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The taccalonolides, novel microtubule stabilizers, and γ -radiation have additive effects on cellular viability

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ABSTRACT

The taccalonolides are novel antimetabolic microtubule stabilizers that have a unique mechanism of action independent of a direct interaction with tubulin. Cytotoxicity and clonogenic assays show that taccalonolide A and radiation act in an additive manner to cause cell death. The taxanes and epothilones have utility when combined with radiotherapy and these findings further suggest the additive effects of microtubule targeting agents with radiation on cellular proliferation are independent of direct tubulin binding and are instead a result of the downstream effects of these agents. These studies suggest that diverse antimetabolic agents, including the taccalonolides, may have utility in chemoradiotherapy.

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1. Introduction

Combination therapy is employed in cancer treatment to optimize anticancer efficacy and to prevent the emergence of drug resistant populations of cancer cells. The combination of radiotherapy and chemotherapy is well established to provide therapeutic benefit in many types of cancers, including head and neck cancers. Chemotherapy has been shown to be an effective addition to radiation treatment in patients with either resectable or non-resectable tumors to reduce both the primary tumor and distant metastases that are not affected by localized treatment [1,2]. The addition of induction or concurrent chemother-

apy with radiation has led to significant improvements in both progression free and overall survival [3]. The combination of chemotherapy and radiation therapy also has utility in the treatment of breast cancer.

Many microtubule targeting agents, including the taxanes and epothilones, have demonstrated radiosensitizing properties both *in vitro* and *in vivo* [4–6]. Classically, the radiosensitization potential of these agents was thought to be due to their ability to arrest cells in the most radiosensitive phases of the cell cycle, G₂ and M [7]. However, some studies show that G₂/M accumulation is not a prerequisite for radiosensitization by microtubule stabilizers, indicating the potential for additional mechanisms of action [8,9].

The benefit of the taxanes in the treatment of head and neck cancers as single agents or in combination with radiation and/or other chemotherapeutic agents is well established [10]. As single agents, docetaxel or radiation treatment consistently show patient response rates between 20% and 50% regardless of the treatment schedule or measured endpoint [11,12]. While these treatments are effective individually, the administration of taxane-

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containing chemotherapy regimens prior to and/or during radiation treatment dramatically improves overall response rates to 85–100% [13]. Patupilone, a non-taxane microtubule stabilizer of the epothilone class, acts as a radiosensitizer in multidrug resistant tumor models [8]. In addition to the well documented radiosensitizing effects of diverse microtubule stabilizers, several microtubule depolymerizers including vinflunine, vinorelbine and the colchicine site agent TZT-1027 radiosensitize cancer cells and have additive antitumor effects when used in combination with radiation in murine models [14,15].

The taccalonolides are a novel class of microtubule stabilizers [16]. They are highly acetylated steroids isolated from plants of the genus *Tacca* that have a unique mechanism of action and the ability to circumvent two clinically relevant forms of taxane resistance, P-glycoprotein-mediated drug efflux and expression of the β III tubulin isotype [17,18]. Consistent with the actions of the taxanes and epothilones, the taccalonolides cause an increase in the density of cellular microtubules and a shift in intracellular tubulin to the polymerized form; however the taccalonolides do not bind directly to tubulin or polymerize purified tubulin *in vitro* [18]. Although the taccalonolides do not bind directly to tubulin, the fact that they disrupt interphase and mitotic microtubules, causing mitotic arrest and apoptosis, suggests that they might be effective with γ -radiation in an additive or synergistic manner. In this study, we show that taccalonolide A (tacca A) and γ -radiation have additive effects on head and neck squamous cancer cells when measured in either short term viability or longer term clonogenic assays. These additive effects between γ -radiation and tacca A treatment are also observed in the clonogenic assay with tacca E or in MCF7 cells, indicating these additive effects with γ -radiation are a generalizable property of taccalonolide treatment. Finally, the additivity between tacca A and γ -radiation is observed regardless of the order of treatment and at doses of tacca A that do not cause mitotic arrest, suggesting that the taccalonolides are not true radiosensitizers and instead contribute to cell death of γ -irradiated cells independent of their ability to cause mitotic arrest prior to irradiation.

2. Materials and methods

2.1. Materials

Taccalonolides A and E (taccas A and E) were isolated as previously described [17]. The chemical identities of tacca A and tacca E were confirmed by nuclear magnetic resonance. Paclitaxel was purchased from Sigma–Aldrich (St. Louis, Mo).

