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In vitro radiosensitisation by trabectedin in human cancer cell lines ☆

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ABSTRACT

Purpose: To examine the potential radiosensitising properties of trabectedin (ET-743, Yondelis©).

Methods and materials: In vitro chemosensitivity was assessed in four tumour cell lines (DU145, HeLa, HT29, HOP62) by the crystal violet method. IC10s and IC50s were established for 1-h, 24-h and 7-day (continuous) exposure times. Radiosensitisation was evaluated by conventional colony assay. BrdUrd DNA-labelling and flow cytometry were used to analyse cell cycle kinetics. The rate of apoptotic induction was assed by annexyn-V labelling.

Results: Mean IC50s were 18.8 nM (10.5 – 30), 2.5 nM (1.5 – 5) and 0.25 nM (0.2–0.8) for 1 h, 24 h and continuous exposure times, respectively. HT29 and HOP62 were the most sensitive cells lines to trabectedin. Radiosensitisation was observed in DU145 and HeLa cells with a dose enhancement factor (DEF) of 1.92 and 1.77 at IC50 dose level, respectively. Trabectedin induced early S phase arrest in all cell lines studied.

Conclusions: Trabectedin, at pharmacologically appropriated concentrations, harbours a significant *in vitro* radiosensitising effect and induces cell cycle changes and apoptosis in several human cancer cell lines. Further studies to define the clinical potential of the combination of trabectedin and radiotherapy are needed.

1. Introduction

Trabectedin (ET-743), a tetrahydroisoquinoline alkaloid isolated from the tunicate *Ectenascidia turbinata*,[1,2] shows *in vitro* and *in vivo* activity against human tumours with evidence of a lack of complete cross-resistance with conventional therapies.[3–8]

A set of phase I studies demonstrated feasibility in pretreated adult cancer patients. The dose limiting toxicities were fatigue and bone marrow toxicity. Phase II studies have

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shown activity in pre treated ovarian cancer,[9] sarcoma[10–13] and breast cancer.[14]

Trabectedin has recently been approved by the European Agency for the Evaluation of Medicinal Products (EMEA) as a second line systemic therapy for patients with sarcoma.

The mechanism of action of trabectedin is under active investigation. Trabectedin binds to the minor groove of DNA, bending it toward the major groove[15,16] and interferes with several transcription factors [17–19] by inhibiting activated, but not constitutive, transcription .[20] Although trabectedin produces protein-linked single-strand breaks (SSB) at very high concentrations,[21] neither DNA-breaks nor DNA-protein cross-links were found by alkaline neutral elution in cells exposed to trabectedin at 50% inhibitory concentrations (IC50).[22] However, recent data clearly shows that exposure to clinically relevant concentrations of trabectedin produces double DNA strand breaks.[23] In contrast to other DNA interacting agents, a set of studies have noted that nucleotide excision repair (NER) -deficient cells are resistant to trabectedin, and restoration of the NER function sensitises cells to the drug.[24,25]

Moreover, cell kinetic studies with trabectedin have demonstrated a late S- and G2M-phase arrest.[22,26] Dynamic gene expression profiling studies conducted in low passage human sarcoma cell lines exposed to clinically relevant concentrations of trabectedin have identified a gene signature associated to response/resistance to trabectedin.[27] Such molecular signatures are mostly represented by DNA repair genes.

A recent study has described moderate, cell-line dependent radiosensitisation by trabectedin.[28] The G2M blockade induced by the drug has been invoked by the authors as a potential mechanism of radiosensitisation.[28]

In this paper, we report the characterisation of *in vitro* trabectedin induced radiosensitisation in a panel of human cancer cell lines. This study also reports the drug related changes in apoptotic rates and in cell cycle distribution.

2. Material and methods

2.1. Cell lines and culture conditions

We used a panel of four human tumoural cell lines obtained from the American Type Culture Collection (ATCC): HeLa (cervical), DU145 (prostate), HT29 (colon) and HOP62 (lung). All cell lines were cultured as monolayers in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal calf serum (FCS, Gibco), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere, trypsinised and passaged once a week.

