore, incubation of PC-3 cells with [3H]adenosine as an initial substrate revealed its subsequent conversion into 3H-labeled inosine/hypoxanthine (Fig. 4A; lower panel), which can be prevented by a specific ADA inhibitor EHNA (10 μM; data not shown).

Reverse-phase HPLC analysis provides an independent line of evidence for the rapid metabolism of exogenous adenosine by cultured PC-3 cells with practically all adenosine being
inactivated within 20-hours of incubation (Fig. 4B). Concurrent measurement of the whole spectrum of intracellular nucleotides and related compounds demonstrated that, along with ATP as a predominant nucleotide constituent, PC-3 cells contain other nucleotides (ADP, UTP, GTP, ITP and AMP), whereas adenosine and other nucleosides are maintained at very low, nearly undetectable, levels (Supplementary Fig. S3). Strikingly, the exposure of PC-3 cells to exogenous adenosine significantly increased the relative amount of intracellular adenosine, accompanied by a concurrent drop in the level of AMP (Fig. 4C). Additional bioluminescent analysis did not reveal any changes in ATP concentrations between the control and adenosine-treated cells (4.5±0.5 vs 5.1±0.3 nmol/mg protein; mean±SEM, n = 5).

To elucidate the link between extracellular and intracellular purine homeostasis, PC-3 cells were incubated for 20 hours with 10 μM [3H]adenosine, lysed and assayed by TLC for the relative amounts of cell-incorporated [3H]adenosine and its 3H-metabolites. As shown in Figure 4D, the majority of the uptaken [3H]adenosine was converted into [3H]ATP and, to some extent, into [3H]ADP/AMP, with the total amount of 3H-labeled nucleosides accounting only for less than 5% of total radioactivity incorporated (Inset). Noteworthy, pretreatment of the cells with an inhibitor of equilibrative nucleoside transporter S-(4-nitrobenzyl)-6-thioinosine (NBTI; 1 μM) or with 1 μM ABT-702 (the inhibitor of adenosine kinase mediating intracellular phosphorylation of adenosine into AMP (16)), did not affect the amount of cell-incorporated 3H-nucleosides and the pattern of their sequential interconversion into [3H]ATP (data not shown). Collectively, incubating PC-3 cells with exogenous adenosine was shown to affect the balance of intracellular purines, most likely via the transport of adenosine and/or its nucleoside metabolites and their subsequent multistep interconversion and transphosphorylation inside the cell.
Adenosine inhibits phospho-AMPK1α and other signaling pathways in PC-3 cells

At this point, the most evident mechanism(s) underlying the inhibitory effects of adenosine on PC-3 invasion may include its cellular uptake and deregulation of intracellular purine homeostasis and signalling pathways. To address this hypothesis experimentally, we compared the phosphorylation levels of key protein kinases in control and adenosine-treated cells by using an antibody-based array (Fig. 5A, B). The subsequent quantitative analysis of the array images revealed the ability of adenosine to decrease the phosphorylation levels of certain kinases, including TOR, JNK pan, MEK1/2, AMPK1α and p38a (Fig. 5C), without affecting the phosphorylation profiles of many other protein kinases (Supplementary Table). Interestingly, the adenosine-mediated inhibition of phospho-AMPK1α is basically consistent with the above HPLC data on decreased AMP/ATP ratio in adenosine-treated PC-3 cells (see Fig. 4C). Western blotting analyses further confirmed that, in comparison with untreated controls, adenosine-treated PC-3 cells are characterized by the down-regulation of phospho-AMPK1α (Fig. 6A). Additional immunofluorescence stainings with anti-phospho-AMPKα1/2 (Thr172) antibodies also demonstrated that along with predominant nuclear fluorescence, substantial punctate cytosolic green staining was observed in the control and, to lesser extent, adenosine-treated PC-3 cells (Fig. 6B, upper panels). Staining patterns with antibodies against total AMPKα1/2 were fairly comparable between the control and treated cells (Fig. 6B, middle panels).

