Article

Calcitriol and Calcidiol Can Sensitize Melanoma Cells to Low–LET Proton Beam Irradiation

Ewa Podgorska [1],[†], Agnieszka Drzal [1],[†], Zenon Matuszak [2] (iD), Jan Swakon [3], Andrzej Slominski [4] (iD), Martyna Elas [1] (iD) and Krystyna Urbanska [1],*

[1] Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7 Street, Krakow 30-387, Poland; ewa.podgorska@doctoral.uj.edu.pl (E.P.); agnieszka.drzal@doctoral.uj.edu.pl (A.D.); martyna.elas@uj.edu.pl (M.E.)
[2] Department of Medical Physics and Biophysics, Faculty of Physics and Applied Computer Science, AGH University of Science and Technology, al. A. Mickiewicza 30, Krakow 30-059, Poland; zenon.matuszak@fis.agh.edu.pl
[3] Polish Academy of Sciences, Institute of Nuclear Physics, Krakow 31-342, Poland; jan.swakon@ifj.edu.pl
[4] Department of Dermatology, Comprehensive Cancer Center Cancer Chemoprevention Program, University of Alabama at Birmingham, Birmingham, AL 35294, USA; aslominski@uabmc.edu
* Correspondence: krystyna.urbanska@uj.edu.pl; Tel.: +48-12-664-6153
† These authors contributed equally to this work.

check for
updates

Abstract: Proton beam irradiation promises therapeutic utility in the management of uveal melanoma. Calcitriol ($1,25(OH)_2D_3$)—the biologically active metabolite of vitamin D_3—and its precursor, calcidiol ($25(OH)D_3$), exert pleiotropic effects on melanoma cells. The aim of the study was to evaluate the effect of both calcitriol and calcidiol on melanoma cell proliferation and their response to proton beam irradiation. Three melanoma cell lines (human SKMEL-188 and hamster BHM Ma and BHM Ab), pre-treated with $1,25(OH)_2D_3$ or $25(OH)D_3$ at graded concentrations (0, 10, 100 nM), were irradiated with 0–5 Gy and then cultured in vitro. Growth curves were determined by counting the cell number every 24 h up to 120 h, which was used to calculate surviving fractions. The obtained survival curves were analysed using two standard models: linear-quadratic and multi-target single hit. Calcitriol inhibited human melanoma proliferation at 10 nM, while only calcidiol inhibited proliferation of hamster lines at 10 and 100 nM doses. Treatment with either $1,25(OH)_2D_3$ or $25(OH)D_3$ radio sensitized melanoma cells to low doses of proton beam radiation. The strength of the effect increased with the concentration of vitamin D_3. Our data suggest that vitamin D_3 may be an adjuvant that modifies proton beam efficiency during melanoma therapy.

Keywords: vitamin D_3; proton beam radiotherapy; melanoma; in vitro

1. Introduction

Because of their high incidence, mortality rates and resistance to the therapy, melanomas are still one the most challenging cancer types for researchers and clinicians [1,2]. Over the last decade, our understanding of the molecular principles regulating melanoma behaviour has improved significantly, leading to new therapies [3,4]. However, even with the new therapeutic approaches the problem of limited efficacy and selective responsiveness of patients still remains [5]. Therefore, new creative approaches and their combinations are required.

Accumulating evidence from a variety of epidemiological and experimental studies confirms in vitro and in vivo anticancer activity of vitamin D_3 [6–9]. Those studies indicate that biologically active vitamin D_3 derivatives may lower the incidence, and inhibit the progression, of various tumours, including melanoma [10–12] and sensitize them to radiotherapy [13–18]. Moreover, there are reports of

an inverse relationship between patient survival and melanoma thickness and $25(OH)D_3$ serum levels, polymorphisms in the genes encoding the vitamin D receptor (VDR), the vitamin D binding protein, expression of the VDR and CYP27B1 expression [19–25] and complex relations with CYP24A1 [26]. Additionally, it was proposed that VDR plays a role in the development and progression of melanocytic tumours [27].

So far, no studies have been published on a combination of vitamin D_3 and proton beam irradiation, a therapy with superior dose distribution compared to photon radiation, which is commonly used in the treatment of uveal melanoma. However, reports on the differences in the level of production of free radicals, cell cycle, cell migration inhibition and apoptotic signalling between photon and proton beam radiotherapy [28–32] suggest possible differences in the effect of their combination with vitamin D_3.

This work aims to examine the influence of calcitriol $(1,25(OH)_2D_3)$ and calcidiol $(25(OH)D_3)$ on the proliferation and response to proton beam radiotherapy of three melanoma cell lines: human SKMEL-188 and hamster BMH Ma and BHM Ab. In the light of existing evidence, we hypothesize that vitamin D_3 can sensitize melanoma cells to radiation, thus enhancing its effectiveness. Two different models, linear-quadratic and single hit multi-target, were fitted to analyse the surviving curves of the tested melanoma cell lines exposed to combined treatment and to get the most comprehensive picture of proton radiation dose response.

2. Results

2.1. Impact of Two Metabolites of Vitamin D3 on Melanoma Cell Proliferation

$1,25(OH)_2D_3$ and $25(OH)D_3$ affected the growth rate of melanoma cells in culture (Figure 1). In the case of calcitriol, only human SKMEL-188 melanoma cells showed a significant decrease in growth rate, after treatment with the concentration of 10 nM, with BHM Ma and BHM Ab hamster lines showing no major changes in the rate of proliferation. Calcidiol, on the other hand, slightly but significantly stimulated proliferation of SKMEL-188 cells at the concentration of 100 nM, with both BHM Ma and BHM Ab hamster cells showing an inhibition of proliferation at 10 and 100 nM concentrations.
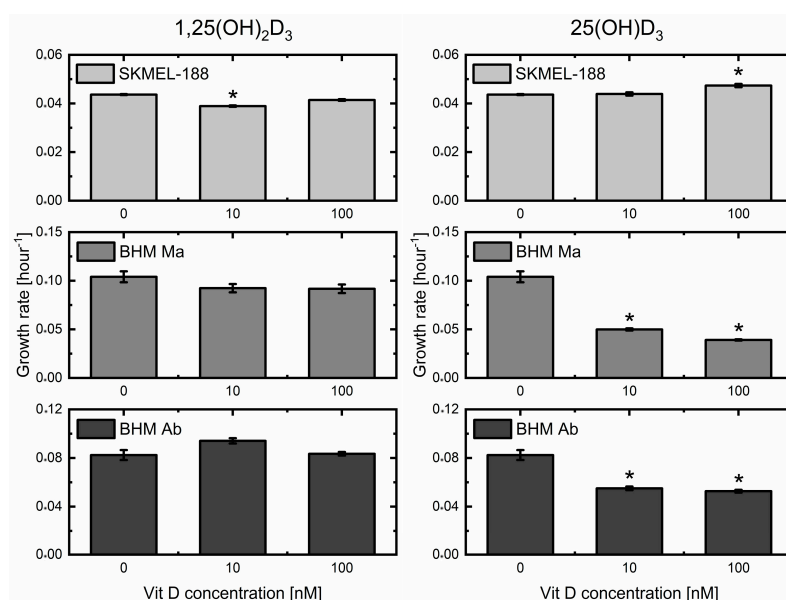

Figure 1. The impact of calcitriol $(1,25(OH)_2D_3)$ and calcidiol $(25(OH)D_3)$ on the proliferation of human SKMel-188 and hamster BHM Ma and BHM Ab melanoma cells. Growth rate was calculated from exponential fitting to cell number, counted every 24 h for 4 days in culture. * denotes statistical significance $p < 0.05$ against control (0 nM of vitamin D_3 derivative).

There were also significant differences in basal growth rates between the tested melanoma cell lines. Both hamster lines showed similar proliferation rates (approximately 0.09 h^{-1}), while the human SKMEL-188 cells divided almost two times slower. Such differences may be one of the factors influencing response to the vitamin D$_3$ metabolites.

