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A switch between cytoprotective and cytotoxic autophagy in the radio sensitization of breast tumor cells by chloroquine and vitamin D

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Abstract

Calcitriol or 1,25 dihydroxy vitamin D₃, the hormonally active form of vitamin D, as well as vitamin D analogs, have been shown to increase sensitivity to ionizing radiation in breast tumor cells. The current studies indicate that the combination of 1,25 dihydroxy vitamin D₃ with radiation appears to kill p53 wild type, estrogen receptor positive ZR-75-1 breast tumor cells through autophagy. Minimal apoptosis was observed based on cell morphology by DAPI and TUNEL staining, Annexin/PI analysis, Caspase-3 and PARP cleavage as well as cell cycle analysis. Induction of autophagy was indicated by increased acridine orange staining, RFP-LC3 redistribution and detection of autophagic vesicles by electron microscopy, while autophagic flux was monitored based on p62 degradation. The autophagy inhibitors, chloroquine and Bafilomycin A1, as well as genetic suppression of the autophagic signaling proteins Atg5 or Atg 7 attenuated the impact of the combination treatment of 1,25 D₃ with radiation. In contrast to autophagy mediating the effects of the combination treatment, the autophagy induced by radiation alone was apparently cytoprotective in that either pharmacological or genetic inhibition increased sensitivity to radiation. These studies support the potential utility of vitamin D for improving the impact of radiation for breast cancer therapy, support the feasibility of combining chloroquine with radiation for the treatment of breast cancer and demonstrate the existence of an “autophagic switch” from

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cytoprotective autophagy with radiation alone to cytotoxic autophagy with the 1, 25 D3 – radiation combination.

Keywords

Breast cancer; vitamin D; radiation; autophagy; chloroquine

Introduction

Radiation therapy is a widely used component of cancer therapy and is a fundamental tool in the treatment of breast cancer (along with surgery and chemotherapy). Exposure to ionizing radiation leads to rapid generation of reactive oxygen species that produce DNA damage [1]. One of the most lethal radiation induced lesions in DNA is the double strand break [2], which can lead to growth arrest or cell death by the activation of various signaling pathways. Although radiation is a very effective modality in the treatment of breast cancer, there is always some likelihood of disease recurrence that may be due to tumor cells that survive through disruptions in cell death (primarily apoptotic) pathways, increased DNA repair capacity and/or overactivation of cytoprotective signaling pathways [3]. Thus, a primary goal of our work has been to develop strategies to enhance the response to radiation therapy using the active form of vitamin D, Calcitriol (1,25D3) as well as analogs of vitamin D [4, 5].

Apart from the established role of vitamin D3 in regulation of calcium homeostasis, numerous studies have shown that the metabolically active form of vitamin D3, 1,25-di hydroxy vitamin D3, (1,25D3) as well as vitamin D analogs, can enhance the response to chemotherapy in a variety of malignancy models[6–9].

Autophagy is an evolutionary conserved process whereby cytoplasmic proteins and cellular organelles are enveloped in autophagosomes and degraded by fusion with lysosomes for amino acid and energy recycling [10]. There is evidence that whereas autophagy can play a critical role in cellular survival [11–14] it can also become a cellular suicide pathway when apoptosis is defective and following extreme stress conditions [15–17]. Furthermore, autophagy is frequently activated in tumor cells following anticancer therapies such as drug treatment and gamma-irradiation[18] and can either contribute to cell death or represent a mechanism of resistance to these treatments[11, 12, 14, 19–23].

A number of studies have demonstrated that radiation induces cytoprotective autophagy[12, 16, 22, 23]. However, studies from our laboratory have indicated that autophagy is the basis for radiation sensitization of MCF-7 breast tumor cells by 1–25 D3[4]. Studies by other investigators have also demonstrated radiation sensitization through the promotion of autophagy[24–26].