We also examined an overall pattern of phosphotyrosine signalling by using 4G10 mAb to tyrosine kinase substrates. Both control and adenosine-treated PC-3 cells displayed similar phosphorylation profiles, with an abundant tyrosine phosphorylation of several protein bands being detected within a molecular weight range of ~50-140 kDa (Fig. 6A; lower panel). These immunoblotting data are consistent with the data on PC3 stainings with 4G10 antibody showing strong green fluorescence in the cortical regions beneath the plasma membranes of both control and treated cells (Fig. 6B, lower panels). Noteworthly, control and adenosine-treated PC-3 cells
also displayed similar patterns of Alexa-546-Phalloidin (Fig. 6B; red), as well as tubulin and vimentin (data not shown) stainings, thus excluding the potential changes of F-actin filaments and the whole cytoskeleton assembly in the treated cells.

**Treatment of MDA-MB-231 cells with adenosine also diminishes their invasion and affects phosphorylation profiles of AMPK1α and other kinases**

Lastly, we evaluated the effects of adenosine on the invasiveness and phospho-kinase profile of another highly invasive cell line, MDA-MB-231 breast cancer cells. These cells also express A2B receptors (ADORA2B) and CD73 (NT5E) at mRNA levels (Fig. 7A), possess high ecto-5’-nucleotidase/CD73 activity (Fig. 7B), and in addition, are capable of further deaminating adenosine (29), with subsequent uptake of generated nucleosides and their interconversion into intracellular [3H]ADP/ATP (data not shown). As in the case of PC-3 cells, pre-treatment of MDA-MB-231 cells with micromolar concentrations of NECA and adenosine, but not with inosine, inhibited their invasion through matrigel-coated inserts (Fig. 7C). Importantly, the Phospho-Kinase Array also revealed significant inhibitory effects of adenosine on phosphorylation profiles of certain kinases in MDA-MB-231 cells, including phospho-AMPK1α (Fig. 7D,E and Supplementary Table). Finally, we employed a small-molecule AMPK inhibitor compound C capable of inhibiting phospho-AMPK activity in PC-3 and other cancer cells in a concentration-dependent fashion (30, 31). Similarly to adenosine-pretreated cells, compound C significantly diminished the number of invaded MDA-MB-231 cells (Fig. 7C), with a similar inhibitory pattern being also detected in PC-3 cells (data not shown), thus providing an independent line of evidence for the potential implication of AMPK-dependent mechanisms in the inhibitory effects of adenosine.
Discussion

Given the emerging role of extracellular purines in tumor growth and metastasis, this study was undertaken to evaluate the in vitro effects of adenosine, other nucleosides and related compounds on the invasive and migration patterns of highly metastatic tumor cell lines. Major findings of this work are summarized as the following: 1) Pre-treatment of human PC-3 prostate carcinoma cells and MDA-MB-231 breast cancer cells with low micromolar concentrations of adenosine and with non-selective adenosine receptor agonist NECA or small-molecule AMPK inhibitor compound C inhibited subsequent cell invasion and migration through the matrigel- and laminin-coated inserts. 2) The inhibitory effects of adenosine presumably involve cellular uptake of adenosine and/or its nucleoside metabolites, their intracellular interconversion into ADP/ATP and eventually, the inhibition of phospho-AMPK1α and other signaling pathways. 3) Strikingly, no differences in the proliferation rates, cytoskeleton assembly, expression of major adhesion molecules (integrin-1β, CD44, FAK), and MMP secretion were detected between the control and treated cells, thus excluding the contribution of these components of the invasion cascade to the inhibitory effects of adenosine.

At first sight, the inhibitory effects of adenosine are unexpected, since high ecto-5'-nucleotidase activity is often considered as a poor prognostic factor, which allows the tumors to avoid immune destruction via the generation of immunosuppressive adenosine and deactivation of tumor-infiltrating inflammatory cells (5, 10, 12, 32). This apparent discrepancy could be resolved based on the following considerations. From the standpoint of adenosine homeostasis and its pharmacological activity, it might be more relevant to examine the ratio of the adenosine-producing capability of ecto-5'-nucleotidase/CD73 to the combined adenosine-utilizing activities (33). In fact, despite the abundant CD73 expression on tumor cells studied, the generated adenosine was shown to be rapidly deaminated and transported into the cells (see Fig. 4), suggesting a rather transient appearance of adenosine in the external milieu as the intermediate
product of the purine-inactivating chain. Therefore, local and transient generation of nanomolar adenosine by tumor-derived CD73 would indeed be sufficient for the failure of mounting effective immune responses via activation of the A2A receptors on cytotoxic T-lymphocytes and other inflammatory cells (14, 32). On the other hand, the overall pattern of purine homeostasis is markedly deregulated in the tumor environment by hypoxia and metabolic stress, as ascertained by data on constitutively elevated levels of intratumoral ATP (7, 34), the accelerated rate of nucleotide turnover (4, 9, 33), and the ability of solid tumors to maintain adenosine gradient, with the highest nucleoside concentrations being reported in the center (14). Therefore, it may be speculated that besides dampening antitumor immunity, the sustained exposure of tumor cells to the elevated adenosine concurrently impairs their invasiveness (present study) and at higher micromolar concentrations also retards tumor growth and proliferation (15, 21). This scenario might provide a partial explanation for the well-known phenomena of the high proliferative and invasive capacities of peripheral tumor cells located in the parenchyma and stroma, whereas the core environment of the tumor is maintained in a relatively stable, "semi-quiescent", state (2).

