



Radiosensitizing effects of trabectedin on human A549 lung cancer cells and HT-29 colon cancer cells

Katrin Manda¹ · Tina Präkelt¹ · Tonja Schröder¹ · Stephan Kriesen¹ · Guido Hildebrandt¹

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Summary

Background and Purpose Trabectedin is a unique alkylating agent with promising effects against a range of solid tumors. In this study, we aimed to examine the cytotoxic and radiosensitizing effects of trabectedin on two human epithelial tumor cell lines *in vitro*, and its effects on DNA repair capacity. **Methods** Cancer cells (A549: human lung cancer cells, HT-29: colon cancer cells) were treated with either trabectedin alone for the determination of their growth, or in combination with radiation for the determination of their metabolic activity, proliferation, and clonogenic survival. Besides, the γ H2AX foci assay was performed for the assessment of ionizing radiation-induced DNA damage and to evaluate the influence of trabectedin on DNA damage repair. **Results** Treatment with trabectedin resulted in a growth-inhibiting effect on both cell lines, with the IC₅₀ values remaining within a low nanomolar range. Analyses of metabolic activity confirmed a cytotoxic influence of trabectedin and a BrdU assay demonstrated an antiproliferative effect. When combined with radiation, incubation with trabectedin was found to enhance the radiosensitivity of the tumor cells. The γ H2AX foci assay resulted in an increased number of DNA double-strand breaks (DSBs) in cells treated with trabectedin. **Conclusion** The results of this study underline the antitumor activity of trabectedin at low nanomolar concentrations. We demonstrated that trabectedin enhanced radiation response in human lung (A549) cancer cells and colon (HT-29) cancer cells. Further studies are necessary to examine trabectedin as a potential candidate for future applications in radiotherapy.

Keywords Trabectedin · irradiation · cancer cells · DNA double-strand breaks

Abbreviations

BrdU Bromodeoxyuridine
DMEM Dulbecco's modified Eagle medium
DMSO Dimethylsulfoxide

DSBs double strand breaks
FBS fetal bovine serum
HR homologous recombination
IC₅₀ drug concentration causing 50% growth inhibition
ID₅₀ radiation dose causing 50% inhibition of surviving fraction
NSCLC non-small cell lung carcinoma
SF4 surviving fractions at 4 Gy

✉ Katrin Manda
katrin.manda@uni-rostock.de

Tina Präkelt
tina.praekelt@med.uni-rostock.de

Tonja Schröder
tonja.schroeder@gmx.de

Stephan Kriesen
stephan.kriesen@uni-rostock.de

Guido Hildebrandt
guido.hildebrandt@uni-rostock.de

¹ Department of Radiotherapy and Radiation Oncology, University Medical Center Rostock, 18059 Rostock, Südring 75, 18059 Rostock, Germany

Introduction

Trabectedin was originally isolated from the marine Caribbean tunicate *Ecteinascidia turbinata* [1]. Today the substance is produced synthetically and distributed as YondelisTM [2]. It is a licensed pharmaceutical in Europe for the second-line treatment of advanced soft-tissue sarcoma and was first authorized for use in 2007. In 2009, trabectedin received marketing authorization for the treatment of patients with relapsed platinum-sensitive

ovarian cancer when used in combination with pegylated liposomal doxorubicin [3]. The drug was also approved by FDA for the treatment of liposarcoma and leiomyosarcoma as a second-line therapy [4]. In addition, trabectedin also holds the FDA orphan drug status for advanced and recurrent soft-tissue sarcoma, and ovarian cancer [5]. Its clinical activity is being evaluated in a variety of neoplasms: prostate, ovarian, pancreatic and breast cancers [6]. As with all chemotherapeutic agents, adverse reactions also occur in patients undergoing treatment with the drug. The most common side effects are neutropenia, nausea, vomiting, AST/ALT increase, anemia, fatigue, thrombocytopenia, anorexia and diarrhea [7]. The most frequent and relevant side effect as well as a dose-limiting factor is hepatotoxicity [8]. Metabolism of the drug by CYP450 enzymes increases the ROS level and leads to the death of hepatocyte cells [9–11]. But there are many ways to help minimize or prevent the adverse effects of trabectedin. For example, in order to reduce hepatotoxicity, premedication with the cytochrome P450 inducer, dexamethasone, is recommended due to its hepatoprotective functions [12, 13].

The mechanism of action of trabectedin is not fully elucidated but seems to differ from traditional alkylating agents [14, 15]. It has been described that trabectedin binds to the DNA minor groove in specific triplets, induces the formation of DNA adducts and bends DNA toward the major groove; whereas a part of it protrudes from the DNA and perhaps interacts with proteins alongside the adduct [14, 16, 17]. In contrast to other available DNA-damaging agents that have been used in cancer chemotherapy to date, trabectedin is less active in cells deficient in repairing DNA, suggesting a role for transcription-coupled nucleotide excision repair (TC-NER) in its cytotoxic activity [18, 19]. Furthermore, trabectedin causes strong perturbations in the cell cycle inhibiting DNA synthesis and creating a marked blockage in G2/M phase [19, 20].

In preclinical studies, trabectedin showed anticancer activities against a number of tumor cell lines including melanoma, non-small cell lung carcinoma (NSCLC) as well as pancreatic, prostate, and breast cancers [21–24].

