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In vitro radiosensitization by eribulin in human cancer cell lines

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ABSTRACT

Background: The objective was to to determine the radiosensitizing properties of eribulin and the potential mechanisms of radiosensitization in cervical (HeLa) and pharyngeal (FaDu) cancer cell lines.

Materials and methods: Cytotoxicity was evaluated by the crystal violet method. The 10% and 50% inhibitory concentration (IC10, IC50) for 24-hour drug exposure were determined. The surviving fraction at 2 Gy (SF2) and the sensitizer enhancement ratio (SER) were calculated from radiation cell survival curves in the presence or absence of eribulin. Combination index (CI) was calculated to determine if there is a true synergistic interaction between eribulin and irradiation. Cell cycle changes were assessed by propidium iodide staining and flow cytometry. Apoptotic cells were detected by annexin V and TUNEL-assay.

Results: Mean IC50s and IC10s were 1.58 nM and 0.7 nM and 0.7 nM and 0.27 nM for HeLa and FaDu cells, respectively. Radiosensitization was observed in both lines with a SER up to 2.71 and 2.32 for HeLa and FaDu cells, respectively. A true synergistic effect was showed with a CI of 0.82 and 0.76 for HeLa and FaDu cells, respectively. Eribulin induced significant G2/M cell arrest and marked apoptosis. Irradiation combined with 3 nM eribulin increased the apoptotic response to radiation in Hela cells.

Conclusion: Eribulin shows a true in vitro radiosensitizing effect in HeLa and FaDu cells by inducing significant G2/M phase arrest. In HeLa, the enhancement radiation-induced apoptosis could be an additional mechanism of radiosensitization. Further studies are needed to evaluate the clinical benefits of concurrent eribulin and radiotherapy as a novel therapeutic strategy for cancer.

Key words: eribulin; radiotherapy; radiosensitization; G2/M arrest; apoptosis

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Introduction

Eribulin mesilate (eribulin) is a structurally simplified synthetic analogue of marine natural compound halichondrin B, isolated predominantly from the marine sponge Halichondria Okadaki [1, 2]. This agent shows a potent in vitro and in vivo activity against a variety of human cancer cell lines

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[3]. Preclinical studies reveal that eribulin inhibits tumour cell proliferation in nanomolar range by disruption of mitotic spindles, which results in irreversible mitotic block, G2/M phase arrest and apoptosis [3, 4].

Eribulin is a non-taxane microtubule dynamics inhibitor with a novel mechanism of action, distinct from conventional tubulin-targeted therapies. This agent inhibits microtubule growth, with no effect on microtubule shortening and induces the sequestration of tubulin into non-productive aggregates [5].

On the basis of phase I studies results, the dose limiting toxicity was neutropenia at a maximum tolerated dose of 1.4 mg/m^2 [6]. Eribulin exhibits a manageable toxicity profile, especially when administered at 1.4 mg/m^2 given as a 2- to 5-minute intravenous infusion on days 1 and 8 of a 21-day cycle [7]. Phase II studies confirm eribulin activity in pretreated breast, prostate and ovarian cancer, non-small cell lung cancer and sarcoma [8–13].

Two phase III clinical trials demonstrated the benefit of eribulin in advanced or metastatic breast cancer as a second line of systemic therapy [14, 15]. Eribulin has more recently been approved for pretreated patients with liposarcoma based on data from a phase III trial [16].

Currently, the standard treatment for head and neck and uterine cervix cancer is cisplatin-based chemoradiotherapy. Drugs that induce arrest in the G2/M phase of the cell cycle, such as taxanes, have also been shown to be effective in this type of tumours [17–19]. Eribulin, as an agent that induces arrest in more radiosensitive phases of the cell cycle, has potential radiosensitizing effects in head and neck and cervical cancer cell lines. In this paper we analyse the radiosensitizing properties of eribulin in cervical and head and neck cancer cell lines and the cell cycle and apoptosis changes induced by eribulin.

Materials and methods

Cell lines and culture conditions

We used HeLa (human cervical cancer) cell line obtained from Puerta de Hierro Institute (IDIPHISA), and FaDu (human pharyngeal carcinoma) cell line acquired from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DMSZ-ACC 784). Cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher), 2 mM glutamine, and antibiotics. Cultures were incubated at 37°C in humidified atmosphere, trypsinized and passaged once a week without exceeding 10 passages. The cultures were tested for mycoplasma (Mycoplasma Gel Detection Kit biotools B&M Labs) routinely.

