Involvement of A2B receptor in DNA damage response and radiosensitizing effect of A2B receptor antagonists on mouse B16 melanoma

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Summary
It is therapeutically important to elucidate the factors involved in the radiation resistance of tumors. We previously showed that ATP is released from mouse melanoma B16 cells in response to γ-irradiation, but the role of adenosine, a metabolite of ATP, is still unclear. Here, we show that the adenosine A2B receptor is involved in DNA damage repair and radioresistance in mouse melanoma B16 cells. The DNA damage response after γ-irradiation was attenuated by pretreatment with A2B receptor antagonists, such as PSB603, while it was enhanced by pretreatment with A2B receptor agonists, such as BAY60-6583. γ-Irradiation decreased the cell survival rate, and pretreatment with PSB603 further reduced the survival rate. On the other hand, pretreatment with BAY60-6583 increased the cell survival rate after irradiation. The DNA damage response and the cell survival rate after γ-irradiation were both decreased in A2B-knockdown cells. In vivo experiments in mice confirmed that tumor growth was suppressed and delayed in the irradiated group pretreated with PSB603, compared with the irradiation-alone group. Our results indicate that adenosine A2B receptor contributes to radioresistance, and could be a new target for the development of agents to increase the efficacy of radiotherapy.

Keywords
radiation therapy; melanoma; adenosine; A2B receptor; DNA repair; γ-irradiation
Introduction

Radiotherapy is an important cancer treatment, together with surgery and chemotherapy, but many tumors, such as malignant melanoma, glioblastoma and thyroid cancer, exhibit radioresistance \(^1\). The antitumor effect of ionizing radiation is mediated by the generation of reactive oxygen species, which damage DNA and induce apoptosis or senescence of cancer cells \(^2\). However, cancer cells that are not killed may acquire enhanced proliferative capacity and radioresistance via upregulation of DNA damage repair. Although many radiosensitizers, including direct radiosensitizers using gold nanoparticles and indirect radiosensitizers targeting DNA damage repair factors, have been investigated in attempts to increase the efficacy of radiation therapy, none has yet entered clinical use \(^3, 4\). Further, the intracellular biochemical processes that occur after irradiation have not been established in detail.

We previously reported that ATP is released from B16 melanoma cells after gamma irradiation and the release is mediated by P2X7 receptor and connexin 43 \(^5, 6\). We also showed that inhibition of P2X7 receptor had a radiosensitizing effect \(^7\). In the present work, we focused on adenosine, which is a metabolite of extracellular ATP, based on a recent report that adenosine is present at high concentrations around tumor tissue \(^8\). Adenosine is a purine nucleoside that is ubiquitous in organs and tissues \(^9\), playing key roles in the central nervous system \(10, 11\), immune responses such as inflammation \(12, 13\), the endocrine system \(14, 15\), pain \(16, 17\), lung function \(18, 19\), and kidney function \(20\). Adenosine is the major endogenous agonist for all human adenosine receptors, and is present at high concentrations in the cancer microenvironment \(21\).

Adenosine receptors are G-protein-coupled receptors, and four subtypes are known: A1, A2A, A2B and A3. The A1, A2A and A3 receptors have a high affinity for adenosine, while A2B receptor has low affinity \(21, 22\). Indeed, A2B receptor is activated only at high concentrations of adenosine, e.g., under conditions of hypoxia or ischemia \(21, 22, 23\). Notably, tumor tissue is generally hypoxic, and A2B receptor is highly expressed in tumor tissue, because hypoxia directly increases the expression of A2B receptor mRNA via a hypoxia-inducible factor 1 (HIF-1\(\alpha\)) binding site at the promoter \(23, 24, 25\). Thus, A2B receptor is likely to be activated in tumor cells. Importantly, recent studies found that A2B receptor activation by the selective agonist BAY 60-6583 promoted tumor growth in melanoma-bearing model mouse \(26\). Moreover, suppression of the growth of various cancers, including bladder cancer and breast cancer, by A2B-selective inhibitors has been confirmed \(27\). Very recently, we reported that A2B receptor is involved in radiation-induced translocation of epidermal growth factor receptor and DNA damage response in human lung cancer cells \(28\). However, the role of adenosine in radiation-induced biological effects has not been established.

Ionizing radiation induces DNA damage, but this may be repaired by cellular systems,
whose induction in tumor cells may lead to accelerated regrowth and acquisition of radioresistance. Indeed, the initial DNA damage response, involving phosphorylation of ataxia telangiectasia mutation (ATM), formation of phosphorylated histone variant H2AX (γH2AX) foci, accumulation of p53 binding protein 1 (53BP1), etc., occurs within 1 hour after irradiation. However, the role of adenosine receptors in these cellular responses to radiation is not known in melanoma.

Therefore, in this study, we examined the radiosensitizing effect of adenosine A2B receptor antagonists, such as PSB603, in a malignant melanoma cell line, since it is reported that PSB603 enhances tumor immunity. Here, we found that PSB603 suppresses the DNA damage repair response and decreased the growth rate of B16 melanoma cells. Furthermore, tumor growth in an in vivo mouse model transplanted with B16 cells was suppressed and delayed in the irradiated group pretreated with PSB603.