Lung and colon cancers are two of the most common malignancies found in many parts of the world and are among the leading causes of cancer [25, 26]. Therefore, in the present study, we aimed to investigate the effects of trabectedin alone and in combination with ionizing radiation on two human epithelial tumor cell lines: A549 (lung adenocarcinoma) and HT-29 (colon adenocarcinoma). We were able to demonstrate the high cytotoxic activity of trabectedin and its potential to operate as a radiosensitizer in the tested cancer cell lines.

Materials and methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Penicillin/Streptomycin (P/S) and Trypsin/EDTA were purchased from GE Healthcare Europe GmbH (Freiburg, Germany). Phosphate buffered Saline (PBS) was obtained from Merck Millipore (Darmstadt, Germany). Trabectedin was ordered from PharmaMar (Madrid, Spain).

Drug preparation

A sterile, lyophilized vial of trabectedin was delivered from PharmaMar GmbH (Madrid, Spain). The drug was dissolved in Dimethylsulfoxide (DMSO) to create a concentrated stock solution of 1 mM and stored in aliquots at -20°C . Aliquots were thawed for each new experiment to avoid degradation, and then diluted with cell culture medium to the desired concentrations. The final concentration of DMSO in the experiments was 1% (v/v) or less, a concentration that had no effect on the control cells.

Cell lines

Human lung cancer cell line A549 and human colon cancer cell line HT-29 were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 . The cells were grown as epithelial monolayers in tissue culture flasks. To maintain exponential growth they were trypsinised and passaged once a week.

Growth inhibition assay

Cells in the exponential growth phase were seeded in 24-well plates at a density of 2×10^3 cells per well. After pre-culture for 24 h, serially increasing concentrations of trabectedin (0.25 nM, 0.5 nM, 1 nM, 2 nM, 6 nM) were added to the wells. The growth inhibitory effect of the drug was evaluated daily by counting the number of cells using a Coulter Counter (Beckman Coulter GmbH, Krefeld, Germany). Each concentration value and the controls (medium, DMSO) were determined in triplicates; experiments were run for 7 days.

Metabolic activity assay

Cells were seeded in 96-well plates at a density of 4×10^2 (A549) or 1×10^3 (HT-29) cells per well. After attaching for 24 h, cells were incubated with increasing concentrations of trabectedin (0.25 nM, 0.5 nM, 1 nM, 2 nM, 3 nM, 4 nM, 6 nM) for 24 h. The metabolic activity was determined using the EZ4U Cell Proliferation Assay (Biomedica GmbH, Vienna, Austria). Optical densities were measured after 2 h

at a wavelength of 492 nm using a microplate reader (Anthos Zenyth 340, Anthos Mikrosysteme GmbH, Krefeld, Germany). A background correction was made and each concentration was assayed six times within the same experiment; three independent experiments were performed.

Ionizing irradiation

Radiation was administered utilizing a Siemens Oncor expression linear accelerator (Siemens, Erlangen, Germany) at a dose rate of 3.75 Gy/min (energy 6 MeV). Cells were irradiated at room temperature, using dose rates between 0 Gy and 8 Gy. Sham irradiated controls (0 Gy) were taken to treatment facility to maintain identical environmental conditions.

Proliferation assay

For measurement of the antiproliferative activity of trabectedin, bromodeoxyuridine (BrdU) incorporation into DNA was detected using a colorimetric cell proliferation ELISA assay (Roche Diagnostics, Mannheim, Germany). Cells were seeded into 96-well plates at a density of 1×10^3 cells per well and allowed to grow for 24 h. Subsequently, trabectedin in increasing concentrations (0.25 nM, 0.5 nM, 1 nM, 2 nM, 3 nM, 4 nM, 6 nM) and BrdU were added to each well. At 2 h later, cells were irradiated with 6 Gy (control 0 Gy) and, following an additional 24 h incubation period, the antiproliferative activity was measured in accordance with the assay's specific instructions.

Clonogenic assay

Radiosensitizing effects were evaluated by performing colony forming assays. Cells (1×10^3) were plated in T25 cell culture flasks and allowed to attach for 2 days. Trabectedin was added at a concentration of 0.25 nM at two different time points before irradiation: 4 h and 0.5 h. The colonies were allowed to grow for 12 days under standard culture conditions with a cell culture medium change on day 7. Subsequently, cells were fixed using 70% ethanol and stained with 1% crystal violet solution. Colonies containing more than 50 cells (about four to five full generations of progeny) were scored manually using an Eclipse TE3000 inverted microscope (Nikon, Tokyo, Japan).

Clonogenic fractions of treated cells were normalized to the plating efficiency of untreated controls and fitted using the linear-quadratic model. ID_{50} was calculated, which is the radiation dose that causes 50% inhibition of colony formation.

γ H2AX foci assay

DNA double-strand breaks (DSBs) were detected by immunofluorescence of γ H2AX foci. Cells were seeded on glass

chamber slides at a density of 1×10^4 cells per well. After attachment, trabectedin was added at different concentrations (2 nM or 6 nM) to each well. Following an incubation period of 4 h, cells were irradiated with 6 Gy (control 0 Gy). The first samples were fixed 30 min after irradiation, the remaining were incubated for 24 h under standard cell culture conditions to allow repair of radiation-induced DSB. Cells were labelled with the Anti-phospho-Histone H2A.X antibody (Merck Chemicals GmbH, Schwalbach, Germany), followed by secondary antibody labelling with Alexa Flour 549 goat anti-mouse IgG1 (Life Technologies, Darmstadt, Germany). The number of foci was counted using an Eclipse TE3000 inverted microscope (Nikon, Tokyo, Japan). 50 cells were scored per slide.