2.2. Cell culture

SCC4 human oral squamous cell carcinoma cells and MCF7 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM or IMEM medium, respectively (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (Hy-

clone; Logan, UT) and 50 μ g/ml gentamicin sulfate (Invitrogen). Cells were used in log phase growth.

2.3. Cell cycle analysis

Cell cycle distribution was evaluated by flow cytometry. Cells were treated with tacca A, Taxol, or γ -radiation for 24 h and harvested by scraping. The cell pellet was collected by centrifugation and resuspended in Krishan's reagent [19]. Cells were then evaluated on a Beckton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ). The propidium iodide content of 20,000 cells was measured and cell cycle distribution was calculated using ModFit software (Verity; Topsham, ME) with 2N, 4N or intermediate DNA content referred to as the G₁, G₂/M or S populations respectively. Each value is the mean of at least three independent experiments.

2.4. γ -Radiation

A Gamma cell 40 ¹³⁷Cs γ -ray source (Atomic Energy of Canada Limited; Ottawa, ON) at a dose rate of 1.191 Gy/min was used to irradiate cells immediately after removal from incubation at 37°.

2.5. High-throughput short term cytotoxicity assay

SCC4 cells were plated in a 384-well view bottom plate at a density of 2000 cells/well (100 μ l of a 20,000 cell/ml suspension) and 24 h later treated with drug in replicates of 16. The cells were irradiated with the indicated dose of γ -radiation 24 h after drug addition. After an additional 24 h, individual cell viability was determined by Invitrogen's live/dead assay kit. Radiation only controls were performed on the same plate as drug treated cells and therefore also analyzed 24 h after radiation treatment. In this assay, viable cells retain calcein-AM and thus are indicated in green while dead cells, which have lost membrane integrity, take up ethidium bromide and are indicated in red. Fluorescence images were acquired with a 20 \times long working distance objective from three fields per well (1/10th of the 30 possible fields in each well) using the Operetta high throughput imaging system (PerkinElmer; Waltham, MA). An algorithm to identify the number of live and dead cells in each frame was developed using Harmony software (PerkinElmer) and validated on control cell populations, including mostly viable untreated cells as well as dead cells resulting from treatment with 0.1% Triton X-100. The algorithm was applied to each frame and the number of live and dead cells for each condition was determined in an unbiased manner. A total of 48 fields were evaluated for each treatment, resulting in a minimum of 10,000 cells counted for each experimental condition. Expected additive values were calculated by adding together the decrease in viable colonies caused by each individual treatment modality with propagated errors. The difference between the additive expected and observed values for each combination of tacca and radiation treatment showed no statistically significant difference with a p value of 0.1, other than one combination as noted.

2.6. Clonogenic assay

SCC4 or MCF7 cells were plated in tissue culture dishes at a predetermined density that resulted in the formation of approximately 200 colonies per plate in the absence of drug or radiation. Tacca A, tacca E or paclitaxel were added 24 h after plating and then cells were irradiated after 24 h of drug exposure, except in Fig. 4B where the order of treatments was reversed. The cell culture medium was replaced 24 h after the final treatment and colonies were allowed to grow for an additional 12 days. Colonies were gently washed with room temperature PBS and then fixed and stained with a 20% methanol, 0.5% crystal violet solution. Excess stain was removed by gently washing with PBS and images of the plates were acquired using the Geliance imaging system (PerkinElmer). The colony counting function of the GeneTools software (Syngene; Frederick, MD) was used to count colonies. The survival fraction was calculated and plotted with the standard error of three independent replicates. *F* ratios were calculated for each pair of values in Fig. 3B and C and the statistical significance at *p* values of 0.005–0.1 were calculated and represented as indicated in the figure legend. Expected additive values in Fig. 4 were calculated by adding together the decrease in viable colonies caused by each individual treatment modality with propagated errors. The difference between the additive expected and observed values for each combination of tacca and radiation treatment showed no statistically significant difference with a *p* value of 0.1.