Cytotoxicity evaluation

Eribulin was used as its commercial form (HALAVEN®), provided by Puerta de Hierro Pharmacy Department. For the evaluation of cytotoxicity and radiosensitivity we used the crystal violet method, a colorimetric cell density assay whose results have been shown to be comparable to those obtained by the more traditional clonogenic assay [20, 21]. As described previously [22], twenty-four hours after plating, cell lines were exposed to different eribulin concentrations. Three replicates were used for each tested condition. After 10 days of incubation, cells were fixed with 1% glutaraldehyde for 10 min, washed in PBS (phosphate-buffered saline) and stained with 1 ml of 0.1% crystal violet solution for 30 min. Wells were rinsed in distilled water and left to dry overnight. For quantitation, crystal violet stain was extracted with 10% acetic acid and the intensity of colour was measured by Multiskan EX, Thermo Scientific Spectrophotometer. The absorbance was read at 590 nm. The surviving fraction was determined by dividing the treated wells absorbance by the control wells.

For the evaluation of chemosensitivity, data from at least three independent experiments performed in triplicate were grouped for each dose and fitted to the log (inhibitor) *vs.* response model of the GraphPad Prism Software. The IC10 and IC50 defined as the eribulin concentrations at which 90% and 50% of cells survive, respectively, were obtained by interpolation of the dose-response curves in both cell lines.

Eribulin concentrations corresponding to IC50 and two additional doses above and below this value were used for the evaluation of radiosensitivity. In HeLa cells, the concentrations tested were 0.75, 1.5 and 3 nM. Only two concentrations were used in FaDu cells, 0.75 and 0.3 nM, since the high percentage of cell death at higher dose produced inconsistent results. Twenty-four hours

after planting, eribulin was added to selected wells at the desired concentration. Cells were irradiated at clinically relevant doses ranging from 2 to 6 Gy after 24 h of drug exposure in a Varian® 6-MV linear accelerator with a source-to-target distance of 100 cm at a dose/rate of 400 cGy/min. The gantry was positioned at 180º and the dose was estimated at the bottom of the multiwell plates. The medium was replaced with fresh medium immediately after irradiation and cells were allowed to grow up for 10 days, after which the cell survival was evaluated as described above. Surviving fraction was calculated by dividing the absorbance of irradiated wells by the control wells. Data from at least three triplicate independent experiments were grouped for each dose and adjusted to the linear-quadratic model:

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

using a least squares algorithm performed with Prism software (GraphPad Inc Software). The α, β, and surviving fraction at 2 Gy (SF2) parameters were obtained from the fitted data. To quantify the magnitude of radiosensitization we calculated SER (sensitizer enhancement ratio) by dividing the radiation dose (Gy) in the absence of drug by the dose (Gy) for radiation plus drug at the 50% survival level. We calculated the combination index (CI) described previously [23], to determine if the interaction between radiation and drug is synergistic according to the formula:

$$CI = \frac{d1}{Dx1} + \frac{d2}{Dx2}$$

Dx1: dose of radiation alone at which 50% of cells survive; **d1**: dose of radiation in eribulin treated cells at which 50% of cells survive; **Dx2**: Concentration of eribulin alone at which 50% of cells survive; **d2**: Concentration of eribulin in irradiated cells at which 50% of cells survive.

A CI bellow 1 implies supra-additive effect and a true synergism between the two agents.

Cell cycle analysis

Cell-cycle distributions from cultures were assessed by propidium iodide (Sigma-Aldrich) staining and flow cytometry. Exponentially growing HeLa and FaDu cells were exposed to 1.5 nM and 0.75 nM eribulin, respectively, which correspond to around the 24 h exposure IC50 concentrations for each cell line. The analysis of the intracellular DNA content was performed 8, 24 and 48 h after drug exposure and the percentage of cells in G0/G1, S and G2/M phases were determined. Cells were trypsinized, washed twice in PBS, fixed in cold 70% ethanol for at least 15min at 4°C, and washed twice with PBS. Samples were re-suspended in PBS containing 10µg/ml propidium iodide and 0.5µg/ml Rnase (Sigma-Aldrich). DNA content was analysed by flow cytometry in a FACS-can (Becton-Dickinson) collecting a minimum of 20,000 events. After excluding doublets and triplets, histograms of the propidium iodide fluorescence (FL2A) were obtained. The resulting data were fitted using the Modfit LT v.3.0 software (Verity Software House, Inc).

Apoptosis analysis

Annexin-V labelling method

Apoptotic cells were evaluated by the FITC-conjugated annexin V staining method (BD pharmingen). The fraction of apoptotic cells was evaluated for untreated and treated cells exposed to eribulin for 24 h. Eribulin concentrations were selected according to the chemosensitivity of each cell line. The concentrations tested were 3 and 1.5 nM for HeLa cells, and 0.75 nM for FaDu cells. Briefly, cells were trypsinized, washed with PBS, and re-suspended in binding buffer with 0.5 µgr/ml FITC-conjugated annexin V. After 20 min incubation at room temperature in the dark, cells were stained with 1 µg/mL propidium iodide and cultured for 10 min prior to flow cytometry analysis in FACS-can (Becton-Dickinson). The percentage of apoptotic cells was obtained from a bivariate histogram of annexin V labelled-cells *vs*. DNA content. Control, eribulin alone, radiation alone and radiation plus eribulin groups were tested for apoptosis. The irradiation doses used were 4 and 6 Gy.

