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1,25-Dihydroxyvitamin D_3 modulates effects of ionizing radiation (IR) on human keratinocytes: *In vitro* analysis of cell viability/proliferation, DNA-damage and -repair☆

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ABSTRACT

We investigated the capacity of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) to protect spontaneously immortalized human keratinocytes (HaCaT) and cutaneous squamous cell carcinoma cells (SCL-1) against the hazardous effects of ionizing radiation (IR). We pretreated HaCaT and SCL-1 cells *in vitro* with $1,25(OH)_2D_3$ (10^{-7} M) over 48 h and then irradiated them once with IR (1 Gy, 2 Gy, and 5 Gy). Using WST-1-assay and crystal violet (CV) assay, we compared viability/proliferation in $1,25(OH)_2D_3$-pretreated cells with controls that were pretreated with the carrier substance ethanol alone. Additionally, we analyzed the effects of $1,25(OH)_2D_3$ on the presence of IR-induced DNA-damage by immunocytochemical detection of γ-H2AX-foci in HaCaT-keratinocytes. We demonstrate that $1,25(OH)_2D_3$ (10^{-7} M) inhibits proliferation of human keratinocytes and that IR (1–5 Gy) has no significant effect on proliferation and viability of HaCaT-keratinocytes and SCL-1 cells. Moreover, we show that IR modulates dose-dependently the number of γH2AX-foci in HaCaT-keratinocytes. Pretreatment of the cells with $1,25(OH)_2D_3$ reduces the number of IR-induced γH2AX-foci after irradiation with 1 Gy and 2 Gy and increases it after irradiation with 5 Gy. To put it in a nutshell, our data support the hypothesis that $1,25(OH)_2D_3$ modulates the effects of low-dose IR (1–5 Gy) on cultured human keratinocytes.

1. Introduction

Increasing evidence indicates that the UV-B-mediated cutaneous photosynthesis of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the active form of vitamin D, represents an evolutionary highly conserved endocrine system that protects the skin against environmental hazards, maybe including ionizing radiation [1]. In recent years, it has been demonstrated that keratinocytes and numerous other cell types possess the enzymatic machinery (CYP27B1) to synthesize $1,25(OH)_2D_3$ [2,3]. Although their function in skin is not fully understood, it is well accepted that the normal differentiation of keratinocytes is partly regulated by $1,25(OH)_2D_3$ [4]. In this study, we analyzed the effects of $1,25(OH)_2D_3$ (10^{-7} M) on proliferation and viability in IR-irradiated human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) using WST-1-assay and crystal violet assay. The key target of ionizing radiation in the cell is DNA [5]. The main class of potentially mutagenic lesions induced by IR is the formation of DNA double-strand breaks (DSBs) [6,7]. The histone variant H2AX was reported to be rapidly phosphorylated at the sites of DNA-damage. This phosphorylated H2AX (γ-H2AX) has been shown to be involved in the recruitment and retention of signaling and repair factor complexes at the sites of DNA DSBs [8]. Phosphorylated H2AX, denoted γ-H2AX, can consequently serve as a marker for IR-induced DSBs. Although further findings indicate that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) exerts photoprotective effects in ultraviolet-B treated human keratinocytes, possible effects of $1,25(OH)_2D_3$ on IR-induced DNA-damage and -repair have not been systematically analyzed until now. It has been shown in further studies that IR leads to a down-regulation of various apoptosis-relevant genes in HaCaT cells pretreated with vitamin D [9]. It can therefore be speculated that vitamin D could prove to be a promising radioprotective substance. The aim of the present study was to investigate the capacity of $1,25(OH)_2D_3$ to protect human keratinocytes (HaCaT) and squamous cell carcinoma cell lines (SCL-1) against the hazardous effects of ionizing radiation. Additionally, we investigated the ability of $1,25(OH)_2D_3$ to influence IR-induced DNA-damage in HaCaT-keratinocytes by immunofluorescence-detection of γ-H2AX-foci.

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2. Materials and methods

2.1. Cell culture

Spontaneously immortalized ("normal") human keratinocytes (HaCaT) and cutaneous squamous cell carcinoma cells (SCL-1) were maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 1% L-glutamine and 10% foetal calf serum (Biochrom). They were grown in a humidified atmosphere of 5% CO_2 at 37 °C. Cell culture medium was changed every 2 days.