2.2. Proton Beam Radiosensitivity of Melanoma Cells

Melanoma cell lines are characterized by different radiosensitivity to proton beam therapy (Figure 2), with the highest level of cell killing seen in BHM amelanotic Ab cells. BHM melanotic Ma and SKMEL-188 cells show similar survival curves. Similar differences in response to radiation between BHM Ma and BHM Ab cells were reported for X ray irradiation, where pigmented cells (BHM Ma) were 2.4 times more radio resistant than the unpigmented (BHM Ab) ones [33]. In the case of proton radiation this characteristic is less pronounced but still marked with the ratio of mean lethal dose of 1.56.
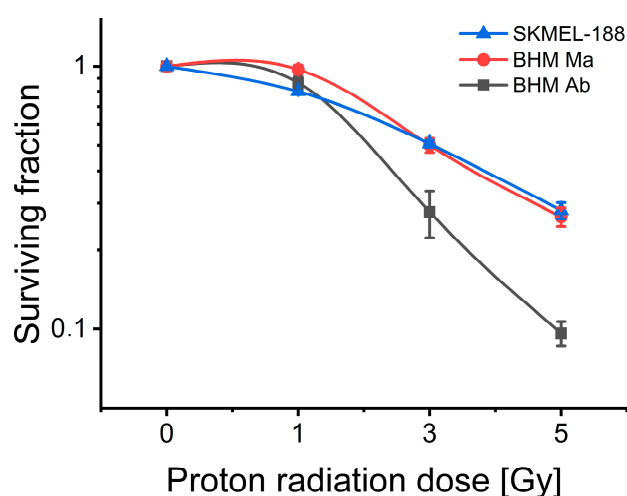

Figure 2. Proton beam radiosensitivity of SKMel-188, BHM Ma and BHM Ab melanoma cells. Surviving fraction was determined from the model described by Buch et al. [34], based on the experimental data from cell number, counted every 24 h for 4 days in culture after irradiation.

2.3. Vitamin D$_3$ Derivatives Influence Proton Beam Radiosensitivity of Melanoma Cells

Changes in both surviving fraction and survival curve shape can be distinguished after pre-treatment with both vitamin D$_3$ metabolites (Figure 3). The most significant effects are seen for both vitamin D$_3$ derivatives in hamster melanoma lines, with a weaker effect in the human line. Furthermore, higher doses of 25(OH)D$_3$ cause in BHM Ma cells a flattening of the survival curve in the region of higher doses (3–5 Gy). Those changes are consistent with the data from fitted survival models. It has been shown that α and β parameters from the linear-quadratic model (LQ model) determine the effectiveness at low and high radiation doses, respectively [35]. Vitamin D$_3$ dose dependent decrease in surviving fraction for proton irradiation dose of 1 Gy, visible on the survival curve, is represented by an increase in the values of parameter α (Figure 4, upper panel). For calcitriol, no significant change in α was detected at 10 nM dose for SKMEL-188 and BHM Ma cells. On survival curves, that concentration was slightly protective for cancer cells, with surviving fraction higher than the control one but the effect was not statistically significant. The most effective dose was 100 nM. Calcidiol has a clear dose dependent effect on the radiosensitivity of the studied melanoma cell lines, with the highest concentration being the most effective one. An increase in parameter β, indicating higher effectiveness of high doses of radiation, occurs only for calcitriol pre-treatment: 10 nM dose in BHM Ma and BHM Ab cells (Figure 4, lower panel). The same dose leads to a noticeable decrease in surviving fraction on survival curves. The tested doses were within the range of the normal serum level of calcitriol, which is between 50–120 nM.
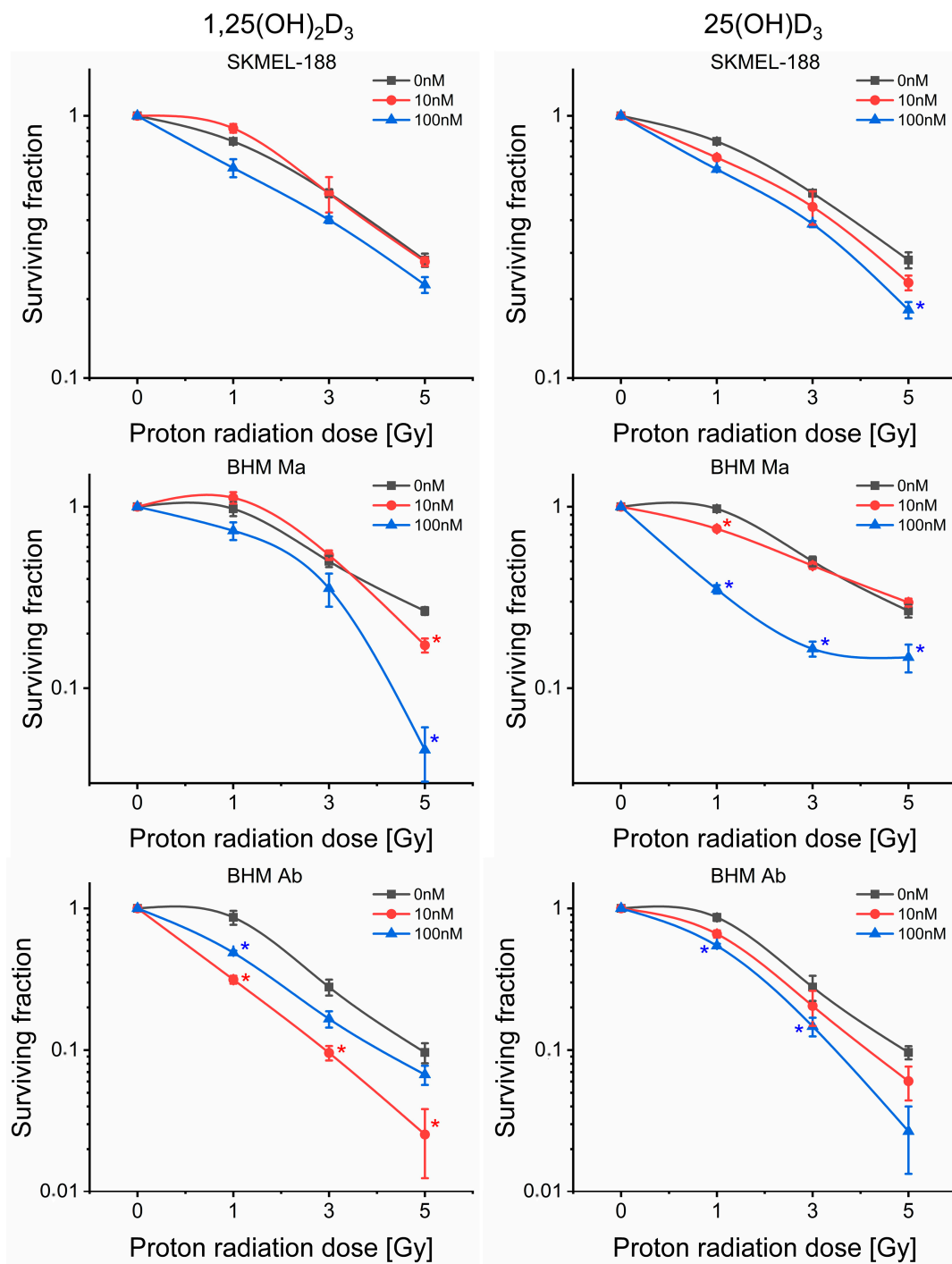
Figure 3. The effect of calcitriol (1,25(OH)$_2$D$_3$) and calcidiol (25(OH)D$_3$) on the cellular response to proton beam irradiation of human SKMel-188 and hamster BHM Ma and BHM Ab melanoma cells. Surviving fraction was determined from the model described by Buch et al. [34], based on the experimental data from cell number, counted every 24 h for 4 days in culture after irradiation. * denotes statistical significance *p* < 0.05 against irradiated control.
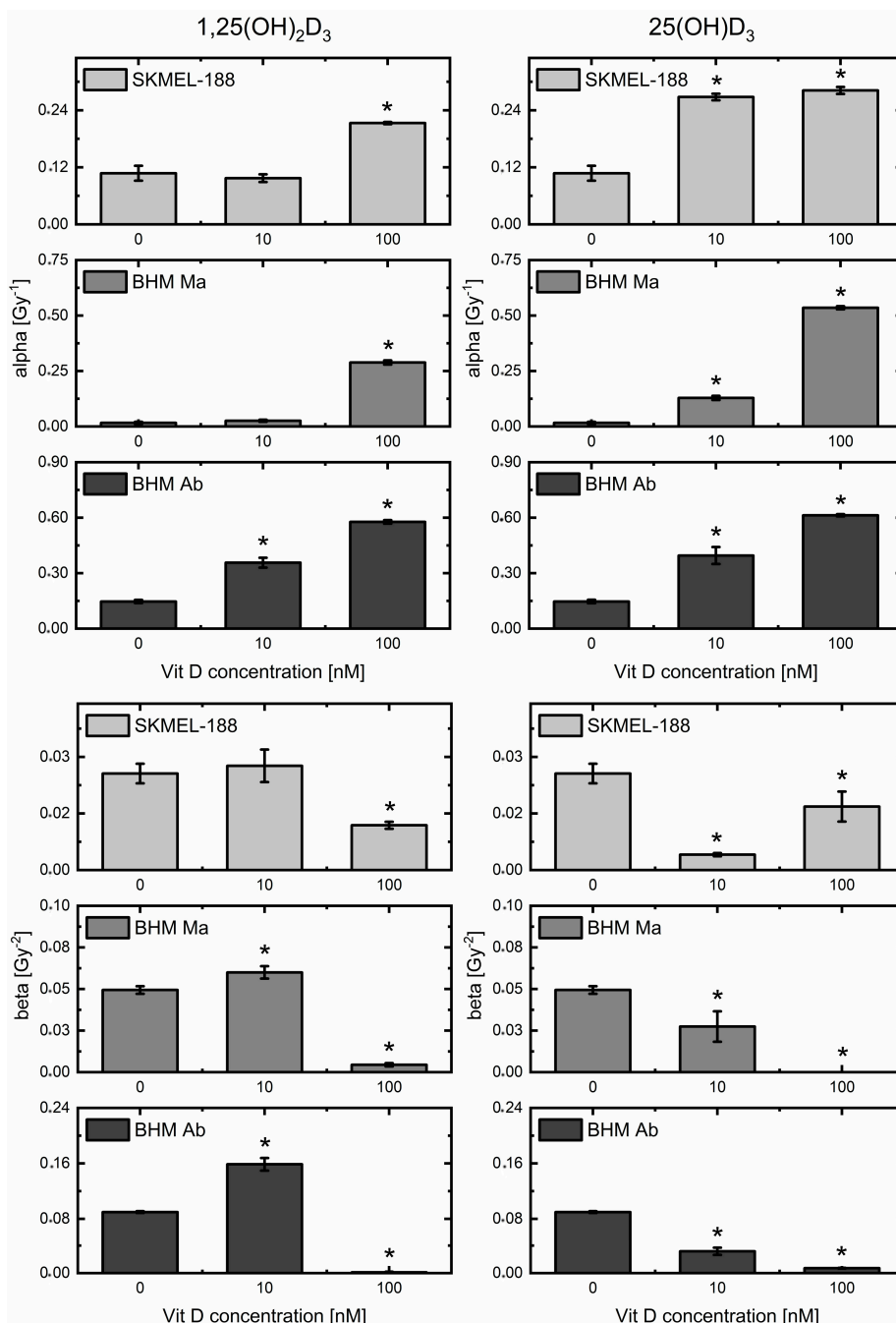
Figure 4. Coefficients α (**upper** panel) and β (**lower** panel) from linear-quadratic model calculated for cells pre-treated with calcitriol (1,25(OH)$_2$D$_3$) and calcidiol (25(OH)D$_3$) and irradiated with proton beam. Surviving fraction was determined from the model described by Buch et al. [34], based on the experimental data from SKMel-188, BHM Ma and BHM Ab cell number, counted every 24 h for 4 days in culture after irradiation. * denotes statistical significance $p < 0.05$ against control (0 nM of vitamin D$_3$ derivative).

