

## Adenine Inhibits B16-F10 Melanoma Cell Proliferation

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Adenine, a purine base, is a structural component of essential biomolecules such as nucleic acids and adenine nucleotides. Its physiological roles have been uncovered. Adenine suppresses IgE-mediated allergy and LPS-induced inflammation. Although adenine is known to inhibit lymphocyte proliferation, the effect of adenine to melanoma cells is not reported. Here, we investigated the growth inhibitory effects of adenine on B16-F10 mouse melanoma cells. Adenine suppressed the proliferation of B16-F10 cells in dose-dependent manner with the maximal inhibitory dose of 2 mM. Adenine treatment induced cell death molecular markers such as PARP and caspase 3 cleavages. Pan-caspase inhibitor z-VAD dramatically rescued the cell death molecular markers, cell proliferation recovered marginally. These results provide the possibility of adenine to be used as an anti-tumor agent.

**Key Words:** Adenine, Melanoma, Cell death, Tumor, Apoptosis

### INTRODUCTION

The purine adenine is a component of biomolecules, such as nucleic acids and adenine nucleotides. It is produced mainly from 5'-methylthioadenosine (MTA) by MTA-phosphorylase (Avila et al., 2004). Adenine production from the MTA degradation contributes 85~97% of total adenine synthesis in dividing human lymphoblastoid cells (Kamatani and Carson, 1981). Adenine promotes survival of Purkinje cells (Watanabe et al., 2003) and erythrocytes in whole blood storage (Simon et al., 1962). In contrast to its survival effects, adenine inhibits the growth of lymphoblast (Hershfield et al., 1977; Snyder et al., 1978) independent of adenine salvage enzyme APRT (Hershfield et al., 1977). In our previous report we showed that adenine inhibits IgE-mediated mast cell activation and allergic reactions *in vitro* and *in vivo* (Silwal

et al., 2015). Adenine also inhibits the immune function of human mononuclear leukocytes (Kishi et al., 1985). Adenine is cell-permeable and convertible to adenosine monophosphate (AMP) via adenine salvage pathway. AMP and ATP pools are replenished during the salvage thereby contributing to its cellular and physiological activities. AMP formation from adenine contributes to activation of AMPK in various cells (Young et al., 2015).

The programmed cell death, or apoptosis, is an orderly and genetically controlled form of cell demise. It is a coordinated and usually energy-dependent process which involves the activation of a group of cysteine aspartyl proteases, caspases (Elmore, 2007). Caspase-3 is an execution caspase in apoptosis that can be activated by external or intrinsic stimuli. The Bcl-2 (B-cell lymphoma-2) family proteins, including both anti apoptotic (Bcl-2, Bcl-XL, and Mcl-1) and pro-apoptotic (Bid, Bax, and Bad) members are particularly

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important in the caspase activation (Cory and Adams, 2002). AMP-activated protein kinase (AMPK) participates in the development and progression of tumors by regulating cellular energy. AMPK is a metabolic checkpoint regulating energy homeostasis, and several AMPK activators are being used in cancer therapy because of its involvement in inhibiting tumor cell growth and inducing apoptosis (Theodoropoulou et al., 2013). AMP activates AMPK by inhibiting dephosphorylation of phosphorylated AMPK as well as acts as an allosteric activator of AMPK (Gowans et al., 2013).

Previous studies showed that adenine is cytotoxic to lymphoblast (Hershfield et al., 1977; Snyder et al., 1978) and has anti-tumor activity for colon (Han et al., 2017), cervical (Lai et al., 2019) and hepatic (Su et al., 2020) cancer cells. However, its effect on melanoma cells is yet to be studied. Melanoma is the most aggressive form of skin cancer which is highly metastatic with the major problem being its pronounced resistance to therapy (Soengas and Lowe, 2003). Cancer therapies target the elimination of tumor cells through apoptotic cell death. The ineffectiveness of chemotherapy in treating melanoma is mainly due to improper functioning of the apoptosis programs. Therefore, the therapeutic approaches that either alone or in combination induce apoptosis are crucial for the proper treatment of melanoma (Lowe and Lin, 2000).

In this study, we investigated the adenine induced cell death on B16-F10 murine melanoma cells. We show that adenine elicits growth inhibition and apoptotic cell death by activation of caspase in B16-F10 melanoma cells.

