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Effects of Kinetin Riboside on Proliferation and Proapoptotic Activities in Human Normal and Cancer Cell Lines

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ABSTRACT

Kinetin riboside (KR) is a N6-substituted derivative of adenosine. It is a natural compound which occurs in the milk of coconuts on the nanomole level. KR was initially shown to selectively inhibit proliferation of cancer cells and induce their apoptosis. We observed that KR inhibited growth (20–80%) of not only human cancer, but also normal cells and that this effect strongly depended on the type of cells. The anti-apoptotic Bcl-2 protein was downregulated, while proapoptotic Bax was upregulated in normal as well as in cancer cell lines, upon exposure to KR. Cytochrome *c* level increased in the cytosol upon treatment of cells with KR. The activity of caspases (ApoFluor® Green Caspase Activity Assay), as well as caspase-3 (caspase-3 activation assay) were increased mainly in cancer cells. The expression of procaspase 9 and its active form in the nucleus as well as in cytosol of KR-treated cells was elevated. In contrast, no effect of KR on caspase 8 expression was noted. The results indicated that non-malignant cells were less sensitive to KR then their cancer analogs and that KR most likely stimulated apoptosis mechanism of cancer cells through the intrinsic pathway. J. Cell. Biochem. 112: 2115–2124, 2011.

KEY WORDS: KINETIN RIBOSIDE; CASPASE-3; APOPTOSIS; CANCER; PROSTATE; BREAST; MELANOMA

Kinetin riboside (9-(β-D-ribofuranosyl)-6-furfurylaminopurine, KR) belongs to N6-substituted derivatives of adenosine. It was identified as a natural product in the endosperm liquid of young fresh coconut fruits [Ge et al., 2005; Barciszewski et al., 2007]. It displays a potent anti-proliferative activity against various human cancer cell lines, especially in human leukemia, melanoma, hepatoma, pancreas carcinoma, and colon adenocarcinoma cells [Cabello et al., 2009]. KR also manifested a cytotoxic effect in mice with melanoma (B16F-10) [Griffaut et al., 2004] and leukemia (P388) cells [Choi et al., 2008]. Moreover, KR was demonstrated to be active against plant tumors [Griffaut et al., 2004]. The cytotoxic effect of KR at micromole concentration on human tumor cells was shown in HL-60 [Ishii et al., 2002; Mlejnek and Dolezel, 2005], M4 Beu, He-La, and HepG2 [Cheong et al., 2009]. A decreased proliferation of cancer cells suggests a possibility of employing KR as an anti-cancer drug. It was necessary, however, to consider the effect of inhibiting carcinoma cell growth on the viability on non-malignant cells. The first results of KR application to non-malignant cells were promising. Human non-malignant skin CCL116 cells appeared to be resistant to the effect of KR and only high (20 μM) concentration resulted in a slight decrease of proliferation [Choi et al., 2008]. However, the recently published report indicates that the effect on other non-malignant cells may be different [Cabello et al., 2009]. In-depth studies of molecular mechanism of apoptosis have found activation of caspase enzymes [Kumar and Vaux, 2002]. Depending on the pathway of apoptosis activation of downstream executor, caspase 3 goes through caspase 8 (extrinsic pathway) or caspase 9 (intrinsic mechanism) [Fulda and Debatin, 2006; Krautwald et al., 2010]. In case of the latter, there is extensive evidence showing the decrease in the intracellular ATP content, disruption of mitochondrial integrity and membrane potential, as well as accumulation of reactive oxygen species. One of the quick

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and easy measures of ongoing damage of mitochondria is the release of cytochrome *c* to the cytoplasm. The structural and functional integrity of mitochondria involved in the intrinsic mechanism of apoptosis is under the control of numerous processes and systems. Among them, an important role is played by constitutively expressed Bcl-2/Bax anti-pro apoptotic proteins. Therefore, very often cancer cells maintain a relatively higher expression of the Bcl-2 family proteins, sometimes called apoptosis-break. On the contrary, the Bax family proteins may show a lower level of expression. Triggering of apoptosis is often accompanied by a decrease in expression of the Bcl-2 family proteins and a parallel increase of Bax family partners. In consequence, Bax released from Bcl-2/Bax complexes stimulates disintegration of mitochondria followed by all the above-mentioned effects, including the leakage of cytochrome *c* to the cytosol. This leads to activation of caspases and execution phase of apoptosis.

It was shown that although the precursor form of caspase-3 is located in the cytoplasm, caspase-3 plays essential roles in the nuclear changes in apoptotic cells [Woo et al., 1998; Zheng et al., 1998]. These results suggested that some cytoplasmic substrates translocate into the nucleus after cleavage by caspase-3, leading to nuclear morphological changes. Kamada et al. [2005] postulated that active caspase-3 is present not only in the cytoplasm, but also in the nuclei of apoptotic cells. An analogous phenomenon was observed with respect to other caspases, including caspase 8 and 9 [von Loo et al., 2002]. In the present study, we investigated the effects of KR on both non-malignant cells and human melanoma, prostate, and breast cancer cells in the presence of 2.5 μM of KR. We also addressed the questions on the subcellular location of different pro- and active caspases, as well as expression of several other proteins, such as Bax, Bcl-2, and cyclin D1, which also play important roles in apoptosis. The results indicated that non-malignant cells were less sensitive to KR then their cancer analogs and that KR most likely stimulated apoptosis mechanism of cancer cells through the intrinsic pathway.

MATERIALS AND METHODS

MATERIALS

Basal reagents were purchased from POCH. Non-essential amino acid solution, BSA, RPMI-1640, sodium bicarbonate solution, sodium pyruvate solution, MEM, HEPES, apo-transferine, crystalline violet, monoclonal mouse antibody against β-actin were obtained from Sigma. FBS, MEM, PBS, bovine pituitary extract BPE, trypsin inhibitor soybean, trypsin with EDTA, penicilin–streptomycin, and cholera toxin, Keratinocyte-SFM were purchased from Gibco. Mammary Epithelial cell Basal Medium (HMEC) and supplement kit were obtained from Clonetics. KR was from Sigma.