Statistical analysis

All data are presented as means \pm Standard Deviation (SD) based on a minimum of three independent experiments. Growth inhibitory analyses were carried out in triplicates per attempt. Metabolic and BrdU assays were performed using 6 wells for each concentration per experiment. The statistical significance of differences was assessed by Student's *t* Test. A value of $p < 0.05$ was considered as statistically significant.

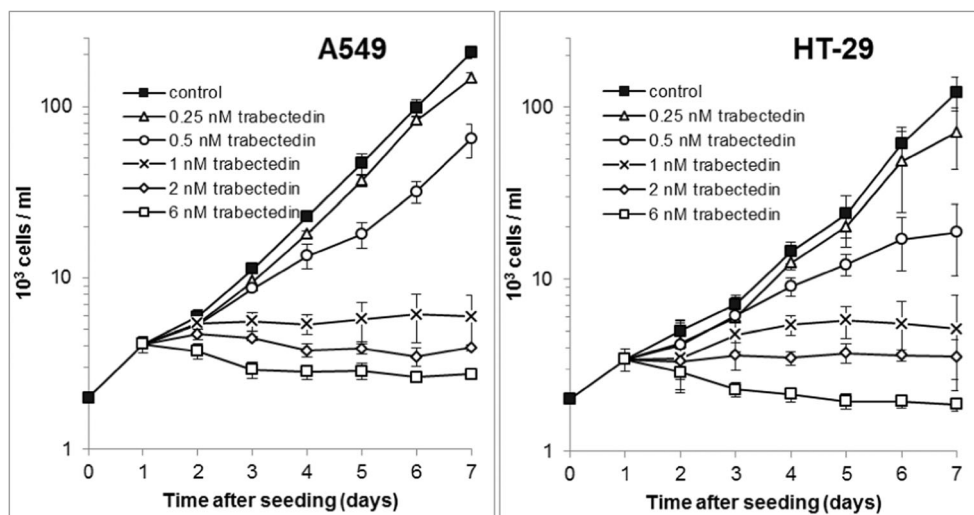
Results

Trabectedin-induced inhibition of cell growth and viability

In order to evaluate the cytotoxic effect of trabectedin on tumor cells, growth curves as well as metabolic activity were analyzed under the influence of the active component. An inhibitory effect of trabectedin on cellular growth of both tumor cell lines, A549 and HT-29, could be demonstrated (Fig. 1). Trabectedin showed a similar growth inhibiting potency on both cell lines; but HT-29 cells were slightly more sensitive. The delay in cell growth was detectable 24 h after trabectedin treatment in a concentration-dependent environment, starting with a concentration of 0.25 nM. The mean IC_{50} values at that time were 9.18 nM (A549) and 9.32 nM (HT-29). Prolonged treatment led to a more pronounced growth inhibitory effect until the 7th and last day of evaluation (mean IC_{50} : 0.38 nM for A549, 0.30 nM for HT-29 cells).

The cellular viability was analyzed by measuring the metabolic activity (Fig. 2). Treatment with trabectedin for 24 h resulted in a concentration-dependent decrease of metabolic activity in both cell lines, beginning at 1 nM trabectedin (Fig. 2a). Significant values could be observed in trabectedin concentrations of 3 nM and higher for A549 cells; for the cell line HT-29, in concentrations of 2 nM and higher. With trabectedin, extension of the treatment time to 48 h resulted

Fig. 1 Inhibitory effect of increasing concentrations of trabectedin on growth of A549 and HT-29 cells over a 7-day period. Cells were seeded on day 0, trabectedin was added after a 24 h cell adhesion period. Error bars indicate the standard deviation for three independent experiments; wells were assayed in triplicates. Asterisks illustrate significance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$



in the reduction of metabolic activity for concentrations up to 1 nM (HT-29) or 2 nM (A549). However, higher concentrations of the drug led to an increase in the metabolic activity again (Fig. 2b). Additionally, treatment of the cells with ionizing radiation had no influence on the metabolic activity (Fig. 2b).

Radiosensitizing effects of trabectedin

Combined treatment with trabectedin and ionizing radiation was evaluated by a conventional colony-forming assay and Fig. 3 illustrates the corresponding dose-response curves. The colony-forming ability of the cells decreased with increasing radiation doses. Administration of 0.25 nM trabectedin, at 0.5 h or 4 h before irradiation, resulted in significant enhancement of radiation response for both cell lines (Fig. 3a). Also, in both cell lines under treatment with 0.25 nM trabectedin, surviving fractions at 4 Gy (SF4) were reduced from 0.55 to 0.37 (A549) or 0.55 to 0.35 (HT-29) compared to administration without the drug. Mean ID₅₀ declined from 3.98 Gy (control) to 3.15 Gy (0.5 h of preincubation) and 3.05 Gy (0.5 h of preincubation) for A549 cells ($p < 0.005$). Cell line HT-29 showed a decrease from 4.28 Gy (control) to 2.77 Gy and 3.24 Gy ($p < 0.005$). For cells pretreated with trabectedin, an extension of the pretreatment time from 4 h to 24 h before ionizing radiation yielded in cell type dependent results (Fig. 3b). While the A549 cells showed reduced survival after irradiation, the HT-29 cells were unable to form colonies after the prolonged pretreatment with the drug.