Materials & Methods

Reagents and Antibodies

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Gibco® fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (U.S.A). The primary antibodies used were 53BP1 antibody rabbit polyclonal (Novus, U.S.A.), phospho-histone H2AX (Ser139) rabbit monoclonal antibody (Cell Signaling Technology, U.S.A.), anti-adenosine A2b receptor rabbit polyclonal antibody (Sigma-Aldrich, U.S.A.). The secondary antibodies used were Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG) (Invitrogen, U.S.A.), goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) (Sigma-Aldrich), and anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology). SiRNA targeting mouse A2B receptor and negative control siRNA (TriFECTa Kit® DsiRNA Duplex) were purchased from Integrated DNA Technology.

Antagonists and Inhibitors

PSB603 (Tocris Bioscience, England) is a selective antagonist of adenosine A2B receptor. CGS15943 (Tocris Bioscience) is a P1 receptor antagonist. PSB36 (Tocris Bioscience) is a selective antagonist of adenosine A1 receptor. SCH442416 (Tocris Bioscience) is a selective antagonist of adenosine A2A receptor. MRS3777 (Tocris Bioscience) is an adenosine A3 receptor antagonist. MRS1706 (Tocris Bioscience) is a selective antagonist of adenosine A2B receptor. MRS1754 (Tocris Bioscience) is a selective antagonist of adenosine A2B receptor. Adenosine (Sigma-Aldrich) is a P1 receptor ligand, and we used it as an agonist of P1 receptor. BAY60-6583 (Tocris Bioscience) is an adenosine A2B receptor agonist. APCP
(adenosine 5’-(α,β-methylene) diphosphate) (Sigma-Aldrich) is an ecto-5’-nucleotidase (CD73) inhibitor.

**Cell Culture**

Mouse melanoma B16 cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO₂ in air at 37 °C.

**Irradiation**

B16 cells were irradiated with γ-rays from a Gammacell 40 ($^{137}$Cs source) (Nordin International, Inc.; 0.72 Gy/min) at room temperature for an indicated time. After irradiation, the cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C.

**Immunofluorescence Staining**

DNA damage response was quantified by immunofluorescence staining as described previously

**Colony Formation Assay**

The survival rate was quantified by colony formation assay as described previously

**RT-PCR**

A2B receptor mRNA was analyzed by RT-PCR as described previously. The primers used for amplification were designed based on the cDNA sequence of mouse A2B receptor.

**Western blotting**

Expression of A2B receptor were analyzed by immunoblotting as described previously. Lysate of B16 cells (8.0 × 10⁴ cells) was mixed with 2 x sample buffer and 10 mM DTT, and incubated at 95 °C for 10 min. The primary antibodies used were rabbit anti-adenosine A2b receptor antibody (1:1000) for detection of A2B receptor. The secondary antibodies used were goat horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5,000) for detection of A2B receptor for 1.5 h at room temperature. As a loading control, the blots were incubated with peroxidase-conjugated anti-β-actin monoclonal antibody (Wako Pure Chemical Industries, Ltd.) (1:50,000) for 60 min at room temperature.

**Small interfering RNA (siRNA) transfection**

B16 cells (5.0×10⁴ cells/well) were incubated in culture for 16 h. The siRNA duplex oligonucleotides (25 nM) for knockdown of mouse adenosine A2B receptor were transfected.
into B16 cells by using Lipofectamine RNAiMAX (invitrogen) and Opti-MEM Reduced Serum Medium (invitrogen) according to the manufacturer’s instructions. Cells were used for Western blotting at 24 hours after transfection, and for immunofluorescence and colony formation assay at 48 hours after transfection.

Animals
Pathogen-free male C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 5 weeks of age. They were housed as described previously. The mice were treated and handled according to the Tokyo University of Science’s institutional ethical guidelines for animal experiments and with the approval of Tokyo University of Science’s Institutional Animal Care and Use Committee (permission numbers S19007, S18009, S17009).

Transplantation Model
B16 melanoma cells (2.0×10^5 cells) were injected into the right hind footpad of 5-week-old male C57BL/6 mice. To examine the effect of A2B receptor antagonist on melanoma growth, 100 µL of PBS or 50 mM PSB603 was intraperitoneally administered to B16 melanoma-bearing mice (22.29±3.21 g) on the 14th, 18th, 21st, and 23rd days after transplantation. Mice were irradiated with 1.0 Gy of γ-rays at 2 h after treatment with PSB603 on the 14th, 18th, 21st, and 23rd days after transplantation. The size of the solid tumor was measured with a caliper twice a week for 25 d, and the tumor volume (V) was calculated according to the following equation:

\[ V (mm^3) = \frac{4}{3} \times 3.14 \times \text{length (mm)}/2 \times \text{width (mm)}/2 \times \text{thickness (mm)}/2 \]

Statistics
Results are expressed as the mean ± standard error (S.E.). The statistical significance of differences between the control and other groups was calculated by using Dunnett’s test. Calculations were done with the Instat version 3.0 statistical software package (Graph Pad Software). The criterion of significance was set at p<0.05.

Results
DNA damage response and cell viability following γ-irradiation
First, we examined γH2AX-53BP1 focus formation in γ-irradiated B16 melanoma cells using immunofluorescence staining. γH2AX-53BP1 focus formation increased in a dose-dependent manner (1.0-8.0 Gy) (Fig. 1A, B) and reached a maximum at 0.5-3 h (Fig. 1C). Based on these results, in this study, we performed 2 Gy of irradiation, because 2 Gy is a dose widely used in radiotherapy. We examined γH2AX-53BP1 focus formation at 30 minutes after irradiation, which is the peak of γH2AX-53BP1 focus formation.