The salient finding of this study is that the effects of adenosine on tumor invasion may extend beyond its receptor-mediated activity. Potential mechanisms of this inhibition involve adenosine metabolism via ecto-adenosine deaminase activity, subsequent cellular uptake and interconversion of transported nucleosides, accompanied by inhibition of several protein kinases, including AMPKα1. AMPK is an energy-sensing serine/threonine kinase consisting of an α-catalytic subunit and regulatory β- and γ-subunits. It is activated under conditions of metabolic stress, such as glucose deprivation, hypoxia and strenuous exercise that deplete intracellular ATP and increase AMP (35). A number of findings suggest that AMPK-mediated reduction in anabolic pathways may ultimately lead to reduced tumor growth. Hence, pharmacological activation of AMPK would be advantageous in treating different endocrine-related tumors, including prostate and breast cancers (35-37). However, challenging this view, other studies have
emerged that lead to the opposite conclusion, stating that inhibition of AMPK reduces cancer growth (35, 36). In particular, the malignant human prostate tissue was shown to display a higher AMPK activity than normal tissue cells. In addition, the down-regulation of AMPK in prostate cancer cell lines, but not in non-tumorigenic prostate epithelial cells, reduced cell proliferation and induced apoptosis (30, 31). These conflicting results presumably depend on the tumor type and may also reflect a fine-tuned biphasic regulation of AMPK function and/or changing role of AMPK at different disease states.

Interestingly, short-term (1-10 min) incubation of human endothelial cells (38) as well as rat liver and intestinal epithelial cells (39) with micromolar concentrations of adenosine or its precursor nucleotides triggered a transient activation of AMPK. However, the mechanisms of these acute effects may differ substantially from the chronic effects of adenosine on AMPK inhibition seen in our experimental settings. In addition, the modulation of AMPK in normal and transformed cells often triggers the opposing effects due to differential deletion of downstream tumor suppressors, thus allowing AMPK activation while mitigating the growth-limiting effects of the enzyme (31, 35). Under conditions of energetic stress and the demand of continuous cell proliferation, AMPK bestows tolerance of cancer cells to nutrient deprivation without restricting cell growth and proliferation (31). In fact, the decreased AMPK activity in prostate cancer cells challenged to certain energy-starved conditions enhances apoptotic cell death during androgen deprivation and hypoxia (30) and also diminishes their invasive capability when incubated in serum-free media containing adenosine (present study).

Current research on the role of adenosine in tumor pathogenesis and inflammation is particularly focused on targeting the releasing pathways of the precursor nucleotide ATP (7, 34), directional regulation of ecto-5'-nucleotidase/CD73 activity by using selective enzyme inhibitors, siRNA gene silencing and anti-CD73 mAb therapies (10, 12, 32), synthesis of phosphorylated adenosine derivatives acting as site-specific CD73-activated prodrugs of A2A receptors (40), as
well as development of non-metabolizable nucleoside analogues acting either as selective adenosine receptor agonists and antagonists (5, 13) or as metabolic inhibitors of intracellular purine homeostasis (16, 20). Yet another approach includes tumor treatment with adenosine as a “kindred” metabolite, however most of the pro-apoptotic and anti-proliferative effects of adenosine reported in studies with different malignant cells required continuous presence of supra-physiological nucleoside concentrations (0.1-20 mM) in the assay mixture (15, 17-19).