Results from the second model applied to survival data, single hit multi-target, are in agreement with data from the LQ model. Changes in the calculated parameters n and D$_0$ are shown in Figure 5. Parameter n, indicating the required number of hits for cell death and being sensitive to the effectiveness of low radiation doses, shows an opposite trend to parameter α from the LQ model. An increase in α goes together with a decrease in n, which is consistent with vitamin D$_3$ metabolites radiosensitizing cells to low doses of proton beam irradiation. The second parameter, mean lethal dose D$_0$, describes

the average effect of radiation, without distinction between low and high doses [36]. Only several groups were characterized by a decrease in that parameter in comparison to the control, representing the process of radiosensitization by the tested vitamin D_3 analogues. Among them, there were groups treated with 100 nM of calcitriol, the most effective treatment in enhancing the efficiency of low radiation doses. This indicates that the highest influence of vitamin D_3 analogues on the averaged effects relates to the response to low doses of proton radiation.
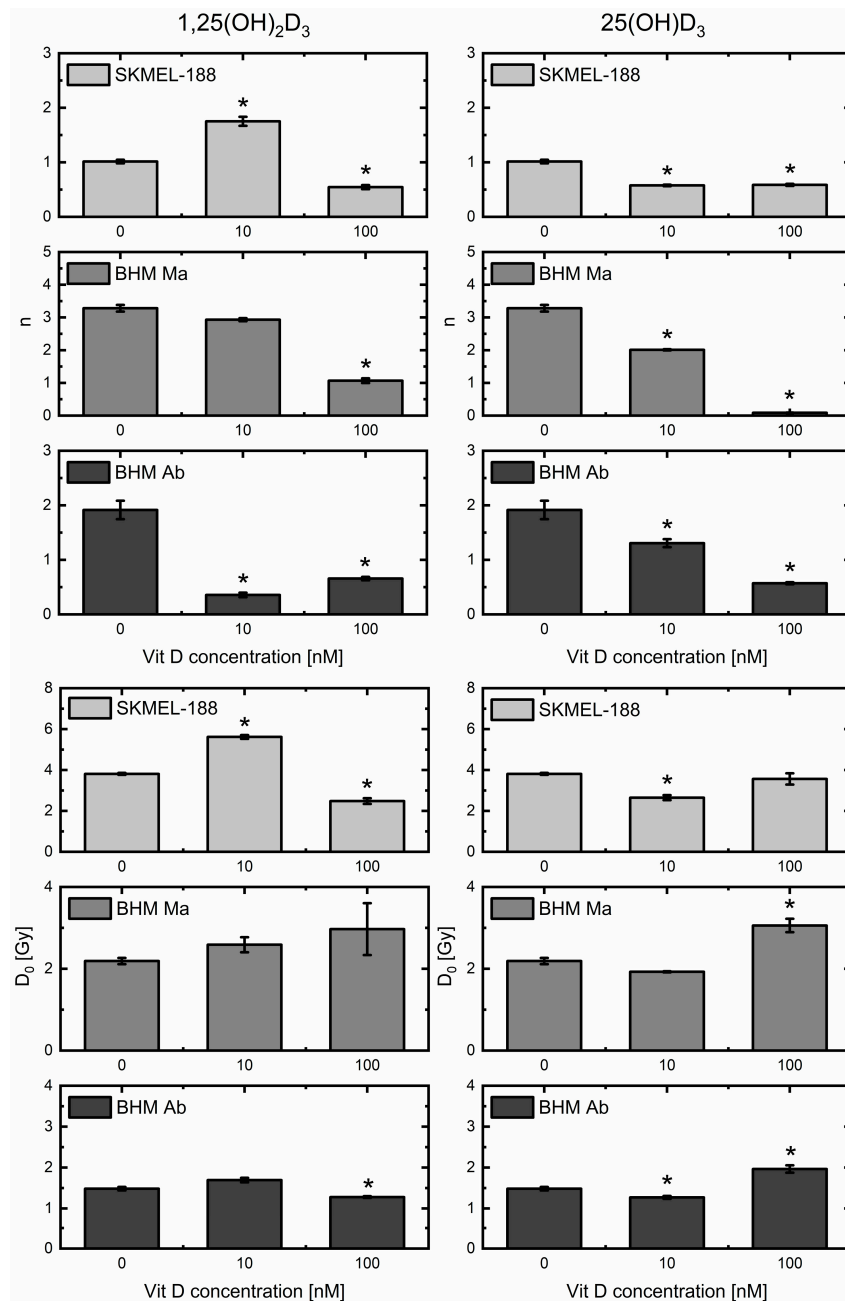

