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Kinetin riboside preferentially induces apoptosis by modulating Bcl-2 family proteins and caspase-3 in cancer cells

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Abstract

Here, we demonstrate that kinetin riboside (KR), a cytokinin analog, induces apoptosis in HeLa and mouse melanoma B16F-10 cells. KR disrupted the mitochondrial membrane potential and induced the release of cytochrome *c* and activation of caspase-3. Bad were upregulated while Bcl-2 was down-regulated under KR exposure. A tumor growth in mice was dramatically suppressed by KR. In contrast, human skin fibroblast CCL-116 and bovine primary fibroblast cells show resistances to KR and no significant changes in Bad, Bcl-X_L, and cleaved PARP were observed. Our data suggest that KR selectively induces apoptosis in cancer cells through the classical mitochondria dependent apoptosis pathway.

Keywords: Kinetin riboside; Apoptosis; Caspase-3; Bcl-2; Cancer

1. Introduction

Plant cytokinins are the N⁶-substituted purine derivatives and serve as plant hormones. They have been shown to be implicated in the promotion of cell division and regulation of several developmental events, including apical dominance, seed germination, leaf senescence, chloroplast differentiation and nutrient mobilization [1]. Natural cytokinins are grouped based on structure into three categories: isoprenoid cytokinins (isopentenyladenine, zeatin, and dihydrozeatin), aromatic cytokinins (benzyladenine, topolin, and methoxytopolin), and furfural derivatives (kinetin and kinetin riboside) [2]. Although the effects of cytokinins have been extensively studied in plant development, little is known about the precise mechanisms of their actions in mammalian cells.

Kinetin (N⁶-furfuryladenine) was detected as a secondary product of DNA damage and plant cell extracts [3]. Recently, kinetin and its riboside have been extracted in the endosperm liquid of

fresh young coconut fruits [4]. Kinetin has been shown to retard senescence in plants, delay aging in normal human fibroblasts and dermal microvascular endothelial cells, and prolong the lifespan of fruit fly [5, 6]. On the other hand, kinetin riboside (9-(β -D-ribofuranosyl)-6-furfurylaminopurine, referred as KR) (Fig. 1) has been shown to exert growth inhibitory effects and also induce apoptosis. It has been implicated in the reduction of intracellular ATP level and mitochondrial membrane potential and the induction of reactive oxygen species, whereas cytokinin protects against mitochondrial disruption and apoptosis in HL-60 cells [7]. In addition, treatment with KR leads to cytotoxic effects on mouse, human, and plant tumor cells [8]. Thus, KR appears to have an important role in the induction of cell death in different types of cancer cells. However, the mechanism by which KR induces apoptosis, especially in cancer cells, still remains largely unknown.

The Bcl-2 family proteins are important players in apoptosis and the interactions between the antiapoptotic members and pro-apoptotic members provide a mechanistic basis in modulating the apoptotic cell death [9, 10]. Dysregulation of the Bcl-2 family can lead to cellular demise or abnormal proliferation, such as neurodegeneration or cancer [11, 12]. Although mutations of Bcl-2 family members are rare in cancer, anti-apoptotic molecules such as Bcl-2 and Bcl-X_L are often overexpressed in cancer cells [13]. Recently, it has been shown that antisense oligonucleotides targeting Bcl-2 result in increment of tumor cell apoptosis [14, 15]. In addition, small molecules inhibiting Bcl-2 and Bcl-X_L have emerged as strong inducer of apoptosis in human leukemia cells [16, 17], suggesting that inhibition of the anti-apoptotic proteins might be an attractive approach for selective killing of cancer cells via induction of apoptosis.

In this study, we have investigated the effects of KR both in cancer and immortalized cells. Our results showed that the treatment with KR triggers mitochondrial dysfunction through the upregulation of the pro-apoptotic proteins Bad and Bak and the down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, which causes the release of cytochrome *c* and the activation of caspase-3, thereby contributing to the cell death in cancer cells, whereas the similar cytotoxic effects of kinetin riboside have not been observed in immortalized cells. In addition, we also showed that kinetin riboside is effective against an *in vivo* tumor model. Our results might provide insightful information on the mechanism of kinetin riboside as a potential chemotherapeutic agent.