Here, we have shown that adenosine is capable of preventing tumor cell invasion at relatively low concentrations (1-10 μM) without any adverse cytotoxic effects, and that these effects persisted for at least 48 ensuing hours even in the absence of stimuli applied. While this study outlines the potential implication of phospho-AMPK1α into inhibitory effects of adenosine, the diminished phosphorylation profiles of other key tumorigenic kinases in adenosine-treated cells (such as p27, p38α, JNK pan and TOR in case of PC-3 and STAT1, STAT4 Akt, eNOS and JNK pan in MDA-MB-231 cells; see the Supplementary Table) suggest the existence of a rather complex and probably tumor type-specific interplay between the extracellular and intracellular purine homeostasis, signaling pathways and tumor behavior. Moreover, significant inhibition of invasion in studies with non-selective agonist NECA (see Figs. 1B and 7C), together with data on predominant expression of the A2B receptors on PC-3 and MDA-MB-231 cells ((26, 28); also present study), and diverse pro- and anti-tumoral roles of A2B receptors in different tumors (13, 26), may also indicate the involvement of A2B-receptor mediated signalling in control of tumor invasion, growth and proliferation, especially at high micromolar concentrations of agonist.

In conclusion, the results presented here confirm the important role of adenosine in tumor pathogenesis and further expand the concepts presented above by hypothesizing the co-existence of dynamic networks coordinately regulating purine homeostasis and signal transduction pathways in cancer cells. Intratumoral adenosine presumably exerts dual functions serving as “immunological barrier” responsible for the failure of mounting an effective immune response
toward malignant cells and at higher nucleoside concentrations, impairing invasion and retarding cell growth and proliferation. Noteworthy, these conclusions mainly rest on comprehensive analyses performed with highly invasive androgen-independent PC-3 prostate carcinoma cells, and also with MDA-MB-231 breast cancer cells representing a form of triple-negative breast cancer with poor prognosis. Therefore, additional *in vitro* and *in vivo* studies with other tumor cell lines and tissues would be required to elucidate the role of adenosine in prostate, breast and other endocrine-related cancers. The complex implication of both extrinsic and intrinsic mechanisms into a tuned adenosine-dependent control of tumor growth and metastasis may open up further research for development of combined therapeutic approaches selectively targeting adenosine receptors on the cell surface and at the same time, modulating intracellular purine homeostasis and related signaling pathways.

**Acknowledgments**

We are grateful to Sergei Samburski for help in performance of reverse-phase HLPC assays and data analysis. We also thank Ruth Fair-Mäkelä for the revision of the text, Sari Mäki for technical assistance and Anne Sovikoski-Georgieva for secretarial help.

**Author contributions**

**Conception and design:** G.G. Yegutkin

**Development of methodology:** S.S. Virtanen, G.G. Yegutkin

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** G.G. Yegutkin, S.S. Virtanen, A. Kukkonen-Macchi, M.Vainio, K.Elima

**Writing, review, and/or revision of the manuscript:** G.G. Yegutkin, S.Jalkanen

**Study supervision:** G.G. Yegutkin, S. Jalkanen, P.L. Härkönen
References


Figure Legends

**Figure 1.** The effect of adenosine and other nucleosides and purinergic agonists on PC-3 cell invasion and migration. PC-3 cells were pre-treated with the indicated concentrations of adenosine (Ado), inosine (Ino) and guanosine (Guan), as well as non-selective (NECA) and A2-selective (CPCA) agonists of adenosine receptors, followed by incubation in invasion or migration assays in the absence of tested compounds. The numbers of invaded and migrated cells were determined as described in Materials and Methods and expressed either as actual cell counts (A) or as a percentage of controls (B) (mean±SEM; n = 4-8). Note, since no differences were observed between vehicle-treated cells incubated without and with 0.02% DMSO, these results were pooled and considered as a common “Control” (Co). *, P < 0.05 as compared to control.