2.2. Chemosensitivity assaya

Trabectedin was provided by Pharma Mar SA (Tres Cantos, Spain) as a white powder to be reconstituted. Drug stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma) at 1 mg/ml concentration and kept in aliquots at –20 °C until use. Additional dilutions were made in distilled water immediately before use, so that 20 μl were added to treatment wells to obtain the final desired concentration.

Cytotoxicity of trabectedin was determined by the crystal violet method, a colourimetric cell density assay described previously,[29] carried out in 24-well multiwell plates. Twenty-four hours after planting, trabectedin at different concentrations was added to each of three replicates treatment wells. To mimic the clinical infusion schedules, three different exposure times were used: 1 h, 24 h and 7 days (continuous). After 7 days of incubation, cells were fixed with 1% glutaraldehyde for 10 min, washed twice in PBS and stained with 1.5 ml of 0.1% crystal violet solution for 30 min. Wells were rinsed in a beaker with a slow stream of distilled water and left to dry overnight. The absorbance was read at 590 nm by dye uptake in 10% acetic acid. The surviving fraction was calculated by dividing the treated wells absorbance by the control wells absorbance.

The IC10 (10% inhibitory concentration) and the IC50 (50% inhibitory concentration) were defined as the trabectedin concentrations at which 90% and 50% of cells survive, respectively. IC10 and IC50 values were obtained by interpolation of the dose response curves in the different cell lines.

2.3. Radiosensitivity assay

Trabectedin radiosensitisation was evaluated by a conventional colony assay. In order to obtain the best conditions for radiosensitisation and resemble the continuous infusion schedules commonly applied in clinical protocols, the dose corresponding to the IC10 and IC50 of continuous exposure for each cell line was chosen for the radiosensitisation experiments. Exponentially growing cells were trypsinised and plated in T60 tissue culture plates. A variable number of cells were used depending on the plating efficiency of each cell line and drug and radiation dose levels. After 24 h, trabectedin was added to the medium to a desired final concentration. Cells were irradiated after 24 h incubation of drug, with a ^{60}Co unit (Theratron, AECL,Canada) at a dose rate of 1.6 Gy/min.

After treatment, colonies were allowed to grow for 14 days, fixed in 70% ethanol and stained with 0.1% crystal violet solution. Colonies containing at least 50 cells were counted to determine cell survival. The surviving fraction was calculated as the ratio of the plating efficiency of irradiated cells to plating efficiency of control cells. Data from at least three duplicate experiments were grouped for each dose and adjusted to the linear-quadratic model (ln SF = –(alfa * dose) – (beta * dose^2)), using a least squares algorithm. Surviving fraction at 2 Gy (SF2) for each survival curve was calculated from the fitted data. Surviving fractions for combined treatment were normalised through dividing by the surviving fraction for drug only. The radiation dose enhancement factor (DEF) was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus drugs (normalised for drug toxicity) at the 50% survival level.

2.4. Cell cycle analysis

Cell-cycle distributions from asynchronous cultures was determined by bromodeoxyuridine (BudR) labelling and flow-cytometric analysis as described previously.[30] Exponentially growing HeLa, DU145, HT29 and HOP62 cells were trea-