3. Results

3.1. Tacca A or γ -radiation cause G_2/M accumulation

Cell cycle progression is known to be inhibited by diverse microtubule targeting agents or γ -radiation. The effects of tacca A or γ -radiation on the cell cycle progression of SCC4 cells was evaluated to determine whether these treatments caused G_2/M accumulation. In control, untreated SCC4 cells, 47.2% of the population were in G_1 , 27.2% in S phase, and 25.6% were in G_2/M (Fig. 1). Treatment of cells with either γ -radiation or tacca A caused a dose dependent accumulation of cells with 4N DNA content, the G_2/M population, within 24 h (Fig. 1). Although concentrations up to 1 μ M tacca A did not significantly affect cell cycle distribution, 2 μ M tacca A doubled the number of cells in G_2/M from 25.6% to 58.0%, with corresponding decreases in the G_1 and S phase populations of cells (Fig. 1). Increasing the concentration of tacca A to 4 μ M further increased

the percentage of cells in G_2/M to 82.2%. A dose of 2 Gy of γ -radiation had no effect on cell cycle distribution and a slight increase in cells in the G_2/M population was initiated with a 4 Gy dose (Fig. 1). A near doubling of the G_2/M population from 25.6% to 49.1% was achieved with 6 Gy of γ -radiation, mostly due to a decrease in the S phase population. 10 Gy of γ -radiation further increased the G_2/M population of cells to 69.9%. These data confirm that SCC4 cells accumulate in G_2/M in response to either tacca A or ionizing radiation treatment.

Propidium iodide-based cell cycle analysis cannot distinguish the G_2 and M phases of the cell cycle, but most adherent cells, including SCC4 cells, appear flat in interphase but undergo a RhoA-dependent change in the actin cytoskeleton during mitosis to become noticeably more spherical [20]. Therefore, the G_2 and M phases of the cell cycle can be easily distinguished by light microscopy. The approximate percentage of cells that were in mitosis after treatment with γ -radiation or tacca A was determined by visually examining the morphology of cells prior to their preparation for flow cytometry. The percentage of spherical mitotic cells observed upon tacca A treatment roughly correlated with the percentage of cells with 4N DNA content as determined by flow cytometry. This finding supports previous reports that the taccalonolides arrest cells in mitosis [16]. In contrast, γ -radiation did not noticeably increase the percentage of spherical cells even at 10 Gy, a concentration that tripled the accumulation of G_2/M cells. This is consistent with reports that γ -radiation leads to G_2 arrest of cells, like SCC4, that express a mutant form of p53 [21]. Therefore, although both tacca A and γ -radiation cause a dose-dependent G_2/M accumulation of SCC4 cells, tacca A causes mitotic arrest while cells appear to accumulate in G_2 in response to γ -radiation.

3.2. Tacca A and γ -radiation inhibit short-term cell viability in an additive manner

The cytotoxic effects of tacca A and radiation were evaluated alone and in combination using a high-throughput fluorescence microscopy assay. The number of live (green) and dead (red) cells were counted following treatment with tacca A, paclitaxel, γ -radiation or a combination of drug and γ -irradiation. The assay clearly differentiates live and dead cells (Fig. 2A) and the high throughput functionality allowed the evaluation of 10,000–22,000 cells for each treatment group. Consistent with the colorimetric readout of the assay shown in Fig. 2A, a histogram showing the raw data of the effects of tacca A treatment alone is presented in Fig. 2B. The histogram retains the green/red coloring indicating live/dead cells remaining on the plate. The percentage of viable cells is a conservative readout, since any cells lost from the plate due to cell death during the experiment are not detected. We calculated a doubling time of 26 h for untreated cells for the duration of the assay, which means that cells were exposed to drug for one population doubling before being subjected to radiation and that viability was analyzed after an additional population doubling. Tacca A alone caused a dose dependent decrease in viable cells from 98% in untreated controls to 91% viability at 0.75 μ M, 80% at 1.25 μ M and 66% at 1.75 μ M tacca A (Fig. 2B; Table 1). A dose-dependent increase in the number of dead cells retained on the plate was also observed (Fig. 2B). The effects of paclitaxel on cell viability were also determined to allow comparisons between the two microtubule stabilizers. In untreated wells, live viable cells represented 98% of the population while treatment with 3 nM paclitaxel resulted in 81% viable cells (Table 1). As with tacca A, the number of total viable cells remaining after paclitaxel treatment diminished in a dose-dependent manner (Table 1). Ionizing radiation alone also caused cell death, with cell viability decreasing to 92% and 80% at 6 Gy and 10 Gy respectively (Table 1). The magnitude of the individual effects of tacca A, paclitaxel or radiation treatment on cell viability was used to select concentrations of each treatment to evaluate in combination. Concentrations of tacca A or paclitaxel and doses of radiation that caused sub-maximal cell death, retaining 65–90% cell viability after treatment with a single modality, were selected. This defined an optimal range to detect additive or synergistic actions of combination treatments.