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Furthermore, cell viability was measured one week after γ-irradiation by colony formation assay. Cell death increased in a dose-dependent manner (1.0-8.0 Gy) (Fig. 1D).

**Involvement of adenosine receptors in DNA damage response and reproductive cell death induced by γ-irradiation**

To confirm the involvement of adenosine receptors in DNA damage response and reproductive cell death induced by γ-irradiation, we evaluated the effect of adenosine receptor antagonists on γH2AX-53BP1 focus formation at sites of DNA damage in γ-irradiated B16 melanoma cells. Co-staining of γH2AX and 53BP1 was significantly reduced by pretreatment with various adenosine receptor antagonists (PSB36, SCH442416, PSB603, MRS3777, CGS15943) (Fig. 2A). Next, we evaluated the effects of these antagonists on reproductive cell death. Pretreatment with PSB603 and CGS15943 significantly enhanced reproductive cell death induced by γ-irradiation (2 Gy) (Fig. 2B). Pretreatment with adenosine (10-100 µM) itself increased γH2AX-53BP1 focus formation following γ-irradiation and attenuated reproductive cell death (Figs. 2C, D). Furthermore, in order to clarify the involvement of extracellular adenosine, we focused on CD73, an extracellular adenosine-generating enzyme. Pretreatment with the CD73 antagonist APCP reduced γH2AX-53BP1 focus formation (Fig. 2E), confirming that extracellular adenosine is involved in the DNA damage response to γ-irradiation. These results indicate that adenosine receptor antagonists may exert their radiosensitizing effect via suppression of the γ-irradiation-induced DNA damage response in B16 melanoma cells.

**Involvement of adenosine A2B receptor in DNA damage response and reproductive cell death induced by γ-irradiation**

As shown in Fig. 2, adenosine A2B receptor appeared to play a key role in DNA damage response and suppression of reproductive cell death following γ-irradiation. Therefore, we initially examined the expression of adenosine A2B receptor in B16 melanoma cells. B16 melanoma cells expressed A2B receptor at both the mRNA and protein levels (Figs. 3A, B). In addition, in order to compare the expression of A2B receptor in normal cells and cancer cells, we examined A2B receptor expression in human keratinocyte HaCaT cells and B16 cells. Although there is a difference between human cells and mouse cells, A2B receptor was more expressed in B16 cells than in HaCaT cells (Supplementary Fig. 1). Co-staining of γH2AX and 53BP1 in γ-irradiated B16 melanoma cells was significantly reduced by pretreatment with adenosine A2B receptor antagonists (PSB603, MRS1706, MRS1754), indicating that γH2AX-53BP1 focus formation at DNA damage sites was reduced (Fig. 4A). Further, pretreatment with the adenosine A2B receptor antagonist PSB603 significantly enhanced reproductive cell death due to γ-irradiation (2 Gy) (Fig. 4B), while pretreatment with the adenosine A2B receptor agonist BAY60-6583 (1 µM) increased γH2AX-53BP1 focus formation.
focus formation and attenuated reproductive cell death (Figs. 4C, D). As shown in Fig. 1B, DNA damage usually repairs within 24 hours after irradiation. However, if the repair reaction does not finished, γH2AX-53BP1 focus remains. The remaining DNA repair focus indicates unrepaired DNA damage. Then, we examined γH2AX-53BP1 focus formation 24 hours after γ-irradiation (4.0 Gy) to elucidate unrepaired DNA damage. In the measurement of remaining DNA repair focus (unrepaired DNA damage), B16 cells were irradiated with 4 Gy, because the number of remaining γH2AX-53BP1 focus at 24 h induced by 2 Gy irradiation is too low to elucidate the effect of inhibitor appropriately. As shown in Fig. 4E, unrepaired DNA damage was still apparent at 24 hours after γ-irradiation in the case of antagonist pretreatment. Thus, the treatment with A2B receptor antagonists caused remaining of unrepaired DNA damage leading to cell death. These results indicate that adenosine A2B receptor antagonists have a radiosensitizing effect via suppression of the γ-irradiation-induced DNA damage response in B16 melanoma cells.

Effect of A2B receptor knockdown on DNA damage response and reproductive cell death induced by γ-irradiation

To confirm the involvement of A2B receptor in DNA damage response and suppression of reproductive death by γ-irradiation, B16 cells were transfected with siRNA targeting A2B receptor. Expression of A2B receptor protein was decreased in the knockdown cells, as determined by Western blotting (Fig. 5A), so we used these cells to evaluate the effect of adenosine A2B receptor knockdown on γH2AX-53BP1 focus formation at DNA damage sites in γ-irradiated B16 melanoma cells. Co-staining with γH2AX and 53BP1 was significantly reduced (Fig. 5B). Furthermore, reproductive cell death induced by γ-irradiation (2.0 Gy) was significantly enhanced (Fig. 5C). These results strongly support the idea that A2B receptor is involved in DNA damage response and suppression of reproductive cell death by γ-irradiation.