2.2. 1,25(OH)$_2$D$_3$-treatment

When treating cells with 1,25(OH)$_2$D$_3$ (10^{-7} M), 1% of BSA and a mix of 1% penicillin/streptomycin was added to the medium. Due to the fact that 1,25(OH)$_2$D$_3$ was solved in ethanol, we compared 1,25(OH)$_2$D$_3$-treated cells with controls that we treated only with ethanol. Another control was effectuated with medium only. Cells were pretreated with 1,25(OH)$_2$D$_3$ 48 h before irradiation with IR. After irradiation, cell culture medium was changed every 2 days with supplementation of 5 μl of 1,25(OH)$_2$D$_3$ resp. ethanol or medium.

2.3. IR-irradiation

Before irradiation, cells were washed with phosphate-buffered saline (PBS) and then irradiated through a thin film of PBS. Depending on the experiments, cells were irradiated with IR-doses between 1 Gy and 5 Gy. After irradiation, cells were provided with fresh medium.

2.4. WST-1 assay

HaCaT-keratinocytes and SCL-1 cells were seeded in 96-well culture plates (3×10^3 cells in 100 μl RPMI + 10% FCS-medium/well) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. In six wells the cells were pretreated with 1,25(OH)$_2$D$_3$ 48 h before irradiation. As independent controls, cells were treated in another six wells with ethanol resp. medium. Cells were then irradiated with IR: 0 Gy, 1 Gy, 2 Gy or 5 Gy. 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples was measured in an ELISA-reader as follows: 10 μl of WST-1-reagent were added to each well and the absorption of the samples was measured 0 h, 1 h, 2 h, 3 h and 4 h after adding the reagent at 450 nm using the ELISA-reader.

2.5. Crystal violet assay

Seeding, treatment and irradiation of HaCaT-keratinocytes and SCL-1 cells was effectuated as described above for the WST-1 assay. Afterwards, medium was removed from the 96-well culture plates and cells were washed 2 times with 200 μl of cold PBS/well and then incubated with 150 μl of 70% ethanol at 4 °C for at least 30 min. After removing the ethanol, cells were incubated with 100 μl of 0.1% crystal violet solution/well for 30 min at room temperature. Crystal violet was then removed and the cells were washed with water. Cell culture plates were dried overnight. The next day, wells were incubated with 200 μl of 70% ethanol for at least 30 min at room temperature to dissolve the crystal violet stain before measuring its absorption at 550 nm in a Titertek Multiskan Plus-Reader.

2.6. γ-H2AX immunofluorescence

HaCaT-keratinocytes were pretreated with 1,25(OH)$_2$D$_3$, ethanol and medium as described above. After irradiation, cells were fixed with 1 ml of formaldehyde in PBS for 15 min, then washed 3× for 10 min with 1 ml of PBS and permeabilized with 2 ml of iced Triton-X-PBS. Cells were washed again 3 times for 10 min with 1 ml of PBS and then blocked with PBS + 5% FCS + 0.02% Triton-X-100 for 30 min at room temperature. Incubation with the primary-antibody (anti-γ-H2AX-AK) in a dilution of 1:200 in PBS-FCS for 1 h at room temperature. Cells were then washed 3 times for 10 min with 1 ml of PBS and incubated with the secondary antibody (Alexa FluorR 488 goat-anti-mouse IgG, Invitrogen) in a dilution of 1:500 in PBS-FCS for 1 h at room temperature. Labeled cell preparations were then examined with a fluorescence microscope equipped with a CCD imaging system. Images were captured using the accompanying image processing software from Cytovision with bandpass filter sets allowing the visualization of the Alexa 488 dye for γ-H2AX identification and DAPI as the nuclear counterstain. γ-H2AX immunofluorescence was effectuated 30 min, 2 h, 6 h and 12 h after irradiation.