Autophagic cell death has been defined based on characteristics such as i) cell death occurring without the involvement of apoptosis and necrosis; (ii) increased autophagic flux, rather than an increase of autophagic markers; and (iii) suppression of autophagy via both pharmacological inhibitors and genetic approaches is able to rescue or prevent cell death [27]. In the present manuscript we have established that autophagic cell death is the mode of radiosensitization by 1,25D3 in ZR-75-1 breast tumor cells. We were further able to demonstrate that autophagy can actually have dual functions in the same experimental system, acting both as a cytoprotective mechanism for radiation alone and a cytotoxic mechanism when radiation is accompanied by 1,25 D3.

RESULTS

Sensitization to ionizing radiation by 1,25-D3 in ZR-75 breast tumor cells

Initial studies assessed the influence of treatment with 1,25-D3 on sensitivity to radiation. Exposure of ZR-75-1 cells to 1,25D3 alone had a modest impact on cell growth; at Day 3 control cells had doubled approximately 2.5 times whereas cells treated with 1,25 D3 had doubled approximately 2 times (1a). This is likely due to 1,25 D3's regulation of proteins involved in cell cycle progression[28, 29]. Figure 1b shows that the combination of 1,25-D3 with radiation resulted in a reduction of viable cells (actual cell killing) that was followed by growth arrest in the residual surviving cell population; in contrast, radiation alone appeared to inhibit cell growth without producing an actual reduction in viable cell number (compared to control growth in 1a)

Radiosensitization of ZR-75-1 breast cancer cells by 1,25 D3 was further assessed in clonogenic survival assays using both single dose (1c) and fractionated dose radiation (1d). When combined with 1,25 D3, there was a pronounced decrease in clonogenicity compared to radiation alone at all doses. As in the studies presented in Figure 1a, 1,25D3 alone had a minimal impact on clonogenicity, indicating that the sensitization to radiation is likely not simply a result of additive toxicity.

Minimal Induction of apoptosis by radiation and 1,25D3+IR

Figure 2 presents studies that were designed to assess whether apoptosis was the basis for the radiosensitization by 1,25 D3. Apoptosis was assessed by the TUNEL assay and DAPI staining (2a), Fluorescence Activated Cell sorting (FACS) analysis for Annexin/Propidium Iodide (PI) staining(2b),western blot analysis for both PARP and Caspase-3 cleavage (2c) and FACS analysis for a sub G1 population (2d). The TUNEL and DAPI staining data indicate that minimal apoptosis occurs with either radiation alone or 1,25D3 + radiation. This result was confirmed by Annexin/PI staining where there is minimal staining in quadrants Q2 and Q4, which are indicative of early and late apoptotic cells; there was also no evidence of necrosis (quadrant Q1). Furthermore, with both radiation alone and 1,25D3+radiation, there is no indication of an increase in the sub G1 population (2d). Cell cycle analysis also indicates that there is no mitotic catastrophe, due to the lack of accumulation of cells with DNA content greater than 4n[30, 31]. Staurosporine was used as a positive control to demonstrate that ZR-75-1 cells have the ability to undergo apoptosis (2a and 2b).

Autophagy induction and autophagic flux by radiation alone and 1,25 D3+radiation

Autophagy has been shown to play an important role in cell survival and cell death[32, 33]. To investigate whether autophagy plays a role in radiation sensitization in the ZR-75-1 cells, cells were stained with acridine orange and the formation of punctate staining was monitored based on visual assessment (3a) as well as quantification using flow cytometry (3b and 3c). Figure 3b presents a representative set of histograms for each treatment while figure 3c summarizes data from two replicate experiments. At 72 hours post irradiation, there is an approximately 4- fold increase in the percent of AVOs (acidic vacuolar organelles) in cells that received radiation treatment alone and an approximately 5-fold increase by the combination treatment; the increase produced by 1,25 D3 alone did not achieve statistical significance(3c). Timecourse data suggest that autophagy is initiated earlier in cells treated with 1,25D3+IR and autophagy is sustained to a higher extent with 1,25D3+IR treatment compared to radiation treatment alone. (Online Resource 1(S1)) The promotion of autophagy by both radiation alone and 1,25-D3 + radiation was confirmed by monodacylcadaverine (MDC)[34] staining (Online Resource 1(S2)) and RFP-LC3 redistribution (3d) Upon autophagy induction, RFP-LC3 displays punctate staining in the