Figure 5. Coefficients *n* (**upper** panel) and D_0 (**lower** panel) from multi-target single hit model calculated for cells pre-treated with calcitriol ($1,25(OH)_2D_3$) and calcidiol ($25(OH)D_3$) and irradiated with proton beam. Surviving fraction was determined from the model described by Buch et al. [34], based on the experimental data from SKMel-188, BHM Ma and BHM Ab cell number, counted every 24 h for 4 days in culture after irradiation. * denotes statistical significance $p < 0.05$ against control (0 nM of vitamin D_3 derivative).

3. Discussion

Current pre-clinical and clinical reports show that vitamin D_3 has overall anticancer properties [6–8]. The finding is also supported by a strong correlation between higher serum 25(OH)D_3 level and lower incidence of breast [37,38], colon [39], lung [40], prostate [41] and melanoma [10] and by the antiproliferative and pro-differentiative action of vitamin D_3 and its analogues towards multiple cancer cell lines [42–47]. Interestingly, not all cancer cell lines do respond to vitamin D_3 [20,48–51], which also included melanoma lines [41,50] and such responsiveness can depend on the culture conditions [11].

In the present study, we demonstrate differential effects of the biologically active form of vitamin D_3, calcitriol and its precursor calcidiol, on the proliferation rate of three melanoma cell lines: human SKMEL-188 and hamster BHM Ma and BHM Ab (Figure 1). Only SKMEL-188 cells responded to lower doses of 1,25(OH)$_2D_3$ with inhibition of growth rate, which was consistent with other reports [22,52]. However, inhibition of proliferation by calcidiol, with no effect in the case of calcitriol in both BHM Ma and BHM Ab cells requires further explanation. It has been proposed that one of the factors influencing the responsiveness of melanoma cell lines to vitamin D_3 is melanin pigmentation [53,54], which would explain the decrease in VDR expression with concomitant decrease in anti-proliferative response to 1,25(OH)$_2D_3$ in human melanoma. It is possible that a similar mechanism could underlie the resistance of BHM Ma, which produces melanin pigment also when cultured in a medium with low tyrosine content [55]. However, since BHM Ab cells were pre-selected for amelanotic phenotype by culturing in DMEM, other factors, including a defect in VDR or a high activity of CYP24A1 that inactivates 1,25(OH)$_2D_3$ could be responsible for the differential effects. It must also be noted that there are alternative receptors for vitamin D_3 derivatives that could affect responsiveness to active forms of vitamin D_3 (reviewed in [9,10]). Therefore, proper understanding of these differential effects would require additional studies, involving the identification and cloning of hamster nuclear receptors for vitamin D_3.

Vitamin D_3 enhances the of anticancer drugs such as doxorubicin, cisplatin, gemcitabine or cyclophosphamide [56–58] and sensitizes cancer cells to ionizing radiation [12–18]. In this paper, we focus on combining calcitriol or calcidiol with proton beam therapy. That mode of cancer therapy is commonly used for the treatment of tumours located near or on critical organs, because of its dosimetric benefits. Thanks to the occurrence of a Bragg peak, there is no exit dose normally deposited by photon therapy within the healthy tissue surrounding the tumour [59]. Accumulating evidence suggests that even with relative biological effectiveness of 1.1, there are significant differences in biological effects on tumour cells between low-energy protons and photon radiation [60]. As regards the combination of proton beam radiotherapy and vitamin D_3 treatment, the most crucial aspects appear to be the increased production of reactive oxygen species (ROS) and the differences in cell cycle inhibition and apoptotic signalling, all implicated in the response to vitamin D_3.

Overall, our results show that vitamin D_3 is a potential radiosensitizing agent in proton beam irradiation of melanoma cells. Radiosensitization is a complex concept that has many different interpretations and is used to describe a variety of interactions at biochemical and biological level. Alterations in radiosensitization are demonstrated in the SF curve; a downward or leftward shift of the curve implies a radiosensitizing interaction, while an upward or rightward shift implies a radioprotective influence on the treated cells.

Generally, radiosensitization is conventionally defined as an increased amount of radiation-induced cell death resulting from exposure to a second agent, after correction for the cytotoxicity of the agent (in our case vitamin D_3, nontoxic in the tested concentrations). Considering the changes in shape and the above-mentioned shift in SF suggests that both calcitriol and calcidiol are radiosensitizing agents, within the range of the concentrations used. Radiosensitization by vitamin D_3 is particularly visible for low radiation doses, for which we a decrease or elimination of the shoulder on the SF curve is observed (Figure 3). The effect of radiosensitization is also visible at high radiation doses of about 5 Gy. The surviving fraction values of the cells are lower than the analogous values for SF curve obtained for the exposure without vitamin D_3. Some of the observed survival-dose dependencies, in particular for

BHM Ma cells pre-treated with 100 nM of calcidiol, do not resemble the commonly found SF curves with the shoulder in the low dose area and with a linear course for zero for high doses. In this case, we observe a gradual disappearance of the effect the radiation dose has on the number of killed cells. The SF curve gradually flattens (upward-bending) and the number of surviving cells does not decrease with increasing dose. The effect is the reason for the unusual values of some coefficients characterizing survival curves of such non-classical forms of SF, for example, close to zero β values (see Figure 4). The phenomenon (upward-bending of SF) has already been observed during the He-3 irradiation of the V79 cells but the authors interpreted it as an artefact [61]. More recent theoretical studies linked that effect mainly with the repair processes in irradiated cells [62,63]. Nevertheless, the theory is formal and does not explain the upward-bending effect of SF curves with the help of probable molecular mechanisms involved in the repair of irradiated cells. In our work, the upward-bending effect was only observed in the presence of vitamin D_3 derivatives. Therefore, it might be due to the radioprotective properties of vitamin D_3. Probable targets of such interaction include ATM and mTor, which are directly related to the process of DNA repair and cell proliferation [64–66]. Thus, the radiosensitization effect of high vitamin D_3 doses would be classified as radiosensitization by targeting the response to DNA damage.

In conclusion, our data suggest that active forms of vitamin D may improve the effectiveness of proton therapy. They also support further in vivo studies on use of vitamin D as adjuvant during radiotherapy of melanoma.

4. Materials and Methods

4.1. Cell Lines

Human SKMEL-188 melanoma cell line, a gift from Dr Chakraborty, Yale University, was established from a human metastatic melanoma and then maintained in our laboratories as a continuous cell line [67,68].

BHM Ma, Bomirski Hamster Melanoma—pigmented subline, is a stable transplantable tumour cell line that was derived by Dr Andrzej Bomirski in Gdansk, Poland, from a spontaneous hamster melanoma in 1959 [69].

BHM Ab, Bomirski Hamster Melanoma—non-pigmented subline, arose in 1963 by spontaneous alteration of a black tumour (BHM Ma). The amelanotic subline of Bomirski Hamster Melanoma results in non-pigmented tumours in hamsters. The tumours are malignant, dedifferentiated, fast-growing and metastasizing [69]. The cells isolated from those tumours by means of a non-enzymatic method undergo rapid pigmentation in primary cultures in media containing high concentrations of L-tyrosine [70,71]. The level of pigmentation depends on the culture medium; in DMEM with 10% of foetal bovine serum (FBS) the cells are pigmented, while in media low in L-tyrosine, such as Ham's F10, they are amelanotic [72]. However, culturing for prolonged period in DMEM leads to the selection for an amelanotic phenotype that is stable in cell culture [73]. This type of preselected cells was used in the experiments.