Influence of trabectedin and radiation on cell proliferation

BrdU was incorporated into DNA to determine proliferation of cells. Both cell lines showed a reduced DNA synthesis when treated with trabectedin alone (Fig. 4). This effect was

concentration-dependent, initiating at 0.25 nM and exhibiting significant values at 0.5 nM (A549) and 1 nM (HT-29) concentrations of trabectedin.

Irradiation alone had a clear effect on the proliferation of tumor cells; the BrdU incorporation fell by an average of 33% (A549) and 40% (HT-29). Cell irradiation delays progression through the G1, S and G2 phases of the cell cycle. These results were anticipated because it is well known that this effect is triggered by DNA damage control points between the individual cell cycle phases following which progression can be blocked or slowed to the next phase [27].

Both cell lines showed that exposure to a combination of trabectedin and 6 Gy radiation had different effects on cell proliferation. In A549 cells, drug concentrations of up to 3 nM did not cause a significantly altered BrdU incorporation after cell irradiation while 4 nM and 6 nM trabectedin concentrations led to a reduction in cell proliferation. For smaller drug concentrations of up to 1 nM, the proliferation of irradiated A549 cells was clearly reduced compared to unirradiated cells. In contrast, after exposure to higher trabectedin concentrations the irradiated cells showed slightly higher measured values than those of the unirradiated samples.

HT-29 cells showed a dose-dependent decrease in BrdU incorporation when treated with a combination of trabectedin and radiation, with an initial significant inhibitory effect coming into play at a concentration of 1 nM trabectedin. In contrast to A549 cells, proliferation rates in the irradiated HT-29 cells were consistently and clearly lower than in the corresponding unirradiated cells.

Influence of trabectedin on DNA double-strand breaks

The influence of trabectedin on the DNA repair capacity of cells was investigated through an analysis of the numbers of DNA double-strand breaks (DSBs) in both cell lines and by

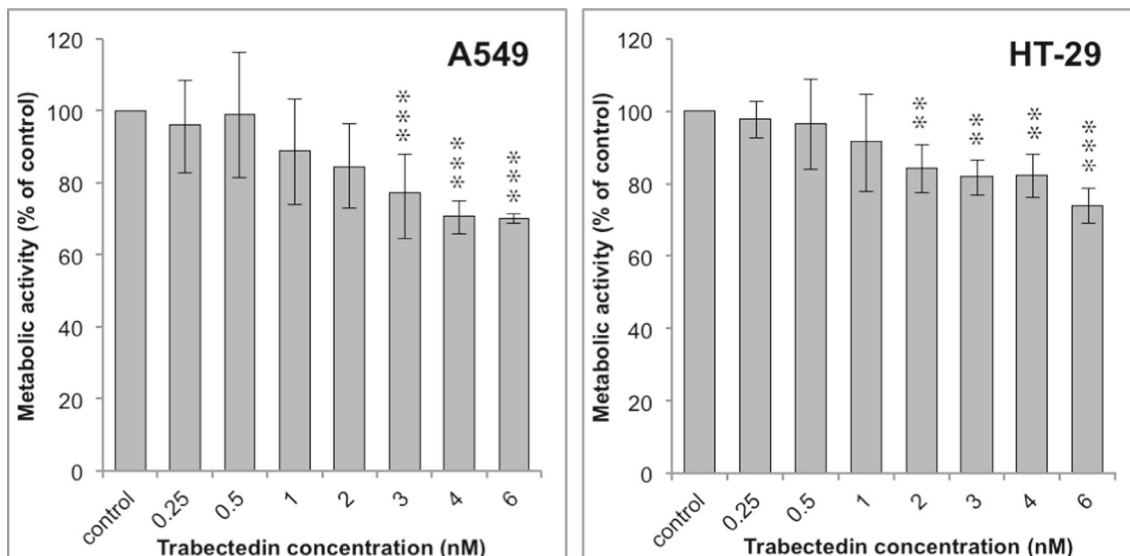
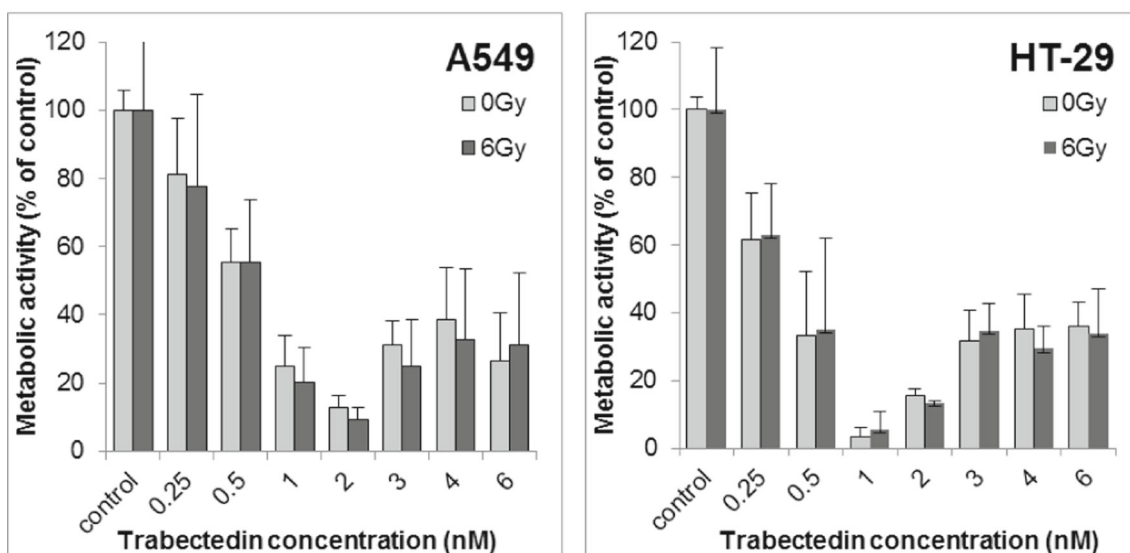
a Trabectedin pretreatment 24 hours**b Trabectedin pretreatment 48 hours**