cytoplasm and can be visualized using confocal microscopy[35]. Quantification of RFP-LC3 puncta is presented in figure 3e. Consistent with acridine orange staining, there is essentially the same number of LC3 puncta per cell for IR and 1,25D3+IR treatment at 72 hours. Transmission electron microscopy images also indicate the presence of autophagosomes with the combination treatment as well as with radiation alone (3f).

Since the presence of autophagic vesicles does not necessarily mean that the autophagosomes have fused with the lysosome and that the contents of the autophagosome have been degraded, autophagic flux was assessed by western blot analysis for p62 degradation. p62 binds directly to LC-3 (Atg8), a critical protein involved in autophagosome formation to facilitate autophagic degradation[36–38]. Consistent with the acridine orange staining and electron microscopy data, there was no p62 degradation with 1,25D3 alone (Online Resource 1(S3)) while p62 degradation is shown with serum starvation, which was used as a positive control. In the case of both radiation alone and 1,25D3 + radiation, p62 degradation appears to be virtually complete within 72 hours (3g).

Residual Surviving cells are in a state of Senescence

In previous studies, we reported that MCF-7 cells undergo a period of growth arrest/senescence following treatment with radiation alone as well as in the residual surviving breast tumor cell population following treatment with vitamin D analog, EB1089 + radiation[4]. Based on cell morphology and β -Galactosidase staining, residual surviving cells are indeed in a state of senescence after both radiation alone and 1,25D3 + radiation treatment (4a). To further confirm β -Galactosidase activity 5-dodecanoylaminofluorescein di- β -D-galactopyranoside (C_{12} FDG), a fluorogenic substrate for β gal activity [39] was analyzed by FACS analysis and fluorescence intensity was measured (4b and Online Resource 2(S4)). For both experimental conditions, senescence is most pronounced at 144 hours post-treatment.

Effects of Pharmacologic Autophagy Inhibition on Cell Viability after IR and 1,25D3+IR

In efforts to confirm that autophagy is the basis for sensitization by 1,25D3, studies were conducted with the autophagy inhibitors chloroquine and bafilomycin A1. Bafilomycin A1 prevents the fusion of the autophagosome with the lysosome[33, 40], while chloroquine prevents the acidification of the lysosome[41]. We identified concentrations of bafilomycin A1 and chloroquine that would have little to no toxic effects, as indicated by trypan blue exclusion as well as the MTT assay (Online Resource 3(S5–S6)). Inhibition of the autophagy induced by either radiation or 1,25D3 + radiation (specifically the formation of acidified autophagic vesicles) was confirmed through an assessment of acridine orange distribution by flow cytometry (Figure 5a and Online Resource 3 (S7)).

Treatment with radiation concurrently with bafilomycin A1 or chloroquine resulted in a significant enhancement of cytotoxicity as indicated by the decline in viable cell number (5b). These observations are consistent with the premise that autophagy induced by radiation alone is cytoprotective. In dramatic contrast, interference with autophagy in 1,25D3+IR treated cells results in reduced radiation sensitivity, which is the expected outcome if autophagy mediates the cytotoxicity of the 1,25 D3-radiation combination treatment. In fact, the cell death induced by the combination treatment is attenuated and cell viability is restored to levels similar to what is observed with IR treatment alone (comparing Figures 5b and 5c). These results were confirmed by monitoring propidium iodide uptake by flow cytometry. Radiosensitization by chloroquine appears to be a result of the promotion of both apoptosis and necrosis (Online Resource 3 (S8–S9)). Moreover, these findings indicate the existence of both cytoprotective and cytotoxic functions of autophagy in response to radiation in the same experimental system.