2. Materials and methods

2.1. Materials

Kinetin riboside and dacarbazine were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against caspase-3, cytochrome *c*, PARP, Bad, Bak, Bcl-2, Bcl-X_L, and β -tubulin were obtained from Santa Cruz Biotech (Santa Cruz, CA). Antibodies against GAPDH and COX IV were obtained from Ambion (Austin, TX) and Molecular Probes (Eugene, Oregon), respectively. Protease inhibitors complete-mini protease tablets were purchased from Roche Applied Science (Indianapolis, IN). Z-VAD-fmk was obtained from Santa Cruz Biotech (Santa Cruz, CA). Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 and fetal bovine serum were purchased from HyClone (Logan, Utah).

2.2. Cell Lines and culture conditions

Human cancer cell lines, HeLa, human skin fibroblast cell line, CCL-116, and mouse melanoma cell line, B16F10 were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37 °C. Bovine primary fibroblasts were isolated from bovine adrenal medulla as previously described [18]. The cells were maintained in DMEM/F-12 containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37 °C.

2.3. Cell viability assay

Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [19]. Cells were seeded onto 96-well plates at a density of about 5×10^3 cells per well in the growth medium and incubated until about 70–80% confluency. Following the treatments as indicated, 15 µl of MTT solution (5 mg/ml) was added to each well, and cells were maintained for 4 h at 37 °C. Hundred microlitres of solubilizing solution (50% dimethylformamide and 20% SDS, pH 4.8) was then added. After an overnight incubation at room temperature, absorbance at 490 nm was measured.

2.4. Measurement of apoptosis

Cells were treated with 4.5 µM kinetin riboside and incubated for 24 h. Whole cells were collected and apoptosis was examined by using Annexin V-FLUOS staining kit (Roche, Penzberg, Germany), which detects phosphatidylserine exposed on the outer surface of the cell membrane. Cells were counter-stained with propidium iodide followed by fluorescence activated cell sorter (FACS) analysis on a flow cytometer (BD LDR II, BD Biosciences, San Jose, CA). For visualization of apoptotic cells, cells were seeded on 22 mm² coverslips within a 6-well plate. After fixation in 3.7% paraformaldehyde, cells were washed with phosphate buffered saline (PBS) and permeabilized with 0.2% Triton X-100, washed again with PBS, and mounted by ProLong Gold antifade reagent with DAPI (Molecular probes, Eugene, Oregon). The stained nuclei were observed and photographed under a fluorescence microscope (Nikon Inc., Melville, NY).

2.5. Detection of the mitochondrial membrane potential ($\Delta\psi_m$)

The $\Delta\psi_m$ was measured by using the Mitochondrial Membrane Potential Detection Kit (Stratagene, Cedar Creek, TX). Briefly, detached cells were stained with JC-1 for 15 min and then measured on a flow cytometer, according to the instruction manual. Data analysis was performed with CellQuest software (Becton Dickinson, Singapore) by measuring both green and red JC-1 fluorescence.

2.6. Caspase-3 activity assay

Caspase-3 activity was measured by the CaspACE Assay System, Colorimetric kit (Promega, Madison, WI). Briefly, in a 6-well tissue culture plate (Falcon, England), cells were seeded at a density of about 2×10^5 cells per well. When the density of cells reached 70–80%, cells were treated with 4.5 μ M kinetin riboside for 24 and 48 h in the absence or presence of the pan-caspase inhibitor Z-VAD-FMK (100 μ M). Cells were incubated for the indicated time periods and then lysed by freeze–thaw. The lysate (100 μ g) was added to the CaspACE assay buffer containing the caspase-3 substrate Ac-DEVD-*p*-nitroaniline (Ac-DEVD-pNA), and incubated for 24 h at 37 °C. Cells treated with DMSO were used as controls. Absorbance at 405 nm was read on a spectrophotometer.

2.7. Subcellular fractionation

Subcellular fractionation was performed as previously described [20]. Briefly, cells were lysed in a isotonic mitochondrial buffer (300 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) containing protease inhibitors, homogenized and centrifuged at 1000g for 10 min to discard nuclei and unbroken cells, and the resulting supernatant was centrifuged at 10,000g for 30 min to obtain the mitochondrial membrane-enriched fractions.