2.7. Statistical analysis

All data are represented as a mean for at least four experiments. Statistical significance was calculated by a two-tailed Student's *t*-test for unpaired samples, using the Microsoft EXCEL software. Mean differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Pharmacological doses of 1,25(OH)$_2$D$_3$ inhibit the growth of spontaneously immortalized HaCaT- and cutaneous squamous cell carcinoma cells (SCL-1)

We confirm that both human HaCaT-keratinocytes (Fig. 1) and SCL-1 cells (Fig. 2) are target cells for 1,25(OH)$_2$D$_3$. As shown by crystal violet (Figs. 1a and 2a) and WST-1 (Figs. 1b and 2b) assays, incubation with 1,25(OH)$_2$D$_3$ (10^{-7} M) results in SCL-1 cells in a significant suppression of cell proliferation, while in HaCaT-keratinocytes, suppression of cell proliferation is less pronounced and not significant.

3.2. Ionizing radiation (1–5 Gy) has no effect on proliferation of HaCaT-keratinocytes and squamous cell carcinoma cells (SCL-1)

Comparing unirradiated controls (0 Gy) and HaCaT (Fig. 1) or SCL-1 (Fig. 2) cells that were irradiated with IR of 1 Gy, 2 Gy or 5 Gy, no significant effect on cell viability or proliferation was found. Results were not significantly different between controls (0 Gy) and cells exposed to 5 Gy of IR. In conclusion, treatment of HaCaT-keratinocytes and squamous cell carcinoma cell lines (SCL-1) with ionizing radiation has no effect on cell proliferation and viability.

3.3. Exposure of HaCaT-keratinocytes to increasing doses of ionizing radiation (1-5 Gy) induces a dose-dependent augmentation of γH2AX-foci formation

Irradiation of HaCaT-keratinocytes with IR (1 Gy, 2 Gy, and 5 Gy) resulted in a dose-dependent and significant increase in the number of γH2AX-foci, as assessed by immunocytochemistry (Fig. 3). In vehicle (ethanol)-treated cells, the mean value of γH2AX-foci/cell increases from 1.53 (0 Gy) up to 66.25 γH2AX-foci/cell (5 Gy), which represents an augmentation of 4330%. Results are statistically significant ($p = 3.924 \times 10^{-29}$, Fig. 3).

3.4. Pretreatment of HaCaT-keratinocytes with 1,25(OH)$_2$D$_3$ (10^{-7} M) over 48 h modulates presence of γH2AX-foci

As we demonstrated in ethanol-treated controls, irradiation of HaCaT-keratinocytes with IR (1 Gy, 2 Gy, and 5 Gy) resulted in a