Concentration ranges of 0–1.75 μ M tacca A, 0–6 nM paclitaxel and radiation doses of 6 and 10 Gy were selected to optimally detect additive or potential synergistic actions of combination treatments. Fig. 2C shows the effects of tacca A treatment alone and in combination with 6 or 10 Gy of radiation. Within each concentration of tacca A the cytotoxic effects of radiation are observed and the ability of the combination to decrease cell viability across the range of concentrations of tacca A is seen. The combination of 1.75 μ M tacca A and radiation caused a decrease in cell viability

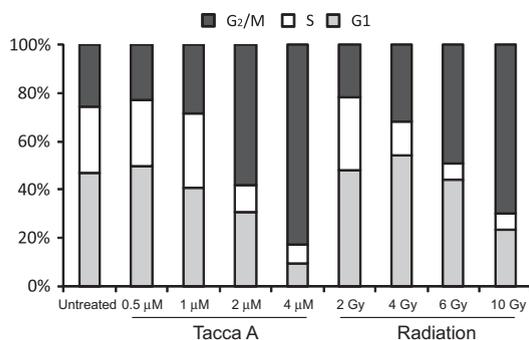


Fig. 1. Dose-dependent effects of tacca A and γ -radiation on cell cycle distribution. The cell cycle distribution of propidium iodide stained SCC4 cells was analyzed 24 h after treatment with tacca A or radiation.