## MATERIALS AND METHODS

### Materials

The following materials were purchased from the indicated commercial sources: adenine, thiazolyl blue tetrazolium bromide (MTT), z-VAD-fmk and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St Louis, MO, USA); fetal bovine serum (FBS) from Gibco/Life Technologies (Grand Island, NY, USA); Cytotoxicity Detection Kit plus (LDH) from Roche Diagnostic GmbH (Mannheim, Germany); Dulbecco's Modified Eagle Medium (DMEM) from Welgene (South Korea); anti-cleaved caspase-3 antibodies from Cell Sig-

nalizing Technology (Beverly, MA, USA); anti-PARP from Santa Cruz (CA, USA); RIPA lysis buffer from ELPIS Biotech (South Korea); ECL chemiluminescence kit from Millipore (Billerica, MA, USA); B16-F10 melanoma cell line from American Type Culture Collection.

### Cell culture

B16-F10 melanoma cells were cultured in DMEM supplemented with 10% FBS and standard concentration of penicillin/streptomycin antibiotics. Cultures were maintained in a standard condition in 5% CO<sub>2</sub> incubator at 37°C. For all experiments, required numbers of cell were incubated for 24 h in appropriate plates. Then various doses of adenine or other compounds were treated for 24 h in a complete growth medium and cells were harvested according to required experimental purposes.

### Cell viability assay

Cell viability was determined using thiazolyl blue tetrazolium bromide (MTT) and cell counting. For MTT assay, cells were seeded into 96-well plates ( $1 \times 10^3$  cells/well) and incubated for 24 h at 37°C. The cells were then treated with various doses of adenine for another 24 h which were then incubated with MTT (5 mg/mL) for 2 h and the formazan produced was dissolved in DMSO. Absorbance was measured at 570 nm in a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Data are presented as percentage of untreated control group. For cell counting assay,  $1 \times 10^5$  cells per mL were seeded in 6-well plates. After incubating overnight, cells were treated with adenine and were trypsinized after indicated time. 10 µL of cell were mixed with equal volume of 0.4% Trypan blue and 10 µL of mixture was used in cell counting using automated cell counter.

### Lactate dehydrogenase (LDH) assay

Non-radioactive colorimetric assay was performed using Cytotoxicity Detection Kit (LDH). B16-F10 cells seeded and incubated overnight in 96-well plates were treated with adenine for 24 h. Then as per the manufacturer's protocol, catalyst and dye solution mixture (reaction mixture) was added to the cells and incubated for 30 min at room tem-

perature, protecting plates from lights. Resulting values were used to calculate LDH release percentage using the following formula: LDH release (cytotoxicity) (%) = (experimental value - low control)/(high control - low control) × 100. To determine the effect of adenine on cell proliferation using LDH assay, B16-F10 cells were seeded in presence or absence of adenine in 96 well plates and were incubated for 24 h.