CANCER CELL LINES

DU145, LNCaP—human prostate cancer; T47D, MCF7—breast cancer, and WM793 and Lu1205—melanoma cell lines were obtained from American Type Culture Collection. Cells Du145, LNCaP, T47D, WM793, Lu1205 were maintained in RPMI-1640 medium (Sigma), MCF7 in MEM (Sigma), supplemented with 5% and 10% heat-inactivated fetal bovine serum (Gibco), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Du145, LNCaP, and T47D cells were also supplemented with 1 mM sodium pyruvate solution (PPA), 1,5 g/L sodium bicarbonate solution (Sigma), 4.5 g/L glucose and 10 mM HEPES (Sigma). MCF7 were also supplemented with 0.1 mM non-essential amino acid solution (Sigma). MCF7 and T47D were supplemented with 0.01 mg/ml insulin (Gibco). All cancer cells were cultured in typical medium for type of cells, with heat-inactivated fetal bovine serum FBS, at 37°C in a humidified atmosphere of 5% CO_2.

NON-MALIGNANT (NORMAL) CELL LINE

Prostate PZ-HPV-7, breast 184A1, and fibroblast CCL110 cells obtained from American Type Culture Collection. PZ-HPV-7 non-tumorigenic prostate epithelial cells were cultured in Keratinocyte-SFM media (Invitrogen), serum free supplemented with 0.2 ng/ml EGF and 30 mg/ml bovine pituitary extract (Gibco). 184A1 breast epithelial cells were cultured in MEGM (Lonza, Clonetics), serum free supplemented with 0.005 mg/ml transferin and 1 ng/ml cholera toxin (Gibco). CCL110 cells were cultured in MEM, supplemented with non-essential amino acid solution (Sigma). Cells were supplemented with 10% FBS. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

PRIMARY FIBROBLAST CELL LINE

Experiments were carried out in a primary line of fibroblasts isolated from tooth pulp originating from premolars and molars (11 teeth) extracted for orthodontic reasons. Following extraction, each tooth was disinfected in 96% ethanol for 60 s and subsequently transversely sectioned with a fissure bur (with a water-cooled turbine) at the level of the neck of the tooth. Thus the prepared tooth was placed in a test tube with sterile PBS and antibiotics (penicillin and streptomycin). Subsequently, the pulp was extirpated from the chamber and canal under sterile conditions. The pulp was rinsed in PBS and the tissue was placed in a collagenase solution (1 mg/ml) and left overnight in a warmer (37°C). After incubation, the tissue-containing test tube was vigorously shaken to isolate the cells. The resultant suspension was centrifuged at 1,000 rpm for 5 min. The cell pellet was suspended in a culture medium (DMEM, 10% FCS) and transferred to culture vessels. The cells were cultured in dishes (Corning) in the DMEM medium (Sigma) in the presence of 10% FBS, 100 μg/ml streptomycin (Polfa) and 100 U/ml penicillin, at pH 7.4. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

CYTOTOXICITY OF KINETIN RIBOSIDE

KR was dissolved in water at concentration 1 mM. The cells were treated with KR (1, 2.5, 5, and 10 μM) for 48 and 96 h. Additionally MCF7 and CCL110 cells were treated with 0.01–2.5 μM KR for 2–6 days. The cells were seeded in medium with 10% FBS, triplicates into 96-microwell plates at density of 1.5×10^3 cells/well. A colorimetric assay was performed according to the manufacturer's instruction (Cytotoxicity Detection Kit, Roche). Absorbance of the colored product—formazane—was measured at 492 nm by an ELISA reader (Synergy HT, BIO-TEK).

CELL PROLIFERATION ASSAY

The cells were seeded in triplicates into 96-microwell plates at the density of 5×10^3 cells/well and 3×10^3 cells/well for long time of incubation (144 h) and cultured in the medium without or with different concentrations of KR, as described above. After the end of incubation, the medium was removed and cells were dyed by 0.5% crystalline violet solution (methanol/water, 1:4) for 4 min, after wash the cells were discharge for 20 min and the absorbance of violet solution was measured at 540 nm by an ELISA reader (Synergy HT, BIO-TEK).

PREPARATION OF NUCLEAR AND CYTOPLASMATIC CELL LYSATES

Nuclear and cytoplasmatic extracts were prepared using the CellLytic™ NUCLEAR™ Extraction Kit (Sigma) according to the producer's protocol and equal amounts of protein were used for immunoblot analysis.

CASPASE-3 ACTIVATION ASSAY

Treatment-induced caspase-3 activation was examined in all tested cells. The cells were seeded in medium with 10% FBS, triplicates into 24-microwell plates at the density of 8×10^4 cells/well for 6–12 h and 4×10^4 cells/well for 12–48 h and incubated in the presence or absence of KR. Staurosporine (1 μM) was used as a positive control for apoptosis. After the treatment without or with KR, the medium was removed and the buffer for caspase-3 was added. The cells were frozen at −80°C overnight. After 20 min incubation at 37°C the substrate for caspase-3 (Ac-DEVD-AFC, Biomol) was added and cells were incubated with reagent. After 1.5 h incubation in RT, inhibitor caspase-3 (DEVD CHO) was added and the fluorescence was detected at 505 nm emission wavelengths after excitation at 400 nm (Hitachi F-2000 reader). In parallel to this experiment, we tested cells numbers with cell proliferation assay.

CASPASE ACTIVITY TEST

All studied cell lines were tested with ApoFluor®Green Caspase Activity Assay (ICN Biomedical, Inc.). Cells (5×10^5; triplicate) were cultured for 48 h in the presence of KR. Staurosporine (1 μM) was used as a positive control for apoptosis (12 and 24 h incubation with staurosporine). After the treatment without or with KR cells were incubated with the ApoFluor®Green reagent according to the manufacturer's recommendation. The cells were harvested, washed to remove any unbound dye and placed in black microtiter plates (NUNK). The fluorescence was detected at 520 nm emission wavelength after excitation at the 488 nm (ELISA reader).

WESTERN BLOT ANALYSIS

Total cellular protein was isolated from the cells according to the procedure described previously [Dulińska et al., 2005]. The Western blot analysis was performed on equal amounts of each protein samples. The identification of proteins was performed with respective antibodies: monoclonal mouse antibody against: β-actin (Sigma), Bcl-2 (Pharmingen), D1 and Bax (Pharmingen), caspase-9 (Cell Signaling), caspase-8 (1C1) (Cell Signaling), caspase-3 (3G2) (Cell Signaling), and cytochrome *c* (136F3) (Cell Signaling).