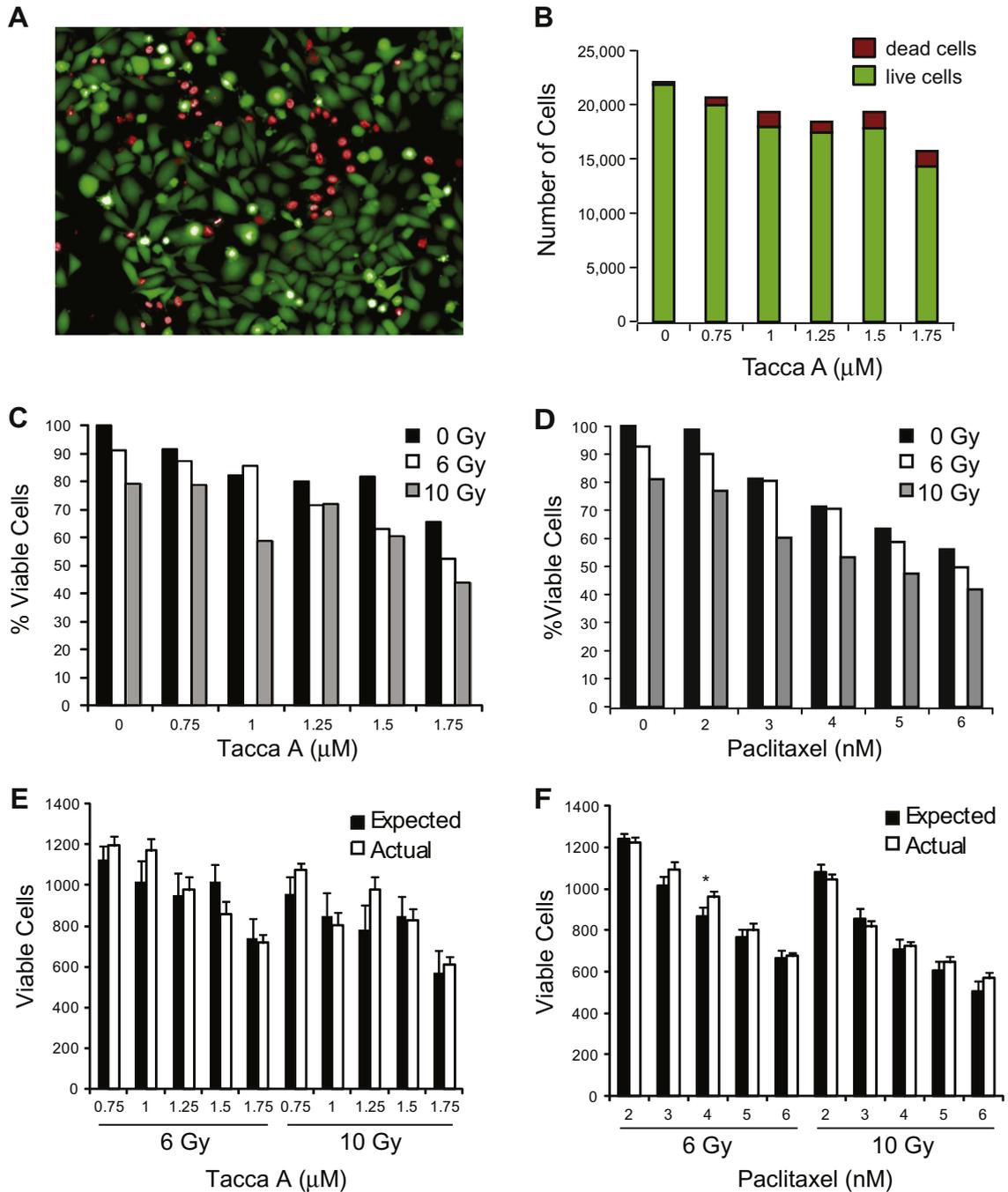


Fig. 2. Effects of γ -radiation and tacca A in the short term viability assay. Representative image of live (green) and dead (red) cells (A). Number of live (green) and dead (red) cells following treatment with tacca A alone (B). Percentage of live cells remaining after treatment with a combination of γ -radiation and tacca A (C) or paclitaxel (D) as compared to untreated cells. The expected number of viable cells after treatment with γ -radiation and tacca A (E) or paclitaxel (F) as calculated from the effects of individual treatments is shown with propagated error in the black bars. The actual number of viable cells from these dual treatments is shown in the white bars. The expected additive and actual values show no statistically significant difference with $p = 0.1$ with the exception of one value, which shows significance at $p = 0.1$ but not at $p = 0.05$ and is depicted by an asterisk.

from 66% with tacca A alone to 52% at 6 Gy and 44% in combination with 10 Gy of radiation (Fig. 2C). Similar effects were observed with paclitaxel (Fig. 2D).

The data were evaluated further to determine whether the effects of combining a microtubule stabilizer with radiation cause additive loss of cell viability or indicated a potential synergistic effect. The predicted

additive effects of tacca A and radiation on cell viability were calculated for each combination from their individual effects and compared to the actual effects measured when the two treatment modalities were combined. This analysis indicates that various combinations of γ -radiation and tacca A have effects that are statistically indistinct from their predicted additive values (Fig. 2E). The predicted additive effects between

Table 1

Percent cell viability of SCC4 cells when treated with single agents in the short term viability assay.

Treatment	Dose	Viability (%)
Untreated	–	98
γ -Radiation	6 Gy	92
	10 Gy	80
Tacca A	0.75 μ M	91
	1 μ M	82
	1.25 μ M	80
	1.5 μ M	82
	1.75 μ M	66
Taxol	2 nM	99
	3 nM	81
	4 nM	71
	5 nM	63
	6 nM	56

These values were used to calculate the expected additive viability of tacca A or paclitaxel treatment when combined with γ -radiation in the short-term cell viability assay. The percent viability values are normalized to the viability of cells that have not been treated with drug or radiation.

paclitaxel and γ -radiation were also determined and compared to measured effects. Additive effects between paclitaxel and γ -radiation were observed with almost every combination treatment (Fig. 2F). These analyses suggest that the combination of tacca A or paclitaxel with radiation cause additive effects on SCC4 cell viability as measured with the high-throughput, short term viability assay and provides no indication of a potential synergistic effect of combining either tacca A or paclitaxel with radiation.

The advantage of the short-term high-throughput assay is its robustness due to the large number of cells that can be counted in each experimental group. A limitation of this assay is that there is no way to evaluate the fate of individual cells that are lost from the plate during the course of the 48 h experiment. An example can be seen in Fig. 2B, where 22,269 cells were evaluated in untreated controls yet the number of cells decreased to 15,711 with 1.75 μ M tacca A treatment. The difference in total cell number upon treatment could be due to cell death and/or an increase in mitotic cells, which are more susceptible to being dislodged from the plate. Another potential shortcoming of this analysis is that radiation induced cell death often requires numerous mitotic events to allow chromosomal damage to accumulate to a point that is lethal, which will not be reflected in this short-term assay where cells have only gone through one mitotic event on average between irradiation and viability measurements. To address these issues, the effects of combining tacca A and radiation in a longer term clonogenic assay were also studied.

3.3. The taccas and γ -radiation have additive effects on colony formation

The colony formation assay was used to evaluate the long-term effects of tacca A and γ -radiation on SCC4 cell growth and viability when used alone or in combination. In untreated controls an average of 165 colonies formed during the assay (Fig. 3A, top left panel). The concentration dependent effects of tacca A treatment alone on colony formation were first determined (Fig. 3B, solid line). Concentrations of tacca A up to 125 nM did not inhibit the colony forming ability of the SCC4 cells (Table 2). However, colony formation was inhibited by 28% with 150 nM tacca A and this inhibition is clearly visible in the treated plates (Fig. 3A, top right panel). A 49% inhibition of survival was observed with 175 nM tacca A and 76% inhibition with 200 nM tacca A (Fig. 3B, solid line; Table 2).