Fig. 2 Metabolic activity of A549 and HT-29 cells following 24 h (a) or 48 h (b) of incubation with trabectedin and (b) in combination with IR, measured via EZ4U assay. The metabolic activity of treated cells was normalized to the values of untreated controls. Error bars indicate the

standard deviation for three independent experiments; wells were assayed in six replicates. Asterisks illustrate significance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$

counting the number of γ H2AX foci consequently formed. Treatment of tumor cells with trabectedin alone resulted in a clear increase in the number of DNA double-strand breaks, as can be seen from the increased count of γ H2AX foci present at 0 Gy (Fig. 5). The γ H2AX foci values of cells treated with different trabectedin concentrations (2 nM, 6 nM) varied only marginally. The statistical analysis showed no significance, but a tendency toward a time-dependent increase in the number of γ H2AX foci formed.

Time-dependent effects on the number of γ H2AX foci could clearly be observed following their exposure to 6 Gy radiation. After 0.5 h of irradiation, due to the acute cell damage suffered in the immediate post-radiation period, the number of γ H2AX foci increased significantly. Owing to the presence of a large number of foci, no differences between cells treated with trabectedin and those not treated with the drug were detected. The ability of a cell to repair DNA DSBs is to be evaluated after it has gone through a prolonged repair time of 24 h. In both trabectedin-treated cell lines, compared to