2.8. Immunoblot analysis

Cells were resuspended in a lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF) containing protease inhibitors on ice for 40 min. The clear cell lysates were obtained after centrifuging for 15 min at 15,000 rpm. The lysates (30 μ g of protein) were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS-T (20 mM Tris–HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween-20) and subsequently incubated with primary antibody followed by a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase, and the immunoreactive bands were visualized by the SUPEX Western blotting detection kit (Neuronex, Korea).

2.9. Tumor engrafts and drug injection

Male C57BL/6 mice were maintained on a 12-h day/ night cycle with lights off at 20:00 h and *ad libitum* access to food and water prior to the day of the experiment. Six-week-old mice were subcutaneously injected with 5×10^6 B16 F-10 cells. After 5 days for tumor growth, each drug was injected to tumor mass directly. Tumor engrafts and drug injection were performed between 16:00 h and 18:00 h. Drug injection was performed once a 3 days for three times. After third injection of drug, mice were kept for 3 days with no injection and tumor mass was removed from each mouse and weighed.

2.10. Statistic analysis

All experiments were independently repeated a minimum of three times. All quantitative data are presented as means $\pm$ SE. Comparisons between two groups were analyzed via student's t test, and values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Cancer cells are more susceptible to kinetin riboside than immortalized cells

Recent studies demonstrated that KR-induced cell death in human myeloid leukemia cells and human and mouse melanoma cells [7, 8]. We thus first examined the cytotoxic effect of KR on HeLa cells. Cells were treated for 48 h with KR at concentrations of 5, 10, and 20 μ M. We found a concentration-dependent decrease in cell viability. The viabilities of cancer cells were decreased by more than 50% in response to KR at 5 μ M (Fig. 2). Next, we examined the cytotoxic effect of KR on CCL-116 cells, which are immortalized skin fibroblast cells, in a concentration-dependent manner. To our surprise, CCL-116 cells exhibited a less than 20% reduction in viability after KR treatment only with highest concentration used (20 μ M) and cytotoxic effects were not apparent at 5 and 10 μ M (Fig. 2). The cytotoxic effect with 20 μ M was markedly less compared to that of what was seen in cancer cells at this concentration. Moreover, no significant effect of kinetin riboside was observed on the viability of CCL-116 cells at 10 μ M in time-dependent manner (data not shown).

We next assessed whether the cytotoxic effect of KR was associated with induction of apoptosis. FACS analysis after labeling the cells with annexin V showed that the induction of apoptosis by KR was evident. As shown in Fig. 3A, a majority of the untreated cells appeared annexin V-negative section, and KR-treated HeLa cells increased annexin V-positive cell populations to 55%. Similar results were obtained from the nuclear fragmentation analysis. Significant nuclear fragmentation attributable to internucleosomal cleavage occurred 24 h after exposure to KR in HeLa cells (Fig. 3C). In contrast, CCL-116 cells treated with KR did not exhibit significant changes in annexin V staining and nuclear fragmentation analyses as compared to untreated control cells (Fig. 3B and C). The results were consistent with KR's cytotoxic effects. Taken together, these results suggest that KR is implicated in the reduction of cell viability and induction of apoptotic cell death in HeLa cells, but not apparent in CCL-116 cells with low concentrations of kinetin riboside.

3.2. Kinetin riboside induces mitochondrial dysfunction and activation of caspase-3 in cancer cells

Since mitochondrial dysfunction is one of important characteristics during apoptotic cell death [21], we examined the mitochondrial membrane potential perturbation under KR treatment. As shown in Fig. 4, the level of the mitochondria membrane depolarization significantly increased when HeLa cells were treated with kinetin riboside for 24 h. Disruption of the mitochondrial membrane potential is usually associated with the diffusion of cytochrome *c* to the cytosol that normally resides in the space between the outer and inner mitochondrial membranes [22]. We monitored the levels of cytochrome *c* that reside in the mitochondrial-enriched fraction. As shown in Fig. 5A, KR caused the levels of cytochrome *c* to decrease in a concentration-dependent manner.