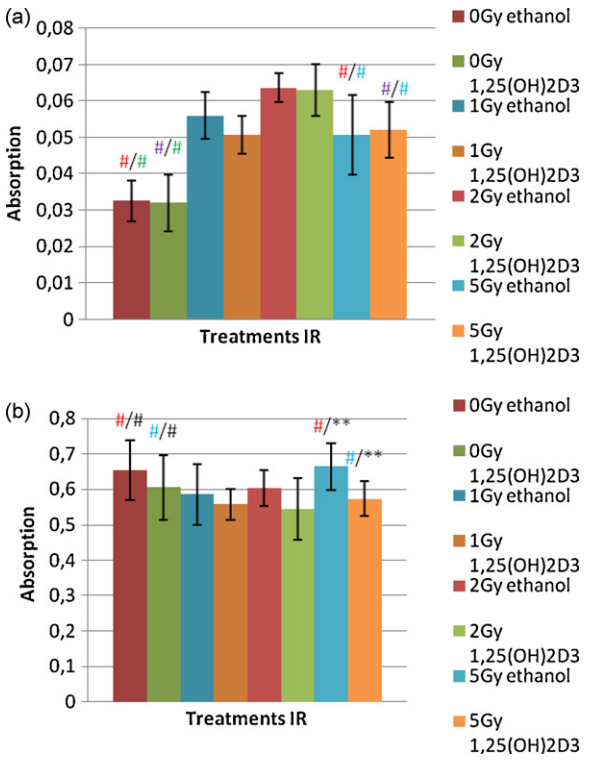

Fig. 1. Pharmacological doses of $1,25(OH)_2D_3$ inhibit the growth of HaCaT-keratinocytes as assessed by crystal violet (a) and WST-1 (b) assay. Low-dose ionizing radiation (1–5 Gy) has no significant effect on proliferation of HaCaT-keratinocytes. HaCaT-keratinocytes were pretreated over 48 h with $1,25(OH)_2D_3$ (10^{-7} M) or ethanol alone (vehicle) and then irradiated once with low-dose ionizing radiation (1–5 Gy). After irradiation, cells were provided with fresh medium. 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples [which is correlated to cell proliferation (crystal violet) and cell viability (WST-1)] was measured in an ELISA-reader. Data shown in (a) and (b) were measured after 48 h. The results show that incubation of HaCaT-keratinocytes with $1,25(OH)_2D_3$ in a concentration of 10^{-7} M results in an inhibition of cell proliferation, even if not all results are significant. After irradiation with 1–5 Gy, there is no significant reduction in proliferation or viability of HaCaT-keratinocytes. ($^*p < 0.05/^{**}p < 0.01/^{\#}p > 0.05$; symbols refer to columns that have been compared in individual analyses).

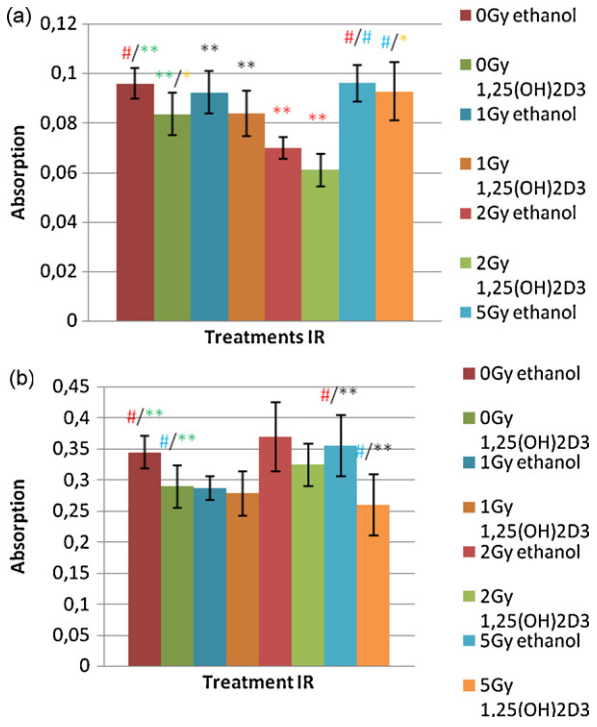

Fig. 2. Pharmacological doses of $1,25(OH)_2D_3$ inhibit the growth of squamous cell carcinoma cell lines (SCL-1) as assessed by crystal violet (a) and WST-1 (b) assay. Low-dose ionizing radiation (1–5 Gy) has no significant effect on proliferation of squamous cell carcinoma cell lines (SCL-1). SCL-1 were pretreated over 48 h with $1,25(OH)_2D_3$ (10^{-7} M) or ethanol alone (vehicle) and then irradiated once with low-dose ionizing radiation (1–5 Gy). After irradiation, cells were provided with fresh medium. 0 h, 6 h, 12 h, 24 h and 48 h after irradiation, the absorption of the samples [which is correlated to cell proliferation (crystal violet) and cell viability (WST-1)] was measured in an ELISA-reader. Data shown in (a) and (b) were measured after 48 h. The results show that incubation of SCL-1 cells with $1,25(OH)_2D_3$ in a concentration of 10^{-7} M results in an inhibition of cell proliferation. After irradiation with 1–5 Gy, there is no significant change in proliferation or viability of squamous cell carcinoma cell lines. ($^*p < 0.05/^{**}p < 0.01/^{\#}p > 0.05$; symbols refer to columns that have been compared in separate analyses).