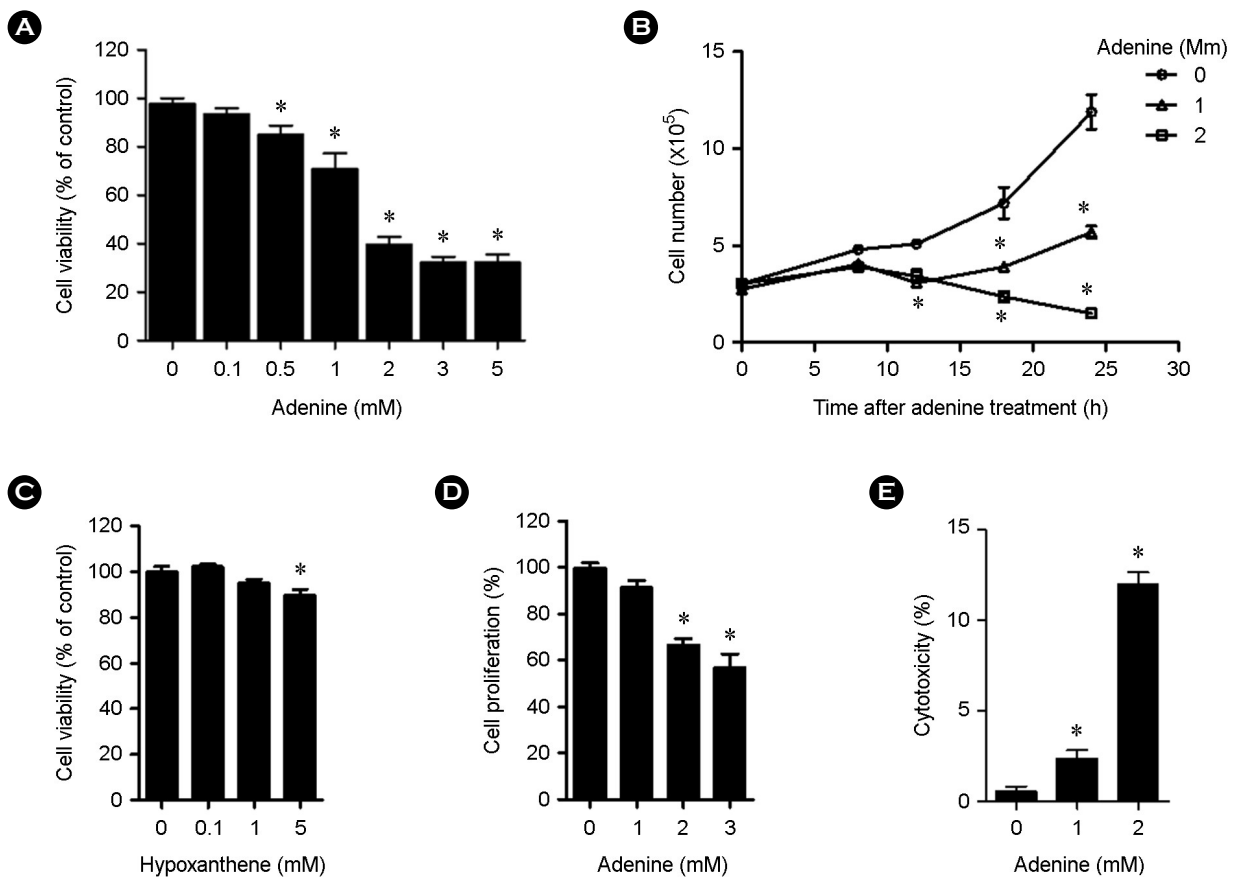
### Western blot analysis

Cells were collected, washed with ice-cold PBS, and then lysed in ice-cold lysis buffer for 30 min on ice. Lysates were micro-centrifuged at 12,000 rpm at 4°C for 20 min and protein concentration was measured using Bradford assay. After SDS-PAGE, proteins in the gel were transferred to a

PVDF membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h and then incubated overnight with primary antibodies at 4°C. After washing the membrane with TBS-T, immune-reactive proteins were detected with horseradish peroxidase-conjugated secondary antibody using chemiluminescence kit. Densitometry analysis was performed using ImageJ software.

### Statistical analysis

Data are presented as means ± SEM. Statistical analysis were performed by using GraphPad Prism software. The data from adenine-treated and adenine-untreated groups of cells were analyzed with Student's *t*-test and  $P \leq 0.05$  was



**Fig. 1. Adenine inhibits cell viability and induces death of B16-F10 melanoma cells.** (A) B16-F10 cells were treated with indicated doses of adenine for 24 h in complete media and cell viability was measured using MTT assay (A) and viable cell count (B). The MTT data are presented as percentage of non-treated control group. (C) Hypoxanthene was treated in similar condition as that of adenine and MTT assay was performed to determine cell viability after 24 h. (D, E) B16-F10 cell treated with indicated doses of adenine for 24 h in complete media and LDH release was determined using LDH assay kit as described in materials and method section. Data are means ± SEM of three independent experiments. Significant difference against control group is indicated as \* $P < 0.05$ .

considered to indicate significance difference.

