Research Square

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# In Vitro Radiosensitization by Eribulin in Human Cancer Cell Lines

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# Abstract

**Objectives:** To determine the radiosensitizing properties of eribulin and to establish the potential mechanisms of radiosensitization in cervical (HeLa) and pharyngeal (FaDu) cancer cell lines.

**Material 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 in both cell lines. The surviving fraction at 2Gy (SF2) and the sensitizer enhancement ratio (SER) were calculated from radiation cell survival curves in 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 analysis. Apoptotic cells were detected by FITC-conjugated annexin-V labelling and flow cytometry and by immunofluorescence with TUNEL-assay.

**Results:** Mean IC50s and IC10s were 1.58nM and 0.7nM and 0.7nM and 0.27nM for HeLa and FaDu cells respectively. Radiosensitization was observed in both lines tested 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 3nM eribulin enhanced radiation induced apoptosis in Hela cells.

**Conclusions**: Eribulin shows a true in vitro radiosensitizing effect in tested cell lines by inducing significant G2/M phase arrest and apoptosis changes. Further studies are needed to assess the clinical benefits of concurrent eribulin and radiotherapy as a novel therapeutic strategy for cancer.


# 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. 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 dynamic's 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/m2 [6]. Eribulin exhibits a manageable toxicity profile, especially when administered at 1.4 mg/m2 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 justify the use 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.

Prior studies have demonstrated in vitro radiosensitizing effect by eribulin in small cell lung cancer and glioma cell lines. G2/M phase arrest and apoptosis have been suggested as potential mechanisms of radiosensitization [16, 17].

Currently, the standard treatment for head and neck and uterine cervix cancer is cisplatin-based chemoradiotherapy (CRT). 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 [18, 19, 20]. Eribulin, as an agent that induces arrest in more radiosensitive phases of the cell cycle, has potential radiosensitizing effects and its study in head and neck and cervical cancer cell lines could justify the initiation of clinical studies in these tumours.

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-Segovia de Arana Institute for Health Research (IDIPHISA), and FaDu (human pharyngeal carcinoma) cell line acquired from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DMSZ-ACC 784). All cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cultures were incubated at 37°C in humidified atmosphere with 5% CO2, trypsinized and passaged once a week.

# Cytotoxicity evaluation

Eribulin was used as its commercial form (HALAVEN®), provided by Puerta de Hierro Pharmacy Department. Crystal violet method, a colorimetric cell density assay described previously [21], was used to determine cytotoxicity and radiosensitivity of eribulin in 24-well plates. Twenty-four hours after planting, cell lines were exposed to different eribulin concentrations. Three replicates were used for each tested condition. After 7–10 days of incubation, cells were fixed with 1% glutaraldehyde (HeLa) or 70% ethanol (FaDu) for 10 min, washed in PBS and stained with 1ml of 0.1% crystal violet solution for 30 min. Wells were rinsed in distilled water and left to dry overnight. For quantitation, stained crystal violet 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. The IC10 and IC50 were defined

as the eribulin concentrations at which 90% and 50% of cells survive, respectively. These values were obtained by interpolation of the dose-response curves in both cell lines.

For the evaluation of radiosensitivity, the eribulin concentrations tested were 3nM, 1.5nM and 0.3nM for HeLa and 0.75nM and 0.3nM for FaDu cells. Cells were planted in 24-well plates. After 24h, eribulin was added to the medium to a desired final concentration. Cells were irradiated from 2Gy to 6Gy after 24h incubation with the drug, with a Varian® 6-MV linear accelerator. The medium was replaced with fresh medium immediately after irradiation and cells were allowed to grow up for 7–10 days, after which the cytotoxicity 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 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 with software Prism (GraphPad Software). Parameters of α, β, and surviving fraction at 2Gy (SF2) were obtained from the fitted data. To quantify the magnitude of radiosensitization we calculated SER (sensitizer enhancement ratio) dividing the radiation dose (Gy) in absence of drug by the dose (Gy) for radiation plus drug (normalized for drug toxicity) at the 50% survival level. We calculated the combination index (CI) described previously [22], to determine if the interaction between irradiation and drug is synergistic according to the formula:

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

Dx1: Dose of agent 1 (irradiation) at which 50% of cells survive (D50)
d1: Dose of agent 1 (irradiation) plus agent 2 (eribulin) at which 50% of cells survive (D50)

Dx2: Dose of agent 2 (eribulin) at which 50% of cells survive (IC50)

d2: Dose of agent 2 (eribulin) plus agent 1 (irradiation) at which 50% of cells survive (IC50)

A CI bellows 1 implies supra-additive effect and a true synergism.

# Cell cycle analysis

Cell-cycle distributions from cultures were assessed by propidium iodide staining and flow-cytometry analysis. Exponentially growing HeLa and FaDu cells were exposed to 1.5nM and 0.75nM eribulin respectively, which correspond to the 24h exposure IC50 concentrations for each cell line. The analysis of the intracellular DNA content was performed 8h, 24h and 48h 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. 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 red fluorescence (FL2A, propidium iodide) 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 FITC-conjugated annexin V staining method. The fraction of apoptotic cells was evaluated for untreated and treated cells exposed to eribulin for 24h. Exponentially growing Hela and FaDu cells were incubated with 3nM and 1.5nM or 0.75nM of eribulin respectively. Cells were trypsinized, washed with PBS, and re-suspended in binding buffer with 0.5µgr/ml FITC-conjugated annexin V. After 20 min incubation at room temperature in the dark, cells were stained with 1µg/ml propidium iodide and cultured for 10 min prior flow cytometry analysis in FACS-can (Becton-Dickinson). The percentage of apoptotic cells was obtained from a bivariate histogram of annexin V labelled-cells (green fluorescence) versus DNA content (red fluorescence). To determine if drug treatment increases the radiation-induced apoptosis, we analysed the effect of combined eribulin, at doses described above with 6Gy or 4Gy irradiation for Hela and FaDu cells respectively.

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 24h, eribulin was added at 3nM and 1.5nM or 0.75nM for HeLa and FaDu respectively. To determine the radiation-induced apoptosis, cells were irradiated after 24h of drug incubation. The medium was replaced with fresh medium immediately after irradiation and cells were left to grow for 48h. HeLa and FaDu Cells were fixed with 4% paraformaldehyde (4%PF) or 70% ethanol respectively for 10min at room temperature. Cells were washed and permeabilized with Proteinase K (20ug/ml) for 10 min. After washing again, cells were fixed with 4%PF for 5 min and incubated with equilibration buffer for 10 min at room temperature in a humidified chamber. Fifty µl of TdT reaction mix containing: equilibration buffer, nucleotide mix and rTdT enzyme, was added. Plates were incubated at 37°C avoiding exposure to light for 60min. The reaction was terminated by adding 2XSSC (Saline sodium citrate) for 15min. Cells were washed with PBS and nuclei were stained with To-Pro (Thermo Fisher) to Stain Nuclei (Blue). Confocal microscopy was used to detect green fluorescence of apoptotic cells (the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3´-OH DNA ends using Terminal Deoxynucleotidyl Transferase (TdT)).

Images were collected with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using 10x and 20x HCX PL APO. The two channels were acquired sequentially to avoid cross-talk between them. The following excitation and emission parameters used were: (488 nm, 500- – 540 nm) for Tunel signal, and (633 nm, 645- – 750 nm) for TO-PRO 3. The gains were adjusted for each channel to avoid saturation in pixels intensity. Laser intensity and detector sensitivity settings remained constant for all image acquisitions for experimental replicas. Image processing was performed with the ASF Leica software.

# Statistics

The data analysis was carried out with statistical program 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 (± SEM).