Effects of Genetic Autophagy Inhibition on Cell Viability in IR and 1,25D3+IR treated cells

To confirm the results generated with pharmacological autophagy inhibition, additional studies were performed using ZR-75-1 cells in which expression of either ATG-5 or ATG-7 was stably suppressed by shRNA. Atg5 combines with Atg12 in a multimeric protein complex that is required for autophagosome formation, whereas Atg7 is an E1-like enzyme involved in activating both LC3II (Atg8) and Atg5[42]. Atg-5 levels were reduced by approximately 77% and Atg-7 levels by approximately 60% (6a). To determine if this knockdown was sufficient to alter autophagic function, autophagic flux was monitored by p62 degradation in ATG-5 and ATG-7 knockdown cells. Indeed, cells in which ATG 5 or ATG 7 had been knocked down displayed decreased autophagic flux compared to vector control cells following serum starvation (Online Resource 4 (S10)).

Initial studies were conducted to confirm that 1,25D3, IR and 1,25D3+IR treatment produced the same relative effects in control cells expressing shRNA against GFP (shRNA GFP control) as they did in the parental ZR-75-1 cells. A single dose of 4 Gy radiation was used for these studies, as we found that this dose allowed us to more effectively distinguish the effects of radiation alone from the combination treatment in these genetically modified cells. Figure 6b shows transient growth inhibition by 1,25 D3 while Figure 6C indicates that indeed 1,25D3 sensitized shRNA control cells to IR treatment. In the cells where ATG-5 was partially silenced, sensitivity to radiation was increased compared to the shRNA control cells, again indicating that autophagy is a mode of cell protection for radiation alone (6d). In dramatic contrast, the impact of 1,25 D3 + radiation is blunted or attenuated when Atg5 is suppressed, which supports the cytotoxic actions of autophagy for the 1,25-D3 + radiation combination treatment (6d).

The studies in the ATG-7 suppressed cells demonstrated an essentially identical outcome as with the pharmacological inhibitors chloroquine and bafilomycin as well as with suppression of Atg5. That is, sensitivity to radiation alone is increased, albeit quite modestly, while radiosensitization by 1,25D3 is markedly attenuated (6e). Knockdown of ATG-5 or ATG-7 had no perceptible effect on the capacity of 1,25D3 treatment alone to moderately inhibit cell growth (Online Resource 4 (S11) and (S12)).

DISCUSSION

The current studies further establish the potential utility of vitamin D as an adjuvant therapy for the treatment of breast cancer and furthermore provide evidence that sensitization to radiation by 1,25D3 occurs through the promotion of autophagy. Consequently, the combination treatment is likely to be effective even in breast tumor cells that might be resistant to apoptosis. Interestingly, 1,25 D3 appears to switch the cells from a cytoprotective to a cytotoxic mode of autophagy. Although either cytoprotective or cytotoxic actions of autophagy have been reported in multiple publications, to our knowledge these are the first studies in the literature to provide evidence for this type of “autophagic switch”

While 1,25 D3 treatment promotes cell killing in response to radiation in ZR-75-1 breast cancer cells, our studies do not support the involvement of apoptosis in the actions of radiation alone or for the combination treatment. Instead our data support the promotion of autophagic cell death as the mode of radiation sensitization by 1,25D3. In confirmation of the conclusion that sensitization to radiation by 1,25 D3 is a consequence of the induction of cytotoxic autophagy, pharmacologic and genetic interference with autophagy resulted in increased cell survival, with viable cell numbers essentially restored to levels observed after treatment with radiation alone. Conversely, when autophagy was blocked in cells irradiated in the absence of 1,25 D3, there was clearly a decrease in cell viability, supporting the

induction of cytoprotective autophagy by radiation alone. Our data further suggest that residual surviving cells are in a state of senescence, which is consistent with studies showing that radiation induces senescence in tumor cells [43, 44].