Immunofluorescence: TUNEL assay

To detect apoptotic cells we also evaluated DNA fragmentation, an indicator of apoptosis by TUNEL assay using the DeadEnd™ Fluorometric TUNEL System kit (Promega G3250). Cells were cultured in 24-well plates. After 24 h, eribulin was added at 3 nM and 1.5 nM or 0.75 nM for HeLa and FaDu, respectively. To determine the radiation-induced apoptosis, cells were irradiated after

24 h of drug incubation. Cells were washed, permeabilized, fixed and incubated with equilibration buffer. Fifty µl of TdT (Terminal Deoxynucleotidyl Transferase) reaction mix was added. To-Pro (Thermo Fisher) was used to stain nuclei (Blue). Confocal microscopy was used to detect green fluorescence of apoptotic cells. Images were collected with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using 10× and 20× HCX PL APO. Image processing was performed with the ASF Leica software.

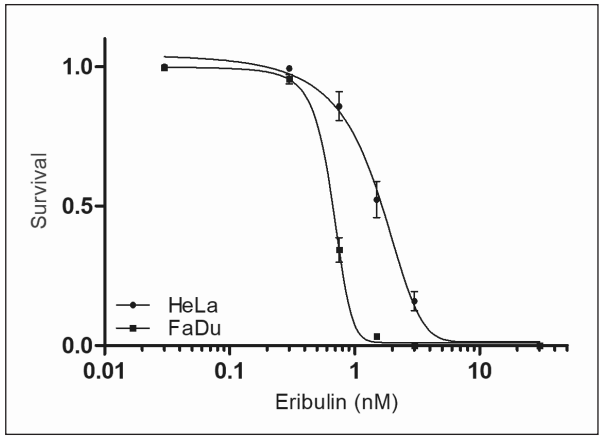

Figure 1. Dose-response curves after 24 h eribulin exposure time for Hela and FaDu cell lines obtained after fitting individual data to the log (inhibitor) *vs.* response model of the GraphPad Prism Software. Each point represents the mean of at least three independent experiments performed in triplicate (± SEM)

Statistics

The data analysis was carried out with SPSS v.19 (IBM). T-Student analysis for paired samples was used to compare means of different groups. Values of $p < 0.05$ were considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

Results

As shown in Figure 1, the growth of both lines was inhibited by eribulin within the nanomolar range. Mean IC50s and IC10s were 1.58 ± 0.21 nM and 0.7 ± 0.16 nM and 0.7 ± 0.05 nM and 0.27 ± 0.03 nM for HeLa and FaDu cells, respectively.

Eribulin enhanced radiation response after 24h exposure in both cell lines (Fig. 2, Tab. 1). In cells treated with eribulin plus irradiation, SF2 values decreased as compared with radiation alone from 0.83 to 0.51 and 0.92 to 0.59 in HeLa and FaDu cells, respectively (Tab. 1). The SER parameter increased in tested cell lines, in a dose-dependent manner reaching a maximum value of 2.71 and 2.32 in HeLa and FaDu cells respectively (Tab. 1).

Pre-treatment with eribulin significantly increased radioinduced cell death at different doses in both cell lines. Cell survival was lower in HeLa cells treated with 1.5 nM eribulin plus irradiation compared to those treated with radiation alone at 2 Gy (59% *vs.* 81%; $p = 0.004$), 4 Gy (36% *vs.* 68%; $p = 0.0001$); and 6 Gy (25% *vs.* 42%; $p < 0.0001$).