REAL-TIME PCR QUANTITATIVE ANALYSIS (RQ-PCR)

The RNA isolation and cDNA synthesis was carried out as previously described elsewhere [Dulińska et al., 2005]. The relative gene expression analysis was done using the two-step RT-PCR with real-time quantitative amplification performed in the continuous fluorescence detection system (DNA Engine Opticon II, MJ Research). Equal amounts of total RNA (400 ng) were reversely transcribed using the Superscript II Kit (Qiagen). The PCR amplification was performed with the QuantiTect™ SYBR® Green PCR Kit (Qiagen) using the SYBR Green as fluorescent dye. The primers were designed to include an intervening intron between the sense and anti-sense primers to eliminate the possibility of amplifying any genomic DNA, and checked for specificity by BLAST searches. The following specific primers (Oligo Company) were used: *GAPDH*: 5′-CCAGGCGCCCAATACGA-3′ and 5′-GCCAGCCGAGCCACATC-3′; *BAX*: 5′-GAAGCTGAGCGAGTGTCT-CAAG-3′ and 5′-GTCCACGGCGGCAATC-3′; *BCL-2*: 5′-ACACCCCC TCGTCCAAGAAT-3′ and 5′-CCAGAGAAAGAAGAGGAGTTA-TAATCCA-3′, conditions were as follows: the amplification initial incubation at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. Subsequently, the melting curve analysis was performed to verify the specificity of the PCR products [Dulińska et al., 2005].

STATISTICAL ANALYSIS

All data on the proliferation and cytotoxicity experiments represent the average of five wells in each experiment ($n = 4$). The variables were summarized as mean ± standard deviation of the mean. In case of the RQ-PCR and Western blotting, each analysis was performed in triplicate. The significance of the differences between the mean values was calculated using the Student's *t*-test (≤ 0.05).

RESULTS

In the present study, we examined the ability of KR to inhibit growth of human melanoma (WM793, Lu1205), breast (MCF7, T47D), prostate (PC-3, Du145) cancer and non-malignant—fibroblast (primary, CCL110), breast (184A1), prostate (PZ-HPV) cells (Fig. 1). Usually, the cells were incubated with KR for 48 h. The effect of KR on growth of the studied cells depended on the KR concentration (Fig. 1). Incubation with 2.5 μM resulted in a reduced viability from 15% (primary fibroblasts) to 25% (CCL110), while incubation with 5 μM KR decreased the viability by 40–50% both for cancer and non-malignant cells. Microscopy analysis demonstrated morphological changes in the cells treated with KR that confirmed the observed decrease in the number of cells after 48 h of incubation with 5 μM KR (Fig. 2). Primary fibroblast cells were less sensitive to KR in comparison to CCL110 and cancer cells. The proliferation of normal primary fibroblasts and CCL110 cells at 2.5 and 5 μM KR varied by 10% and 20%, respectively.

In the case of human breast cancer (MCF7, T47D) and primary 184A1 cells, incubation with 1 μM KR did not have any significant effect on the viability of non-malignant cells, whereas at the 5 μM KR concentration, the viability dropped to 40% (Fig. 1B). In breast cancer cells, 2.5 μM KR resulted in a considerable decrease in the growth of T47D (60%) and MCF7 (70%). Under the same conditions,

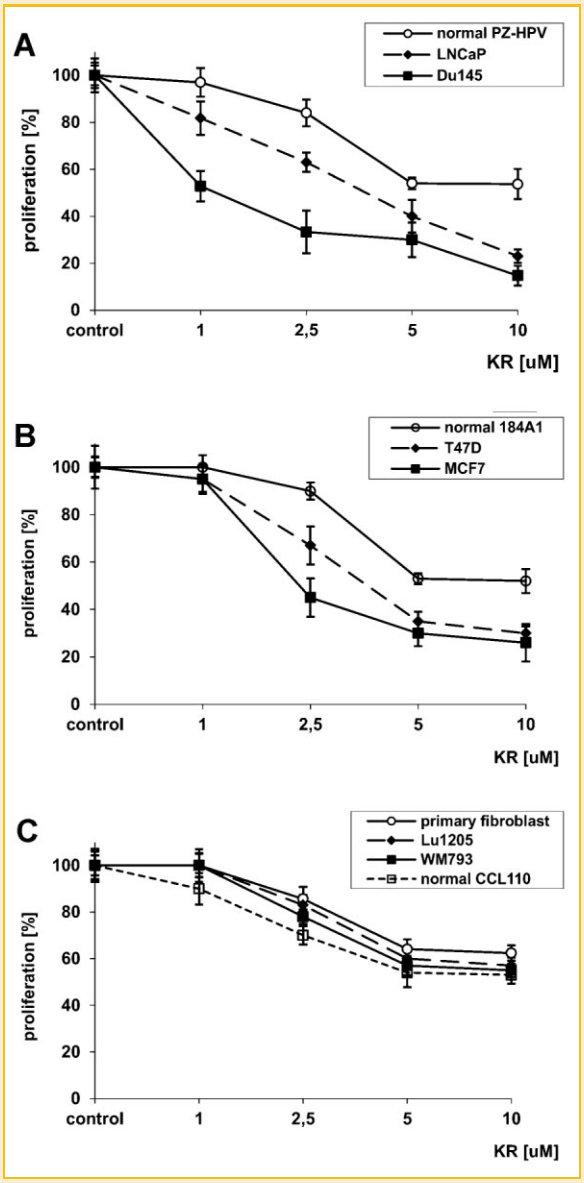

Fig. 1. The effect of KR on proliferation of human cell treated with different concentration of kinetin riboside (1, 2.5, 5, 10 μM) for 48 h incubation: (A) prostate cell lines—cancer LNCaP, Du145, and non-malignant PZ-HPV, (B) breast cell lines—cancer MCF7, T47D, and non-malignant 184A1, (C) melanoma cell lines Lu1205, WM793, non-malignant CCL110 cell line, and primary fibroblast. The cells were cultured in corresponding medium containing 10% FCS (control) and were treated with KR for 48 h. The details are described under the Materials and Methods Section. The results are given as the percentage of the control proliferation. Data represent mean values ± SD.