ted with 2, 5, 1.5 and 1.8 nM of trabectedin, which correspond to the 24 h exposure IC50 concentrations for each cell line. The percentage of cells in G0-G1, S and G2-M phases were assessed in absence of trabectedin exposure times. Control or treated cells were pulse-labelled with 10 μM BUdR for 20 min, trypsinised, and fixed in 70% ethanol. Nuclei were extracted by incubation with 0.04% pepsin in 0.1 M ClH for 60 min at 37 °C and washed twice in PBS containing 0.5% Tween-20 and 0.5% bovine serum albumin (PBS-TB). DNA was partially denatured with 2 M ClH for 7 min. After neutralising the samples with two volumes of 0.1 M $Na_2B_4O_7$, nuclei were washed three times in PBS-TB and incubated with mouse anti-BUdR monoclonal antibody (1:25, Becton Dickinson, San Jose, CA), for 60 min at room temperature. Then, nuclei were incubated with a secondary FTIC-labelled goat anti-mouse IgG antibody (whole molecule, Sigma) for 30 min at room temperature. After washing with PBS-TB, samples were re-suspended in PBS containing 10 μg/ml propidium iodide and 0.5 μg/ml Rnase. The stained samples were analysed in a FACScan flow cytometer (Becton Dickinson), collecting a minimum of 10,000 events. After excluding doublets and triplets, bivariate histograms of BUdR (green fluorescence) versus DNA content (red fluorescence) were obtained, and the data analysed using the Lysis II software (Becton Dickinson).

2.5. *Apoptosis quantification*

Apoptotic cells were detected by FTIC-conjugated Annexyn V labelling method. Exponentially growing cells were treated at the same trabectedin doses as described above for the flow cytometry experiments. The percentage of apoptotic cells was evaluated for untreated cells and also after 24, 48 and 72 h for trabectedin treated cells. After the incubation period, cells were washed and resuspended in Binding buffer containing 100 mM HEPES/NaOH, pH 7.5, 1.4 M NaCl and 25 mM CaCl2 (Becton Dickinson). Cells were stained with 0.5 μgr/ml Annexyn V-FTIC (Becton Dickinson) and 1 μg/ml propidium iodide (Sigma), incubated for 10 min and analysed immediately in a FACScan flow cytometer (Becton Dickinson). The percentage of apoptotic cells was obtained from a bivariate histogram of Annexyn V labelled-cells (green fluorescence) versu DNA (red fluorescence). In order to evaluate the mechanism of radiosensitisation and determine if drug treatment increases the radiation-induced apoptosis, we tested the effect of combined trabectedin, at IC50 doses, with irradiation on apoptosis induction at 72 h, on DU-145 and HeLa cells.

3. Results

As shown in Fig. 1, all cell lines were sensitive to trabectedin within the nanomolar range. Mean IC50s were 18.8 nM (10.5 – 30), 2.5 nM (1.5 – 5) and 0.25 nM (0.2–0.8) for 1 h, 24 h and continuous exposure times, respectively.

Enhanced radiation response was observed in HeLa and DU145 cells (Table 1, Fig. 2) after exposure to IC10 and IC50 concentrations of trabectedin. SF2s for control and treated cells were 0.81 and 0.54 Gy for HeLa cells and 0.84 and 0.59 Gy for DU145 cells, respectively. The DEF, at trabectedin IC50 concentration, were 1.77 and 1.92 for HeLa and DU145 cells lines. HOP62 cells were only slightly radiosensitised by trabectedin with a DEF of 1.2, for IC50 dose. Trabectedin lacked radiosensitising effects on HT29 cells. There was an increase of the DEF on DU145 and HeLa cells, as the trabectedin dose increased from the IC10 to IC50 values, suggesting that trabectedin induces radiosensitisation in a concentration dependent manner.