Although the amount of autophagy induced appears to be essentially the same in both IR and 1,25D3+IR treatment, we speculate that 1,25 D3 increases the rate and extent of autophagic flux in radiation treated cells and that this increase mediates the radiosensitizing effects of 1,25D3. This hypothesis is based on the fact that p62 is degraded earlier and to a greater extent in 1,25D3+R treated cells compared to IR treatment alone. It is important to emphasize that this experimental system should provide the appropriate experimental model system to address the question of what factors might distinguish cytoprotective from cytotoxic autophagy, which is currently a fundamental question in this field [27].

Our future studies will be designed to elucidate the pathways by which 1,25 D3 sensitizes breast cancer cells, and establish what factors determine whether autophagy will be cytotoxic or cytoprotective. It is known that autophagy is a multi-step process that appears to be regulated by various signaling pathways [25, 45]. Moreover, activation of ER stress and mTOR signaling pathways have been shown to be involved in autophagy induction[46, 47]. Both pathways have been shown to be activated in response to radiation[48] and studies have shown that AKT/mTOR signaling is involved in 1,25D3's effects on cell proliferation[49, 50]. The capacity of 1,25D3 to promote radiosensitization may involve its modulation of proteins in one or both of these autophagy pathways.

Materials and Methods

Cell Lines

The p53 wild-type (WT) ZR-75-1 human breast tumor cell line was obtained from ATCC. The ZR-75-1 ATG5^{-/-} and ATG7^{-/-} cells were generated as indicated below.

RNA interference

The siRNA sequence to target Atg5 was obtained from a previous publication[51]: The sequence of the siRNA was: GCAACTCTGGATGGGATTG. It was ordered as a hairpin oligonucleotide with the sense and antisense sequence separated by a loop sequence and restriction sites at the 5' and 3' ends to facilitate cloning into System Bioscience's pSIH-H1-puro lentiviral shRNA vector (which uses the H1 promoter to drive expression). With the shRNA inserted into this vector, lentivirus was produced in HEK 293TN cells co-transfected with the following vectors that encode the necessary packaging components: pPack-Rev, pPack-Gag and pPack-VSVg (purchased from Systems Biosciences as a mix). The virus shed into the medium was then used to infect the ZR75 cells. The latter were selected in medium with 1 ug/ml puromycin to enrich the infected cells. A 70% KD of Atg5 in these cells was observed following selection. shRNA control cells were generated in similar fashion except that the siRNA sequence used was against the irrelevant *Aequorea victoria* green fluorescent protein (GFP).

Mission shRNA lentiviral transduction particles for Atg 7 (Sigma NM_006395), were purchased as a set of 5 different shRNA viral particles. After infecting the ZR75 target cells with each of the 5 different viral populations, each at 3 different MOI's, the cells were checked for Atg7 expression and the culture that displayed the greatest decrease in Atg7 expression, was selected. The Sigma particles that worked best were #TRCN0000007584 (at an MOI of 0.5), with shRNA directed against the following sequence in the 3'UTR of Atg7: GCCTGCTGAGGAGCTCTCCA. The transduced cells were selected with in medium with 1 ug/ml puromycin to obtain stable cell lines.

Cell Culture and Treatment

All ZR-75-1 derived cell lines were grown from frozen stocks in basal RPMI 1640 supplemented with 5% FCS, 5% BCS, 2 mmol/L l-glutamine, and penicillin/streptomycin (0.5 mL/100 mL medium). ZR-75-1/ATG5^{-/-} and ATG-7^{-/-} cells were maintained using (1 µg/ml) puromycin (Sigma p8833) for resistance. All cells were maintained at 37°C under a humidified, 5% CO₂ atmosphere. Cells were exposed to γ-IR using a ¹³⁷Cs irradiator. In our studies, cells were exposed to 100 nmol/L 1,25 Vitamin D3 (Sigma D1530) alone or concurrently with radiation treatment. In the cases where the radiation doses were fractionated, four fractions of 2 Gy radiation were administered over two consecutive days (two fractions separated by 6 hours on days 1 and 2).