**Figure 2.** The effect of adenosine on adenylyl cyclase activity and proliferation of PC-3 cells. Adenylyl cyclase was assayed by measuring cAMP level after incubation of PC-3 cells with increasing concentrations of adenosine (0-500 µM) (A) or with the indicated concentrations of adenosine (Ado), other nucleosides and adenosinergic agonists (B). Cells were also pre-treated with A2B antagonist alloxazine (Allox) prior to the addition of adenosine. *, P < 0.05 as compared to corresponding controls. C, a quantitative PCR analysis shows the abundant mRNA expression for ecto-5'-nucleotidase/CD73 (NT5E) and for A2B (ADORA2B), but not A1 (ADORAI), A2A (ADORA2A) and A3 (ADORA3) adenosine receptors in PC-3 cells. The average mRNA expression of each gene was presented as a percentage of β-actin mRNA expression measured from the same sample. D, proliferation of PC-3 cells was determined after a 20-hour incubation with [methyl-³H]thymidine in the absence (Control) and presence of the indicated concentrations of adenosine and other compounds (µmol/L, except for LPS expressed as ng/ml).
The bars show the amount of cell-incorporated radioactivity (mean±SEM) from three independent experiments. *, $P < 0.05$ as compared to control.

**Figure 3.** Treating PC-3 cells with adenosine does not affect the expression of major adhesion molecules. A, representative histograms for PC-3 cells pre-treated without (Control; $Co$) and with 10 μM adenosine ($Ado$) and subsequently stained with antibodies against integrin β1, CD44 and also with isotype-matched control antibody (shown in grey). B, mean fluorescence intensities of integrin β1- and CD44-positive PC-3 cells were determined either immediately after the treatment with adenosine or after an additional 2-hour incubation on matrigel-coated wells (mean±SEM; $n = 3$). C, Western blot analyses of indicated adhesion molecules in control and adenosine-treated PC-3 cells which were activated by seeding them onto matrigel-coated wells. For each experiment, the blots were probed with an anti-β-tubulin mAb to normalize for protein loading. The arrows indicate the position of molecular mass markers (kDa).

**Figure 4.** Chromatographic analyses of the extra- and intracellular purine-converting pathways in PC-3 cells A, TLC analyses of the time courses of metabolism of 10 μM $[^3]$HAMP (upper panel) and $[^3]$Hadenosine (lower panel) and the formation of their metabolites by cultured PC-3 cells. B, aliquots of conditioned media were collected 0, 2 and 20 hours after the incubation of PC-3 cells with 10 μM of adenosine, followed by the separation of nucleosides by reverse-phase HPLC. The peaks corresponding to adenosine ($Ado$), inosine ($Ino$) and hypoxanthine ($Hyp$) were monitored by absorption at 258 nm and indicated in the chromatograms shown. C, PC-3 cells were incubated without (control) and with 10 μM adenosine for 20 hours. Intracellular nucleotides and adenosine ($Ado$) were determined in PC-3 cells lysates and expressed as percentages of intracellular ATP concentration (mean±SEM; $n = 3$). *, $P < 0.05$ as compared to the control. D, TLC analyses of cell-incorporated $[^3]$Hadenosine and its $[^3]$H-metabolites.
determined after 20-hour incubation of PC-3 cells with 10 μM [3H]adenosine. The results are expressed as percentages of total radioactivity uptaken by the cells (mean±SEM; n = 3).

Figure 5. The effect of adenosine on the kinase phosphorylation profile in PC-3 cells. Control (Co) and adenosine-treated (Ado) PC-3 cells were seeded for 2 hours onto Matrigel-coated wells, lysed and assayed for a panel of 46 phospho-kinases using an antibody-based array. A, a representative array from the control cells shows a phosphorylation profile of the kinases studied. The phosphorylation level of each kinase is presented as duplicate spots and positive control spots are located in the corners of the membranes (a1-2, g1-2 and a17-18). B, Relative phosphorylation levels of the indicated protein kinases. The array signals from scanned X-ray film images from two independent experiments are shown. C, spot pixel densities were quantified after data normalization to the internal controls and background intensity. The results show mean ± SEM from 3 independent experiments, where each sample represents the pooled lysates from two different assays. $P < 0.05$ as compared to control. For complete densitometric analyses of phospho-kinases in control and adenosine-treated PC-3 cells see Supplementary Table.