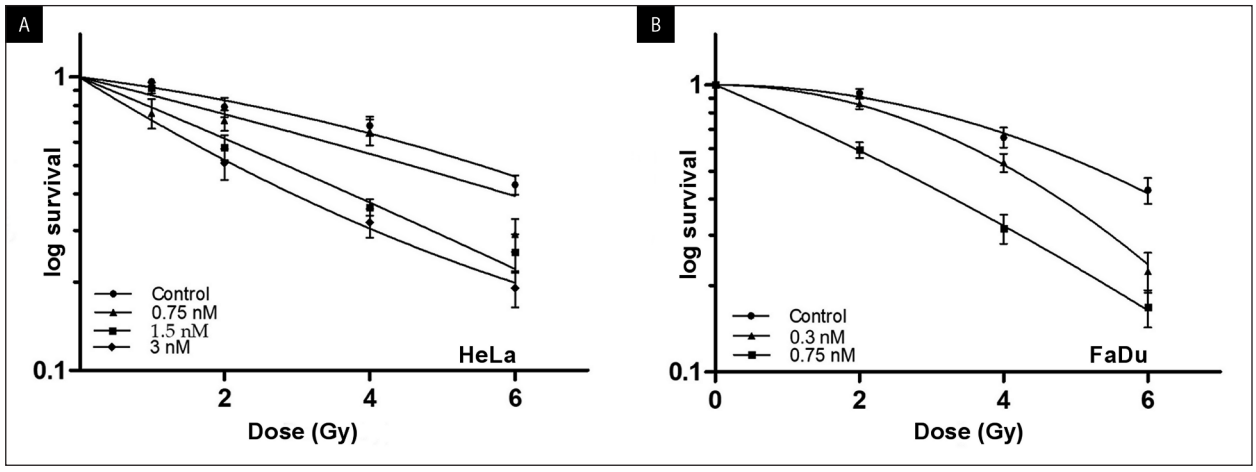

Figure 2. Radiation cell survival curves obtained after adjusting individual data to a linear quadratic model for control (radiation alone) and irradiated HeLa (**A**) and FaDu (**B**) cells pretreated with eribulin. X-axis represents cell survival on a logarithmic scale after normalizing for the effect of eribulin. Each point represents the mean of at least three independent experiments performed in triplicate ± standard error of the mean (SEM)

Table 1. Radiation survival curve parameters (alpha, beta and SF2) for control and treated cells with eribulin

	Alpha (95% CI)	Beta (95% CI)	SF2	SER
HeLa				
Control	0.07 (0.008–0.13)	0.009 (–0.004–0.02)	0.83	
0.75 nM	0.14 (0.04–0.23)	0.003 (–0.01–0.02)	0.74	1.27
1.5 nM	0.24 (0.15–0.31)	0.003 (–0.02–0.02)	0.61	2.05
3 nM	0.35 (0.25–0.45)	–0.01 (–0.03–0.008)	0.51	2.71
FaDu				
Control	2.19×10^{-16}	0.02 (0.01–0.04)	0.92	
0.3 nM	$1{,}69 \times 10^{-16}$	0.04 (0.02–0.05)	0.85	1.41
0.75 nM	0.25 (0.16–0.32)	0.009 (0–0.03)	0.59	2.32

SF2 — surviving fraction at 2 Gy; SER — sensitizer enhancement ratio; CI — confidence interval

There were also a statistically significant lower survival in FaDu cells treated with 0.75 nM eribulin plus irradiation compared to those treated with radiation alone at 2 Gy (59% *vs.* 93%; $p < 0.0001$); 4 Gy (31% *vs.* 66%; $p < 0.0001$); and 6 Gy (16% *vs.* 34%; $p = 0.02$).

In chemosensitivity assays there were a leftward shift of the eribulin dose-response curve as the irradiation dose was increased. The IC50 of eribulin decreased when combined with various doses of irradiation in both, HeLa (1.5 nM for eribulin alone *vs.* 0.9 nM, 0.7 nM and 0.5 nM for the combination with 2, 4 and 6 Gy, respectively) and FaDu cells (0.66 nM for eribulin alone *vs.* 0.58 nM, 0.48 nM and 0.22 nM for the combination with 2, 4 and 6 Gy, respectively).

The following parameters were used for the calculations of CI in Hela cells: Dx1 = 5.69 Gy; d1 = 2.78 Gy; Dx2 = 1.5 nM; and d2 = 0.5 nM. In FaDu cells the parameters were: Dx1 = 5.87 Gy; d1 = 2.53 Gy; Dx2 = 0.66 nM; and d2 = 0.22 nM. The calculated CI for HeLa and FaDu cells was 0.82 and 0.76, respectively, indicating a true synergistic effect.

As shown in Figure 3, 1.5 nM and 0.75 nM eribulin for HeLa and FaDu cells, respectively, induced a significant arrest in the G2/M phase of the cell cycle at 24 or 48 h after exposure to the drug. In HeLa cells, the mean percentage of cells in G2/M phase increased gradually from 13.93% of control up to 31.57% ($p = 0.03$) and 61.44% ($p = 0.01$) after 24h and 48 h of drug exposure, respectively. In FaDu cells, the mean percentage of cells in G2/M phase increased gradually from 10.76% of control up to 56.61% ($p = 0.003$) and 61.76% ($p = 0.001$) after 24 and 48 h of drug exposure, respectively.