Tumor growth-inhibitory effect of the combination of PSB603 and γ-irradiation in a mouse model of melanoma

To determine whether adenosine A2B receptor antagonist treatment is effective in vivo, the radiosensitizing effect of PSB603 on the proliferation of B16 melanoma cells implanted in the footpad of C57BL/6 mice was examined. At 14, 18, 21 and 23 days after transplantation, mice were treated with PSB603 (100 µL of 50 µM solution/head i.p.) and given whole-body irradiation (1.0 Gy) 2 hours later (total radiation dose, 4.0 Gy). Tumor volumes were also measured on the 14th, 18th, 21st, 23rd, and 25th days after transplantation. As shown in Figs. 6A and B, no statistical significant suppression of tumor growth was seen with radiation therapy alone or PSB603 alone, though irradiation or PSB603-treatment tended to suppress tumor growth. However, statistical significant suppression of tumor growth was seen in the
combination group at 25 days after transplantation. This indicates that PSB603 acts as a radiosensitizer not only in vitro, but also in vivo.

**Discussion**

Adenosine A2B receptor is known to be activated in the presence of high concentrations of adenosine, and is overexpressed in tumor tissue as compared to normal cells. We previously found that ATP is released extracellularly after radiation exposure, and is involved in the radiation-induced DNA damage repair response. Therefore, we hypothesized that adenosine, which is a metabolite of ATP and an antagonist of A2B receptor, might serve as a tumor-specific radiosensitizer. Here, we tested this idea using B16 melanoma cells, which are radioresistant, as an in vitro model, and B16-inoculated mice as an in vivo tumor model.

We have previously shown that P2X7 receptor-dependent ATP release occurs 1-10 minutes after γ-irradiation in B16 melanoma cells. The released ATP promotes focus formation of the DNA damage repair marker γH2AX, with peaks at 30 min and 6 h after irradiation. We speculated that the peak at 30 min could be due to the rapid ATP release. Here, we found that adenosine receptor antagonists suppressed the DNA damage response (γH2AX focus formation and 53BP1 accumulation). Thus, adenosine formed by hydrolysis of ATP could be involved in the induction of DNA damage repair by γ-irradiation.

γ-Irradiation (1.0-8.0 Gy) also caused dose-dependent reproductive death of B16 cells, and the effect was enhanced by pretreatment with various A2B receptor antagonists, including PSB603. On the other hand, γ-irradiation-induced reproductive death was suppressed by adenosine itself or by BAY60-6583, an A2B receptor agonist. These findings support the view that A2B receptor activation is involved in the recovery of cancer cells from irradiation-induced damage.

In this study, we mainly focused on PSB603 as an A2B receptor antagonist, because it showed low cytotoxicity to B16 cells at the concentration of 10 µM used in this study, which was sufficient to suppress the activation of A2B receptor by γ-irradiation in B16 cells. Also, we focused on BAY60-6583 as an A2B receptor agonist, because it is effective at the low concentration of 0.5 µM. In our study, based on immunofluorescence and colony formation assay (data not shown), we selected 1.0 µM BAY60-6583 as a suitable concentration to activate the A2B receptor.

As shown in Figs. 2A and 2B, all the adenosine receptor antagonists inhibited the DNA damage response, but only PSB603, MRS3777 and CGS15943 showed significant enhancements of reproductive cell death, as measured by colony formation assay. These results indicate that A2B receptor and A3 receptor are involved in the survival of cancer cells, in accordance with reported findings. However, MRS 3777 and CGS 15943 reduced the survival rate of non-irradiated groups, so we focused on PSB603 for this study. PSB603 was
also used for the *in vivo* study, because it showed the most potent effect among the A2B receptor antagonists examined (Figs. 4A and 4B).

It has been reported that PSB603 suppresses tumor growth in melanoma and prostate cancer\(^{41, 42}\). However, there is no report of the effect of the combination of \(\gamma\)-irradiation and PSB603 *in vivo*. Therefore, we examined the effect of PSB603 as an adenosine receptor antagonist in a B16 melanoma tumor-bearing mouse model *in vivo*. Melanoma growth was not significantly suppressed by irradiation alone or PSB603 alone. However, significant inhibition of tumor growth was observed in the 1.0 Gy irradiation and PSB603 combination treatment group. This suggests that PSB603 acts as a radiosensitizer *in vivo*.

In this study, we used only mouse B16 melanoma. To investigate whether the involvement of A2B receptor in DNA damage response is only observed in radioresistant melanoma, we will need further comparison between human melanoma cells and human normal skin cells such as keratinocytes, and comparison between human radioresistant melanoma and radiosensitive melanoma. These investigations will help to apply our findings to radiotherapy of human melanoma.

CD73 is a cell membrane enzyme that is involved in the formation of extracellular adenosine, and has roles in tumor growth, angiogenesis and chemoresistance\(^{43, 44, 45}\). Furthermore, the hypoxia-inducing factor HIF-1 is increased by irradiation, and it induces CD73\(^{43}\). Here, we found that pretreatment with APCP, which is an inhibitor of CD73, suppressed the DNA damage repair response after \(\gamma\)-irradiation. This is consistent with the observation that inhibitors of A2B receptor can suppress tumor growth. However, it should be borne in mind that the expression level of CD73 is cell-type-dependent\(^{46, 47, 48}\), so there might be differences in the concentration of adenosine in the tumor microenvironment, depending upon the type of tumor.