Figure 6. The effect of adenosine on phospho-AMPK activity and overall pattern of phosphotyrosine signalling in PC-3 cells. PC-3 cells pre-treated without (Control; Co) and with 10 μM adenosine (Ado) were activated by seeding onto matrigel-coated wells. A, Western blotting analysis of PC-3 cell lysates stained with antibodies against total and phosphorylated forms of AMPK, phosphotyrosine kinases (4G10), and β-tubulin as a control for the protein loading. The arrows indicate the positions of the molecular mass markers (kDa). B, AMPK, phospho-AMPK, and phosphotyrosine (4G10) stainings of PC-3 cells grown on matrigel-coated coverslips. The sections were incubated with the indicated antibodies and subsequently stained
with appropriate secondary Alexa<sub>488</sub>-conjugated antibodies (green). For the actin cytoskeleton staining, the cells were co-stained with Alexa<sub>566</sub>-Phalloidin (shown in red). Nuclei were stained with blue-fluorescent DAPI. Scale bar, 50 µm.

**Figure 7.** The effect of adenosine on the invasion pattern and phospho-kinase profile of breast cancer cells. A, a quantitative PCR analysis of MDA-MB-231 cell lysates shows the abundant mRNA expression for A<sub>2B</sub> (ADORA2B), but not other adenosine receptor subtypes (ADORA1, ADORA2A and ADORA3), and also for ecto-5'-nucleotidase/CD73 (NT5E) (n = 3). The average mRNA expression for each gene was presented as a percentage of β-actin mRNA measured from the same sample. B, TLC analysis of the time course of [<sup>3</sup>H]AMP (10 µM) hydrolysis and formation of <sup>3</sup>H-nucleoside metabolites by MDA-MB-231 cells. C, Invasion of MDA-MB-231 cells after their pre-treatment with indicated concentrations (µM) of adenosine (Ado), inosine (Ino), NECA and compound C (Comp C). The bars show the numbers of invaded cells (mean±SEM; n = 3-5). *, P < 0.05 as compared to control vehicle-treated cells. D, lysates from control (Co) and adenosine-treated (Ado) MDA-MB-231 cells were assayed for phosphorylation levels of protein kinases. The array signals from scanned X-ray film images from two independent experiments are shown. E, the results from panel D were analyzed using the image analysis software, normalized for background intensity and expressed as mean ± SEM (n = 3). *, P < 0.05 as compared to the control. For complete quantitative densitometric analyses of major kinases see Supplementary Table.
Figure 3

A

Counts

\[
\begin{align*}
\text{Co} & \quad \text{Ado} \\
\text{\(\beta_1\) integrin-FITC} & \quad \text{\(\beta_1\) integrin-FITC} \\
\text{CD44-FITC} & \quad \text{CD44-FITC}
\end{align*}
\]

B

Mean fluorescence

<table>
<thead>
<tr>
<th>Staining</th>
<th>integrin (\beta_1)</th>
<th>CD44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Matrigel</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

C

- **Integrin \(\beta_1\)**: 102 kDa
- **tubulin**: 52 kDa
- **CD44**: 76 kDa
- **tubulin**: 52 kDa
- **p-FAK (Y397)**: 150 kDa, 102 kDa
- **FAK**: 150 kDa, 102 kDa
Figure 4
Figure 5

A

Membrane A

Membrane B

B

Target (coordinate)
TOR (c1-2)
JNK pan (a7-8)
MEK1/2 (b3-4)
AMPKα1 (b7-8)
p27 (d15-16)
p38a (a3-4)
Lyn (d3-4)

Co Ado Co Ado

Exp 1 Exp 2

C

Optical density (ODu/mm²)

- Control - Ado

TOR (S2446) (T183/Y185)
JNK pan (T202/Y204)
MEK1/2 (S218/S222)
AMPKα1 (T174)
p27 (T157)
p38a (T180/Y182)
Lyn (Y397)

*
Figure 6

Panel A: Western blots showing the levels of p-AMPK (Thr174), AMPK, 4G10, and tubulin in control (Co) and Ado-treated cells. The molecular weights (kDa) are indicated.

Panel B: Immunofluorescence images of control (Co) and Ado-treated cells stained for p-AMPK, AMPK, 4G10, and tubulin. The scale bar represents 50 μm.
Figure 7
Molecular Cancer Research

Adenosine Inhibits Tumor Cell Invasion via Receptor-independent Mechanisms

Sanna S. Virtanen, Anu Kukkonen-Macchi, Minna Vainio, et al.

Mol Cancer Res  Published OnlineFirst July 30, 2014.

Updated version
Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-14-0302-T

Supplementary Material
Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2014/07/31/1541-7786.MCR-14-0302-T.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.