As shown in Figure 4, eribulin induced apoptosis was evaluated by annexin V labelling in both cell lines. A statistically significant induction of apoptosis was observed with eribulin as compared with untreated cells at doses of 1.5 nM ($p = 0.003$) and 3 nM ($p = 0.0006$) in HeLa cells and 0.75 nM ($p = 0.007$) in FaDu cells with respect to control. In cell cultures that received combined treatment with eribulin and radiation, there were also an increase in apoptosis with respect to the untreated cells, which reached 45.2% and 54.7% at 1.5 nM + 6 Gy and 3 nM + 6 Gy, respectively, in HeLa cells; and 8.3% at 0.75 nM + 6 Gy in FaDu cells. Therefore, there was a 5- to 7-fold increase in apoptosis in the samples that received both treatments with respect to untreated cells. The increase in apoptosis in the samples treated with the drug and radiation was mainly due to an additive effect of both agents, with no statistically significant differences between eribulin alone and the combined treatment. However, in the HeLa cell line treated with eribulin 3 nM + 6 Gy, the percentage of apoptosis was 8.93% higher than the sum of both agents separately, suggesting a potentiation of radiation-induced apoptosis in the presence of eribulin.

We also conducted a TUNEL-assay to qualitatively confirm the induction of apoptosis by eribulin. As shown in Figure 5, the results confirm those obtained with the annexin V labelling assay.

Discussion

Previous preclinical studies have shown a significant cytotoxic activity of eribulin in the nanomolar range in several tumour cell lines [3, 24–26]. In this study, IC50 ranged from 1.58 nM to 0.7 nM