4.2. Cell Culture

For each cell line, a different culture medium was used: for BHM Ma—RPMI, for BHM Ab—DMEM and for SKMEL-188 Ham's F10 medium supplemented with glucose, L-glutamine and pyridoxine hydrochloride. The cells were cultured in 75 cm^2 flasks in culture medium supplemented with 10% of FBS (Gibco—Thermofisher Scientific, Waltham, MA, USA) and antibiotics (Sigma, St. Luis, MO, USA) and grown until 70% confluent. The calcitriol or calcidiol (Sigma, St. Louis, MO, USA) was dissolved in 100% ethanol to obtain 100 µM stocks. After that stocks were diluted in media to obtain 10 and 100 nM. Concentration of ethanol in media never exceeded 1%. The cells were incubated with vitamin D compounds for 24 h before irradiation. To study the influence of calcitriol or calcidiol on cell proliferation, cell growth curves were determined by manual counting the cells growing with vitamin D_3 metabolites at different concentrations every 24 h up to 120 h. Counts were performed

with the use of haemocytometer, by triplicate by one analyst under a 40× objective according to the standard methodology. Influence on cell response to proton radiation was tested by cultivating cells for 24 h prior to irradiation in a culture medium with 10% FBS, antibiotics and vitamin D_3 metabolites as indicated.

4.3. Irradiation Procedure

The irradiation was performed at the Institute of Nuclear Physics of the Polish Academy of Sciences (IFJ PAN) in Krakow uses he Proteus C-235 cyclotron produced proton beam. The 230 MeV proton beam, after degradation to 70 MeV, was delivered to the treatment room with a small field horizontal beam line. The passive scattering technique and rotating energy modulator were used to forming the irradiation field. A 40 mm diameter, fully modulated proton beam with energy of 61 MeV, spread-out Bragg Peak (SOBP) with 31.5 mm range and 31.5 mm modulation (measured in water), was used for cell irradiation. Dose of 1, 3, or 5 Gy at dose rates of 1 Gy/min, 2 Gy/min and 6.6 Gy/min respectively, were delivered to the samples. The dose averaged LET_d calculated at the depth of 15.8 mm in the SOBP that is, at the centre of the cell container position, was 2.8 keV/μm. The dosimetry was performed according to the recommendations of IAEA TRS-398 protocol [74]. A semiflex ionisation chamber with 0.125 cm^3 active volume and a PTW reference class UNIDOS Webline electrometer calibrated at the IFJ PAN with ^{60}Co radiation source were used. A dedicated PMMA phantom with a holder for the Eppendorf container was used during irradiation. Cells were irradiated in Eppendorf tubes positioned in the phantom, orthogonally to the direction of the proton beam. Cell suspension in phosphate buffer saline (PBS) at 1 × 106 cells/mL was transported on ice between the facilities, including the untreated (non-irradiated) control. Cells were transferred to the culture medium and placed at 1 × 105 cell/mL in 24-well plates. Every 24 h, for 5 days, cells from 6 wells were removed and counted. The experiment was repeated 3 times for each cell line tested.

4.4. Data Analysis

Data from cell counting was used to determine cell growth rates, doubling times and surviving fractions. Since the cells in most of the experimental groups exhibited logistic growth, the first step was to establish the duration of exponential growth phase (96 h). Then, growth rates (*gr*):

$$N_t = N_0 e^{gr \cdot t} \tag{1}$$

and doubling times ($t_{doubling\ time}$):

$$t_{doubling\ time} = \frac{\ln(2)}{gr} \tag{2}$$

where calculated by exponential function fitting, using the nonlinear least squares method. Additionally, time delay for irradiated groups was calculated as previously described [34]. Those values were used to calculate surviving fractions:

$$SF = 2^{-\frac{t_{delay}}{t_{doubling\ time}}} \tag{3}$$

Establishing the relationship between the physical conditions of irradiation and its biological effects is a starting point for any radiobiological experiment. Such relations are known as survival curves and presented as the dependence of cell survival probability (Surviving Fractions—SF) on the absorbed radiation dose (in Gy). Many theoretical models of ionizing radiation–induced cell killing have been proposed and described in literature but because the fundamental mechanisms leading to lethal cell damage are not well understood, the models have semi-empirical character and many limitations [36]. Our experimental survival curves were analysed within framework of two classical models describing the response of cells populations to ionizing radiation (IR). The models have a few basic assumptions in common: cell inactivation is treated as a multistep process, cells are killed by energy absorption deposited in a sensitive volume of the cell and radiation-induced lethal events

have Poisson distribution. The first one is a "molecular model," widely known as the linear-quadratic model (LQ):

$$SF(D) = e^{-(\alpha D + \beta D^2)} \tag{4}$$

describing the logarithmic plot of SF in linear and quadratic dose–dependent terms, where D is the absorbed dose. The experimentally determined parameters α and β are interpreted as rates of cell annihilation by a single-hit and double-hit mechanism, respectively. Molecular interpretation of LQ model is based on the following assumptions: DNA is a critical target, radiation produces the breakage in DNA strands, the broken bonds in DNA strand can be repaired and the critical damage leading to cell death is a double strand break (DSB), resulting in non-repairable lesions. The linear part (α) represents a lethal lesion produced by a direct induction of DSB (one track hit) and the quadratic term (β) is a result of two single strand breaks (SSB) which could be repaired (a sub-lethal lesion).

The second model used for analysis of our survival curves is a multitarget–single hit (MTSH) model:

$$SF(D) = 1 - \left(1 - e^{-\frac{D}{D_0}}\right)^n \tag{5}$$

$D_0 = 1/k$ is the dose for $1/e$ survival in the linear portion of the plot, where k—inactivation constant for each target (or mean lethal dose), n—target multiplicity, number of targets per cell that must be inactivated for cell death.

Radiation survival curves are presented as log-linear plots of surviving fraction and dose, respectively; surviving fraction is plotted along the vertical axis (normalized to unity when $D = 0$), radiation dose is plotted along the horizontal axis.

Both models were fitted to the dose dependence of survival fraction, using the nonlinear least squares method. All analyses were carried out with in-house written Matlab (2014b, MathWorks, Natick, MA, USA) scripts. Optimization of the fitting procedures (both for growth and survival curves) was conducted until mean R-squared from all curves was higher than 0.9. Besides parameters values, their standard errors were calculated and used for the calculation of weighted mean and its error within experimental groups.

4.5. Statistical Analysis

Results were presented as weighted mean and weighted mean error. All analyses were performed with STATISTICA 13 software (Stat-Soft Inc., Tulsa, OK, USA), with the use of weights computed during the fitting procedure. For each parameter, Shapiro-Wilk normality test and Levene's test were used to determine a normal distribution and equality of variances, respectively. Depending on the data, a one-way ANOVA, followed by post-hoc Tukey's HSD test or Kruskal–Wallis H test, followed by Dunn's test was performed. p values smaller than 0.05 were considered statistically significant.

5. Conclusions

We conclude that the effect of vitamin D_3 metabolites on the proliferation and response to proton radiation in the studied melanoma cell lines is not straightforward. As regards cell proliferation, the impact of melanin content and other factors such as VDR, CYP27A1, CYP27B1 and CYP24A1 gene expressions as well as VDR localization and interaction with other receptors should be considered in the differential responses of cells to calcitriol and calcidiol. The studied vitamin D_3 derivatives have proven to be potent radiosensitizers in proton therapy. Radiosensitization by vitamin D_3 was particularly visible for low radiation doses. Interestingly, for higher doses the observed upward-bending of SF curves in the presence of vitamin D_3 implies involvement of repair mechanisms and interaction between VDR and radiation-induced signalling pathways. Our results indicate the need for a further investigation of the underlying molecular mechanisms and suggest that vitamin D_3 can be a promising agent, capable of modifying proton beam therapy efficacy and thus offering a new option in cancer therapy.