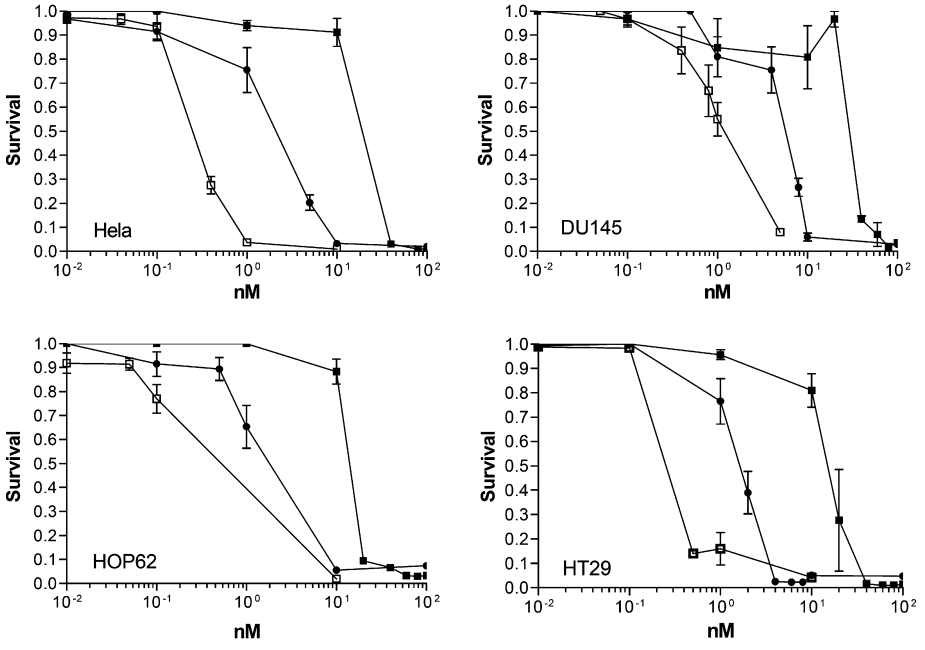

Fig. 1 – Dose response curves for 1 h (closed squares), 24 h (closed circles) and 7 day (open squares) ET749 exposure times. Each data point represents the mean of at least three triplicate experiments. Errors bars represent the 95% confidence interval (CI).

Table 1 – Radiation survival curve parameters (alpha, beta and SF2) for control and treated cells at IC10 and IC50 doses

		Alpha (95% CI)	Beta (95% CI)	SF2	DEF
DU145	Control	0.026 (–0.022-0.075)	0.02 (0.017-0.041)	0.84	
	IC10	0.12 (0.050-0.189)	0.02 (0.009-0.045)	0.7	1.44
	IC50	0.225 (0.112-0.339)	0.01 (–0.017-0.05)	0.59	1.92
HELA	Control	0.008 (–0.034-0.052)	0.04 (0.036-0.06)	0.81	
	IC10	0.188 (0.103-0.274)	0.01 (–0.004-0.039)	0.64	1.28
	IC50	0.234 (0.093-0.375)	0.03 (–0.013-0.079)	0.54	1.77
HOP62	Control	0.33 (0.184-0.485)	0.06 (–0.009-0.13)	0.4	
	IC10	0.503 (0.373-0.633)	0.01 (–0.041-0.062)	0.35	1.2
	IC50	0.54 (0.395-0.689)	-0.01 (–0.068-0.046)	0.35	1.2
HT29	Control	0.133 (0.024-0.242)	0.007 (–0.016-0.032)	0.74	
	IC10	0.119 (0.012-0.226)	0.01 (–0.012-0.039)	0.74	0.85
	IC50	0.015 (–0.024-0.056)	0.03 (0.028-0.046)	0.83	0.8

SF2: surviving fraction to 2 Gy; DEF: drug enhancement factor.

As shown in Fig. 3, trabectedin decreased the percentage of cells in the G0-G1 phase and it induced accumulation in the S phase, which peaked after 24 h exposure. At later exposure times, this blockage was followed by a moderate G2M phase arrest evidenced after 72 h of trabectedin incubation. These effects were most pronounced in DU145 and HeLa cells. The mean percentage of cells in the S phase, after 24 h of drug exposure, increased from 37.8 to 71%, 54.7 to 85%, 46.9 to 61.3% and 36.6 to 57.4% for control and treated HeLa, DU145, HOP62 and HT29 cell lines, respectively. The S phase delay was associated with a decreased uptake of BudR, after 72 h of trabectedin treatments supporting an inhibition of DNA synthesis (Fig. 4).