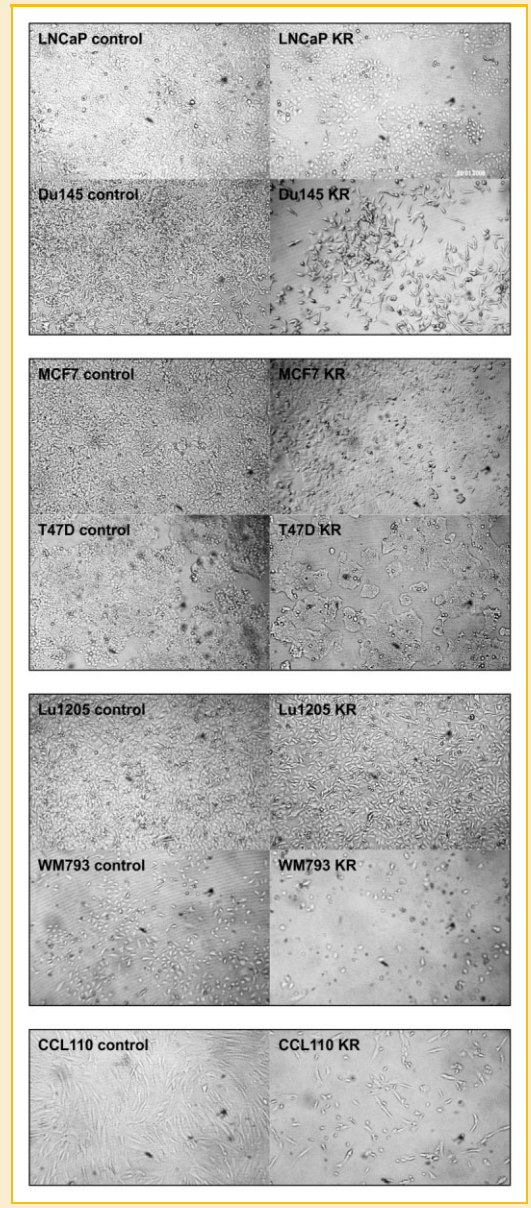

Fig. 2. The microscopy examination of cells after 48 h incubation with 5 μM KR in human cancer cell lines and in human non-malignant cells. The details are described under the Materials and Methods Section. The results are given as the percentage of the control proliferation. Data represent mean values ± SD.

KR had little effect (10%) on normal cells (184A1). In the case of human prostate cancer (Du145 and LNCaP) and non-malignant (PZ-HPV) cells after 48 h of incubation with 1 μM KR, Du145 was demonstrated to be the most sensitive cell line, with a drop in the viability of 50% (Fig. 1A). A further increase in KR concentration to 2.5 μM resulted in inhibition of the LNCaP viability, which reached 60%, at the same time maintaining a high viability rate of PZ-HPV cells (90%). On the other hand, 5 and 10 μM KR resulted in a significantly decreased proliferation of malignant cells (60–80%). Under the same conditions, we observed up to 40% decrease of normal cells proliferation.

To achieve a better insight into inhibition of proliferation of the cell lines most sensitive to KR (cancer MCF7 and a non-malignant CCL110), they were incubated with 0.01–2.5 μM KR for 144 h (not shown). The most significant difference was observed for a short incubation time (48 h), but longer incubation (144 h) produced the same effect. One can see that KR in the range of 0.01–1 μM resulted in a drop in the viability ranging from 10% (48 h) to 40%(144 h) in normal CCL110 and malignant cells, respectively. On the other hand, 2.5 μM KR resulted in a drastic decrease of the viability from 30% (48 h) to 80% (144 h) in melanoma cell line.

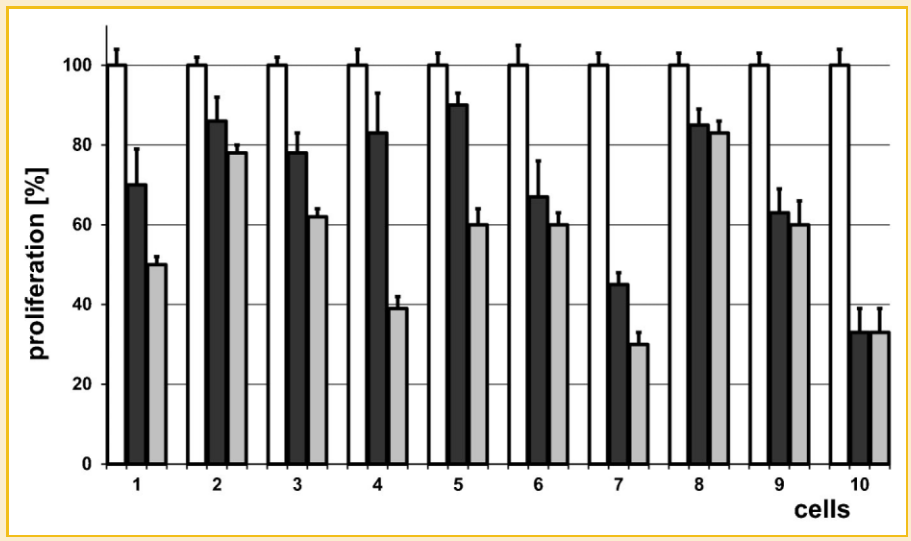

Fig. 3. The effect 2,5 μM KR inhibition on proliferation of human cell lines over ■—48 h and □—96 h time of incubation. We used 1—skin fibroblast CCL110; 2—primary fibroblast; 3—cancer WM793; 4—cancer Lu1205; 5—normal breast 184A1; 6—cancer T47D; 7—cancer MCF7; 8—normal prostate PZHPV; 9—cancer LNCaP; 10—cancer Du145.

To compare the response of various cells, all were treated under the same conditions (2.5 μM KR) for 48 and 96 h (Fig. 3). After 48 h, the least sensitive cells were found to be normal breast 184A1, followed by normal prostate cell line PZ-HPV, primary fibroblasts, Lu1205, WM 793, non-malignant CCL110, LNCaP, and T47D. Du145 and MCF7 cells showed the highest sensitivity to KR. After 96 h, a further 30% decrease in cell viability was observed for normal breast cells 184A1. A similar decrease of proliferation was noted in melanoma WM793 (20%) and normal skin CCL110 (20%) cells, but primary fibroblast cell line was stable and resistant to KR (80%). The highest decrease of proliferation was observed in melanoma Lu1205 and prostate Du145 cells (40%).