Abbreviations

ATM	Ataxia-telangiectasia mutated kinase
BHM	Bomirski hamster melanoma
CSDA	Continues slowing down approximation
DSB	Double strand break
IR	Ionizing radiation
LQ	Linear-quadratic
mTor	Mammalian target of rapamycin
MTSH	Multitarget—single hit
SF	Surviving fraction
SOBP	Spread-out bragg peak
SSB	Single strand break
VDR	Vitamin D receptor

References

1. Matthews, N.H.; Li, W.-Q.; Qureshi, A.A.; Weinstock, M.A.; Cho, E. Epidemiology of Melanoma. In *Cutaneous Melanoma: Etiology and Therapy*; Codon Publications: Brisbane, Australia, 2017.

2. Slominski, A.T.; Carlson, J.A. Melanoma resistance: A bright future for academicians and a challenge for patient advocates. *Mayo Clin. Proc.* **2014**, *89*, 429–433. [CrossRef] [PubMed]

3. Rajkumar, S.; Watson, I.R. Molecular characterisation of cutaneous melanoma: Creating a framework for targeted and immune therapies. *Br. J. Cancer* **2016**, *115*, 145–155. [CrossRef] [PubMed]

4. Su, M.Y.; Fisher, D.E. Immunotherapy in the Precision Medicine Era: Melanoma and Beyond. *PLoS Med.* **2016**, *13*, e1002196. [CrossRef] [PubMed]

5. Levesque, M.P.; Cheng, P.F.; Raaijmakers, M.I.G.; Saltari, A.; Dummer, R. Metastatic melanoma moves on: Translational science in the era of personalized medicine. *Cancer Metastasis Rev.* **2017**, *36*, 7–21. [CrossRef] [PubMed]

6. Ordonez-Moran, P.; Larriba, M.J.; Pendas-Franco, N.; Aguilera, O.; Gonzalez-Sancho, J.M.; Munoz, A. Vitamin D and cancer: An update of in vitro and in vivo data. *Front. Biosci.* **2005**, *10*, 2723–2749. [CrossRef] [PubMed]

7. Feldman, D.; Krishnan, A.V.; Swami, S.; Giovannucci, E.; Feldman, B.J. The role of vitamin D in reducing cancer risk and progression. *Nat. Rev. Cancer* **2014**, *14*, 342–357. [CrossRef] [PubMed]

8. Grant, W.B. Roles of Solar UVB and Vitamin D in Reducing Cancer Risk and Increasing Survival. *Anticancer Res.* **2016**, *36*, 1357–1370. [PubMed]

9. Slominski, A.T.; Brozyna, A.A.; Zmijewski, M.A.; Józwicki, W.; Jetten, A.M.; Mason, R.S.; Tuckey, R.C.; Elmets, C.A. Vitamin D signalling and melanoma: Role of Vitamin D and its receptors in melanoma progression and management. *Lab. Investig.* **2017**, *97*, 706–724. [CrossRef] [PubMed]

10. Slominski, A.T.; Brożyna, A.A.; Skobowiat, C.; Zmijewski, M.A.; Kim, T.K.; Janjetovic, Z.; Oak, A.S.; Jozwicki, W.; Jetten, A.M.; Mason, R.S.; et al. On the role of classical and novel forms of vitamin D in melanoma progression and management. *J. Steroid Biochem. Mol. Biol.* **2018**, *177*, 159–170. [CrossRef] [PubMed]

11. Wasiewicz, T.; Szyszka, P.; Cichorek, M.; Janjetovic, Z.; Tuckey, R.C.; Slominski, A.T.; Zmijewski, M.A. Antitumor effects of vitamin D analogues on hamster and mouse melanoma cell lines in relation to melanin pigmentation. *Int. J. Mol. Sci.* **2015**, *16*, 6645–6667. [CrossRef] [PubMed]

12. Polar, M.K.; Gennings, C.; Park, M.; Gupta, M.S.; Gewirtz, D.A. Effect of the vitamin D_3 analog ILX 23-7553 on apoptosis and sensitivity to fractionated radiation in breast tumour cells and normal human fibroblasts. *Cancer Chemother. Pharmacol.* **2003**, *51*, 415–421. [CrossRef] [PubMed]

13. Sundaram, S.; Sea, A.; Feldman, S.; Strawbridge, R.; Hoopes, P.J.; Demidenko, E.; Binderup, L.; Gewirtz, D.A. The combination of a potent vitamin D_3 analog, EB 1089, with ionizing radiation reduces tumour growth and induces apoptosis of MCF-7 breast tumour xenografts in nude mice. *Clin. Cancer Res.* **2003**, *9*, 2350–2356. [PubMed]

14. Wang, Z.; Liu, J.; Si, S.; Xu, B.; Wang, Y.; Ren, Y.; Dai, Z.; Cui, D.; Su, X.; Song, S. Vitamin D improves the sensitization of lung cancer to radiotherapy. *Int. J. Clin. Exp. Med.* **2017**, *10*, 4921–4928.

15. Sundaram, S.; Gewirtz, D.A. The vitamin D_3 analog EB 1089 enhances the response of human breast tumor cells to radiation. *Radiat. Res.* **1999**, *152*, 479–486. [CrossRef] [PubMed]

16. Chaudhary, M.; Sundaram, S.; Gennings, C.; Carter, H.; Gewirtz, D.A. The vitamin D_3 analog, ILX-23-7553, enhances the response to Adriamycin and irradiation in MCF-7 breast tumor cells. *Cancer Chemother. Pharmacol.* **2001**, *47*, 429–436. [CrossRef]

17. Weitsman, G.E.; Koren, R.; Zuck, E.; Rotem, C.; Liberman, U.A.; Ravid, A. Vitamin D sensitizes breast cancer cells to the action of H_2O_2: Mitochondria as a convergence point in the death pathway. *Free Radic. Biol. Med.* **2005**, *39*, 266–278. [CrossRef] [PubMed]

18. Dunlap, N.; Schwartz, G.G.; Eads, D.; Cramer, S.D.; Sherk, A.B.; John, V.; Koumenis, C. 1α,25-Dihydroxyvitamin D_3 (calcitriol) and its analogue, 19-nor-1α,25(OH)$_2$D$_2$, potentiate the effects of ionising radiation on human prostate cancer cells. *Br. J. Cancer* **2003**, *89*, 746–753. [CrossRef] [PubMed]

19. Slominski, A.T.; Brozyna, A.; Jozwicki, W.; Tuckey, R.C. Vitamin D as an adjuvant in melanoma therapy. *Melanoma Manag.* **2015**, *2*, 1–4. [CrossRef] [PubMed]

20. Wu, W.; Beilhartz, G.; Roy, Y.; Richard, C.L.; Curtin, M.; Brown, L.; Cadieux, D.; Coppolino, M.; Farach-Carson, M.C.; Nemere, I.; et al. Nuclear translocation of the 1,25D$_3$-MARRS (membrane associated rapid response to steroids) receptor protein and NFκB in differentiating NB4 leukemia cells. *Exp. Cell Res.* **2010**, *316*, 1101–1108. [CrossRef] [PubMed]

21. Tan, C.; De Noronha, R.G.; Roecker, A.J.; Pyrzynska, B.; Khwaja, F.; Zhang, Z.; Zhang, H.; Teng, Q.; Nicholson, A.C.; Giannakakou, P.; et al. Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. *Cancer Res.* **2005**, *65*, 605–612. [CrossRef] [PubMed]

22. Skobowiat, C.; Oak, A.S.W.; Kim, T.-K.; Yang, C.H.; Pfeffer, L.M.; Tuckey, R.C.; Slominski, A.T. Noncalcemic 20-hydroxyvitamin D_3 inhibits human melanoma growth in in vitro and in vivo models. *Oncotarget* **2016**, *8*, 9823–9834. [CrossRef] [PubMed]