Evidence of apoptosis was noted at the IC50 of trabectedin. The percentages of apoptotic cells after this incubation period were 18% and 43% for HeLa and DU145 cells, respectively, which means an increment of 12 and 8.9 times with respect to control cells. In HOP62 and HT29 cells there was a less pronounced apoptotic response to trabectedin, with corresponding figures of 8.2% and 12.4% and increments of 2.7 and 2 times with respect to control cells, respectively (Fig. 5). The effect of combined ET743 and irradiation exposures on apoptosis was only additive and due mainly to ET743 treatment (Fig. 6). The combination of trabectedin and radiation lacks effect on the increase of apoptotic induction (Fig. 6).

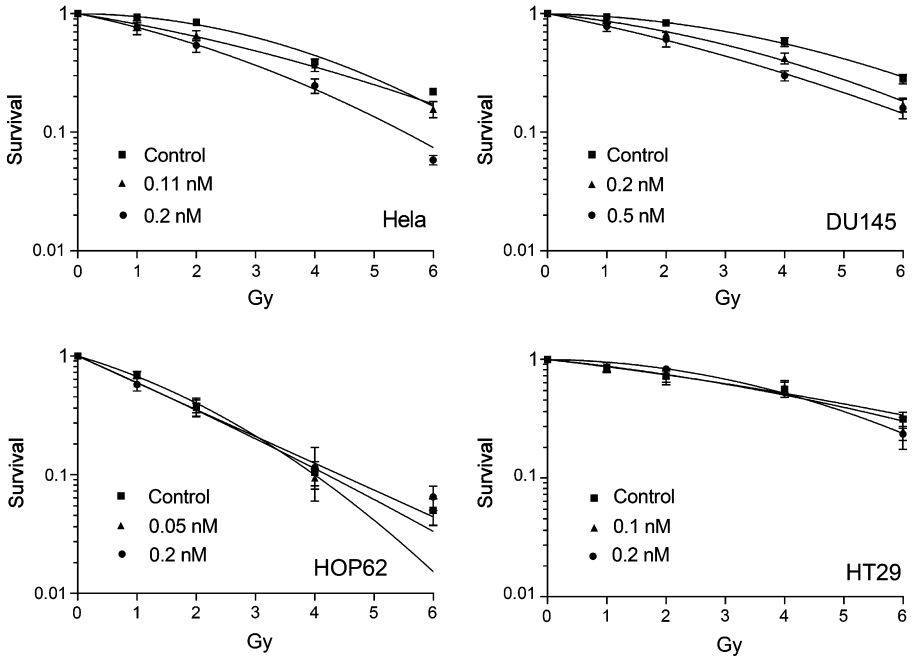

Fig. 2 – Radiation survival curves, generated by adjusting individual data to linear quadratic method, for control (squares), IC10 (triangles) and IC50 (circles) ET749 doses for each cell line. Each data point represents the mean of at least three triplicate experiments. Errors bars represent the 95% confidence interval (CI).