# Results

As shown Fig. 1, the growth of both lines was inhibited by eribulin within the nanomolar range. Mean IC50s and IC10s were 1.58 ± 0.21nM and 0.7 ± 0.16nM and 0.7 ± 0.05nM and 0.27 ± 0.03nM for HeLa and FaDu cells respectively for 24h continuous exposure time. Eribulin had greater anti-proliferative activity in FaDu cell line.

Eribulin enhanced radiation response after 24h exposure in both cell lines (Fig. 1, Table 1). The corresponding values for SF2 after drug incubation as compared to the control for HeLa and FaDu cell lines decreased from 0.83 to 0.51 and 0.92 to 0.59, respectively. 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.

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

| | | Alpha (IC95%) | Beta (IC95%) | 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 | 0 | 0.02 (0.01–0.04) | 0.92 | |
| | 0.3 nM | 0 | 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 2Gy; SER, sensitizer enhancement ratio; IC, confidence interval. | | | | | |

Pre-treatment with eribulin significantly increased radioinduced cell death at different concentrations in both cell lines. In HeLa cells, there was a lower cell survival at 2, 4 and 6Gy irradiation combined with eribulin at 1.5nM ($p = 0.004$, $p = 0.0001$ and $p = 0.002$, respectively) and 3nM ($p = 0.005$, $p = 0.0001$ and $p < 0.0001$, respectively) as compared to irradiation alone. In FaDu cell line there was also a statistically significant increase in growth inhibition of irradiated cells after exposure to 0.75nM eribulin, at doses of 2Gy ($p < 0.0001$), 4Gy ($p < 0.0001$) and 6Gy ($p = 0.02$).

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 cells (1.5nM for eribulin alone versus 0.9nM, 0.7nM and 0.5nM for the combination with 2, 4 and 6Gy, respectively) and in FaDu cells (0.66nM for eribulin alone versus 0.58nM, 0.48nM and 0.22nM for the combination with 2, 4 and 6Gy, respectively).

The calculated CI for HeLa and FaDu cells was 0.82 and 0.76 respectively, indicating a true synergistic effect.

As shown in Fig. 2, eribulin induced a significant arrest in the G2/M phase of the cell cycle at 24 or 48h after exposure to the drug. In HeLa and FaDu cells, the mean percentage of cells in G2/M phase increased gradually from 13.93% and 10.76% of control up to 31.57% ($p = 0.03$) and 61.44% ($p = 0.01$) and 56.61% ($p = 0.003$) and 61.76% ($p = 0.001$) after 24h and 48h exposure times respectively.

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

Apoptosis induced by eribulin was confirmed by TUNEL assay (Fig. 4)

# Discussion

Previous preclinical studies have shown a significant cytotoxic eribulin activity in the nanomolar range in several tumour cell lines. In this study, IC50 ranged from 1.58nM to 0.7nM after 24h exposure time which is within the values reported in the literature [3, 16, 23, 24].

We demonstrate a significant extra cell killing after irradiation combined with eribulin at IC50 concentrations as compared with the irradiation alone ranging from 17–32% and 18–34% for HeLa and FaDu cells respectively. Our results are in accordance with those found in a recent study [16] where they reported an increase in growth inhibition of 29% and 37% for irradiated H446 and H841 small lung cancer cell lines respectively exposed to 0.625nM eribulin.

The present study demonstrates the in vitro radiosensitizing effect of eribulin in the two human cell lines tested in a dose-dependent manner. We show an enhancement of radiation response in pretreated cells

with a SER up to 2.71 and 2.32 for HeLa and FaDu cells respectively which means that combined treatment is more than twice as effective as irradiation alone. To further examine the eribulin induced radiosensitization we have calculated the extra cell killing of combined treatment at a clinically relevant fraction dose of 2Gy. At the IC50 concentrations, the ratio of the SF2 between untreated and treated cells were 1.36 and 1.56 for Hela and FaDu respectively, which correspond to an increase of 36% and 56% in tumour cell death after each fraction of 2Gy. This effect would be magnified in a conventional radiotherapy treatment of 25–30 fractions and could have a significant effect on local tumour control. Our results demonstrate a true synergistic interaction between irradiation and eribulin with a combination index of 0.82 and 0.76 for Hela and FaDu cells respectively, both below 1. We can affirm that radiosensitizing effect is even more pronounced in head and neck line. Our results agree with those previously published regarding the radiosensitizing potential of eribulin. Helfrich et al. [16] found a remarkable enhancement of irradiation response by eribulin in lung cancer cell lines. The same results were obtained by Miki et al. [17] using U87MG glioma cells in a clonogenic assay.