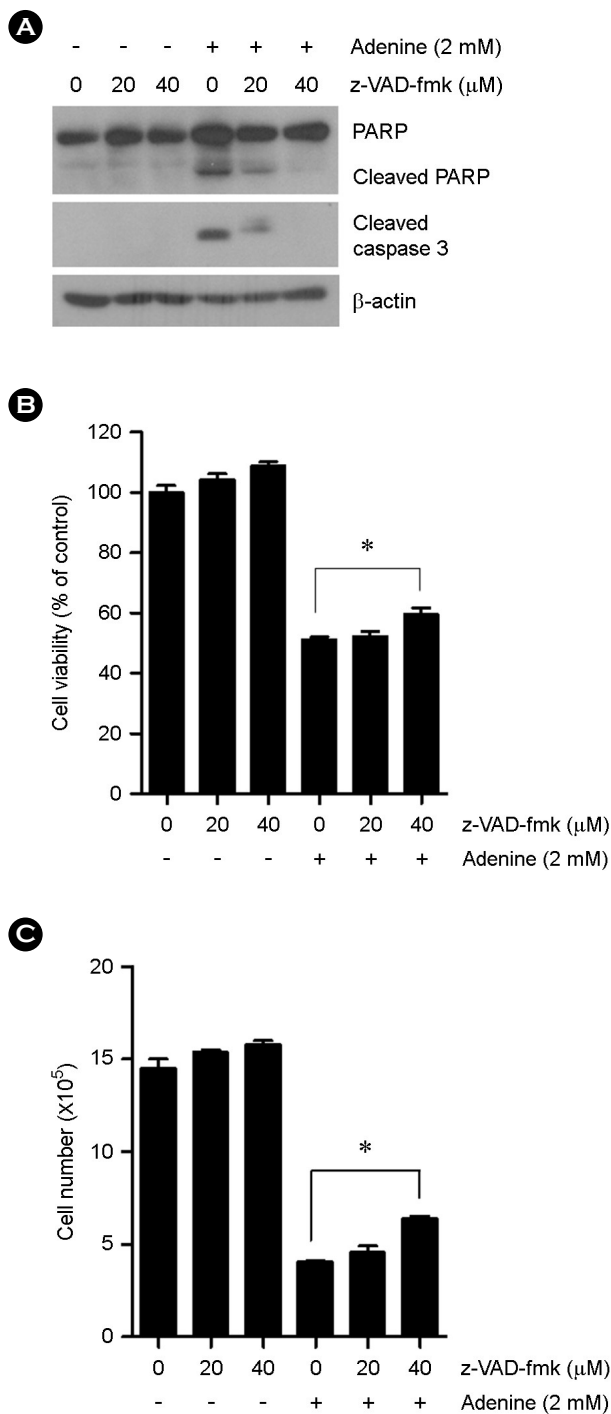
## RESULTS

### Adenine inhibits the cell growth and cell viability in B16-F10 melanoma cells

The effect of adenine on growth of B16-F10 melanoma cells was evaluated by MTT assay and cell counting. Adenine treatment caused a dose-dependent decrease in cell viability as compared to untreated cells with maximal inhibition at 2 mM dose (Fig. 1A). Adenine treatment caused reduction in cell number in time dependent manner as seen on cell counting assay (Fig. 1B). Adenine treatment had no significant effect on cell numbers till 8 h but significant drop in numbers were observed after 12 h of treatment suggesting adenine inhibits cell growth and causes cell death after 12 h (Fig. 1B). To know if the effect is adenine specific, a structural analog of adenine, hypoxanthine, was used. Hypoxanthine did not inhibit the B16-F10 melanoma cell viability (Fig. 1C). Lactate dehydrogenase (LDH) assay was performed to assess the cell proliferation and cytotoxicity by measuring total LDH and LDH released in medium after adenine treatment respectively. Adenine treated at the time of cell seeding and measuring the LDH after 24 h showed inhibited level of LDH confirming inhibition of cell proliferation by adenine (Fig. 1D). Adenine caused excessive release of LDH which suggest that it is cytotoxic as it affects the membrane integrity of the B16-F10 cells (Fig. 1E). These results revealed that adenine decreases proliferation and kill the B16-F10 melanoma cells.

### Adenine induces apoptosis in B16-F10 melanoma cells

To confirm if the cell death induced by adenine in apoptotic cell death, Western blotting was performed to see molecular cleavages of caspase-3 and PARP proteins. We measured cleavage formation of caspase-3, an executor caspase that is activated through both intrinsic and extrinsic apoptosis pathways (Taylor et al., 2008). As shown, expression of cleaved form of caspase-3 was induced by adenine treatment (Fig. 2A). In addition, the activation of caspase-3 leads to the cleavage of several proteins, one of which is PARP. Although PARP is not essential for cell death, the cleavage



**Fig. 2. Adenine induces apoptosis of B16-F10 melanoma cells.** (A) B16-F10 cells were pre-incubated with pan-caspase inhibitor z-VAD-fmk for 1 h and exposed to adenine for 24 h. Cell lysates were used for Western blotting to detect indicated proteins. (B) MTT assay and (C) Cell count assay were performed with cells pre-incubated and treated with z-VAD and adenine as described above. Data are means  $\pm$  SEM of three independent experiments. Significant difference against control group is indicated as  $*P < 0.05$ .

of PARP is another hallmark of apoptosis (Kaufmann et al., 1993). Adenine increased the expression of cleaved PARP (Fig. 2A).