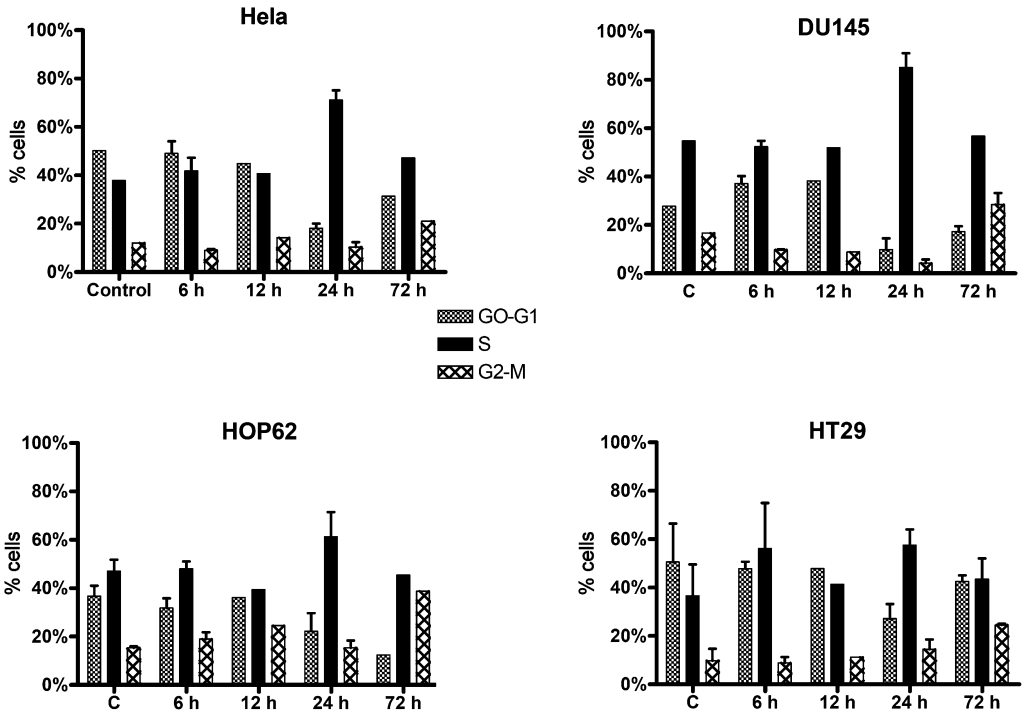

Fig. 3 – Cell cycle changes evaluated at different times after IC50 ET749 exposure. Bars represent the percentage of cells in each phase of cell cycle. Errors bars represent the 95% confidence interval (CI).

4. Discussion

Although there is some variability in the literature, depending on the cell line, exposure time and type of chemosensitivity assay used, most studies have reported a cytotoxic activity of trabectedin in the nanomolar to subnanomolar range concentrations.[3–6,22,26,31,32] In the present study, a 7 day continuous exposure to trabectedin led to IC50 ranging from 0.2 to 0.8 nM in the human cell lines tested. Those trabectedin concentrations are reachable and sustainable in patients plasma well below the recommended dose.[11,33–38] In fact, plasma peak concentrations between 1.14 and 25 ng/ml have been reported across the different schedules tested in clinical trials.

Several studies have reported that the antiproliferative effects are directly related to trabectedin exposure times.[2,3,32] In this study we have also found a reduction of approximately one order of magnitude on trabectedin IC50 concentration for 1 h, 24 h and 7 day exposition times.

This study demonstrates an *in vitro* radiosensitising effect of trabectedin in two of the four human cell lines tested. At the IC50 concentrations, the DEF were 1.92 and 1.77 for DU-145 and HeLa cell, respectively. To further examine the trabectedin induced radiosensitisation we have characterised its antiproliferative effects in combination with clinically relevant fraction dose of 2 Gy by calculating the ratio of SF2 of trabectedin in untreated versus treated cells. As reported in Table 1, at the IC50 concentrations, these ratios were 1.5

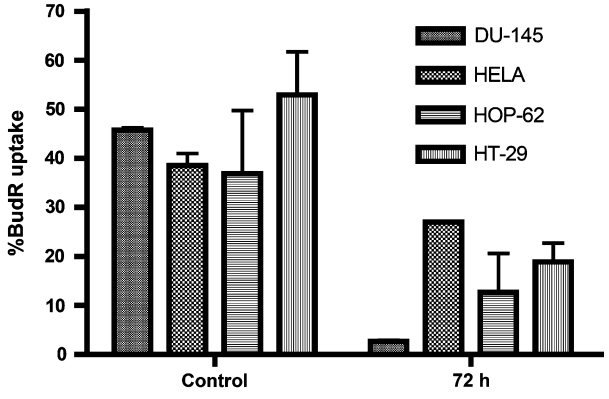

Fig. 4 – Percentage of bromodeoxyuridine uptake for control and ET749-treated cells with IC50 dose for 72 h. Errors bars represent the 95% confidence interval (CI).