The effects of radiation treatment alone on survivability were also evaluated and the data presented as the solid line of Fig. 3C. A 1 Gy dose inhibited colony formation 15%, 2 Gy caused 22% inhibition, 4 Gy caused 61% inhibition and a 6 Gy dose inhibited colony formation 89% (Table 2). Consistent with the effects of the short-term assay, tacca A or radiation alone inhibit the long-term viability of SCC4 cells as measured by the colony forming assay, albeit both drug and radiation effects are observed at lower doses.

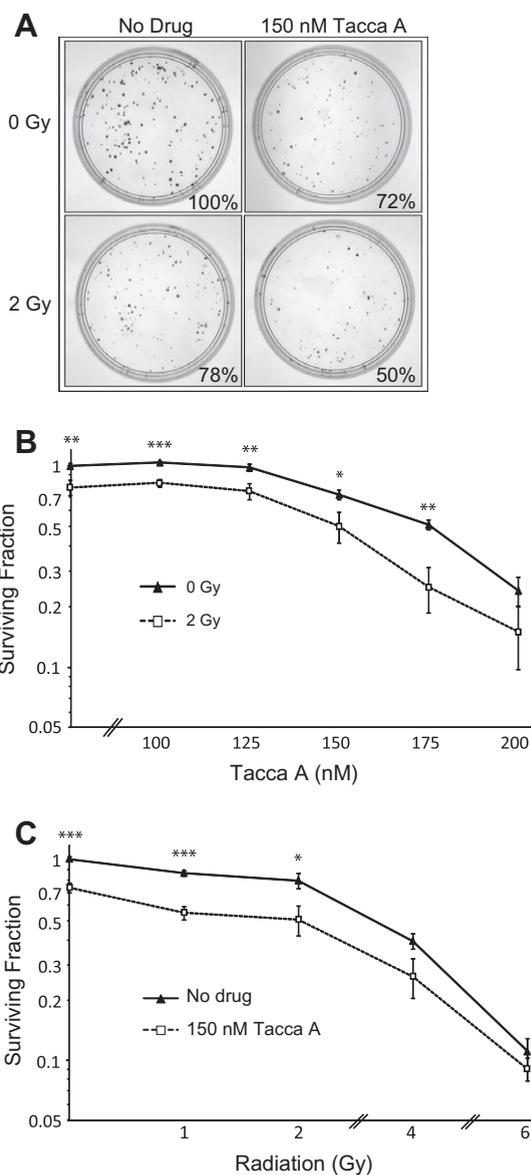


Fig. 3. Effects of γ -radiation and tacca A on colony formation. (A) Images of crystal violet-stained colonies formed after treatment of SCC4 cells with 150 nM tacca A, 2 Gy γ -radiation or a combination of the two treatments. The percentage of colonies formed with each treatment as compared to untreated cells is included for each image. (B) The surviving fraction of SCC4 cells after treatment with 2 Gy γ -radiation and indicated concentration of tacca A is graphed. (C) The surviving fraction of SCC4 cells after treatment with 150 nM tacca A and the indicated concentration of γ -radiation is graphed. Designations of ***, ** and * denote statistical significance with *p* values of 0.005, 0.05 and 0.1 respectively.

The effects of combinations of tacca A and radiation treatment on colony formation were analyzed. Cells were treated with low concentrations of tacca A 24 h prior to irradiation and colonies counted 12 days afterward. The results show that 2 Gy γ -radiation significantly enhanced the effectiveness of tacca A treatment up to a concentration of 175 nM (Fig. 3B, compare solid to dotted lines). Similarly 150 nM tacca A significantly decreased colony formation when combined with doses of 1 or 2 Gy γ -radiation (Fig. 3C, compare solid to dotted lines). Not surprisingly, the added benefit of low concentrations of tacca A pretreatment were not