To determine the mechanism of apoptosis, we applied the caspase activity test (ApoFluor® Green Caspase Activity Assay) to all ten cell lines. The cells were incubated with 2.5 μM KR for 6–48 h. In general, no effect of KR was noted when apoptosis studies were performed on primary fibroblast or normal prostate cell PZ-HPV (Fig. 4). Both the primary breast cells 184A1 and non-malignant CCL110 cells responded to treatment with KR with a markedly increased apoptosis, as shown by a high caspase activity after treatment with KR (Fig. 4). Because caspase-3 is a valuable and easily assayed marker for apoptosis, we analyzed its expression in prostate (Du145), melanoma (Lu1205), and breast (T47D) cancer, as well as non-malignant cells treated with KR (Fig. 5). Caspase-3 activity was compared to the controls (proliferation test after 6, 12, 24, and 48 h). The cell lines did not respond in the same manner. The highest increase in caspase-3 activity was observed in CCL110 and Lu1205 cell lines (Fig. 5C). The threefold increase in the activity was also determined for prostate cancer cells Du145 (Fig. 5A), with a slight increase in the activity in non-malignant PZ-HPV cells. In breast cancer cells, a low signal of caspase-3 activity was observed, while no activity changes were observed in non-malignant cells (Fig. 4B). It was accompanied by an appreciable increase of the expression of Bax and a slight decrease in Bcl-2 expression on day 2 (Fig. 6). The expression of the Bax and Bcl-2 genes was studied in all human normal and cancer cells. A similar effect—an increased expression of Bax and a decreased expression of the Bcl-2 genes after treatment with KR—was observed in all cell lines, but the extent was different. A quantitative analysis of gene expression of BAX and BCL-2 in melanoma cells showed a marked difference in the expression of these genes in tumor cells compared with normal cells. We observed a twofold increase in the expression of BAX in Lu1205 cells, while the expression of BCL-2 was decreased (Figs. 6 and 7). Similar associations were apparent in breast cancer MCF7 cells. The least sensitive line with respect to both changes in the proliferation after treatment with KR, as well as the expression of Bax and Bcl-2 was normal prostate cell line PZ-HPV (Figs. 3, 6, and 7). Apparently, the observed changes in BAX and BCL-2 expression did not unanimously correlate with caspases activity. Lack of expression of BAX in DU145 after treatment with KR was not surprising and also untreated cells did not show this product. Despite the observed decrease in proliferation in LNCaP, prostate cancer cells were not found to correlate with the expression of BAX and BCL-2 in these cells.

We also examined the effect of KR treatment on the levels of Bcl-2 protein and Bax in normal cell line CCL110 and in melanoma cell lines WM793 and Lu1205. The data showed that the proapoptotic protein Bax significantly increased only at 2.5 μM KR, but Bcl-2 was downregulated during KR exposure after 96 h (Fig. 6C). In the case of breast cells, both normal and cancer cells responded with an increased Bax gene expression and a lower expression of Bcl-2 in all the cells treated with KR, but significant changes were observed for MCF-7 cells (Fig. 6B). We did not observe significant changes in the cells of the prostate after treatment with KR in comparison to untreated cells (Fig. 6A).

To determine the mode of action of KR, its effect on apoptosis process in correlation with caspase-3,8,9 and cyt *c* expression level was assessed. The function of casp-3 strongly depended on the interaction with casp-9 or casp-8. After treatment of melanoma cells

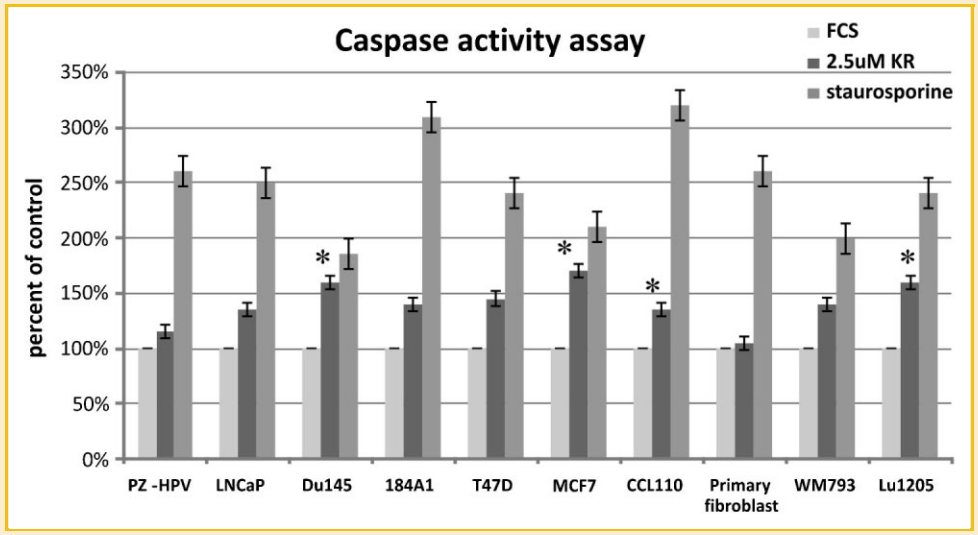

Fig. 4. Global caspase activity—apoptosis pathway. Apoptosis of melanoma—CCL110, primary fibroblast, WM793, Lu1205, breast—184A1, T47D, MCF7 and prostate PZ-HPV, LNCaP, Du145 cell lines—in effect of 2.5 μM kinetin riboside (KR) treatment. The rate of apoptosis was estimated by the measurement of global cellular caspase activity following 48 h incubation of cells with 2.5 mM KR or vehicle alone. The data represents the mean of four independent experiments each conducted in triplicate, expressed as the percentage of activity of the control. Significantly different from the corresponding control $^{*}P < 0.05$.

with KR, the level of nuclear fraction of casp-9 in both CCL110 and Lu1205 significantly increased, which correlated with higher level of casp-3, being even more pronounced in Lu1205 cancer cell line than in normal cell line CL110 (Fig. 8). In addition, the expression of the cytosol fraction of cyt *c* was higher both in normal and cancer melanoma cell line. Because the expression of casp-3 in MCF7 cell was lost, to observe the interaction involved in apoptosis process we used normal 184A1 and T47D cell line. In the case of breast cells, this interaction was less clear. The higher expression of cyt *c* seen was in T47D line and we also noted an increased level of casp-3 in this cell line. By contrast, the level of casp-3 in the cytosol fraction in normal PZ-HPV prostate cells was not seen, what probably correlates with the observed responses of those cells. We did not note any changes in proliferation and apoptosis processes in this cell line. In apoptotic cells, caspases-3, -8, and -9 were present in the cytosol fraction. Caspases-3 were present in the nuclear fraction. The selective localization of procaspases in different subcellular compartments may play an important, but yet unknown, role in their activation. We did not observe significant changes in casp-8 expression.