A2B receptor is reported to be involved in the growth of bladder urothelial carcinoma and prostate cancer via the MAPK pathway, which includes ERK\(^{49, 50}\), but the A2B receptor is a G protein-coupled receptor whose downstream signals include cAMP, JNK, p38, etc.\(^{50}\). Therefore, the involvement of these molecules should also be investigated in future studies. Interestingly, CD73 is involved in tumor metastasis\(^{51, 52}\), so it may also be worthwhile to investigate the effect of A2B receptor inhibitors on tumor metastasis.

In recent years, clinical trials of the combined use of an immune checkpoint inhibitor and radiotherapy have been reported\(^{53, 54}\). The A2B receptor has various functions in immune cells\(^{55, 26}\), and PSB603 was reported to enhance tumor immunity and to suppress tumor growth and metastasis\(^{42}\). Therefore, A2B receptor antagonists might be candidates for use in combination therapy with immune checkpoint inhibitors and radiation. It may also be worth investigating whether the bystander effect\(^{56}\) is relevant to the radiosensitizing effect of A2B receptor inhibitors.

In conclusion, our results indicate that PSB603 suppresses the DNA damage repair response.
and decreases the growth rate of B16 melanoma cells. Furthermore, tumor growth in a mouse model transplanted with B16 cells was suppressed and delayed in the irradiated group pretreated with PSB603. Thus, A2B receptor antagonists may be promising candidates for use as radiosensitizers in radiation therapy of melanoma.

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**Conflict of Interest**
The authors declare no conflict of interest.

**Supplementary Materials**
The online version of this article contains supplementary materials.
References
1) Satyamoorthy K, Chehab NH, Waterman MJ, Lien MC, El-Deiry WS, Herlyn M, Halazonetis TD. Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells. Cell Growth Differ., 11, 467–474 (2000).
2) Yuan W, Yuan Y, Zhang T. Wu S. Role of Bmi-1 in regulation of ionizing irradiation-induced epithelial-mesenchymal transition and migration of breast cancer cells. PloS ONE, 10, e0118799 (2015).
3) Jain S, Hirst DG, O’Sullivan JM. Gold nanoparticles as novel agents for cancer therapy. Br. J. Radiol., 85, 101–113 (2012).
4) Cooper DR, Bekah D, Nadeau JL. Gold nanoparticles and their alternatives for radiation therapy enhancement. Front. Chem., 2, 86 (2014).
5) Ohshima Y, Tsukimoto M, Takenouchi T, Harada H, Suzuki A, Sato M, Kitani H, Kojima S. γ-Irradiation induces P2X7 receptor-dependent ATP release from B16 melanoma cells. Biochim. Biophys. Acta., 1800, 40–46 (2010).
6) Ohshima Y, Tsukimoto M, Harada H, Kojima S. Involvement of connexin43 hemichannel in ATP release after γ-irradiation. J. Radiat. Res., 53, 551-557 (2012).
7) Tanamachi K, Nishino K, Mori N, Suzuki T, Tanuma SI, Abe R, Tsukimoto M. Radiosensitizing effect of P2X7 receptor antagonist on melanoma in vitro and in vivo. Biol Pharm Bull., 40, 878-887 (2017).
8) Di Virgilio F, Adinolfi E. Extracellular purines, purinergic receptors and tumor growth. Oncogene, 36, 293-303 (2017).
9) Gassi S, Merighi S, Sacchetto V, Simioni C, Borea PA. Adenosine receptors and cancer. Biochim Biophys Acta., 1808 1400-1412 (2011).
10) Vecchio EA, White PJ, May LT. The adenosine A2BG protein-coupled receptor: Recent advances and therapeutic implications. Pharmacol Ther., 198, 20-33 (2019).
11) Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous System. Annu Rev Neurosci., 24, 31–55 (2001).
12) Fredholm BB, Chen JF, Masino SA, Vagueois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu Rev Pharmacol Toxicol., 45 385–412 (2005).
13) Fredholm BB. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ., 14, 1315–1323 (2007).
14) Haskó, G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov., 7, 759–770 (2008).
15) Dong Q, Ginsberg HN, Erlanger BF. Overexpression of the A1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. Diabetes Obes Metab., 3, 360–366 (2001).
16) Figler RA, Wang G, Srinivasan S, Jung DY, Zhang Z, Pankow JS, Ravid K, Fredholm B,
Hedrick CC, Rich SS, Kim JK, LaNoue KF, Linden J. Links between insulin resistance adenosine A2B receptors, and inflammatory markers in mice and humans. Diabetes, 60, 669–679 (2011).

17) Zylka MJ. Pain-relieving prospects for adenosine receptors and ectonucleotidases. Trends Mol Med., 17, 188–196 (2011).

18) Sawynok J. Adenosine receptor targets for pain. Neuroscience, 338, 1–18 (2016).

19) Wilson CN, Nadeem A, Spina D, Brown R, Page CP, Mustafa SJ. Adenosine receptors and asthma. Handb Exp Pharmacol., 193, 329–362 (2009).

20) Zhou Y, Schneider DJ, Blackburn MR. Adenosine signaling and the regulation of chronic lung disease. Pharmacol Ther., 123, 105–116 (2009).