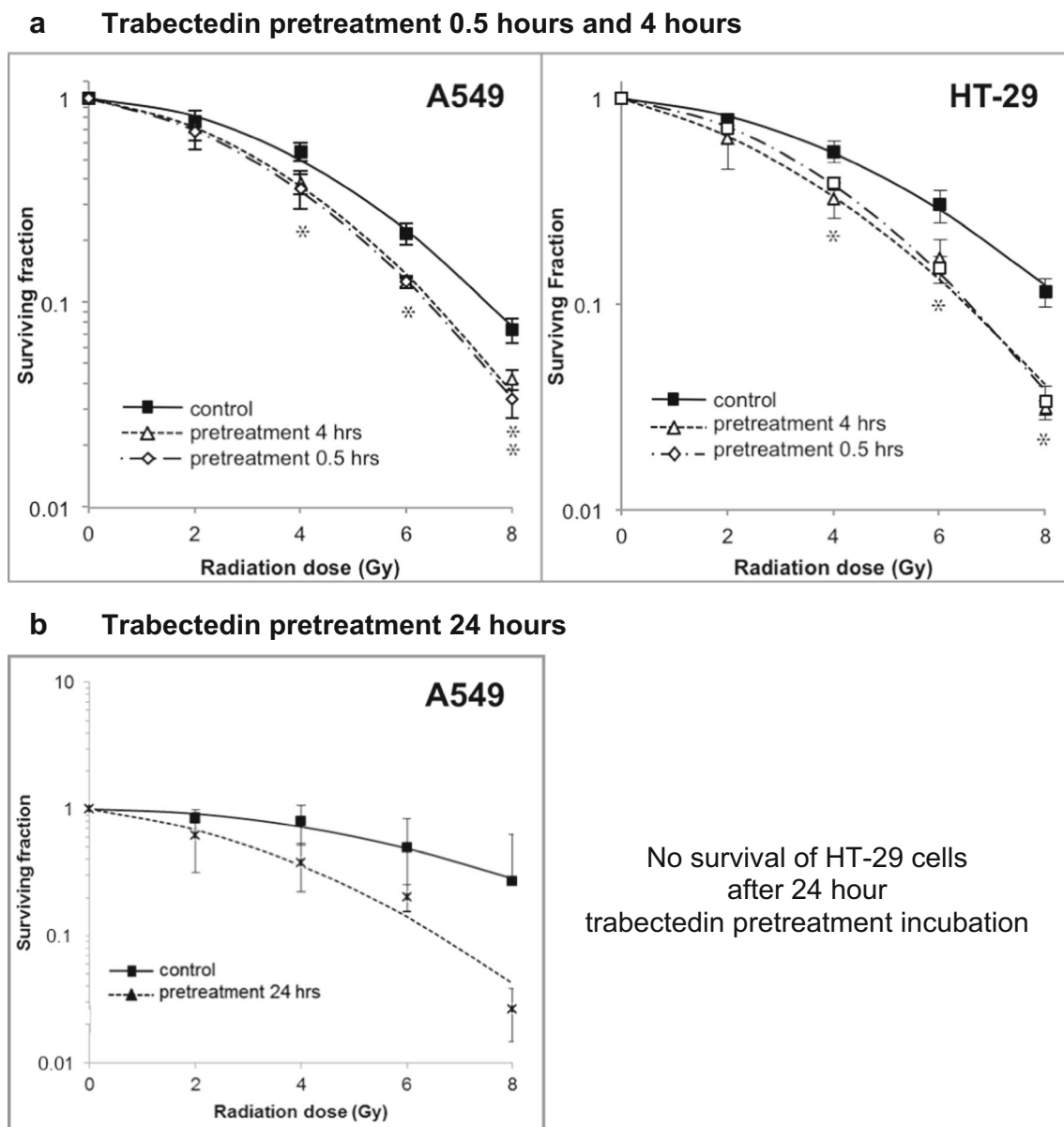


Fig. 3 Effect of 0.25 nM trabectedin on clonogenic survival of A549 and HT-29 cells when pre-treated (a) 0.5 h, 4 h or (b) 24 h before radiation exposure. Clonogenic fractions of treated cells were normalized to the

values of untreated control (0 nM trabectedin, 0 Gy). Error bars indicate the standard deviation for three independent experiments; each assayed in duplicate flasks. Asterisks illustrate significance: * $P < 0.05$; ** $P < 0.005$

unirradiated cells, the number of γ H2AX foci showed no increase in the number of residual DNA DSBs 24 h after radiation.

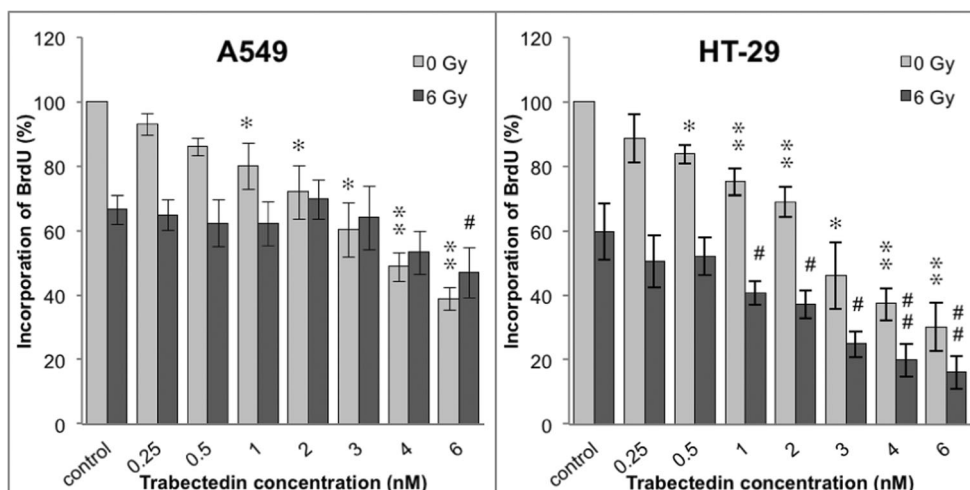
Discussion

Due to its activity against several types of tumor cells including melanoma, NSCLC as well as pancreatic, prostate and breast cancer cells trabectedin is a promising chemotherapeutic agent [21–24]. Treatment in combination with radiotherapy appears useful, but currently trabectedin is administered to patients as a monotherapy for soft-tissue sarcoma. It is also

used in combination with pegylated liposomal doxorubicin as treatment for relapsed platinum-sensitive ovarian cancer [28]. Additionally, trabectedin is approved for the treatment of liposarcoma and leiomyosarcoma as a second-line therapy [4].

In the present study, we investigated the effects of treatment with trabectedin alone and in combination with ionizing radiation *in vitro*. Two human tumor cell lines, A549 (lung cancer) and HT-29 (colon cancer), were treated with trabectedin concentrations between 0.25 nM and 6 nM, which are attainable in patients' plasma concentrations [29]. According to Taamma *et al.*, peak plasma concentrations observed in patients treated with the recommended dose of 1500 $\mu\text{g}/\text{m}^2$ trabectedin as a 24-h continuous infusion are approximately 2.3 nM [29].