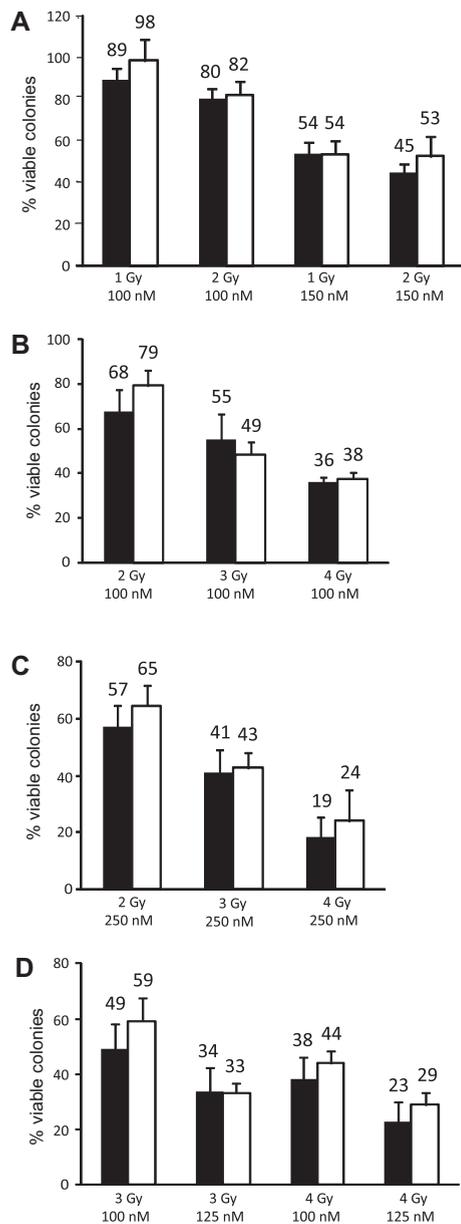


Fig. 4. Effects of γ -radiation and tacca treatment are additive in the clonogenic assay. (A) Tacca A was added to SCC4 cells 24 h prior to γ -radiation exposure. (B) SCC4 cells were exposed to γ -radiation 24 h prior to tacca A addition. (C) Tacca A was added to MCF7 cells 24 h prior to γ -radiation exposure. (D) Tacca E was added to SCC4 cells 24 h prior to γ -radiation exposure. The percentage decreases in colony formation for individual treatments were added together with propagated error to calculate the expected additive decreases in colony formation for the combination treatment, which are depicted by the black bars. The experimentally observed decreases in colony formation after combination treatments were determined and depicted by the white bars. The expected additive and actual values for each treatment combination show no statistically significant difference with $p = 0.1$.

statistically significant at higher doses of γ -radiation since these 4 and 6 Gy doses cause a substantial inhibition of colony formation on their own.

The additive effects of tacca A and radiation treatment in the clonogenic assay were evaluated. The effects of one combination are presented in Fig. 3A, where 150 nM tacca A as a single agent decreased viability to

72% of untreated control (top right panel) and 2 Gy γ -radiation decreased viability to 78% when used alone (bottom left panel). The combination, shown in the bottom right panel, caused 50% viability, demonstrating an additive effect of tacca A and radiation in this assay. The expected additive percent inhibition of colony formation for each combination of tacca A and radiation was calculated by adding together their individual effects with propagated error. The results in Fig. 4A show that the actual inhibition of colony formation generated by cells treated with 100 or 150 nM tacca A 24 h prior to exposure with 1 or 2 Gy γ -radiation are not statistically different than the predicted additive values, suggesting that these two treatment modalities have additive effects on cellular viability in the clonogenic assay.

The fact that tacca A and radiation treatments have strictly additive and not synergistic effects on cellular viability suggests that tacca A is not a true radiosensitizer. To further analyze the relationship between tacca A and radiation, the order of treatments was reversed such that SCC4 cells were subjected to γ -radiation 24 h prior to tacca A treatment. We found that the combination of these two treatments retained their additivity in the clonogenic assay when the order of administration was reversed. This is demonstrated by the fact that the actual effect on viability of cells irradiated prior to treatment with tacca A is not statistically significant from their predicted additive effects (Fig. 4B). This finding supports the hypothesis that tacca A is not strictly radiosensitizing cells, but is instead contributing toward a decrease in cellular viability through a distinct mechanism that is complementary to radiation treatment regardless of the order in which the two treatments are administered. Additional clonogenic experiments using MCF7 cells treated with tacca A prior to γ -radiation (Fig. 4C) and SCC4 cells treated with tacca E prior to γ -radiation (Fig. 4D) demonstrated that the additivity between these two treatment modalities is a generalizable property of the taccas in disparate cell lines. Therefore, the data generated with the longer term clonogenic assay are consistent with the results obtained in the short term viability assay in the sense that they both demonstrate that the taccas and γ -radiation have additive effects on cellular viability.