To check whether the cell cycle was arrested, the effect of 2.5 μM KR exposure on the level of cyclin D1 in cancer and non-malignant cell lines was analyzed. It turned out that human breast cancer cell lines (MCF7 and T47D) and non-malignant breast cells 184A1 treated with KR showed a decreased expression of cyclin D1 (Fig. 6B). Melanoma cells showed a decreased expression of cyclin D1 after treatment with 2.5 μM KR. Our results indicate different sensitivity to KR of not only cancer, but also normal cells.

DISCUSSION

We analyzed melanoma, prostate, and breast cancer as well as primary cell lines against different concentrations of KR. Exposing the cells to high concentrations of KR in the range of 5–10 μM indicates that proliferation of both non-cancer and cancer cells are inhibited by the employed substance (Fig. 1). In the case of prostate and mammary gland cancer cells, the proliferation is dependent upon the type of cells, but also upon the degree of their malignancy. The lowest decrease in proliferation was noted in normal cells, whereas the proliferation of cancer cells was clearly inhibited, with the observed difference amounting to several score percent. The reaction of fibroblasts and melanoma cells to high KR concentrations (5 and 10 μM) was entirely different (Fig. 1C). All the four types of cells: CCL110, primary fibroblasts, Lu1205, and WM793 reacted in a similar manner, demonstrating a strongly comparable degree of proliferation inhibition. Inhibition as a consequence of incubation with KR was markedly weaker as compared to mammary gland and prostate cancer cells, since it did not exceed 50% after 48 h incubation with the KR (Fig. 1A,B). The analysis of cell proliferation profile within the range of low KR concentrations leads to similar conclusions (not shown). After 48 h of incubation with 2.5 μM KR, a decrease of proliferation in all the fibroblast and skin tumor cell lines did not exceed 30%; only extending the incubation time up to 96 h allowed for discrimination between the cells with respect to the degree of proliferation inhibition. This is not the effect we expected, since non-selective inhibition of proliferation of normal and cancer cells may disqualify KR as a subject of further studies aiming at its usage as an anti-tumor drug. This was confirmed by the results of time-dependent inhibition obtained for MCF7—as the strongest-reacting tumor cell line of all the lines used in the present experiment—and for CCL110, as the most sensitive normal cell line of all the lines presently used (not shown). Recent studies demonstrated that KR induces cell death in cancer cells, but the cytotoxic effect of KR on immortalized cells—CCL116 was not significant [Ishii et al., 2002]. The viability of HeLa cells was decreased by more than 50% in response to 5 μM KR, but non-

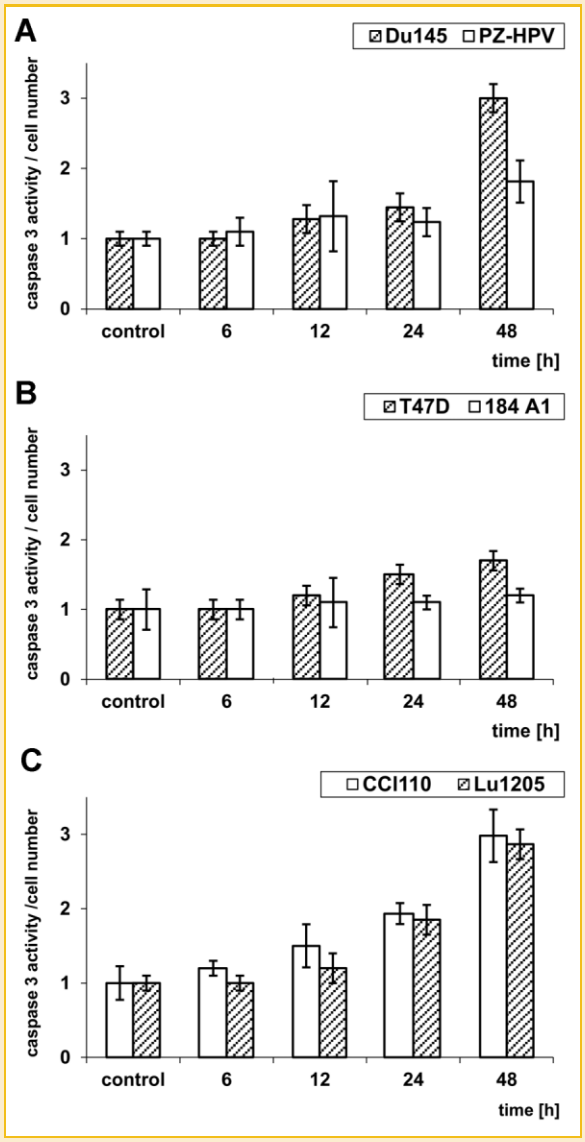

Fig. 5. Caspase-3 activity in both normal and cancer cell lines, after 2.5 μM KR incubation over 6–48 h. Caspase-3 activity was determined relative to the controls, in proportion to the number of signal-yielding cells (proliferation test after 6, 12, 24, and 48 h): (A) prostate cell lines, (B) breast cell lines, (C) melanoma cell lines. The data represent the mean of three independent experiments, each conducted in duplicate expressed as the percentage of caspase 3-activity per cell number.

cancer cells CCL116 exhibited a less than 20% reduction in viability after 20 μM KR treatment. However, the cytotoxic effect was not apparent at 5 and 10 μM. Also, no significant effect of 10 μM KR was observed on the viability of CCL116 cells [Choi et al., 2008]. Each type of cells (melanoma, prostate, and breast) used in the experiment represents a non-malignant, cancer and metastasis cell line. One can see a relationship between KR cytotoxicity and the degree of cells malignancy. For example, primary fibroblasts are almost resistant to KR. However, there is also a marked difference in the sensitivity to KR between two malignant melanoma cell lines WM793 and Lu1205 (Fig. 3). A similar but more pronounced effect was observed for prostate-derived cells PZ-HPV, LNCaP, and Du145. While analyzing the two remaining systems (the prostate and breast), or the mammary gland and prostate cells, we can unequivocally state that they react in a manner dependent upon the type of cell. The use of low concentrations allows for selective inhibition of mammary gland and prostate cancer cells independently of the time of their incubation with the KR, since the effect of inhibition of proliferation of, for example, prostate cells is maintained at the same level after 48 and 96 h of incubation (Fig. 3).