23. Brozyna, A.A.; Jozwicki, W.; Janjetovic, Z.; Slominski, A.T. Expression of vitamin D receptor decreases during progression of pigmented skin lesions. *Hum. Pathol.* **2011**, *42*, 618–631. [CrossRef] [PubMed]

24. Brozyna, A.A.; Jóźwicki, W.; Janjetovic, Z.; Slominski, A.T. Expression of the vitamin D-activating enzyme 1α-hydroxylase (CYP27B1) decreases during melanoma progression. *Hum. Pathol.* **2013**, *44*, 374–387. [CrossRef] [PubMed]

25. Brozyna, A.A.; Jóźwicki, W.; Slominski, A.T. Decreased VDR expression in cutaneous melanomas as marker of tumor progression: New data and analyses. *Anticancer Res.* **2014**, *34*, 2735–2744. [PubMed]

26. Brożyna, A.A.; Jochymski, C.; Janjetovic, Z.; Jóźwicki, W.; Tuckey, R.C.; Slominski, A.T. CYP24A1 expression inversely correlates with melanoma progression: Clinic-pathological studies. *Int. J. Mol. Sci.* **2014**, *15*, 19000–19017. [CrossRef] [PubMed]

27. Chagani, S.; Kyryachenko, S.; Yamamoto, Y.; Kato, S.; Ganguli-Indra, G.; Indra, A.K. In vivo Role of Vitamin D Receptor Signaling in UVB-Induced DNA Damage and Melanocyte Homeostasis. *J. Investig. Dermatol.* **2016**, *136*, 2108–2111. [CrossRef] [PubMed]

28. Girdhani, S.; Sachs, R.; Hlatky, L. Biological Effects of Proton Radiation: What We Know and Don't Know. *Radiat. Res.* **2013**, *179*, 257–272. [CrossRef] [PubMed]

29. Gerelchuluun, A.; Hong, Z.; Sun, L.; Suzuki, K.; Terunuma, T.; Yasuoka, K.; Sakae, T.; Moritake, T.; Tsuboi, K. Induction of in situ DNA double-strand breaks and apoptosis by 200 MeV protons and 10 MV X-rays in human tumour cell lines. *Int. J. Radiat. Biol.* **2011**, *87*, 57–70. [CrossRef] [PubMed]

30. Jasińska-Konior, K.; Pochylczuk, K.; Czajka, E.; Michalik, M.; Romanowska-Dixon, B.B.; Swakoń, J.; Urbańska, K.; Elas, M.; Jasinska-Konior, K.; Pochylczuk, K.; et al. Proton beam irradiation inhibits the migration of melanoma cells. *PLoS ONE* **2017**, *12*, e0186002. [CrossRef] [PubMed]

31. Grosse, N.; Fontana, A.O.; Hug, E.B.; Lomax, A.; Coray, A.; Augsburger, M.; Paganetti, H.; Sartori, A.A.; Pruschy, M. Deficiency in homologous recombination renders mammalian cells more sensitive to proton versus photon irradiation. *Int. J. Radiat. Oncol. Biol. Phys.* **2014**, *88*, 175–181. [CrossRef] [PubMed]

32. Antoccia, A.; Sgura, A.; Berardinelli, F.; Cavinato, M.; Cherubini, R.; Gerardi, S.; Tanzarella, C. Cell Cycle Perturbations and Genotoxic Effects in Human Primary Fibroblasts Induced by Low-energy Protons and X/γ-rays. *J. Radiat. Res.* **2009**, *50*, 457–468. [CrossRef] [PubMed]

33. Śniegocka, M.; Podgórska, E.; Plonka, P.M.; Elas, M.; Romanowska-Dixon, B.; Szczygiel, M.; Żmijewski, M.A.; Cichorek, M.; Markiewicz, A.; Brożyna, A.A.; et al. Transplantable melanomas in hamsters and gerbils as models for human melanoma. Sensitization in melanoma radiotherapy—From animal models to clinical trials. *Int. J. Mol. Sci.* **2018**, *19*, 1048. [CrossRef] [PubMed]

34. Buch, K.; Peters, T.; Nawroth, T.; Sänger, M.; Schmidberger, H.; Langguth, P. Determination of cell survival after irradiation via clonogenic assay versus multiple MTT Assay—A comparative study. *Radiat. Oncol.* **2012**, *7*, 1. [CrossRef] [PubMed]

35. Franken, N.A.P.; Oei, A.L.; Kok, H.P.; Rodermond, H.M.; Sminia, P.; Crezee, J.; Stalpers, L.J.A.; Barendsen, G.W. Cell survival and radiosensitisation: Modulation of the linear and quadratic parameters of the LQ model (Review). *Int. J. Oncol.* **2013**, *42*, 1501–1515. [CrossRef] [PubMed]

36. Bodgi, L.; Canet, A.; Pujo-Menjouet, L.; Lesne, A.; Victor, J.-M.; Foray, N. Mathematical models of radiation action on living cells: From the target theory to the modern approaches. A historical and critical review. *J. Theor. Biol.* **2016**, *394*, 93–101. [CrossRef] [PubMed]

37. Mehta, R.G.; Hussain, E.A.; Mehta, R.R.; Das Gupta, T.K. Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D5, a synthetic analog of Vitamin D. *Mutat. Res.* **2003**, *523–524*, 253–264. [CrossRef]

38. Welsh, J.; Wietzke, J.A.; Zinser, G.M.; Byrne, B.; Smith, K.; Narvaez, C.J. Vitamin D-3 Receptor as a Target for Breast Cancer Prevention. *J. Nutr.* **2003**, *133*, 2425S–2433S. [CrossRef] [PubMed]

39. Lamprecht, S.A.; Lipkin, M. Chemoprevention of colon cancer by calcium, vitamin D and folate: Molecular mechanisms. *Nat. Rev. Cancer* **2003**, *3*, 601–614. [CrossRef] [PubMed]

40. Nakagawa, K. 1,25-Dihydroxyvitamin D$_3$ is a preventive factor in the metastasis of lung cancer. *Carcinogenesis* **2004**, *26*, 429–440. [CrossRef] [PubMed]

41. Krishnan, A.V.; Medicine, S.; Feldman, D. Vitamin D Analogs in Cancer Prevention and Therapy. **2003**, *164*. [CrossRef]

42. Szyszka, P.; Zmijewski, M.A.; Slominski, A.T. New vitamin D analogs as potential therapeutics in melanoma. *Expert Rev. Anticancer Ther.* **2012**, *12*, 585–599. [CrossRef] [PubMed]

43. Moreno, J.; Krishnan, A.V.; Feldman, D. Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid Biochem. Mol. Biol.* **2005**, *97*, 31–36. [CrossRef] [PubMed]

44. Banerjee, P.; Chatterjee, M. Antiproliferative role of vitamin D and its analogs—A brief overview. *Mol. Cell. Biochem.* **2003**, *253*, 247–254. [CrossRef] [PubMed]

45. Thill, M.; Woeste, A.; Reichert, K.; Fischer, D.; Rody, A.; Friedrich, M.; Köster, F. Vitamin D inhibits ovarian cancer cell line proliferation in combination with celecoxib and suppresses cyclooxygenase-2 expression. *Anticancer Res.* **2015**, *35*, 1197–1203. [PubMed]

46. Chen, J.; Wang, J.; Kim, T.K.; Tieu, E.W.; Tang, E.K.Y.; Lin, Z.; Kovacic, D.; Miller, D.D.; Postlethwaite, A.; Tuckey, R.C.; et al. Novel vitamin D analogs as potential therapeutics: Metabolism, toxicity profiling and antiproliferative activity. *Anticancer Res.* **2014**, *34*, 2153–2164. [PubMed]