dose-dependent and significant increase in the number of γH2AX-foci. In $1,25(OH)_2D_3$-pretreated HaCaT cells, the mean value of γH2AX-foci/cell increases from 1.14 (0 Gy) up to 83.84 γH2AX-foci/cell (5 Gy), which represents an augmentation of 7354.4%. Results are statistically significant ($p = 1.05849 \times 10^{-29}$, Fig. 4). In unirradiated controls, there was no significant difference in the number of γH2AX-foci/cell between $1,25(OH)_2D_3$-pretreated HaCaT-keratinocytes and controls that were pretreated with ethanol only (Fig. 4, $p = 0.212235888$). After irradiation with 1 Gy and 2Gy, γH2AX-foci formation in ethanol controls is significantly higher than in $1,25(OH)_2D_3$-pretreated HaCaT-keratinocytes (Figs. 4 and 5). Our results after irradiation with 5 Gy demonstrate the contrary: γH2AX-foci formation in $1,25(OH)_2D_3$-pretreated HaCaT-keratinocytes is significantly higher than γH2AX-foci formation in ethanol controls (Fig. 4). Analysis of the time course shows that the number of γH2AX-foci increases significantly during the first 6 h after irradiation. A reduction is noticed between 6 h and 12 h after irradiation (Fig. 5).

4. Discussion

Hager et al. showed in 2001 that $1,25(OH)_2D_3$ suppresses the growth of squamous cell carcinoma cell lines (larynx and tongue carcinoma) by p21- and p27-regulated cell cycle arrest in G_0/G_1 [10]. In agreement with these findings, we here demonstrate that $1,25(OH)_2D_3$ in a concentration of 10^{-7} M also inhibits proliferation and reduces viability in cutaneous squamous cell carcinoma cells. In line with the results of Hosomi et al. and Smith et al., we could furthermore confirm antiproliferative and prodifferentiating effects of $1,25(OH)_2D_3$ on HaCaT-keratinocytes [11,12]. In several recent studies it was demonstrated that $1,25(OH)_2D_3$

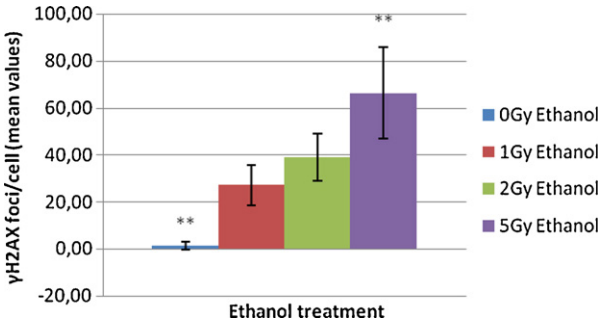

Fig. 3. Ionizing radiation leads to a dose-dependent increase in the number of γH2AX-Foci in HaCaT-keratinocytes. Cells were pretreated over 48 h with ethanol and then irradiated once with low-dose ionizing radiation (1–5 Gy). After irradiation, cells were provided with fresh medium. 30 min, 2 h, 6 h and 12 h after irradiation, γ-H2AX-foci were detected by immunofluorescence and counted semiquantitatively by two independent examiners under the fluorescence microscope. Data shown were measured after 2 h. The results show a dose-dependent augmentation in the number of γH2AX-Foci with increasing IR-intensity. ($^*p < 0.05/^{**}p < 0.01/^{\#}p > 0.05$; symbols refer to columns that have been compared in separate analyses).

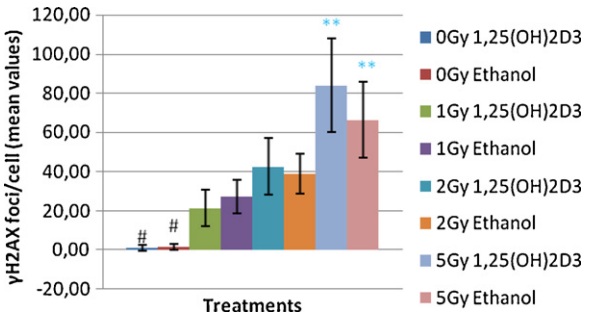

Fig. 4. 1,25-dihydroxyvitamin D_3 reduces the number of IR-induced γH2AX-Foci in HaCaT-keratinocytes. Cells were pretreated over 48 h with $1,25(OH)_2D_3$ (10^{-7} M) or ethanol alone (vehicle) and then irradiated once with low-dose ionizing radiation (1–5 Gy). After irradiation, cells were provided with fresh medium. 30 min, 2 h, 6 h and 12 h after irradiation, γ-H2AX-foci were detected by immunofluorescence and counted semiquantitatively by two independent examiners under the fluorescence microscope. Data shown were measured after 2 h. The results show a reduction in the number of IR-induced γH2AX-Foci after pretreatment with $1,25(OH)_2D_3$ (10^{-7} M). ($^*p < 0.05/^{**}p < 0.01/^{\#}p > 0.05$; symbols refer to columns that have been compared in separate analyses).