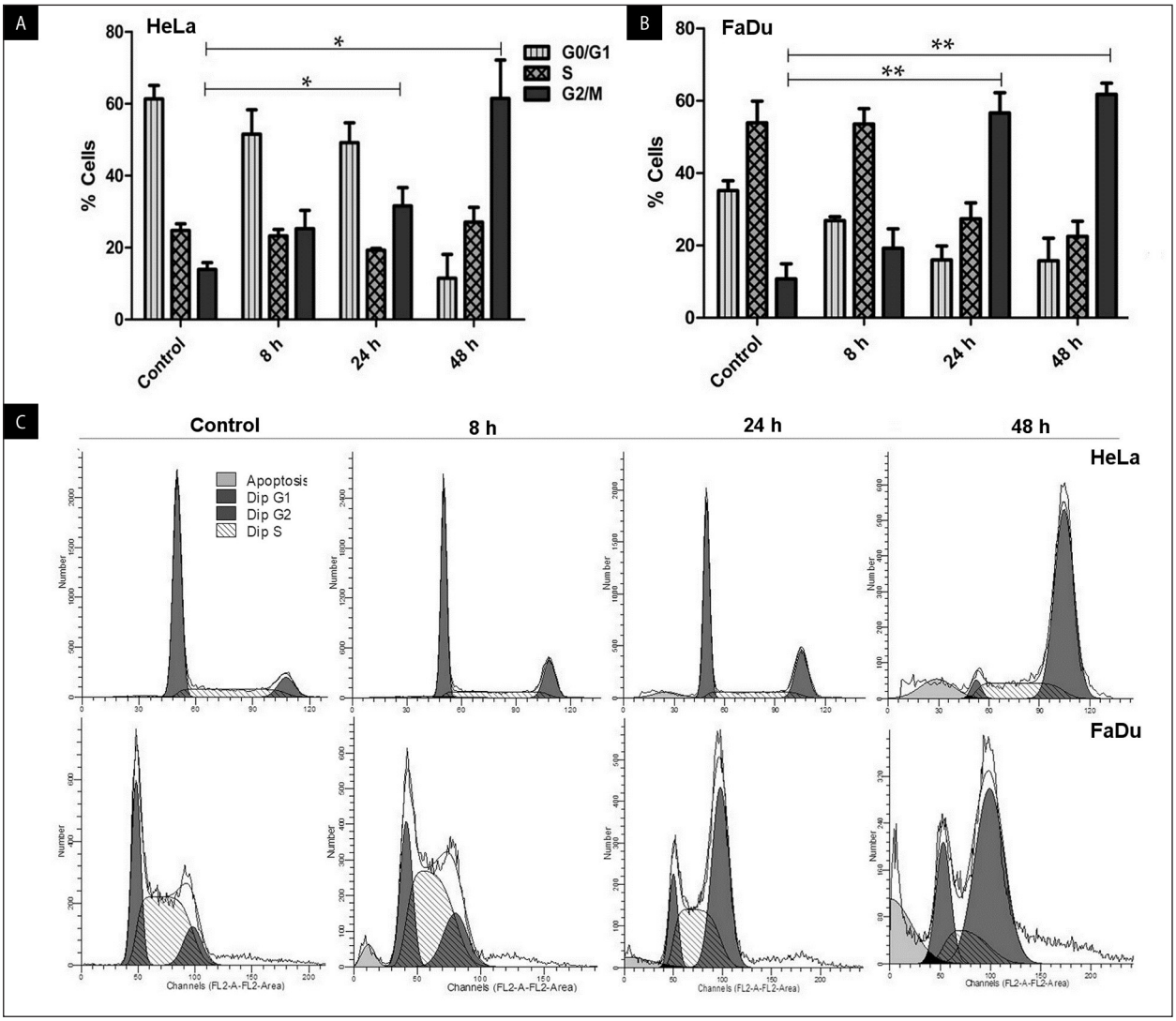

Figure 3. Time course of cell cycle changes after 1.5 nM and 0.75 nM eribulin exposure for Hela (**A**) and FaDu (**B**) cells respectively. Bars represent the mean percentage of cells in each phase of cell cycle of at least three independent experiments for each cell line ±SEM. c, Cell cycle analysis by propidium iodide stained cells showing the changes over a 48 h period of time. Graphs represent histograms of FL2A (X axis, DNA content). Data were fitted using the Modfit LT v.3.0 software (Verity Software House, Inc) to calculate the percentage of cells in each phase of the cell cycle. The most representative experiment for each cell line is shown

after 24 h exposure time which is within the values reported in the literature [3, 24–26].

We found a significant increase in radioinduced cell death after combined treatment with eribulin plus irradiation as compared to radiation alone ranging from 17-32% and 18-35% for HeLa and FaDu cells, respectively. Our results are in accordance with those found in two prior publications [24, 27]. Helfrich et al. [24] reported an increase of cell growth inhibition up to 29% and 37% in irradiated H446 and H841 lung cancer cell lines pretreated with 0.625 nM eribulin, respectively, compared to those treated with radiation alone. Miki et al. [27] also showed a remarkable increase of cell death after concurrent treatment with 0.1-5 nM eribulin and 8 Gy radiation in U87MG and U251MG glioblastoma cell lines.

The present study demonstrates the in vitro radiosensitizing effect of eribulin in the two human cell lines tested in a dose-dependent manner. Our results show that radiosensitization occurs in a range of clinically relevant irradiation doses, with an increase in radiosensitivity in the presence of the drug more than 2 times higher than with radiation alone. A CI of less than 1 indicates that this effect is supraadditive, a term defined by Steel