21) Roberts VS, Cowan PJ, Alexander SI, Robson SC, Dwyer KM. The role of adenosine receptors A2A and A2B signaling in renal fibrosis. Kidney Int., 86, 685–692 (2014).

22) Fredholm BB, Irenius E, Kull B, Schulte G. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem Pharmacol., 61, 443-448 (2001).

23) J Stagg and MJ Smyth. Extracellular adenosine triphosphate and adenosine in cancer, Oncogene, 29, 5346-5358 (2010).

24) Mendichovszky I, Jackson A. Imaging hypoxia in gliomas. Br J Radiol., 84, 145-158 (2011).

25) Feoktistov I, Ryzhov S, Zhong H, Goldstein AE, Matafonov A, Zeng D. Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. Hypertension, 44, 649–654 (2004).

26) Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. FASEB J, 20, 2242–2250 (2006).

27) Iannone R, Miele L, Maiolino P, Pinto A, Morello S. Blockade of A2B adenosine receptor reduces tumor growth and immune suppression mediated by myeloid derived suppressor cells in a mouse model of melanoma. Neoplasia, 15, 1400–1414 (2013).

28) Kitabatake K, Yoshida E, Kaji T, Tsukimoto M. Involvement of adenosine A2B receptor in radiation-induced translocation of epidermal growth factor receptor and DNA damage response leading to radioresistance in human lung cancer cells. Biochim. Biophys. Acta.-General Subjects (in press).

29) Cekic C, Sag D, Li Y, Theodorescu D, Strieter RM, and Linden J. Adenosine A2B receptor blockade slows growth of bladder and breast tumors. J Immunol., 188, 198–205 (2011).

30) Betz H. Importance of the phase of resistance of the organism during the application of a lethal dose of X-rays. C. R. Seances Soc. Biol. Fil., 144, 1439–1442 (1950).

31) Mladenov E, Magin S, Soni A, Iliakis G. DNA double-strand break repair as determinant of cellular radiosensitivity to killing and target in radiation therapy. Front. Oncol., 3, 113
32) Bekker-Jensen S, Mailand N. Assembly and function of DNA double-strand break repair foci in mammalian cells. DNA Repair (Amst.), 9, 1219–1228 (2010).
33) Ataian Y, Krebs JE. Five repair pathways in one context: chromatin modification during DNA repair. Biochem. Cell Biol., 84, 490–494 (2006).
34) Nakatsukasa H, Tsukimoto M, Harada H, Kojima S. Adenosine A2B receptor antagonist suppresses differentiation to regulatory T cells without suppressing activation of T cells. Biochem Biophys Res Commun., 409, 114-119 (2011).
35) Kume H, Tsukimoto M. TRPM8 channel inhibitor AMTB suppresses murine T-cell activation induced by T-cell receptor stimulation, concanavalin A, or external antigen re-stimulation. Biochem Biophys Res Commun., 509, 918-924 (2019).
36) Borrmann T, Hinz S, Bertarelli DC, Li W, Florin NC, Scheiff AB, Müller CE. 1-Alkyl-8-(piperazine-1-sulfonyl)phenylxanthines: development and characterization of adenosine A2B receptor antagonists and a new radioligand with subnanomolar affinity and subtype specificity. J Med Chem., 52, 3994-4006 (2009).
37) Eckle T, Krahn T, Grenz A, Köhler D, Mittelbronn M, Ledent C, Jacobson MA, Osswald H, Thompson LF, Unertl K, Eltzschig HK. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. Circulation, 115, 1581-1590 (2007).
38) Gao ZG, Balasubramanian R, Kiselev E, Wei Q, Jacobson KA. Probing biased/partial agonism at the G protein-coupled A(2B) adenosine receptor. Biochem Pharmacol., 90, 297-306 (2014).
39) Van der Hoeven D, Wan TC, Gizewski ET, Kreckler LM, Maas JE, Van Orman J, Ravid K, Auchampach JA. A role for the low-affinity A2B adenosine receptor in regulating superoxide generation by murine neutrophils. J Pharmacol Exp Ther., 338, 1004-1012 (2011).
40) Taliani S, La Motta C, Mugnaini L, Simorini F, Salerno S, Marini AM, Da Settimo F, Cosconati S, Cosimelli B, Greco G, Limongelli V, Marinelli L, Novellino E, Ciampi O, Daniele S, Trincavelli ML, Martini C. Novel N2-substituted pyrazolo[3,4-d]pyrimidine adenosine A3 receptor antagonists: inhibition of A3-mediated human glioblastoma cell proliferation. J Med Chem., 53, 3954-3963 (2010).
41) Wei Q, Costanzi S, Balasubramanian R, Gao ZG, Jacobson KA. A2B adenosine receptor blockade inhibits growth of prostate cancer cells. Purinergic Signal, 9, 271-280 (2013).
42) Kaji W, Tanaka S, Tsukimoto M, Kojima S. Adenosine A(2B) receptor antagonist PSB603 suppresses tumor growth and metastasis by inhibiting induction of regulatory T cells. J Toxicol Sci., 39, 191-198 (2014).
43) Adair TH. Growth regulation of the vascular system: an emerging role for adenosine. Am J Physiol Regul Integr Comp Physiol., 289, 283–296 (2005).
44) Koszalka P, Pryszlak A, Golun ska M, Kolas a J, Stasi lojc G, Sklada nowski AC, Big da JJ.
Inhibition of CD73 stimulates the migration and invasion of B16F10 melanoma cells in vitro, but results in impaired angiogenesis and reduced melanoma growth in vivo. *Oncol Rep.*, **31**, 819–827 (2014).