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.The plasma Cmax after eribulin i.v. bolus at the maximum tolerated dose (MTD:1.4 mg/m2) is 1μM, higher than the concentration required for radiosensitization [6]. This indirect data suggests that the use of eribulin in radiochemotherapy regimens is clinically feasible and should be evaluated in clinical trials. Moreover, the prolonged half-life of the drug provides sustained plasma concentrations above the concentrations needed to attain cytotoxicity for one week, which could result in a treatment benefit with daily radiotherapy fractions.

To identify the exact mechanisms of the interaction between irradiation and drug is challenging. A modification of radiation cell survival curves in 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. 1, Table 1). These changes could suggest drug interference with radiation-induced DNA damage repair mechanisms. Prior study [17], reported an over-expression of histone H2AX, a marker of DNA damage, 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. Our work is also supported by others that also demonstrate an increase in G2/M phase cells after drug treatment [3, 16, 17].

As shown in Fig. 2, the G2/M arrest is greater in the FaDu cell line at 24h exposure time, which could explain the greater radiosensitizing effect in this line as compared to HeLa cells. Based on the results of our study we can conclude that drug-induced cell cycle changes constitute the main mechanism of radiosensitization by eribulin.

Our results demonstrate that eribulin induces marked apoptosis in HeLa and FaDu cells (Fig. 3,4). Furthermore, we found that in HeLa cells treated with 3nM eribulin and 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 other study with docetaxel whose radiosensitization mechanisms are similar to eribulin, in which an increased radiation-induced apoptosis is described with high drug concentrations [25]. We suggest that radiation-induced apoptosis is involved in the mechanism of eribulin radiosensitization depending on the concentration of the drug and the type of cell line tested.

Recent data [16] confirm that eribulin induces the apoptotic caspases – 3/7 in all cell lines tested and enhances the radiation-induced apoptotic cells. Another study [17] reports an increase of cleaved caspase-3 after 10nM eribulin exposure but suggests that combined treatment induces cell death through a caspase-independent mechanism.

# Conclusion

We demonstrate that eribulin exerts a true in vitro radiosensitizing effect through a synergistic interaction between irradiation and drug. In our study the main mechanism of radiosensitization is the cell cycle arrest in G2/M phase induced by drug. The enhancement radiation-induced apoptosis could be an additional mechanism of radiosensitization.

The results of this investigation are very promising but further studies are needed to assess the clinical benefits of the combination of eribulin and radiotherapy as a novel therapeutic strategy for cancer.

# Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests

### Funding

None

### Author Contributions Statement

Data collection (RB), study design (JR), investigation (RB, RC, SR,MJC), cells irradiation (PS), analysis (RB, JR), writing – original draft (RB), writing – review & editing (JR). All authors read and approved the final manuscript