47. Kozielewicz, P.; Grafton, G.; Kutner, A.; Curnow, S.J.; Gordon, J.; Barnes, N.M. Novel vitamin D analogues; cytotoxic and anti-proliferative activity against a diffuse large B-cell lymphoma cell line and B-cells from healthy donors. *J. Steroid Biochem. Mol. Biol.* **2016**, *164*, 98–105. [CrossRef] [PubMed]

48. Humeniuk-Polaczek, R.; Marcinkowska, E. Impaired nuclear localization of vitamin D receptor in leukemia cells resistant to calcitriol-induced differentiation. *J. Steroid Biochem. Mol. Biol.* **2004**, *88*, 361–366. [CrossRef] [PubMed]

49. Agadir, A.; Lazzaro, G.; Zheng, Y.; Zhang, X.-K.; Mehta, R. Resistance of HBL100 human breast epithelial cells to vitamin D action. *Carcinogenesis* **1999**, *20*, 577–582. [CrossRef] [PubMed]

50. Seifert, M.; Rech, M.; Meineke, V.; Tilgen, W.; Reichrath, J. Differential biological effects of 1,25-dihydroxyVitamin D$_3$ on melanoma cell lines in vitro. *J. Steroid Biochem. Mol. Biol.* **2004**, *89–90*, 375–379. [CrossRef] [PubMed]

51. Reichrath, J.; Rech, M.; Moeini, M.; Meese, E.; Tilgen, W.; Seifert, M. In vitro comparison of the vitamin D endocrine system in 1,25(OH)$_2$D$_3$-responsive and -resistant melanoma cells. *Cancer Biol. Ther.* **2007**, *6*, 48–55. [CrossRef] [PubMed]

52. Slominski, A.T.; Janjetovic, Z.; Kim, T.K.; Wright, A.C.; Grese, L.N.; Riney, S.J.; Nguyen, M.N.; Tuckey, R.C. Novel vitamin D hydroxyderivatives inhibit melanoma growth and show differential effects on normal melanocytes. *Anticancer Res.* **2012**, *32*, 3733–3742. [PubMed]

53. Sun, J.; Yin, M.; Zhu, S.; Liu, L.; Zhu, Y.; Wang, Z.; Xu, R.X.; Chang, S. Ultrasound-mediated destruction of oxygen and paclitaxel loaded lipid microbubbles for combination therapy in hypoxic ovarian cancer cells. *Ultrason. Sonochem.* **2016**, *28*, 319–326. [CrossRef] [PubMed]

54. Janjetovic, Z.; Brozyna, A.A.; Tuckey, R.C.; Kim, T.K.; Nguyen, M.N.; Jozwicki, W.; Pfeffer, S.R.; Pfeffer, L.M.; Slominski, A.T. High basal NF-κB activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D$_3$ derivatives. *Br. J. Cancer* **2011**, *105*, 1874–1884. [CrossRef] [PubMed]

55. Sarna, M.; Zadlo, A.; Czuba-Pelech, B.; Urbanska, K. Nanomechanical phenotype of melanoma cells depends solely on the amount of endogenous pigment in the cells. *Int. J. Mol. Sci.* **2018**, *19*, 607. [CrossRef] [PubMed]

56. Wietrzyk, J.; Nevozhay, D.; Filip, B.; Milczarek, M.; Kutner, A. The antitumor effect of lowered doses of cytostatics combined with new analogs of vitamin D in mice. *Anticancer Res.* **2007**, *27*, 3387–3398. [PubMed]

57. Pelczynska, M.; Switalska, M.; Maciejewska, M.; Jaroszewicz, I.; Kutner, A.; Opolski, A. Antiproliferative activity of vitamin D compounds in combination with cytostatics. *Anticancer Res.* **2006**, *26*, 2701–2705. [PubMed]

58. Ma, Y.; Yu, W.D.; Trump, D.L.; Johnson, C.S. 1,25D$_3$ enhances antitumor activity of gemcitabine and cisplatin in human bladder cancer models. *Cancer* **2010**, *116*, 3294–3303. [CrossRef] [PubMed]

59. Newhauser, W.D.; Zhang, R. The physics of proton therapy. *Phys. Med. Biol.* **2015**, *60*, R155–R209. [CrossRef] [PubMed]

60. Fokas, E.; Kraft, G.; An, H.; Engenhart-Cabillic, R. Ion beam radiobiology and cancer: Time to update ourselves. *Biochim. Biophys. Acta Rev. Cancer* **2009**, *1796*, 216–229. [CrossRef] [PubMed]

61. Folkard, M.; Prise, K.M.; Vojnovic, B.; Newman, H.C.; Roper, M.J.; Michael, B.D. Inactivation of V79 cells by low-energy protons, deuterons and helium-3 ions. *Int. J. Radiat. Biol.* **1996**, *69*, 729–738. [CrossRef] [PubMed]

62. Kundrät, P. Detailed analysis of the cell-inactivation mechanism by accelerated protons and light ions. *Phys. Med. Biol.* **2006**, *51*, 1185–1199. [CrossRef] [PubMed]

63. Kundrát, P.; Lokajíček, M.; Hromčíková, H. Probabilistic two-stage model of cell inactivation by ionizing particles. *Phys. Med. Biol.* **2005**, *50*, 1433–1447. [CrossRef] [PubMed]

64. Trémezaygues, L.; Seifert, M.; Vogt, T.; Tilgen, W.; Reichrath, J. 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. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 324–327. [CrossRef] [PubMed]

65. Murray, P.J.; Cornelissen, B.; Vallis, K.A.; Chapman, S.J. DNA double-strand break repair: A theoretical framework and its application. *J. R. Soc. Interface* **2016**, *13*, 20150679. [CrossRef] [PubMed]

66. Murray, D.; Mirzayans, R.; Parliament, M. New insights into patient radiosensitivity in relation to the biology of the ATM protein. *Transl. Cancer Res.* **2016**, *5*, S809–S814. [CrossRef]

67. Janczyk, A.; Wolnicka-Glubisz, A.; Chmura, A.; Elas, M.; Matuszak, Z.; Stochel, G.; Urbanska, K. NO-dependent phototoxicity of Roussin's black salt against cancer cells. *Nitric Oxide Biol. Chem.* **2004**, *10*, 42–50. [CrossRef] [PubMed]

68. Brindell, M.; Kuliś, E.; Elmroth, S.K.C.; Urbańska, K.; Stochel, G. Light-induced anticancer activity of [RuCl$_2$(DMSO)$_4$] complexes. *J. Med. Chem.* **2005**, *48*, 7298–7304. [CrossRef] [PubMed]

69. Bomirski, A.; Słominski, A.; Bigda, J. The natural history of a family of transplantable melanomas in hamsters. *Cancer Metastasis Rev.* **1988**, *7*, 95–118. [CrossRef] [PubMed]

70. Słominski, A. Rapid melanization of Bomirski amelanotic melanoma cells in cell culture. *Biosci. Rep.* **1983**, *3*, 189–194. [CrossRef] [PubMed]

71. Słomiński, A. Some properties of Bomirski Ab amelanotic melanoma cells, which underwent spontaneous melanization in primary cell culture—Growth kinetics, cell morphology, melanin content and tumorigenicity. *J. Cancer Res. Clin. Oncol.* **1985**, *109*, 29–37. [CrossRef] [PubMed]

72. Słominski, A.; Moellmann, G.; Kuklinska, E.; Bomirski, A.; Pawelek, J. Positive regulation of melanin pigmentation by two key substrates of the melanogenic pathway, L-tyrosine and L-dopa. *J. Cell Sci.* **1988**, *89*, 287–296. [PubMed]

73. Słomiński, A.; Bomirski, A. Phenotypic changes of Ab hamster melanoma during long-term culture. *Anticancer Res.* **1985**, *5*, 403–409. [PubMed]

74. Boberek, M.; Swakoń, J.; Stolarczyk, L.; Olko Pawełand Waligórski, M. A monitoring system for the 60 MeV radiotherapy proton beam at IFJ PAN using a scintillating screen and a CCD camera. *Rom. Rep. Phys.* **2014**, *66*, 5–15.