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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 antimitotic 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 antimitotic agents, including the taccalonolides, may have utility in chemoradiotherapy.

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 chemotherapy 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, G_2 and M [7]. However, some studies show that G_2/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 βIII 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 generalizeable 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 (Hyclone; Logan, UT) and 50 μg/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 G_1, G_2/M or S populations respectively. Each value is the mean of at least three independent experiments.

2.4. γ-Radiation

A Gamma cell 40 ^{137}Cs 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 μl 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× 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.