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Not applicable


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# Figures

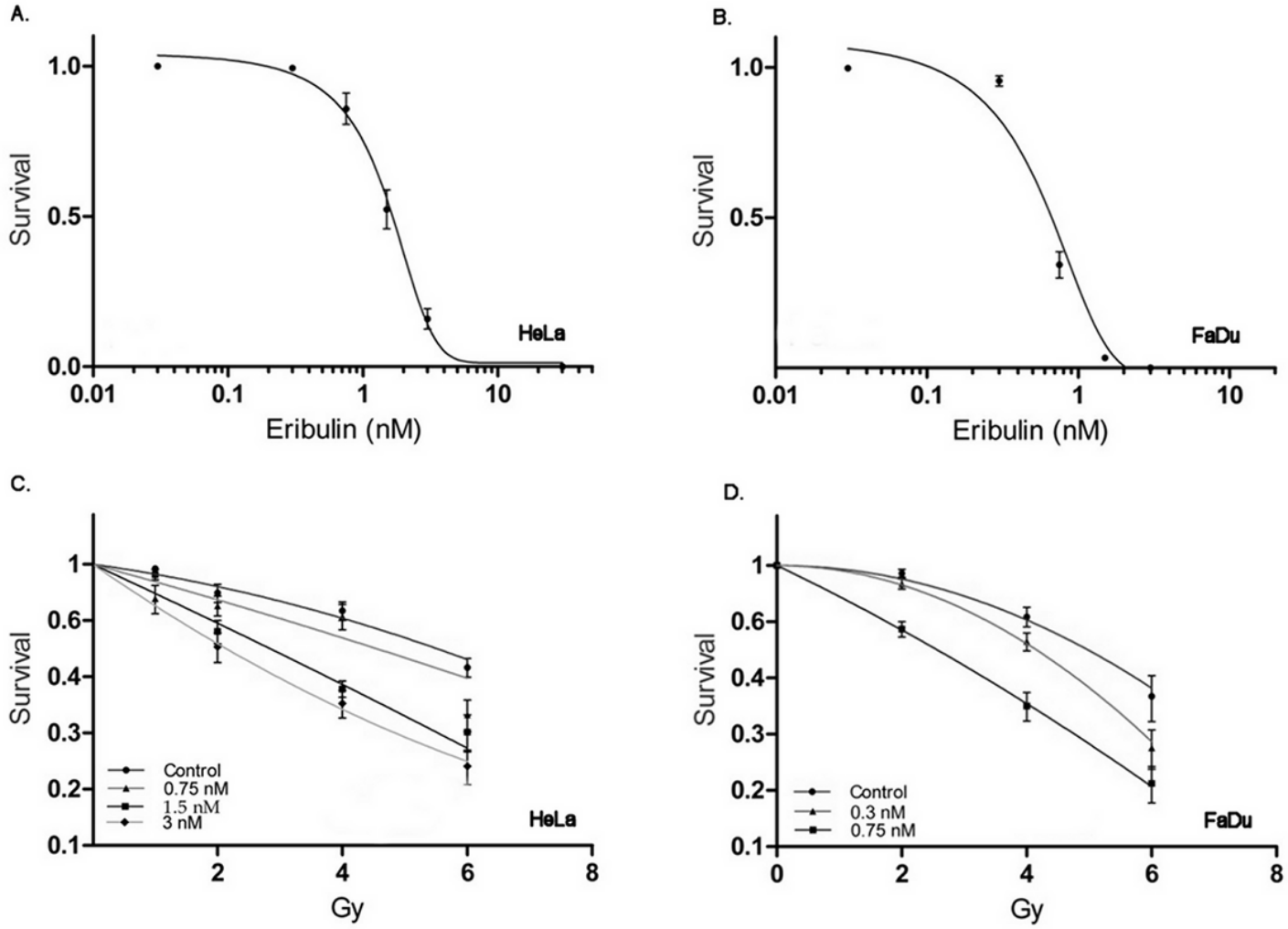
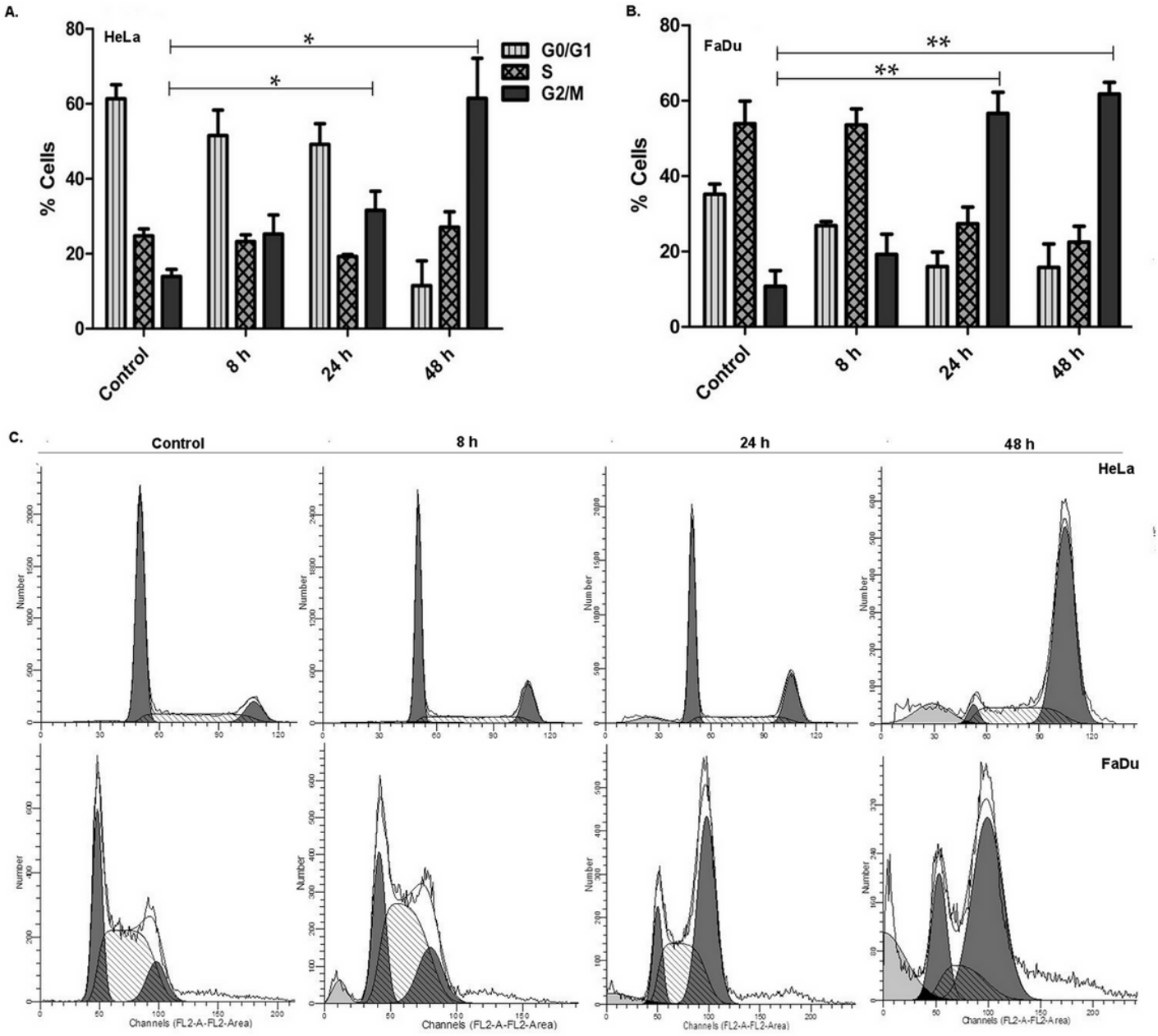