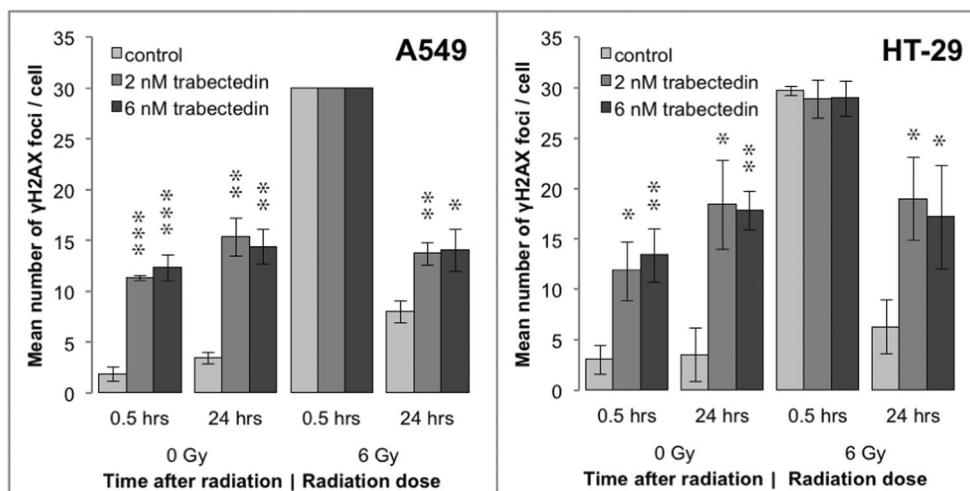
Fig. 4 BrdU incorporation into A549 and HT-29 cells after 26 h incubation with trabectedin. Cells were irradiated after 4 h pre-treatment with trabectedin. Values were normalized to the untreated control (0 nM trabectedin, 0 Gy). Error bars indicate the standard deviation for three independent experiments; wells were assayed in triplicates. Significance illustrated by asterisks (0 Gy) and octothorpes (6 Gy): */# $P < 0.05$; **/### $P < 0.005$



In our study, treatment with trabectedin demonstrated concentration-dependent cell growth inhibition and reduction in cell viability in both, the lung cancer cell line A549 and the colon cancer cell line HT-29; also, HT-29 cells were slightly more sensitive to the drug. Consistent with previous reports [21–24], the IC_{50} values were within the nanomolar range. We verified a decrease in IC_{50} values with varying durations of incubation, suggesting a time-dependent effect, as described previously on tumor cells explanted from patients [23]. The metabolic assay, which was performed, confirmed the cytotoxic effect of trabectedin with a concentration-dependent decrease in metabolic activity in both cell lines, beginning at 1 nM trabectedin. Prolongation of the treatment with trabectedin, from 24 h to 48 h, resulted in a remarkable reduction in metabolic activity when up to 1 nM of the drug was used for HT-29 cell line or 2 nM for A549 cell line. However, higher concentrations of the drug led to an increase in metabolic activity again. A similar effect has been detected in other tested substances as well. In retinal cells exposed for 48 h to 0.125 mg/mL of ranibizumab, the MTT assay showed a

significant decrease in metabolic activity, but it increased again after the dose had been doubled to 0.25 mg/mL [30]. Also, in medulloblastoma cells treated with a low concentration of 5-aza-2'-deoxycytidine for three days, the metabolic activity of the cells decreased, as measured in a WST-1 assay; but higher concentrations of the drug led to an increase in the metabolic activity again [31]. In D283-Med cells treated with 5-aza-2'-deoxycytidine, the metabolic activity steadily decreased when concentrations of the drug were brought down to 1 μ M level. However, there was a marked increase in the activity when the drug concentrations were increased to 3 μ M and 5 μ M. In DAOY cells exposed to 5-aza-2'-deoxycytidine, the inhibition of metabolic activity at concentrations of 1 μ M and 3 μ M and reduction in the inhibition at 5 μ M concentration were observed [31]. In RAW264.7 cells treated with increasing concentrations of TiO_2 nanoparticles (100 nm sizes), Xiong et al. (2013) observed heightened metabolic activity at concentrations of 10 μ g/mL and 20 μ g/mL followed by a decrease in the activity in doses above 100 μ g/mL [32]. In the past, it was assumed that conversion of tetrazolium salts

Fig. 5 Effect of trabectedin on DNA DSB repair capacity of A549 and HT-29 cells after a repair time of 0.5 or 24 h. Error bars indicate the standard deviation for three independent experiments. Asterisks illustrate significance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$



(MTT, WST-1) occurred exclusively through mitochondrial succinate dehydrogenases. Today it is known that tetrazolium salts can be converted by multiple oxidoreductases located both, inside and outside the mitochondria. Rather, studies in recent years suggest that the reduction is mainly dependent on reduction equivalents NADH and NADPH, which contain pyridine, and only partially on succinate [33–35]. Generally, the amount of formazan product is proportional to the number of metabolically active viable cells. But culture conditions, for example depletion of essential nutrients such as glucose, which alter the metabolism of the cells, can influence the rate of reduction of tetrazolium salts into formazan [35]. Blockhuys *et al.* (2019) assumed that the effects observed by them on the metabolic activity were not due to an increased metabolic activity, but rather to an enhanced cellular ingress of tetrazolium salts and their conversion at the mitochondrial level [36]. A further reason for the rise in metabolic activity, as we found again in our 48 h after higher drug concentrations could be an alteration of the drug in high concentrations, such as clustering of the molecules, which would reduce the effect of the substance. The linear dose-response relationships, which were demonstrated both in growth (Fig. 1) and in proliferation (Fig. 4), however, speak against this possibility. There is no conclusive explanation for this observation at this time. Further treatment of the cells with ionizing radiation had no influence on the metabolic activity.