Cell viability and clonogenic survival

Cell viability was determined by trypan blue exclusion at various time points after treatment. Cells were harvested using trypsin, stained with 0.4% trypan blue dye (Sigma T8154), and counted using phase contrast microscopy. For clonogenic survival studies, cells were plated in triplicate in six well tissue culture dishes at the appropriate density for each condition. After 14 days, the cells were fixed with 100% methanol, air-dried and stained with 0.1% crystal violet (Sigma C3886). For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls, which were taken as 100% survival.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay for Apoptosis

The method of Gavrieli et al.[52] was used as an independent assessment of apoptotic cell death in combined cytopins containing both adherent and nonadherent cells. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the In situ Cell Death Detection kit (Roche 11373242910, 03333566001), where strand breaks are end labeled with fluorescein-dUTP by the enzyme terminal transferase. Cells were then fixed to glass slides using DAPI-containing Vectashield mounting medium (Sigma D9542). Pictures were taken using an Olympus inverted fluorescence microscope. All images presented are at the same magnification.

Western Blot Analysis

After the indicated treatments, cells were washed in PBS and lysed using 500–1000 µL M-PER mammalian protein extraction reagent (Thermo Scientific #78501) containing protease and phosphatase inhibitors for 5 minutes on a shaker. Protein concentrations were determined by the Lowry method and equal aliquots of protein (40 µg) were separated using 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and using Licor blocking buffer. Membranes were immunoblotted with respective antibodies and then incubated with respective Licor secondary antibodies. Proteins were visualized using the Licor imaging system. Primary antibodies used were anti-p62 (SQSTM1 – Santa Cruz sc-28359), anti-ATG5 (APG5 – Biosensis R-111-100), anti-ATG7 (APG7 – Santa Cruz sc-33211, anti-β actin (Santa Cruz sc-47778) anti α-tubulin (Santa Cruz sc-5286), anti-caspase 3 (Cell Signaling 9665), anti-parp (Cell signaling 46D11). All primary antibodies presented were used at a 1:1000 dilution. All primary antibodies presented were used at a 1:1000 dilution.

Detection and Quantification of Autophagic Cells by staining with Acridine Orange

As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining[18]. Cells were seeded in six well tissue culture dishes and treated as described above for the cell viability study. 72 hrs hours following initial treatment, cells

were incubated with medium containing 1 $\mu\text{g/mL}$ acridine orange (Invitrogen A3568) for 15 minutes; the acridine orange was then removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope. All images presented are at the same magnification. The number of cells with increased acidic vesicular organelles (AVO) was determined by Flow cytometry. Cells were trypsinized, harvested and analyzed by BD FACSCanto II using BD FACSDiva software. A minimum of 10,000 cells within the gated region were analyzed

Detection of Autophagic Cells by staining with monodansylcadaverine (MDC)

The autofluorescent agent monodansylcadaverine (Sigma Chemical) was used as a specific autophagolysosome marker[34]. Cells were seeded in six well tissue culture dishes and treated as described above for the cell viability study. 72 hrs hours following initial treatment, cells were incubated with medium containing 1 $\mu\text{g/mL}$ MDC for 15 minutes; the MDC was then removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope. Again, all images presented are at the same magnification.

Cell Cycle Analysis by propidium iodide staining

For the cell cycle analysis, tumor cells treated as described above were trypsinized, fixed with 70% ethanol, and stained with propidium iodide 72 hours post treatment. Protocol was adapted from Z. Darzynkiewicz et al [53]. A minimum of 20,000 cells/events were analyzed.

FACS Analysis for Annexin V and Propidium iodide positive cells to monitor apoptosis and necrosis

For the FACS analysis, cells treated as described above were collected and labeled fluorescently for detection of apoptotic and necrotic cells by adding 500 μL of binding buffer, 5 μL of Annexin V-FITC and 5 μL of Propidium Iodide to each sample. Samples were mixed gently and incubated at room temperature in the dark for 15 min. The number of cells with increased annexin/pi staining was determined by flow cytometry and analyzed by BD FACSCanto II using BD FACSDiva software. A minimum of 10,000 cells within the gated region were analyzed.