We also examined the involvement of caspase-3 in response to the treatment of KR. Our results showed a gradual increase in the cleaved form of caspase-3 (Fig. 5B). To confirm that caspase-3 is involved in KR-induced cell death, we measured the activity of caspase-3 (Fig. 5C),

demonstrating in cancer cells caspase-3 was highly activated by the treatment of KR. Furthermore, after cells treated with a pan-caspase inhibitor (z-VAD-fmk), the activity of caspase-3 was completely blocked (Fig. 5C). On the other hand, no significant increase in caspase-3 activity in CCL-116 cells was observed under KR exposure (Fig. 5C), which is consistent with the earlier results showing little apoptosis in these cells (Figs. 2 and 3). Next, we examined the involvement of caspase activities in KR-induced cell death by using MTT assay and little decrease in the cell viability was seen in the presence of z-VAD-fmk (Fig. 5D). The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro-form to active forms mediate the cleavage of PARP. We found that kinetin riboside treatment also resulted in a cleavage of 116 kDa PARP to 85 kDa in HeLa cells, but not in CCL-116 cells. As shown in Fig. 5E, the cleaved form of PARP increased upon KR treatment in a concentration-dependent manner. Taken together, our data suggest that KR-induced apoptosis via caspase-dependent apoptotic pathway in cancer cells.

3.3. Kinetin riboside induces an increase in the levels of proapoptotic proteins Bad and Bak and decrease in Bcl-2 level

Since the Bcl-2 family controls apoptosis at the mitochondria by maintaining a balance between pro- and anti-apoptotic members, we examined KR's effect on the levels of Bcl-2 family proteins in HeLa cells. Our results showed that the pro-apoptotic proteins Bad and Bak increased dramatically at concentrations of 4.5 and 9 μ M. On the contrary, Bcl-2 was down-regulated during KR exposure, but the degree of Bcl-X_L down-regulation was marginal at concentration of 9 μ M (Fig. 6A). On the other hand, CCL-116 treated by KR did not exhibit significant changes in the levels of Bad and Bcl-X_L as compared to untreated control cells (Fig. 4B). In addition, Bak and Bcl-2 were not detected in control and the KR-treated CCL-116 cells until 24 h (data not shown), suggesting that dysregulation of the Bcl-2 family proteins by KR is involved in the apoptotic cell death in cancer cells.

3.4. Kinetin riboside suppresses tumor growth in vivo

Melanoma shows drug-resistance against various chemotherapeutic agents through the dysregulation of apoptosis [23]. Thus we examined KR's cytotoxicity in melanoma cells. To this end, we used B16F-10 mouse melanoma cells derived from C57/BL6 mice and analyzed its viability after kinetin riboside treatment. Similar to what was observed in HeLa and CCL-116 cells (Fig. 2), the treatment of melanoma cells with kinetin riboside led to an increased cell death in a concentration-dependent manner (Fig. 7). More than 80% of the cells underwent cell death in response to kinetin riboside at 1 mM (Fig. 7). On the other hand, bovine primary fibroblasts derived from adrenal medulla exhibited about 20% of reduction in cell viability under the same treatment condition. The result was consistent with what was seen in CCL-116 cells (Fig. 2).

To test that KR's cytotoxicity observed was not limited to the established cell lines, we have investigated the effect of KR on growing mass of melanoma cells *in vivo*. Tumor was induced after subcutaneous injection with B16F-10 cells on C57/BL6 mice, and then directly injected with kinetin riboside in a concentration-dependent manner. As shown in Fig. 8, KR-induced a significant reduction of tumor mass compared to that of control. The most effective anti-melanoma response was elicited at 40 mg/kg of kinetin riboside, and similar results were

obtained after injection with 20 mg/kg dacarbazine which is anti-melanoma drug (Fig. 8). Collectively, these results indicate that kinetin riboside suppresses tumor growth *in vivo* as well as *in vitro*.

4. Discussion

KR exhibits an antiproliferative effect due to its ability to induce apoptosis in cancer cells [7, 8], but the mechanisms conferring KR-induced cell death in cancer cells remains elusive. Here, we reported that KR induces apoptotic cell death through a differential modulation of the Bcl-2 family proteins and activation of caspases. Moreover, our data suggest that KR showed significantly higher cytotoxic effects in cancer cells, suggesting that cancer cells were more sensitive to kinetin riboside than immortalized and primary fibroblast cells.