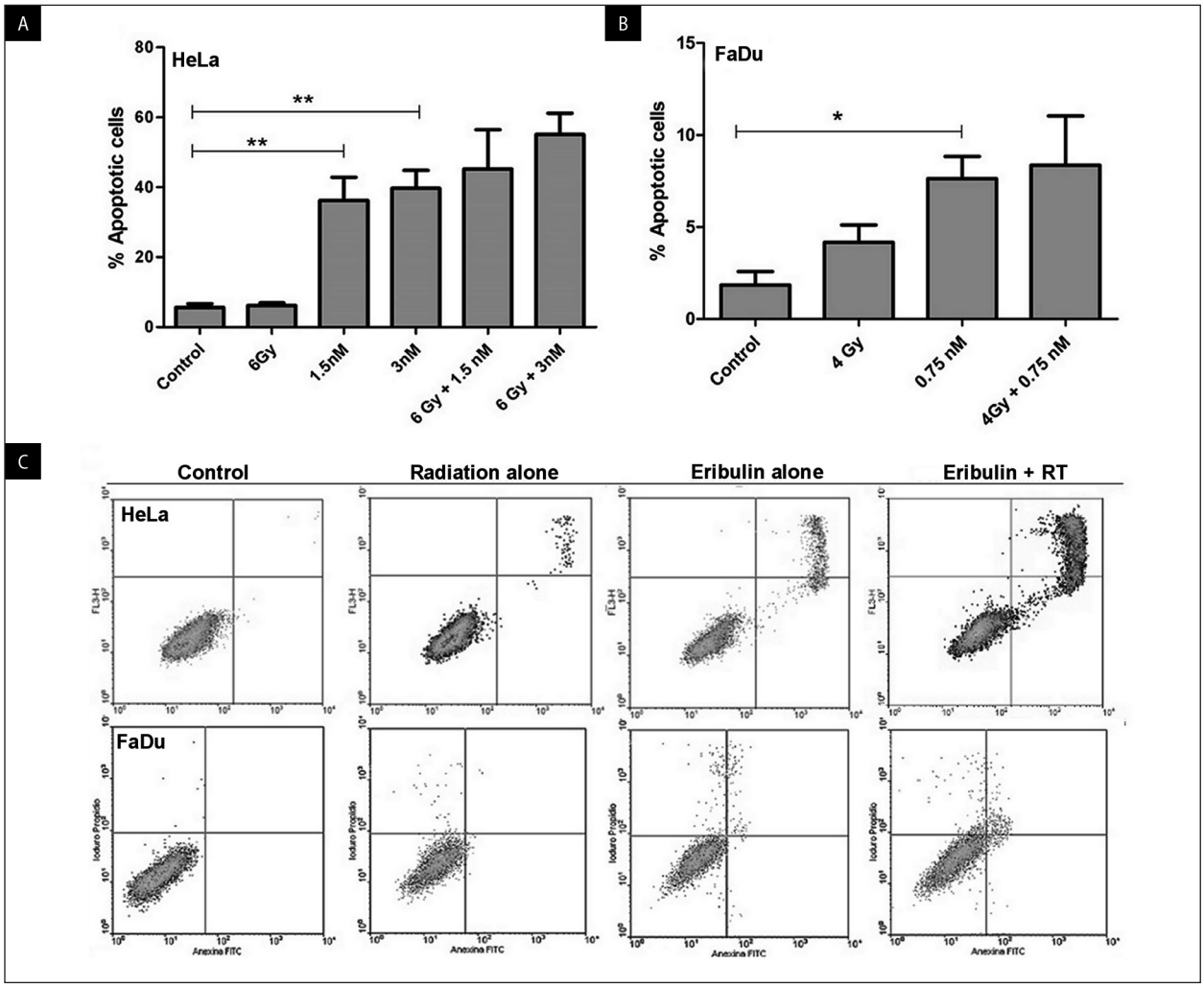

Figure 4. Mean percentage of apoptotic HeLa (**A**) and FaDu (**B**) cells for control, radiation alone, eribulin alone or combined treatment. Bars represent the mean percentage of apoptotic cells of at least three independent experiments for each cell line ± SEM; **C.** Bivariate histograms of annexin V (X axis) *vs*. propidium iodide (Y axis) generated by flow cytometry. Cells were plated 48 h prior to irradiation (6 Gy for HeLa, 4 Gy for FaDu). Twenty-four hours before irradiation, cells were pre-treated or not with eribulin (3 nM for Hela, 0.75 nM for FaDu). After 24 h of incubation, apoptosis was evaluated. Figures in each histogram represent the percentage of early (lower right quadrant) and late (upper right quadrant) apoptosis. The most representative experiment is shown *$p < 0.05$, **$p < 0.005$

et al. [28] which implies that the effect of the combination of the two agents is greater than the mere sum of both agents separately. Our results agree with those previously published regarding the radiosensitizing potential of eribulin. Helfrich et al. [24] found a remarkable enhancement of radiation response by eribulin in small cell lung cancer cell lines; clonogenic survival of H841 cells was significantly decreased by the combination of 0.31 nM eribulin with 2–4 Gy radiation as compared to radiation or eribulin alone. Miki et al. [27] also showed a reduction in clonogenic survival after concurrent treatment with eribulin and radiation in respect to radiation alone in U87MG glioblastoma cells.

Although extrapolation of results from basic experimentation to the clinic should be done with caution, the eribulin concentrations required to radiosensitization in our study are in the nanomolar range which can be reached in the plasma of patients treated with eribulin. A phase I study [6] reported that the plasma concentration of eribulin, administered at the maximum tolerable dose (1.4 mg/m^2), remained above 1 ng/mL up to 4 days after its intravenous administration. The prolonged half-life of the drug provides sustained plasma concentrations above the concentrations needed to attain cytotoxicity for several days, which could result in a treatment benefit with daily radiotherapy fractions.