Caspase-3 with caspase-6 and -7 belong to the effective caspases family, involved in apoptosis [Janicke et al., 1998; Budihardjo et al., 1999; Earnshaw et al., 1999; Fesik and Shi, 2001; Shi, 2002]. Furthermore, one can ask whether caspase-3 is active in cells treated with KR. Some articles point to massive activation of caspase-3 following incubation with KR [Choi et al., 2008; Cheong et al., 2009]. Treatment with different doses of KR for 48 h did not result in induced caspase-3 activity in HepG2 cells, but in a significant degree of DNA fragmentation and cell death. This observation was also noted for human myeloid leukemia cells (HL-60) [Cheong et al., 2009]. In the mitochondrial pathway of apoptosis, caspase activation is closely linked to permeabilization of the outer mitochondrial membrane by proapoptotic members of the Bcl family [Decaudin et al., 1998; Green and Kroemer, 2004]. Numerous cytotoxic stimuli and proapoptotic signal-transducing molecules converge on mitochondria to induce outer mitochondrial membrane permeabilization. This permeabilization is regulated by proteins from the Bcl-2 family, mitochondrial lipids, proteins that regulate bioenergetic metabolite flux and components of the permeability transition pore [Green and Kroemer, 2004]. Upon disruption of the outer mitochondrial membrane, a set of proteins normally found in the space between the inner and outer mitochondrial membranes is released, including cytochrome *c* [Saelens et al., 2004]. Once in the cytosol, these apoptogenic proteins trigger the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors [Saelens et al., 2004]. The release of cytochrome *c* from mitochondria directly triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex [Cain et al., 2000]. Caspase-3 then cleaves key substrates in the cell to produce many of the cellular and biochemical events of apoptosis. In our studies, activity of the global cellular caspases increased proportionally to the result obtained in proliferation inhibition experiments. These results were correlated with caspase-3 activity for several cell lines. In normal breast cells 184A1 and in breast cancer cells, we observed no caspase-3 activity after 48 h of incubation, while other caspases were activated (Fig. 5). No significant changes on the protein level were observed in caspase-3 and also casp-8 and 9 in treated and non-treated breast cells (184A1 and T47D) (Fig. 8), but we noted an increased total caspases activity (Fig. 4), what suggests that in this regulation other types of caspases may participate. Prostate cells responded differently; the expression of cyt *c* significantly increased in cytosol in Du145 cancer cells without activation of this caspase in PZ-HPV normal cells (Fig. 8). Triggering of apoptosis is often accompanied by a decrease in the expression of the Bcl-2 family proteins and a parallel increase of the Bax family partners. In consequence, Bax released from the Bcl-2/Bax complexes stimulates

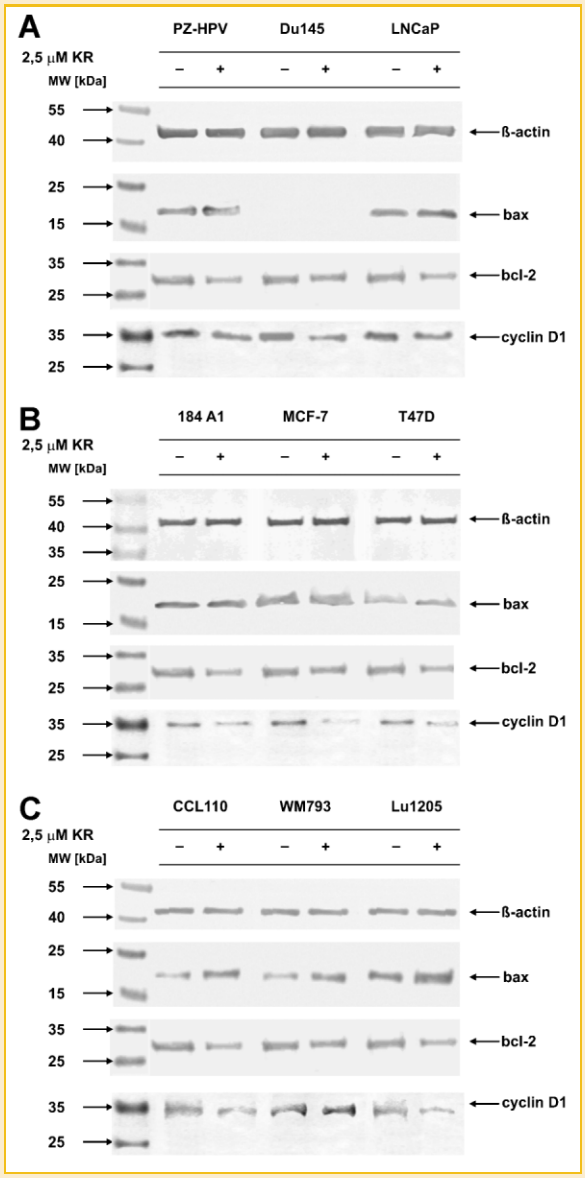

Fig. 6. Effect of 2.5 μM KR on the expression of Bcl-2, Bax, and cyclin D1 proteins in whole cell protein extracts in: (A) human normal PZ-HPV and cancer cell lines Du145 and LNCaP, (B) breast cell lines, non-malignant 184A1 and cancer MCF7 and T47D and (C) fibroblast cell lines CCL110 and in melanoma cell lines WM793, Lu1205 after 48 h incubation. The upper line shows the expression of the house-keeping gene β-actin at the same conditions. Equivalent amount of protein cell lysates were separated by electrophoresis (PAGE–SDS, 10% gel) and probed by Western blotting with individual antibodies. The details are described under the Materials and Methods Section.