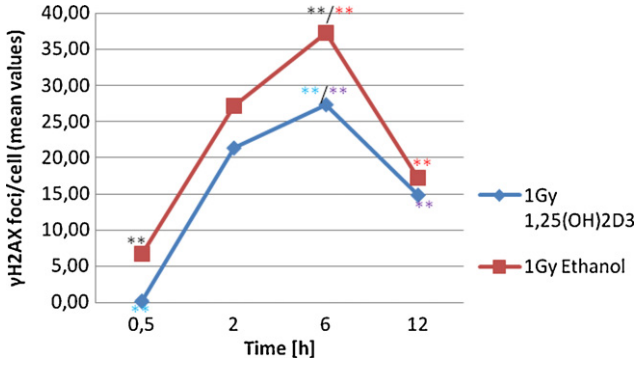

Fig. 5. 1,25-dihydroxyvitamin D_3 reduces the number of γH2AX-foci in HaCaT-keratinocytes 12 h after irradiation with low-dose IR. Cells were pretreated over 48 h with $1,25(OH)_2D_3$ (10^{-7} M) or ethanol alone (vehicle) and then irradiated once with low-dose ionizing radiation (1–5 Gy). Data shown: 1 Gy. After irradiation, cells were provided with fresh medium. 30 min, 2 h, 6 h and 12 h after irradiation, γ-H2AX-foci were detected by immunofluorescence and counted semiquantitatively by two independent examiners under the fluorescence microscope. Our results show that γ-H2AX-foci formation increases during the first 6 h after irradiation. Later, we observe a reduction in the number of γ-H2AX-foci. ($^*p < 0.05/^{**}p < 0.01/^{\#}p > 0.05$; symbols refer to columns that have been compared in separate analyses).

protects human keratinocytes against UV-B-induced cell-damage. However, effects of $1,25(OH)_2D_3$ on IR-induced DNA-damage and -repair have not been systematically analyzed until now. In our experiments, no cytotoxic effects of relatively low-dosed IR on human keratinocytes were noticed, as assessed by analysis of cell proliferation and viability at early time points (until 48 h after irradiation) using WST-1 and crystal violet assays. In contrast, we here report significant findings analysing a marker of DNA-damage (DNA double-strand breaks) after irradiation with low-dose IR: exposure of HaCaT-keratinocytes to IR (1 Gy, 2 Gy, and 5 Gy) resulted in a dose-dependent and significant increase in the number of γH2AX-foci. Next, we aimed in our experiments to investigate putative protective effects of $1,25(OH)_2D_3$ in HaCaT-keratinocytes and squamous cell carcinoma cells (SCL-1). We demonstrate that pretreatment of these cells with $1,25(OH)_2D_3$ reduces the presence of IR-induced γH2AX-foci after irradiation with 1 Gy and 2 Gy. In contrast, after irradiation of the cells with 5 Gy, γH2AX-foci formation in $1,25(OH)_2D_3$-pretreated HaCaT-keratinocytes is significantly higher than γH2AX-foci formation in ethanol-treated controls. Moreover, observation of the time course revealed that the number of γH2AX-foci increases significantly during the first 6 h after irradiation. A reduction is noticed between 6 h and 12 h after irradiation. As γ-H2AX has been shown to be involved in the recruitment and retention of signaling and repair factor complexes at the sites of DNA DSBs, this finding suggests the beginning of DNA-repair in this period of time. To put it in a nutshell, our data demonstrate that $1,25(OH)_2D_3$ differentially modulates hazardous effects of IR in cultured human keratinocytes in a dose- and time-dependent manner. The underlying molecular mechanisms that cause this differential, dose-dependent effect are unknown and deserve future analysis. It can be speculated whether this differential effect of vitamin D analogs may be used clinically on one hand to protect against relatively low IR and, on the other hand, to sensitize tumor cells against therapeutically applied higher doses of IR.

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