45) Yan A, Joachims ML, Thompson LF, Miller AD, Canoll PD, Bynoe MS. CD73 promotes glioblastoma pathogenesis and enhances its chemoresistance via A2B adenosine receptor signaling. *J Neurosci.*, **39**, 4387-4402 (2019).

46) Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka M, Kuch-roo VK, Strom TB, Robson SC. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med.*, **204**, 1257-1265 (2007).

47) Lappas CM, Liu PC, Linden J, Kang EM, Malech HL. Adenosine A2A receptor activation limits graft-versus-host disease after allogenic hematopoietic stem cell transplantation. *J Leukoc Biol.*, **87**, 345-354 (2010).

48) Naganuma M, Wiznerowicz EB, Lappas CM, Linden J, Worthington MT, Ernst PB. Cutting edge: Critical role for A2A adenosine receptors in the T cell-mediated regulation of colitis. *J Immunol.*, **177**, 2765-2769 (2006).

49) Wei Q, Costanzi S, Balasubramanian R, Gao ZG, Jacobson KA. A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signal.*, **9**, 271–280 (2013).

50) Zhou Y, Chu X, Deng F, Tong L, Tong G, Yi Y, Liu J, Tang J, Tang Y, Xia Y, Dai Y. The adenosine A2b receptor promotes tumor progression of bladder urothelial carcinoma by enhancing MAPK signaling pathway. *Oncotarget*, **8**, 48755-48768 (2017).

51) Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, Dwyer K, Stagg J, Smyth MJ, Darcy PK. Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. *Proc Natl Acad Sci U S A.*, **110**, 14711–14716 (2013).

52) Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci USA.*, **107**, 1547–1552 (2010).

53) Grimaldi AM, Simeone E, Giannarelli D, Muto P, Falivene S, Borzillo V, Giugliano FM, Sandomenico F, Petrillo A, Curvietto M, Esposito A, Paone M, Palla M, Palmieri G, Caracò C, Ciliberto G, Mozzillo N, Ascierto PA. Abscopal effects of radiotherapy on advanced melanoma patients who progressed after ipilimumab immunotherapy. *Oncoimmunology*, **3**, e28780 (2014).

54) Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, Kurata T, Chiappori A, Lee KH, de Wit M, Cho BC, Bourhaba M, Quantin X, Tokito T, Mekhail T, Planchard D, Kim YC, Karapetis CS, Hriet S, Ostoros G, Kubota K, Gray JE, Paz-Ares L, de Castro Carpeño J, Faivre-Finn C, Reck M, Vansteenkiste J, Spigel DR, Wadsworth C, Melillo G, Taboada M, Dennis PA, Özgüroğlu M. Durvalumab after Chemoradiotherapy in Stage III Non–Small-Cell Lung Cancer. *N Engl J Med.*, **377**, 1919-1929 (2017).
55) Morello S, Miele L. Targeting the adenosine A2b receptor in the tumor microenvironment overcomes local immunosuppression by myeloid-derived suppressor cells. *Oncoimmunology*, 3, e27989 (2014).