Combined with ionizing radiation, trabectedin demonstrated an *in vitro* radiosensitizing effect on both tumor cell lines under examination. Assays of colony-forming cells presented a statistically significant decrease in the long-term survival of tumor cells, when treated with 0.25 nM trabectedin 0.5 h and 4 h before irradiation. Pretreatment with trabectedin for a longer time (24 h) indicated that only A549 cells could survive irradiation, showing the radiosensitizing effect of the drug. These findings, however, are in conflict with the previous results for A549. Although detectable in other cell lines (ECV304, H292, and CAL-27), Simoens *et al.* observed that trabectedin had no radiosensitizing effect on A549 cells that had been treated with concentration of 1.3 nM or 1.8 nM of the drug 24 h prior to the radiation exposure [20]. In the present study, however, we were able to demonstrate a clear radiation sensitizing effect of trabectedin on the A549 cells. Romero *et al.* also described trabectedin as lacking a radiosensitizing effect on HT-29 cells that were treated with 0.2 nM concentrations of the drug for 24 h [22]. Apparently, the HT-29 cells were unable to survive such a long period of incubation with trabectedin. During growth and measurement of metabolic activity, HT-29 cells had already shown an increased sensitivity compared to A549 cells. In this study we have been able to demonstrate that trabectedin has a radiosensitizing effect on HT-29 cells too, in a shorter preincubation period of 0.5 h or 4 h. Time-dependent radiosensitization effects were previously reported on various

other substances [37–39]. Concerning the mechanism of radiosensitization effect of trabectedin, it was previously shown that the drug caused disorganization of microtubule filaments [24]. This effect induces an arrest of cells in late S- and G2/M-phase, which is demonstrated in cell kinetic studies by Simoens *et al.* [20]. It is well known that in these cell cycle phases, tumor cells are very radiosensitive; and this could explain the radiosensitizing effect of trabectedin. The arrest of cells in late S- and G2/M-phase might also be the reason for the reduced DNA synthesis, which was observed as a dose-dependent decrease in BrdU incorporation in our study for both cell lines treated with trabectedin alone. This effect grew stronger when cells were also treated with radiation.

To investigate the influence of trabectedin on the induction of DNA DSBs and their repair after ionizing radiation, the γ H2AX-foci formation method was used. The number of foci that are formed correlate directly with the number of DNA DSBs [40]; alternatively, a decrease in the number of foci reflects the repair of DNA DSBs [41]. Our study demonstrated that trabectedin had a strong influence on the number of residual DNA DSBs in cancer cells. Of the radiation-induced DNA lesions, DSBs appear to be most frequently associated with cell lethality [42]. The present study demonstrated that trabectedin had a distinct influence on DNA DSBs in both A549 and HT-29 cells. Treatment with trabectedin at nanomolar concentrations increased the number of DNA DSBs significantly. HT-29 cells displayed more DNA DSBs than in the A549 cell line. The mechanism of action of trabectedin and its cellular effects are discussed by both Takebayashi *et al.* [18] and Soares *et al.* [43], although with conflicting results from the cell lines and trabectedin concentrations investigated. Takebayashi *et al.* could not detect DNA DSBs in human colon carcinoma cell line HCT116, which was treated with distinctly higher trabectedin concentrations of 1 μ M, 5 μ M or 10 μ M for up to 15 h [18]. However, Soares *et al.* described a concentration-dependent increase in the number of DNA DSBs in HeLa cells and lymphoblastic leukemia CEM cells after a one-hour incubation with trabectedin in concentrations of 10 nM and 20 nM [43]. An enhanced number of DNA DSBs in both cell lines, after treatment with trabectedin, indicates that our results support the findings of Soares *et al.*, although with lower drug concentrations and different cell lines. Soares *et al.* offer a mechanical explanation for the poor repair of trabectedin-DNA adducts that are formed; and this can induce secondary DNA damage, for example, the formation of DNA DSBs. Casado *et al.* report that trabectedin induces the formation of DNA DSBs in human cells and activates homologous recombination (HR) repair, which is caused by the DNA interstrand cross-linking agent mitomycin C, in a similar way to that explained by Soares *et al.* [44].

Following the treatment of both cell lines with trabectedin and ionizing radiation, the number of γ H2AX foci showed no

increased number of residual DNA DSBs 24 h after radiation, compared to unirradiated cells. This suggests that the repair capacity of DNA DSBs after exposure to ionizing radiation seems to be unaltered despite being treated with trabectedin. This phenomenon cannot explain the increased radiation sensitivity of the cells treated with trabectedin, as demonstrated in the assay of colony formation cells.

Our results demonstrated that trabectedin showed a direct cytotoxic effect on both A549 and HT-29 tumor cells within a nanomolar range. An interesting finding of this study was the ability of the drug to act as a radiosensitizer for both types of tumor cells when treated 0.5 h or 4 h before irradiation. This finding provides crucial evidence of the increased potential of trabectedin for therapeutic use in radiotherapy in the near future. For further elucidation of the clinical relevance of using trabectedin combined with ionizing radiation, future *in vivo* investigations should be considered.

Author's contributions K.M. designed the study. T.P. and T.S. performed experiments, as well as acquired and analyzed the data. S.K. developed the radiation setup and performed dosimetry. K.M. and T.P. wrote and drafted the manuscript. G.H. critically revised the manuscript and contributed to materials and tools. All authors read and approved the final manuscript.

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Data availability The data supporting this study are provided in the results section of this paper. Further datasets used and/or analyzed during the current study are available and stored by the authors at the University Medical Center Rostock.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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