Adjuvant Senolytic Treatment to Prevent Breast-to-Bone Metastasis

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INTRODUCTION: Chemotherapeutic treatment of breast cancer with Doxorubicin (DOX) can induce tumour and stromal cell senescence leading to therapy-resistance(1). Senescence-associated secretory phenotype (SASP) promotes the secretion of pro-inflammatory and tumorigenic factors causing systemic inflammation(2). Combined, this can result in immune suppression, tumour growth and secondary spread, often to the bones. The spine is one of the most common secondary sites for bone metastasis(3). Our objective is to target senescent cancerous and stromal cells using a combination of DOX and senolytic drugs (o-Vanillin, RG-7112). The removal of senescent cells may reduce systemic inflammation, improve therapeutic efficacy, and prevent metastasis, thus, allowing for physiological cell regeneration and tissue repair.

METHODS: We analyzed chemotherapy-induced senescence using immunohistochemistry (IHC) stain $p16^{INK4a}$ for both breast cancer (MDA-MB-231 GFP-tagged) cells, and primary spine osteoblasts from patient donor lumber spines (Institutional REB and biohazard approvals are in place) in monolayer. We then combined DOX with senolytics (o-Vanillin, RG-7112) to assess spheroid viability and growth in both a monoculture spheroid system and a 3D co-culture bone-like tumour microenvironment model *in vitro*. IHC staining of $p16^{INK4a}$, and Ki-67 were performed for evaluation of senescence and proliferation. Images (Figure 1) were captured using an Invitrogen™ EVOS™ M5000 for spheroid area tracking and AlamarBlue assays were run for evaluating metabolic activity. Cytokine Array assays using collected culture media will assess SASP release in future experiments.

RESULTS SECTION: IHC in monolayer showed increased concentrations of DOX will significantly induce senescence over 72 hours of treatment in both MDA-MB-231 cell lines and primary spine osteoblasts ($p < 0.0001$, n=5, one-way ANOVA). Monoculture spheroids showed a significant decrease in area and metabolic activity when DOX was combined with senolytics as compared to a non-treated control and DOX alone ($p < 0.0001$, n=4, 2way ANOVA, $p < 0.0001$, n=4, one-way ANOVA). IHC $p16^{INK4a}$ of co-culture spheroids showed DOX induces senescence in both cell types significantly as the concentration is increased from 0-1µM ($p = 0.0030$, $p = 0.0058$, n=5, one-way ANOVA). Co-culture treated with the combination of DOX and senolytics showed a significant reduction in senescence over 14days of triple treatment compared to DOX alone for both MDA-MB-231 and osteoblast cells ($p = 0.0003$, $p = 0.0001$, n=4, one-way ANOVA). Ki-67 IHC staining of co-culture spheroids after 14days of treatment showed percent proliferation was significantly decreased in the combination treatment, and the total cell count was also significantly decreased ($p = 0.0006$, $p = 0.0204$, n=4, 2way ANOVA). Fluorescence intensity of co-culture spheroids after 14days of treatment showed that the combination treatment has significantly reduced fluorescence intensity/surface plot and AlamarBlue metabolic activity as compared to DOX alone and control ($p = 0.0001$, $p = 0.0006$, n=4, one-way ANOVA, unpaired t-test (see figure 1)). Data was analyzed using GraphPad Prism 10.0 with paired or unpaired t-tests to compare two groups, multiple pairwise comparisons (one- or 2way ANOVA) were run to evaluate multiple groups. All experiments were run four-five independent times with different osteoblast donors per '*n*' done in triplicate. Statistical analysis was done with p-value set to < 0.05 presenting data as +/- SEM.

DISCUSSION: Chemotherapeutic treatment of patients with breast cancer may be optimized by the addition of senolytics. The above results demonstrate an *in vitro* reduction of tumour senescence, growth, and viability of cancer cells within the tumour microenvironment model. Senescent cells if not removed can lead to increased inflammation, angiogenesis, and overall disease progression. Future work will probe the effects of combining senolytics with DOX in an *in vivo* animal model to address the possible effects in a full body system.

SIGNIFICANCE/CLINICAL RELEVANCE: This study may offer a better understanding of cancer cell senescence and its relation to chemo-resistance, chemo-sensitization, enhanced growth, and metastasis. On the social and economic front, cancer kills 84,600 patients annually in Canada of which about 7% are caused by breast cancer. Unfortunately, a flare-up of disease with skeletal metastasis is a common complication, even following initial successful intervention and is the cause of debilitating pain and loss of function. We hypothesize that chemotherapy-induced cell senescence is a leading cause of secondary tumour growth and metastasis with the above results demonstrating a possible method of reducing tumour size, inflammation, and potential for increased disease burden.

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IMAGES AND GRAPHS:

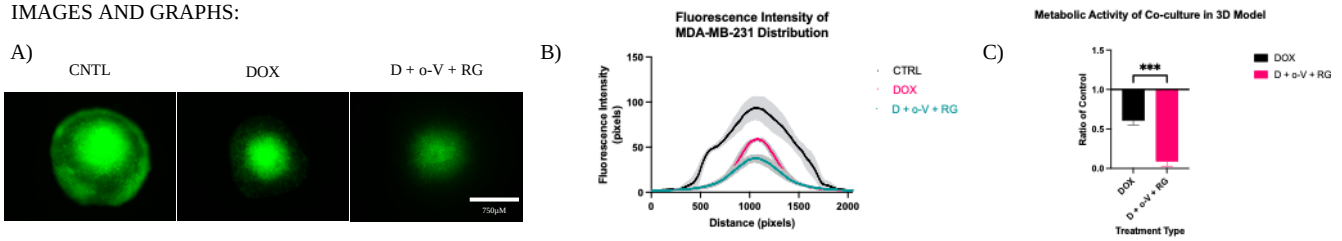

Figure 1. Fluorescence intensity on Day 21 following 14days of treatment with standard media, DOX [0.5µM] alone, or in combination with o-Vanillin [100µM], and RG-7112 [5µM]. A) Representative images of MDA-MB-231 GFP spheroids in co-culture, B) Fluorescence intensity distribution with plotted distance one-way ANOVA **** = $p < 0.0001$, C) Alamar Blue assay with 6h incubation unpaired t-test *** = $p < 0.0006$.