56) Hamada N, Maeda M, Otsuka K, Tomita M. Signaling pathways underpinning the manifestations of ionizing radiation-induced bystander effects. *Curr Mol Pharmacol.*, 4, 79–95 (2011).
Figure 1. Formation of nuclear γH2AX/53BP1 foci and decrease of viability of B16 cells after γ-irradiation. B16 cells were irradiated with various doses of γ-rays (1.0-8.0 Gy) and incubated for 0.5 h (B). Cells were irradiated with γ-rays (2 Gy) and incubated for 0.5-24 h (C). After incubation, co-localized foci of 53BP1 (green) and γH2AX (red) in nuclei (blue) were counted (A). The data represent means ± S.E. (n=31-37). (D) Cells were irradiated with various doses of γ-rays (1.0-8.0 Gy), incubated for 24 h, then seeded in 60 mm dish (4.0×10^2 cells/dish) and further incubated for 1 week. After incubation, crystal violet-stained colonies (>50 cells/colony) were counted. The data represent means ± S.E. (n=6). A statistically significant difference is indicated by *** (P<0.001) or ** (P<0.01). (Color figure can be accessed in the online version.)
Figure 2. Involvement of adenosine receptors in formation of nuclear γH2AX/53BP1 foci and in reproductive cell death after γ-irradiation of B16 cells. (A, C, E) Involvement of adenosine receptors in the formation of γH2AX/53BP1 foci in nuclei after γ-irradiation of B16 cells. Cells were treated with PSB36 (5 μM), SCH442416 (10 μM), PSB603 (10 μM), MRS3777 (10 μM), CGS15943 (10 μM) or APCP (10 μM) for 30 min prior to irradiation, or treated with adenosine (10, 100, 1000 μM) for 15 min prior to irradiation. Cells were irradiated with 2.0 Gy of γ-rays, and incubated for 0.5 h. After incubation, co-localization of 53BP1 with γH2AX was detected by immunostaining and co-localized foci of 53BP1 (green) and γH2AX (red) in nuclei (blue) were counted. The data represent means ± S.E. (n=30-74). (B, D) Radiosensitizing effect of adenosine receptor antagonists in B16 cells. Cell survival was measured by colony formation assay. All cells were irradiated with 2.0 Gy of γ-rays, incubated for 24 h, then seeded in 60 mm dish (4.0×10^2 cells/dish) and further incubated for a week. After incubation, crystal violet-stained colonies were counted (>50 cells/colony). The data represent means ± S.E. (n=3-9). A significant difference from non-irradiated cells is indicated by ### (P<0.001). A significant difference from irradiated cells is indicated by *** (P<0.001) or ** (P<0.01).
Figure 3. Expression of A2B receptor in B16 cells. Total RNA and protein were extracted from B16 cells. A2B receptor mRNA (256 bp) and protein (52 kDa) were detected by RT-PCR and Western blotting, respectively.
Figure 4. Involvement of A2B receptor in the formation of nuclear γH2AX/53BP1 foci and in reproductive cell death after γ-irradiation. (A, C) Involvement of A2B receptor in the formation of γH2AX/53BP1 foci in nuclei after γ-irradiation of B16 cells. Cells were treated with PSB603 (10 μM), MRS1706 (5 μM) or MRS1754 (0.1 μM) for 30 min prior to irradiation, or treated with BAY60-6583 (1.0, 5.0, 10 μM) for 15 min prior to irradiation. Cells were irradiated with 2.0 Gy of γ-rays, and incubated for 0.5 h. (E) Cells were treated with PSB603 (10 μM), MRS1706 (5 μM), MRS1754 (0.1 μM), or CGS15943 (10 μM) for 30 min prior to irradiation with 4.0 Gy of γ-rays, and incubated for 24 h. After incubation, co-localization of 53BP1 with γH2AX was detected by immunostaining, and co-localized foci of 53BP1 (green) and γH2AX (red) in nuclei (blue) were counted. The data represent means ± S.E. (n=30-40). (B, D) Radiosensitizing effect of A2B receptor antagonists in B16 cells. Cell survival was measured by colony formation assay. All cells were irradiated with 2.0 Gy of γ-rays, incubated for 24 h, then seeded in 60 mm dish (4.0×10² cells/dish), and further incubated for a week. After incubation, crystal violet-stained colonies were counted (>50 cells/colony). The data represent means ± S.E. (n=3-9). A significant difference from non-irradiated cells is indicated by ‡ (P<0.05). A significant difference from irradiated cells is indicated by *** (P<0.001), ** (P<0.01) or * (P<0.05).
Figure 5. Effect of A2B receptor knockdown on the formation of nuclear γH2AX/53BP1 foci and on reproductive cell death after γ-irradiation. siRNA duplex oligonucleotides for knockdown of mouse adenosine A2B receptor were transfected into B16 cells. (A) At 24 hours after transfection, A2B receptor protein (52 kDa) was detected by Western blotting. The data represent means ± S.E. (n=3). A significant difference from scramble siRNA-transfected cells is indicated by ** (P<0.01). (B, C) At 48 hours after transfection, the cells were irradiated with 2.0 Gy of γ-rays and incubated for 30 min (B) or 24 h (C). (B) After incubation, co-localization of 53BP1 with γH2AX was detected by immunostaining, and co-localized foci of 53BP1 (green) and γH2AX (red) in nuclei (blue) were counted. The data represent means ± S.E. (n=31-65). A significant difference from non-irradiated scramble siRNA-transfected cells is indicated by ### (P<0.001). A significant difference from irradiated scramble siRNA-transfected cells is indicated by *** (P<0.001). (C) The cells were seeded in 60 mm dish (4.0×10^2 cells/dish) and incubated for 1 week. After incubation, crystal violet-stained colonies (>50 cells/colony) were counted. The data represent means ± S.E. (n=3). A significant difference from non-irradiated scramble siRNA-transfected cell is indicated by ### (P<0.001). A significant difference from irradiated scramble siRNA-transfected cell is indicated by *** (P<0.001) or * (P<0.05).
Figure 6. Radiosensitizing effect of PSB603 in vivo. B16 melanoma cells (2.0 x 10^5 cells) were injected into the hind footpad of 5-week-old male C57BL/6 mice. To examine the effect of A2B receptor antagonist on melanoma growth, PSB603 was dissolved in PBS at the concentration of 50 μM and intraperitoneally administered to the B16 melanoma-bearing mice on the 14th, 18th, 21st, and 23rd days after transplantation. Mice were irradiated with γ-rays (1.0 Gy) at 2 h after treatment with PSB603 on the 14th, 18th, 21st, and 23rd days after transplantation. The size of the solid tumor was measured with a caliper twice a week for 25 days, and the tumor volume (V) was calculated from the following equation: V (mm^3) = 4/3 x 3.14 x length (mm)/2 x width (mm)/2 x thickness (mm)/2. (A) Change in tumor volume (left) and representative photographs of the tumor (right). (B) Tumor volume 25 days after transplantation. Data are averages ± S.E. of multiple determinations (n=6). A significant difference from irradiated cells is indicated by * (P<0.05). (Color figure can be accessed in the online version.)