Cleavage form of caspase-3 suggests that adenine induces the caspase-dependent apoptosis in B16-F10 cells. To confirm this, we used a pan-caspase inhibitor z-VAD and results shows that it reduced the adenine induced cleaved-PARP and -caspase-3 (Fig. 2A). Cell viability analysis with MTT assay (Fig. 2B) and viable cell count data (Fig. 2C) showed z-VAD (40  $\mu$ M) significantly rescued B16-F10 cell death resulted from adenine treatment. These results suggested that adenine induces caspase-dependent apoptosis in B16-F10 cells.

## DISCUSSION

We showed in this study that adenine induces cell death of B16-F10 melanoma cells by activating caspase-3. We demonstrated that adenine reduced the cell viability and caused apoptotic cell death. Adenine inhibited cell growth as measured by MTT assay (Fig. 1A) and viable cell counting (Fig. 1B). Also, adenine induced the cleavage of PARP in B16-F10 cells with a dose of 2 mM or more (Fig. 2A). Proteolytic cleavage of PARP is a marker of apoptosis. Apoptosis is mediated by proteases called caspases (Kerr et al., 1972; Taylor et al., 2008). Caspase-3 is primarily responsible for the cleavage of PARP during cell death (Thornberry, 1997). In the present study, we found that adenine treatment increased the caspase-3 cleavage (Fig. 2A). Blocking apoptosis with pan-caspase inhibitor z-VAD partially protected B16-F10 cells from adenine induced cell death (Fig. 2). These results suggested that adenine killed the melanoma cell death partly by inducing caspase-dependent apoptosis.

Adenine is cell-permeable and can be converted to AMP in cells by an enzyme APRT (Kamatani and Carson, 1981). AMPK activation leading to apoptosis may be cell-type dependent (Zadra et al., 2014). The AMP thereby activates AMPK to promote various physiological effects. Several reports suggest that adenine could be a novel and simplest AMPK activator (Cheng et al., 2015). AMPK activation has also been linked with induction of cell cycle arrest and cell death (Vincent et al., 2015). Anti-tumor effects of adenine

for colon (Han et al., 2017), cervical (Lai et al., 2019) and hepatic (Su et al., 2020) cancer cells are mediated from adenine-induced cell cycle arrest and AMPK activated. Therefore, the cytotoxic effect of adenine on melanoma cells shown here may be through AMPK activation of adenine in melanoma cells.

Another mechanism of adenine induced cytotoxicity could be transmethylation as in W1-L2 cells (Kishi, 1985), adenine inhibits S-adenosylhomocysteine hydrolase and causes accumulation of S-adenosylhomocysteine and growth inhibition where S-adenosylhomocysteine is a well-known transmethylation inhibitor.

Adenine showed cytotoxicity in B16-F10 melanoma cells (Fig. 1). Although its cytotoxic mechanism was inferred that excess production of adenine nucleotides through adenine salvage pathway makes an imbalance on the ratio of purine /pyrimidine nucleotides, the cytotoxicity is independent of the salvage pathway (Hershfield et al., 1977; Snyder et al., 1978). In contrast, adenine protects rat Purkinje cells in rat cerebellar primary cultures and erythrocytes in the whole blood preservation (Watanabe et al., 2003; Simon et al., 1962). Also, patients deficient in adenine phosphoribosyltransferase (APRT) and knock-out mice of the gene are viable (Engle et al., 1996). Therefore, the cytotoxicity of adenine could depend on cell types, cellular environments and the concentration of adenine.

In conclusion, this study is the first to report that adenine inhibits cell growth in B16-F10 melanoma cells partly by inducing apoptotic cell death. This study can provide evidence that adenine could be a valuable resource for the development of new agents for cancer treatment including melanoma.

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## CONFLICT OF INTEREST

The authors have no conflict of interest with regards to this study.

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