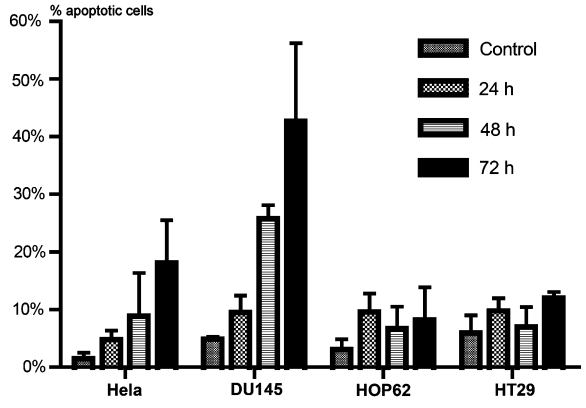

Fig. 5 – Percentage of apoptotic cells at different times after exposure to IC50 ET749 concentration. Errors bars represent the 95% confidence interval (CI).

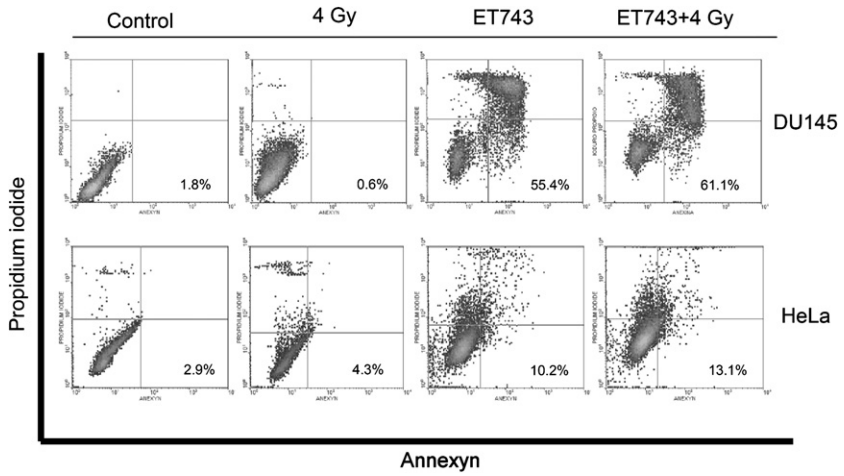

Fig. 6 – Percentage of apoptosis in DU145 and HeLa cell lines, evaluated by annexyn V staining, for control, irradiated, ET749-treated and irradiated in presence of ET749 cells. Graphs represent bivariate histograms of propidium iodide (Y axis) versus annexyn V (X axis), generated by flow cytometry. Figures in each histogram represent the percentage of early (lower right quadrant) and late (upper right quadrant) apoptosis. The most representative of two experiments is shown.

and 1.42 for HeLa and DU145 cells lines respectively. An enhancement of radiation response around 1.5 means a 50% increase in tumoural cell killing after each fraction of 2 Gy in presence of the drug. This effect would be magnified in a conventional radiotherapy treatment of 25 to 35 fractions and could have a significant clinical impact on tumour control.

The trabectedin concentrations needed to attain radiosensitisation are within the subnanomolar range (0.2 nM for HeLa and 0.5 nM for DU-145 cells); such concentrations are consistently reachable in patients well below the recommended dose. A plasma Cmax around 1 nM/ml has been described in patients treated with trabectedin given intravenously as a 24 h infusion at a dose of 900 mcg/m^2. Such a dose level is 40% below the recommended dose of 1.5 mg/m^2 .[34,35] Moreover, when trabectedin is given as a daily intravenous 1 h infusion per five consecutive days, a rational schedule for combination with radiotherapy, sustained plasma concentrations in the range of 1.06 ng/ml are reachable at 144 μg/m^2 dose level.[39]

Modelling the exact mechanism of drug-radiation interactions is difficult. The key characteristic of radiosensitisation is a modification of the radiation survival curve in the presence of the drug, evidenced by changes in alfa and beta parameters, as has been advocated by others authors.[40] In this study we show a clear modification of radiation cell survival curve shape of HeLa and DU145 cells, suggesting a true *in vitro* interaction between ET743 and radiation (Fig. 2 and Table 1).