**Figure 1**

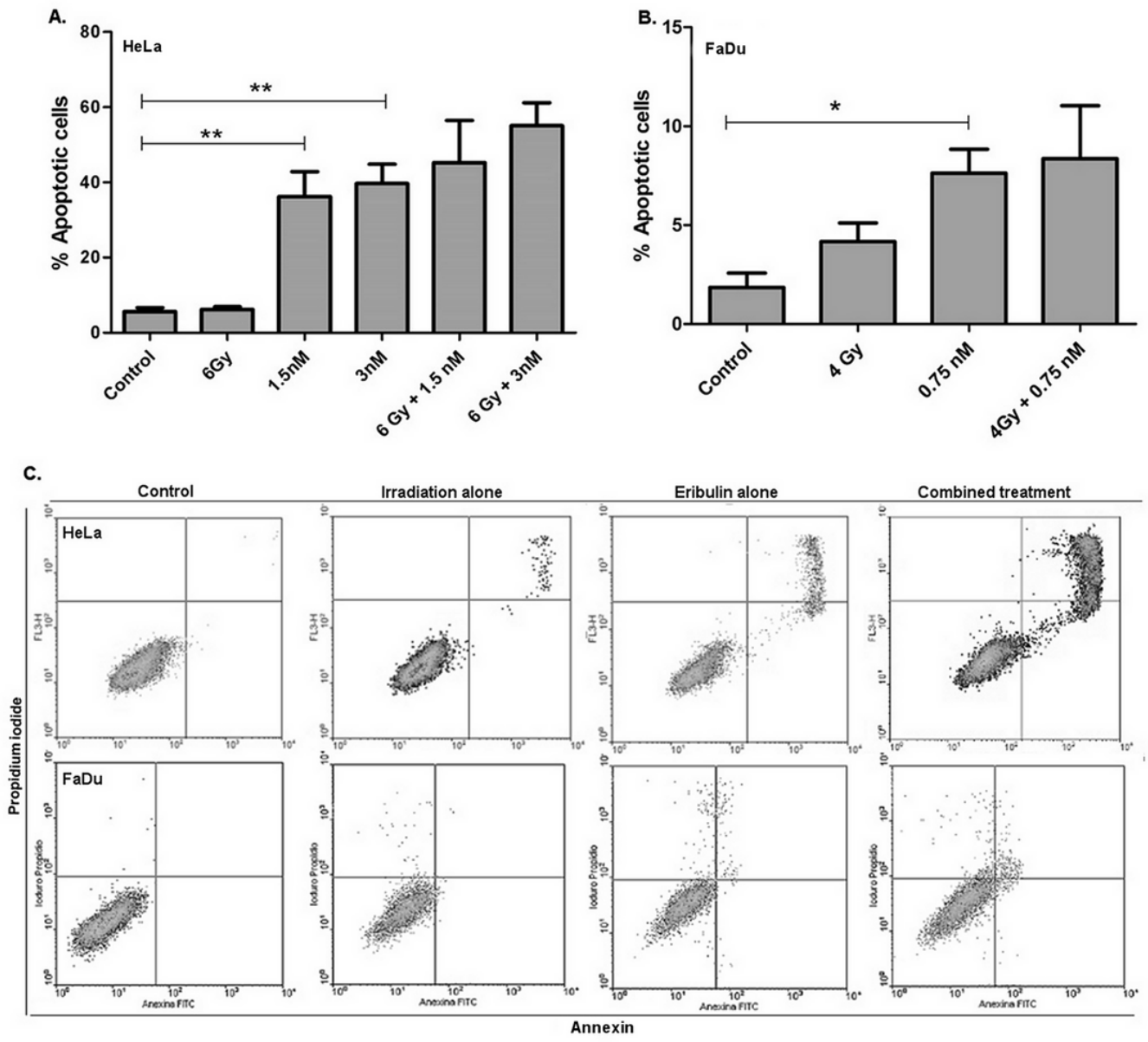
Cytotoxicity evaluation.(A, B), Dose-response curves after 24h exposure time. (C, D), Radiation cell survival curves obtained after adjusting individual data to a linear quadratic model for control (irradiation

alone) and treated cells. Each point represents the mean of at least three triplicate experiments (± SEM).