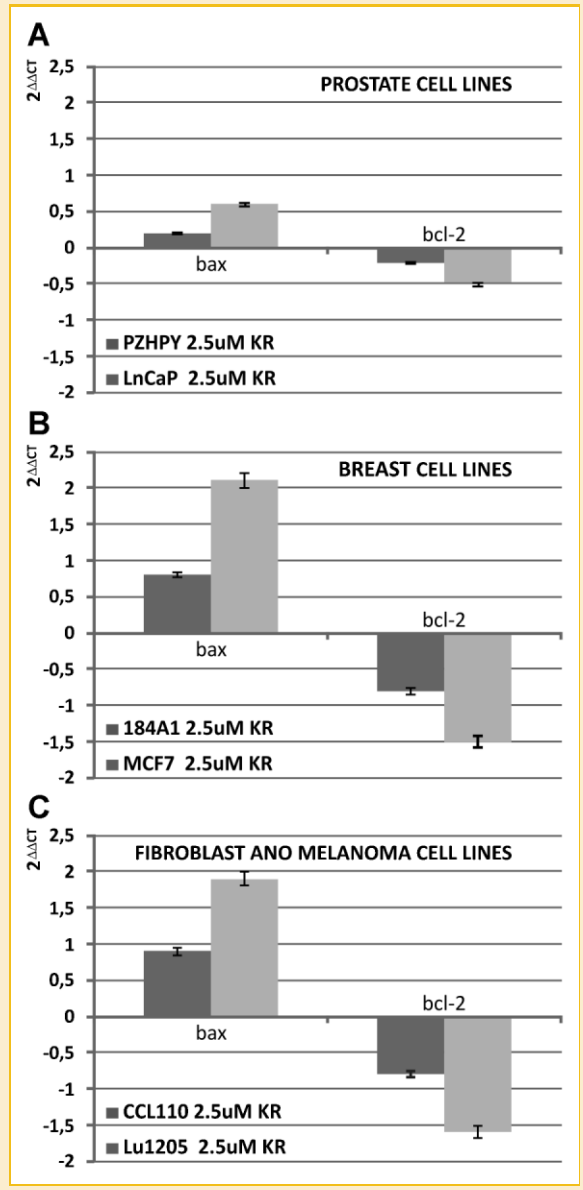

Fig. 7. The expression of bax and bcl-2 transcripts in various human cells. The results of the quantitative real-time PCR illustrating the differential expression of BAX and BCL-2 transcripts in human cells. The total RNA samples were prepared from untreated cell lines and from cells treated with RK. The details are described under the Materials and Methods Section. Columns represent the means ± SEM of three independent experiments, each conducted in triplicate. Significantly different from the corresponding control $^{*}P < 0.001$.

disintegration of mitochondria, followed by all of the above-mentioned effects, including the leakage of cytochrome *c* to cytosol. This leads to activation of caspases and execution phase of apoptosis, what we observed in prostate and melanoma cancer cells. It was shown that although the precursor form of caspase-3 is located in the cytoplasm, caspase-3 plays essential roles in the nuclear changes in apoptotic cells [Woo et al., 1998; Zheng et al., 1998]. These results suggested that some cytoplasmic substrates translocate into the nucleus after cleavage by caspase-3, leading to nuclear morphological changes. Kamada et al. [2005] postulated that active caspase-3 is present not only in the cytoplasm, but also in the nuclei of apoptotic cells. The analogous phenomenon was observed with respect to other caspases, including caspase 8 and 9 [von Loo et al., 2002]. In the present study, we investigated the effects of KR on both non-malignant cells and human melanoma and breast cancer cells in the presence of 2.5 μM of KR. We also addressed the questions on the subcellular location of different pro- and active caspases, as well as the expression of several other

Fig. 8. Effect of KR on the expression of cyt *c* in cytosolic fraction and of caspase-3, -8, -9 protein in nuclear and cytosolic fraction normal and cancer of prostate, breast and melanoma cells. The cells were exposed to 2.5 mM KR in appropriate medium for 2 days. Equivalent amount of protein cell lysates were separated by electrophoresis (PAGE–SDS, 10% gel) and probed by Western blotting with individual antibodies. The details are described under the Materials and Methods Section.

proteins, such as Bax, Bcl-2, cyclin D1, which also play important roles in apoptosis. The results indicated that non-malignant cells were less sensitive to KR then their cancer analogs and that KR most likely stimulated the apoptosis mechanism of cancer cells through the intrinsic pathway.

KR may exert cellular effects that promote apoptosis in addition to regulating cell-cycle entry, because it causes alterations in mitochondrial membrane potential as a prelude to apoptosis [Cabello et al., 2009]. Mitochondria are an important part of the apoptotic machinery [Danial and Korsmeyer, 2004; Green and Kroemer, 2004]. The mechanisms of action of chemically diverse small molecules on specific mitochondria loci, such as respiratory chain, DNA biogenesis, potassium channels, the Bcl-2 family proteins and the permeability transition pores were reported [Jacobson et al., 1993; Clintock et al., 2002; Lailer et al., 2007]. The mitochondrial integration of the numerous signals arising from different cellular compartments and functional pathways during apoptosis is essential for the completion of this cell death program. The integration is controlled by Bcl-2 proteins [Shiroff et al., 2007; Marzo and Naval, 2008]. Bax is a proapoptotic protein of this family, which interacts with anti-apoptotic proteins from the same family (Bcl-2, Bcl-Xl) [Er et al., 2006; Laidler et al., 2007]. We analyzed the expression of Bcl-2 and Bax proteins in all cancer lines (melanoma, prostate, and breast) and in primary cells. Bax was highly upregulated and Bcl-2 was downregulated in fibroblast CCL110 and in melanoma cells, but all cell lines showed an increase of Bax protein level (except Du145-Bax deficient) and a decrease of Bcl-2 level. Specifically, the cyclin D1 gene (CCND1) plays an integral part in cell growth and survival control [Xiong et al., 1991; Fasanaro et al., 2002; Murray, 2004; Utikal et al., 2005]. We have shown that cyclin D1 is downregulated in all the tested cell lines. The same observation was noted in human melanoma cells [Tiedemann et al., 2008].

We noted that KR inhibits the growth of human normal and cancer cells with different potency. Some of non-malignant cells were less sensitive to KR. It was observed that the inhibition depended on cell type and duration of KR treatment. KR induced caspase-3 activity in several cell lines. However, Bcl-2 protein was downregulated, while the expression of Bax was upregulated in both cell lines upon their exposure to 2.5 μM KR. At the same time, both types of the cells manifested a decrease in the expression of cyclin D1. It seems that KR is a selective anti-proliferative agent. In conclusion, KR inhibits proliferation of normal and cancer cells in a different manner, depending on malignancy degree. It has been demonstrated that non-malignant cells are more resistant to apoptosis induced by KR. The inhibition of normal cells depends on the cell type.

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