In an attempt to elucidate the mechanism involved in the trabectedin induced radiosensitisation, we have examined the changes in the cell cycle kinetics and apoptotic induction. Our results show a marked S-phase blockage as well as a moderate G2M arrest after protracted *in vitro* exposure to IC50. These results are in line with previously reported studies.[3,5,21,22,26,41] The delay in cell progression trough the S-phase is common to DNA damaging agents.[21] Moreover, our data shows that the drug induced growth delay is associated with an inhibition of DNA synthesis. This effect could interfere with the DNA repair mechanisms triggered after irradiation of the cells in the presence of trabectedin. In a recent study,[42] flow cytometric assays showed progressive accumulation of cells in the G2M phase in NER-proficient cells, but not in NER-deficient cells.

It is well known that G2M is the most sensitive cell cycle phase to radiation. For instance, this interaction may be the basis for Paclitaxel related radiosensitisation. In our study, cells were irradiated after 24 h of drug incubation. As shown in Fig. 3, at the irradiation time we observed S-phase arrest and not an accumulation of cells in G2M. Thus the late and moderate G2M cell cycle arrest noted in our kinetic study does not sustain the implication of such an event in the enhancement of the radiation sensitivity by trabectedin.

As shown in Fig. 5, cell cycle changes were associated with induction of apoptosis at later incubation times, which were more pronounced in DU-145 and HeLa cells. A similar finding was reported in other study ,[5] in which Ewing's sarcoma cells underwent apoptotic changes after 72 h of subnanomolar trabectedin concentrations.

These studies, and our own results, are in accordance with the model proposed by Gajate et al.[41] of two different signalling pathways activated by ET743 in a dose-dependent manner. At higher doses (10–100 ng/ml) a transcription-independent process, involving JNK and caspase 3 activation, led to early apoptosis.[41] At lower doses (1–10 nM), similar to that used in our study, ET743 induces an S and G2M arrest, growth inhibition and late apoptosis, trough a transcription-dependent process.[41]

Our data shows no significant differences in the apoptotic rates between cells exposed to trabectedin alone and the combination of trabectedin and radiotherapy (Fig. 6), suggesting that increase of apoptosis is not involved in the mechanism of radiosensitisation.

The two cell lines, HeLa and DU-145, in which trabectedin induced radiosensitisation has been seen, harbours a mutant p53. Recent data [43] have also indicated extreme sensitivity to trabectedin in mutant p53 sarcoma cell lines exposed to the drug under 1 nM concentrations.

Recently, the possibility has emerged of using DNA repair inhibitors to optimise the therapeutic use of DNA-damaging agents currently used in the treatment of tumours.[44] In this sense, experimental and clinical data demonstrates that an efficient Nucleotide Excision Repair pathway modulates the sensitivity to trabectedin.[25] However, two recent studies have reported the critical role of DNA-double strand breaks (DNA-DSB) formed during the processing/repair of the trabectedin-induced lesions.[23,45] In addition, cells lacking homologous recombination repair pathway were extremely sensitive to the drug.[45] The fact that DNA-DSB is the most important lesion induced by radiation indicates that shared mechanisms of DNA damage and repair could be implicated in the radiosensitising effect of trabectedin. Nonetheless, the impact of trabectedin on DNA repair pathways induced by radiation therapy needs further characterisation.

5. Conclusions

Our study demonstrated that trabectedin induced radiosensitisation in a panel of human cancer cell lines at pharmacologically appropriated concentrations.

Additional *in vitro* and *in vivo* studies to validate this finding and to establish the combination schedule are warranted.

Conflict of interest statement

Jose Maria Jimeno and Juan Carlos Tercero are employes of Pharma Mar. Jose Antonio Lopez-Martin was employee of Pharma Mar from the year 2000 to 2003. Jesus Romero, Irma Zapata, Sofia Cordoba, Alejandro de la Torre, Juan Antonio Vargas, Rafael Molerón and Ricardo Sanchez-Prieto have no potential confilcts of interest to disclose.

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