Dysregulation of apoptosis is linked to developments of most cancers. Thus, an induction of apoptosis in cancer cells emerges as a valuable approach in combating cancers. Several molecular mechanisms associated with pro- or anti-apoptotic molecules have been described: (1) direct induction of pro-apoptotic molecules, (2) modulation of antiapoptotic molecules, or (3) restoration of tumor suppressor gene functions [24]. Recent studies have shown that a number of anti-cancer drugs induce apoptosis of cancer cells through the activation of the cytochrome *c*/caspases pathway and the mitochondrial membrane dysfunction [25, 26]. Our results showed that KR disrupts the mitochondrial membrane potential (Fig. 4) and induces the activation of cytochrome *c*/caspase-3 pathway in cancer cells (Fig. 5). Interestingly, KR showed little cytotoxicity and apoptosis in CCL-116 immortalized fibroblast cells, which is an established cell line having no cancer marker, and primary fibroblast cells (Figs. 2, 3, 5, and 7), indicating that the normal cells were more resistant to KR under our test condition.

Members of the Bcl-2 family proteins are critical regulators of the apoptotic pathway. Bcl-2 and Bcl-X_L are upstream regulatory molecules in the apoptotic pathway [27]. Bcl-2 and Bcl-X_L have been shown to form heterodimeric complexes with pro-apoptotic members Bax and Bak and thereby neutralizing their pro-apoptotic effects. Therefore, alterations in the levels of pro- or anti-apoptotic members are considered to be a decisive factor in determining whether cells will undergo apoptosis. Here, we showed that Bcl-2 protein level was significantly decreased in KR-treated HeLa cells (Fig. 6A). Bak was upregulated in these cells 24 h after KR treatment (Fig. 6A). The results suggest that the upregulation of Bak and down-regulation of Bcl-2 might swing the rheostat of homeostasis and contribute to KR-induced apoptosis in cancer cells.

Several studies have reported that melanoma cells show chemoresistance to a variety of therapeutic drugs [23]. The alkylating agent dacarbazine is the most frequently used chemotherapeutic drug against malignant melanoma, but it allows complete remissions in only 5% of patients [28]. Other chemotherapeutical agents, including nitrosoureas (carmustine, lomustine), taxanes (taxol, docetaxel), vinca alkaloids (vincristine, vinblastine), and platinum-associated drugs (cisplatin, carboplatin), failed in large randomized studies [23]. For the abovementioned reasons, new therapeutic strategies or drugs are required to improve the therapeutic windows. In present study, we found that KR dramatically suppressed the proliferation of melanoma in mice (Fig. 8), which might be accompanied by its cytotoxicity in cancer cells (Fig. 7). Especially, the

effect of KR at concentration of 40 mg/kg was almost similar to one of dacarbazine at 20 mg/kg (Fig. 8).

Caspases are cysteinyl aspartate-specific proteases, which are activated during apoptosis in a self-amplifying cascade. It is known that two major pathways of caspase cascade activation have been characterized. One is initiated by ligation of death receptors and the activation of caspase-8 [29]. In the other pathway, cytochrome *c* is released from mitochondria in response to a variety of apoptotic stimuli [30]. Here, we showed that the KR treatment resulted in the release of cytochrome *c* into cytosol that activates caspase-3 in a dose-dependent manner (Fig. 5). The activation of caspases in the mitochondrial pathway requires mitochondrial outer membrane permeabilization [21,22], which was also detected by the KR treatment in HeLa cells (Fig. 4), in agreement with a previous report [7]. Therefore, we suggest that KR induces apoptotic cell death through the mitochondria-dependent pathway in cancer cells.

In conclusion, we first have presented that KR exhibits selective inhibition of cancer cells. KR-mediated cell death was correlated with apoptosis in cancer cells. Second, the treatment with KR showed the upregulation of Bad and Bak and down-regulation of Bcl-2 in HeLa cells. Third, we have demonstrated the changes of mitochondrial membrane potential, release of cytochrome *c* from mitochondria, and activation of caspase-3 upon kinetin riboside exposure, which might be due to disrupting the balance of Bcl-2 family proteins. Finally, KR showed its efficacy in an induced-melanoma model *in vivo*, suggesting that KR selectively triggers cancer cell death via inducing classical apoptotic pathway, and suppresses a proliferation of melanoma cells *in vitro* and *in vivo*. In view of these results, kinetin riboside could be considered as a potential candidate drug for treating cancers including malignant melanoma.