4. Discussion

This study demonstrates that taccas A and E, unique microtubule stabilizers that do not bind directly to tubulin, and γ -radiation have additive effects on cellular proliferation *in vitro*. This is similar to the observed additive effects of other diverse microtubule stabilizing drugs with radiation, including both epothilones and taxanes in several *in vitro* studies. In addition, this finding is consistent with the ability of numerous tubulin-binding drugs to act in an additive or synergistic manner with radiation in both murine models and in patients. The ability of two different taccas to enhance the effects of γ -radiation in an additive fashion in both oral and breast cancer cell lines suggests that this is a generalizable effect of the taccas on cancer cells. Thus it appears that the ability of diverse microtubule targeting drugs to enhance the effects of radiation is not related to the direct binding of the drug to tubulin and, instead, is a result of the downstream effects of inhibiting microtubule dynamics.

The ability of microtubule disruptors to have radiosensitizing effects has been linked to the mitotic accumulation caused by the interruption of mitotic spindle dynamics. Our data show that irradiation or treatment with the microtubule stabilizer tacca A interrupts normal cell cycle progression causing accumulation of cells in G_2/M . Interestingly however, the additive cytotoxic effects of the two treatment modalities were noted with low concentrations, below 1 μ M of tacca A, which did not affect cell cycle distribution. These data are consistent with reports that the radiosensitization properties of at least some microtu-

Table 2

Percent cell viability of SCC4 cells when treated with single agents in the clonogenic assay.

Treatment	Dose	Viability (%)
Untreated	–	100
γ -Radiation	1 Gy	85
	2 Gy	78
	4 Gy	39
	6 Gy	11
Tacca A	100 nM	100
	125 nM	98
	150 nM	72
	175 nM	51
	200 nM	24

These values were used to calculate the expected additive viability of tacca A treatment when combined with γ -radiation in the clonogenic assay. The percent viability values are normalized to the viability of cells that have not been treated with drug or radiation.

bule targeting agents are independent of the drug's ability to cause G₂/M arrest [8,22]. The microtubule stabilizer patupilone radiosensitizes cells at concentrations that do not cause G₂/M accumulation and further evaluation showed that these low concentrations of patupilone caused a transient accumulation of cells in S phase that was further amplified by radiation treatment [8]. These data, together with our results showing identical additive effects of the two treatment modalities regardless of the order they are applied, suggest that the exact mechanism(s) by which microtubule targeting agents enhance the effects of radiation may not be strictly due to mitotic accumulation and that these two treatment modalities likely work through independent mechanisms of action to inhibit cancer cell proliferation.

For decades the clonogenic assay has been the assay of choice for drug radiosensitization studies. An advantage of measuring long-term cell viability after radiation using the clonogenic assay is that irradiated cells can undergo one or more rounds of cell division after DNA damage, suggesting that the full effects of radiation would be missed in a short term viability assay. However, the nature of the clonogenic assay requires extremely low cell densities at the time of treatment, which often necessitates significantly different dosing than is used in other measurements of drug efficacy. Additional disadvantages include the low robustness and subjectivity involved in colony counting. In previous studies, a side-to-side comparison demonstrated that less laborious, high-throughput assays, such as the colorimetric SRB assay, were comparable to the clonogenic assay in terms of detecting radiosensitization [23]. However, in both the clonogenic and SRB assays, the cells were plated at a low density that allowed for approximately six population doublings. In the current study, the additive effects of tacca A and γ -radiation in the clonogenic assay were compared to a short-term assay utilizing viability dyes and high-throughput analysis that measured the viability of individual cells 24 h after irradiation. The absolute dose of radiation and drug had to be optimized separately for this assay as compared to the clonogenic assay in order to use doses that give a significant individual effect on cellular viability but do not have such a large effect as to

obscure any potential additive or synergistic effects between the two treatment modalities. We attribute the differences in dosing requirements for the two assays largely to differences in plating density, as we have observed density dependent differences in the potency of the taccalonolides on cellular viability even within an individual assay (unpublished observation). While we cannot rule out the possibility that the differences in the concentration of tacca A and γ -radiation used in each experiment could cause cell death through distinct mechanisms, each test clearly showed that tacca A enhanced the effects of γ -radiation in an additive manner regardless of the absolute dose and measured output. The finding that both short-term and long-term cell viability assays show identical results with regard to the additivity of the taccalonolides and γ -radiation suggests that the higher-throughput and more robust short-term assay may be valuable as an appropriate substitute for the clonogenic assay in these types of studies.

Conflicts of interest

None declared.

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