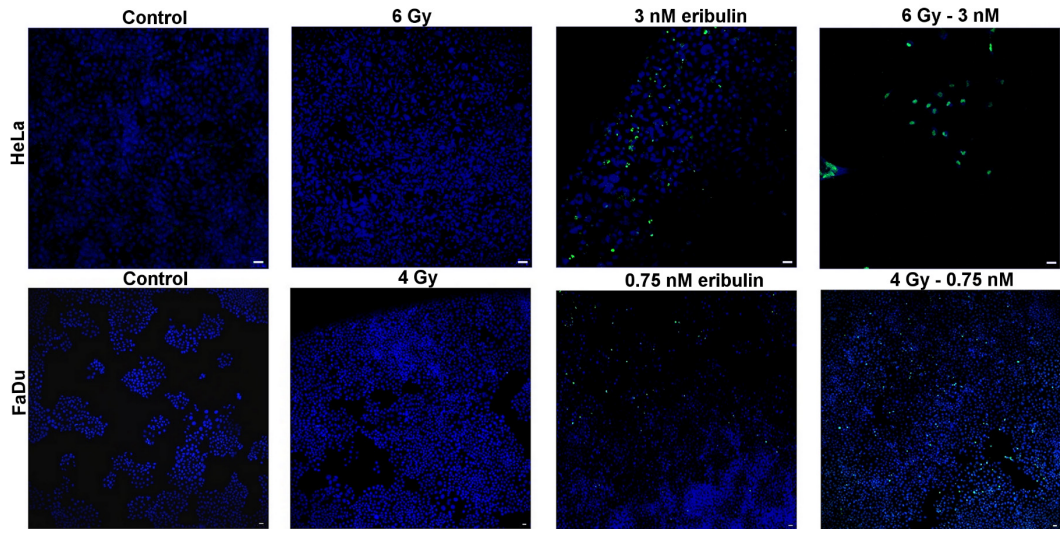

Figure 5. Representative microphotographs of TUNEL-assay showing the merge of DNA staining (blue nuclei) and apoptosis by TUNEL detection (green nuclei). Cells were plated 48 h prior to irradiation (6 Gy for HeLa, 4 Gy for FaDu). Twenty-four hours before irradiation, cells were pre-treated or not with eribulin at the indicated concentrations. Cells were incubated for 48 h prior to the analysis. Bars represent 50 μm

The p53 tumour suppressor protein seems to be a critical determinant in the radiosensitizing effect of other agents as Palbociclib [29]; however, HeLa and FaDu cells have low expression and non-functional p53, respectively, so we suggest that eribulin exerts its radiosensitizing effect independent of p53-status.

To identify the exact mechanisms of the interaction between radiation and drug is challenging. A modification of radiation cell survival curves in the presence of drug is key to demonstrate a true radiosensitization effect. In this study we show a clear modification of radiation cell survival curve shape with an increase in α parameter and a decrease in β parameter (Fig. 2, Tab. 1). These changes could suggest drug interference with radiation-induced DNA damage repair mechanisms. H2AX phosphorylation is a marker of both DNA damage and repair. Miki et al. [27] reported an over-expression of histone H2AX evident after combined treatment in glioma cells respect to each agent separately.

It is well known that G2/M is the most radiosensitive cell cycle phase. Our results show that eribulin induces a marked accumulation of cells in the G2/M phase of the cell cycle in both cell lines. Eribulin-induced G2/M arrest has been previously reported by other authors [3, 24, 27]. After 24 h in vitro exposure to 1.25 nM eribulin, Helfrich et al. [24] showed a significant G2/M arrest ranged from 28–34% in a panel of four small cell lung cancer cell lines. These results are very similar to those obtained in our work.

Recent data [24] confirm that eribulin activates the apoptotic caspases-3/7 in all cell lines tested and enhances radiation-induced apoptosis. In accordance with these findings, our results demonstrate that eribulin induces apoptosis in HeLa and FaDu cells (Fig. 4, 5). Furthermore, we found that in HeLa cells treated with 3 nM eribulin plus irradiation, the apoptosis percentage is 8.93% higher than the sum of both agents separately, which shows that this drug induces an increase in radiation-induced apoptosis. Although the difference does not reach statistical significance due to the small sample size, this effect is considered relevant in the mechanisms of action of this drug. A similar finding was reported in another study with docetaxel whose radiosensitization mechanisms are similar to eribulin, in which an increased radiation-induced apoptosis is described with high drug concentrations [30].

Based on the results of our study and other works, we can conclude that drug-induced cell cycle changes constitute the main mechanism of radiosensitization by eribulin. We also suggest that radiation-induced apoptosis is involved in the mechanisms of eribulin radiosensitization depending on the concentration of the drug and the type of cell line.

Previous studies [24, 27] including ours, show the radiosensitizing effect of eribulin in several pre-

clinical tumour models which suggest the universal character of this drug as a radiosensitizer.

Conclusions

We demonstrate that eribulin exerts a true in vitro radiosensitizing effect through a synergistic interaction between radiation and drug. In our study the main mechanism of radiosensitization is the cell cycle arrest in G2/M phase induced by eribulin. The enhancement radiation-induced apoptosis could be an additional mechanism of radiosensitization. Further studies are needed to assess the clinical benefit of the combination of eribulin and radiotherapy as a novel therapeutic strategy for cancer.

Conflicts of interest

All authors declare that they have no conflict of interest.

Funding

None declared.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Data and material supporting this work are available and comply with field standards.

Author contributions

Data collection — R.B., study design — J.R., investigation — R.B., J.R., R.C., S.R., M.J.C., cell irradiation — PS, analysis — RB, JR, writing: original draft — R.B., writing: review and editing — J.R. All authors read and approved the final manuscript.

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