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- Figure 1 Chemical structure of kinetin riboside.
- Figure 2 Effect of kinetin riboside on cell viability. CCL-116 and HeLa cells were treated with kinetin riboside (5–20 μM) for 48 h, and their viabilities were analyzed by the MTT conversion assay. Results are means $\pm$ SE values from three separate experiments.
- Figure 3 Kinetin riboside induces apoptotic cell death in HeLa cells, but not in CCL-116 cells. (A and B) Cells treated with or without 4.5 μM kinetin riboside for 24 h were co-stained with fluorescent annexin V and propidium iodide and then examined for apoptosis by flow cytometry. (C) Nuclear fragmentation, one of typical apoptotic features, was assessed by nuclei staining with Hoechst33258 after cells were treated with or without 4.5 μM kinetin riboside for 24 h. Images were visualized using a fluorescent microscope and captured with a CCD camera. Arrows indicate nuclear fragmentation.
- Figure 4 Kinetin riboside induces a loss of mitochondrial membrane potential. HeLa cells were treated with or without 4.5 μM kinetin riboside for 24 h, harvested, and then the mitochondrial membrane potential was analyzed by flow cytometer using a voltage-sensitive fluorescent dye, JC-1. In apoptotic cells, the mitochondrial membrane potential collapses, and JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Results are means $\pm$ SE value from three separate experiments. * $P < 0.05$, values compared with control.
- Figure 5 Kinetin riboside induces a release of cytochrome *c* and activation of caspase-3. (A) HeLa cells were treated with or without kinetin riboside (4.5 and 9 μM) for 24 h. The mitochondrial fraction was analyzed by immunoblotting with an antibody against cytochrome *c*. COX IV was used as a mitochondrial protein control. (B) HeLa cells were treated with or without kinetin riboside (4.5 and 9 μM) for 24 h. The cleaved caspase-3 in whole cell extracts was determined by immunoblotting with an antibody against caspase-3. (C) HeLa and CCL-116 cells were treated with 4.5 μM kinetin riboside for 24 h in the presence or absence of 100 μM pan-caspase inhibitor Z-VAD-fmk, and then caspase-3 activity was measured as described in Section 2. Results are means $\pm$ SE value from three separate experiments. (D) HeLa cells were treated with 4.5 μM kinetin riboside for 24 h in the presence or absence of 100 μM pan-caspase inhibitor Z-VAD-fmk. Cell viability was analyzed by the MTT conversion assay. Results are means $\pm$ SE value from three separate experiments. * $P < 0.05$. (E and F) HeLa and CCL-116 cells were treated with or without kinetin riboside (4.5 and 9 μM) for 24 h. The cleaved PARP in whole cell extracts was determined by immunoblotting with an antibody against PARP.
- Figure 6 Fig. 6. (A and B) Effects of kinetin riboside treatment on expression of Bcl-2 family proteins. HeLa and CCL-116 cells were treated with or without kinetin riboside (4.5 and 9 μM) for 24 h. Bad, Bak, Bcl-2, and Bcl-X_L were analyzed by immunoblotting with specific antibodies.

Figure 7 Differential effects of kinetin riboside on cancer or normal cells. B16F10, permanent melanoma cell line and bovine primary fibroblast were seeded onto 6-well plates at a density of about 1×10^5 cells per well and maintained for 24 h prior to treat with kinetin riboside (1 μ M–1 mM) for 24 h. Cell viability was analyzed by the MTT conversion assay. Results are means $\pm$ SE value from three separate experiments. * P < 0.05, values compared with bovine primary fibroblast under the same concentration.

Figure 8 B16F10-induced-melanoma on mice decreased gradually by dose-dependent injection of kinetin riboside. (A) Representative pictures of drug-treated melanomas after hair removing. Red arrows indicate tumor mass. (B) Tumor masses were removed from each mouse and weighed. * P < 0.01; ** P < 0.005. Dac., Dacarbazine; KR, Kinetin riboside. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8