Transmission Electron microscopy

TEM services, including sample fixation, embedding, ultra-microtomy and staining were provided by the VCU Department of Anatomy and Neurobiology Microscopy Facility. Sections were imaged via Jeol JEM-1230 transmission electron microscope (EM) equipped with a Gatan UltraScan 4000SP 4K $\times$ 4K CCD camera. The magnification of each image is indicated by the scale bar at the bottom of the micrograph.

Cytochemical detection of β -galactosidase staining

β -galactosidase-positive cells were detected by the method of Dimri *et al.*[54] Briefly, the monolayers of cells were washed two times with PBS and then fixed with 2% formaldehyde 1 0.2% glutaraldehyde (prepared in PBS) for 5 min. The cells were then washed again two times with PBS. After the last wash, staining solution was added [1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) in dimethylformamide (20 mg/ml stock), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl_2] and the cells were incubated in a 37°C for 24 hr. After incubation the cells were washed two times with PBS and visualized using an Olympus inverted microscope.

FACS analysis of β -galactosidase activity

Cells were treated as described above and analyzed using floursent β -galactosidase activity marker C12FDG. The protocol was adapted from Debacq-Chainiaux et al.[39]

RFP-LC3 Redistribution

The MCF7 RFP-LC3 construct was a generous gift from Dr. Keith Miskimins. ZR-75-1 cells were stably transfected with RFP-LC3 using the standard effectene (Qiagen) protocol. Cells were treated as described above and visualized using a Leica Confocal laser scanning microscope. Cells were counterstained with DAPI to visualize. Five fields were counted for each treated condition to determine the average number of LC3 puncta per cell.

Statistical Analysis

Statistical differences were determined using StatView statistical software. The data were expressed as the means $\pm$ SE (as standard error of the mean) Comparisons were made using a one-way ANOVA followed by Tukey Kramer post-hoc test. $P \leq 0.05$ were taken as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations list

1,25D3	1,25 dihydroxyvitamin D3
DAPI	4',6-diamidino-2-phenylindole
AO	Acridine Orange
AVOs	Acidic Vacuolar Organelles
CQ	Chloroquine
IR	Radiation
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
EM	Transmission Electron Microscopy
BAF	Bafilomycin A1
SS	Serum Starvation
PI	Propidium Iodide
FACS	Fluorescence Activated Cell sorting

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Fig 1. Sensitization to ionizing radiation by 1,25D3

a–b Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following post initial treatment. **a** Cells were exposed 100nM 1,25D3 continuously. **b** Cells received fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation. **c–d** Clonogenic survival assays with both single dose and fractionated radiation, with or without 100nM 1,25 D3. Clonogenic survival was assessed after 14 days. Values shown are from a representative experiment with triplicate samples for each condition. * $p < .05$ compared to control # $p < .05$ compared to IR alone (**b**) * $p < .05$ compared to IR (**c–d**) Data are representative of an average of three experiments

2d

	G1	S phase	G2/M	Sub-G1
Control	65.8	14.3	12.5	7.5
1,25 D ₃	60.2	14.5	16.7	8.6
IR	49.3	12.1	34	4.6
1,25 D ₃ +IR	45.1	11.1	38.1	5.8

Fig 2. Minimal Induction of apoptosis by radiation and 1,25D₃+IR

Cells were treated with 100nM 1,25D₃ alone, fractionated doses of radiation (4×2 Gy) or 1,25D₃ concurrently with radiation. **a** TUNEL assay and DAPI stained images were taken 72 hours post initial drug treatment. **b** Annexin V and PI positive cells were analyzed by FACS. Exposure to 500nM staurosporine for 24 hours was used as a positive control. **c** Western blotting for cleavage of caspase-3 and PARP 72 hours post initial treatment. Alpha tubulin was used as a loading control. **d** Cell cycle distribution by Flow Cytometry 72 hours post initial treatment. Data are representative of an average of three experiments.