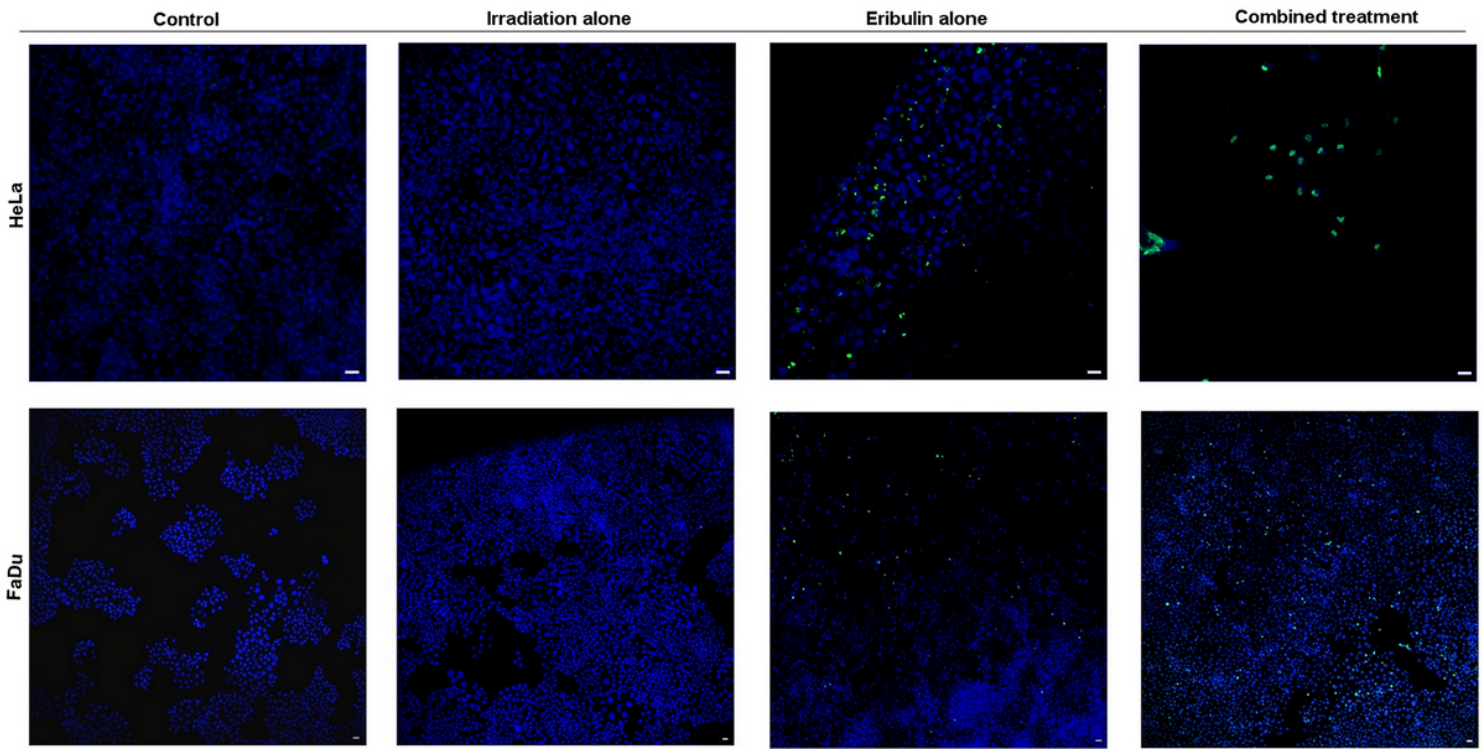

## Figure 2

Cell cycle analysis. Time course of cell cycle changes after 1.5nM and 0.75nM 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 48h period of time. Graphs represent histograms of red fluorescence, 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




## Figure 3

Apoptosis Assay. Mean percentage of apoptotic HeLa (A) and FaDu (B) cells for control, irradiation 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) versus propidium iodide (Y axis) generated by flow cytometry. 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




## Figure 4

Representative microphotographs of TUNEL assay showing the merge of DNA staining (blue nuclei) and apoptosis by TUNEL detection (green nuclei). Bars represent 50µm.