3d

3e

3f

3g

Fig 3. Autophagy induction and autophagic flux after IR and 1,25D3+IR

Cells were exposed to 100nM 1,25D3 alone, radiation alone (4×2 Gy) administered over a period of 2 days or 1,25D3 concurrently with radiation. **a** Acridine orange images were taken at 72 hours post initial treatment (1,25D3,IR or 1,25D3+IR) using an inverted fluorescent microscope. Cells with positive staining for AVOs was monitored using flow cytometry 72 hours post treatment **b** Representative histograms of positive acridine orange staining for each treatment. **c** Percentage of cells with positive acridine orange staining represented as the mean $\pm$ SE. **d–e** RFP-LC3 was assessed by confocal microscopy 72 hours post treatment. **f** The presence of autophagic vacuoles was confirmed by electron microscopy at 72 hours. Autophagosomes are indicated by the red arrows **g** Autophagic flux was based on the decline in p62 levels monitored by western blotting post-irradiation. β -Actin was utilized as a loading control and serum starvation was used as a positive control for autophagic flux. Values shown are from a representative experiment with triplicate samples for each condition. * $p < 0.05$ compared to control. Data are representative of an average of three experiments

4a

4b

Fig 4. Residual surviving cells in a state of senescence

Cells were exposed to 100nM 1,25D3, radiation (4×2 Gy) administered over a period of 2 days or 1,25D3 concurrently with radiation. Cells were stained with β -galactosidase and images were taken at 3 and 6 days post initial drug treatment or post irradiation. **a** Data are representative images from three experiments **b** Cells were exposed to 1,25D3, radiation alone or 1,25D3+IR and samples were stained as described in materials and methods and analyzed by FACS analysis at days 3 and 6 post treatment. Values shown are from a representative experiment with triplicate samples for each condition.

Fig 5. Effects of pharmacologic autophagy inhibition on cell viability in IR and 1,25D3+IR treated cells

a Cells were exposed to 4×2 Gy radiation alone or concurrently with Bafilomycin A1 or Chloroquine and percentage of cells with positive AVO staining was monitored using flow cytometry at 72 hours post treatment. **b** Cells were exposed to radiation in the absence or presence of either Bafilomycin A1 or Chloroquine and viable cell number was assessed by trypan blue exclusion at days 3 and 6 post treatment. **c** Cells were exposed to 1,25D3 concurrently with 4×2 Gy radiation in the absence or presence of either Bafilomycin A1 or Chloroquine and viable cell number was assessed by trypan blue exclusion at days 3 and 6 post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * $p < 0.05$ compared to IR (**b**) ** $p < 0.05$ compared to 1,25D3+IR (**c**). Data are representative of an average of three experiments

Fig 6. Effects of genetic autophagy inhibition on cell viability in IR and 1,25D3+IR treated cells
a ZR-75-1 cells were stably transfected with either a lentivirus encoding shRNA against GFP (control) or with a lentivirus for silencing expression of ATG5 or ATG-7. ATG-5 levels are shown by western blotting comparing control cells to those with ATG-5 or ATG-7 knockdown. Beta actin was used as loading control **b–c** shRNA control cells were treated continuously with 1,25D3 (**b**), a single dose of 4 Gy or concurrently with 1,25D3 and IR (**c**) and cell viability was assessed by trypan blue exclusion. **d–e** Cell viability was assessed by trypan blue exclusion in cells receiving a single dose of 4 Gy IR or 1,25D3+IR in ATG-5 silenced cells (**d**) or ATG-7 silenced cells (**e**). Values shown are from a representative experiment with triplicate samples for each condition. * $p < .05$ compared to IR ** $p < .05$ compared to 1,25D3+IR. Data are representative of an average of three experiments