Inhibition of Sorcin Protein to Improve the Efficacy of Doxorubicin in the Treatment of Metastatic Breast Cancer

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Abstract

Doxorubicin, and the liposomal formulation Doxil, are among the first-line chemotherapeutic agents used in the treatment of a variety of cancers, including metastatic breast cancer [1]. Although doxorubicin has proven to be an effective cytotoxic agent, one major challenge in current therapy is the development of cancer cell resistance to this drug [2,3]. One possible mechanism by which this resistance can occur is through overexpression of the calcium-binding protein, sorcin, which has the potential to directly bind doxorubicin, thereby limiting its cytotoxic effects [4,5,6]. Therefore, the focus of this research is to utilize the compound dihydromyricetin (DMY), a known inhibitor of sorcin [7,8] to potentially increase the overall efficacy of doxorubicin. In this study, a series of cytotoxicity assays were conducted to assess cytotoxic effect of doxorubicin against metastatic breast cancer cells (MDA-MB-231) as well as non-cancerous breast cells (MCF-10A), both with and without the inhibitor. A significant increase in cytotoxic capabilities of doxorubicin was observed in the metastatic cell line when coupled with DMY. Furthermore, western blot analysis was used to probe for the overexpression of sorcin in both non-cancerous breast cells as well as the metastatic cell line. Future work will involve the incorporation of doxorubicin and DMY into a unique, targeted-liposomal formulation which will ultimately be tested in cytotoxicity assays.

Background

Soluble resistance-related calcium binding protein (sorcin) is a highly conserved calcium-binding protein known to be overexpressed in multitude of cancers, including metastatic breast cancer [5]. This protein can bind with high affinity to doxorubicin (Fig. 2, top), a commonly used chemotherapeutic agent used in the treatment of this specific type of cancer (Fig. 1) [7]. The interaction between the protein and doxorubicin can limit the nuclear uptake of the drug, and ultimately limit its cytotoxic properties. Sorcin induced chemoresistance frequently indicates poor clinical outcome, and as a result, more research efforts are aimed at reducing protein expression through use of a known inhibitor of sorcin, dihydromyricetin, or DMY (Fig. 2, bottom) [6]. In fact, the data presented here supports previous work which reports an increase in the efficacy of doxorubicin against breast cancer cells when co-treated with DMY [9]. Due to the promising nature of these results, the novel aspect of this study will ultimately involve the combination of the drug and inhibitor in a unique, targeted-liposomal formulation (described in greater detail in conclusions and future work).

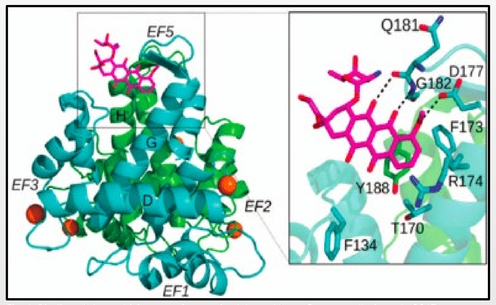

Fig. 1: Binding of doxorubicin to sorcin protein. Adapted from *Genovese, Ilari, et al. (2017)*

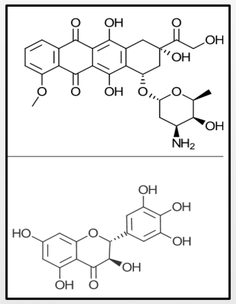

Fig. 2: The structures of doxorubicin (top) and of dihydromyricetin (bottom) generated from the ChemDoodle program.

Methods

For the cytotoxicity experiments, all individual preparations of free doxorubicin (Dox), free DMY, and the combination (Dox+DMY) were dissolved in DMSO to create a working 4mM stock solution. These solutions were then sterile filtered and diluted in the respective cell-line dependent maintenance media. Serial dilutions of each stock solution were prepared to the necessary concentrations for this study (0.01 µM 0.1 µM, 1.00 µM, 10 µM, and 100 µM). Each cell line (MCF-10A or MDA-MB-231) was plated on a 96-well tissue culture treated plate at 10,000 cells/well and incubated for 24 hours. The cells were then treated with the varying concentrations of each aforementioned preparations. Following a 48 hour incubation, an MTT assay was conducted to assess cell viability, which was analyzed by using an Infinite M200 fluorescence plate reader at λ=570nm. The data analyzed was graphed using OriginPro software.

For the western blot, lysates of both MDA-MB-231 and MCF-10A cells were prepared and analyzed using an SDS-PAGE method . The nitrocellulose membrane was subsequently blocked in a 5% milk/TBST solution for a minimum of one hour. After a series of washes, the membrane was incubated in the primary anti-sorcin antibody (1:200) and anti-GAPDH (1:5,000) for a minimum of one hour. The membrane was then incubated in the secondary antibody for a minimum of one hour (goat anti-rabbit, goat anti-mouse 1:15,000) followed by several washes with TBST. Finally, 1-Step NBT/BCIP was then applied to the membrane until bands were observed. The results were then analyzed and quantified using ImageJ software.

Cytotoxicity and Western Blot

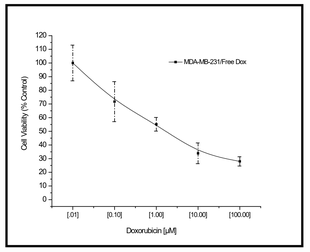

Fig. 3: Cytotoxicity graph of metastatic breast cancer cells (MDA-MB-231) treated with free doxorubicin over 48 hours. The resulting IC-50 value was approximately 2.4 µM ± 0.2.

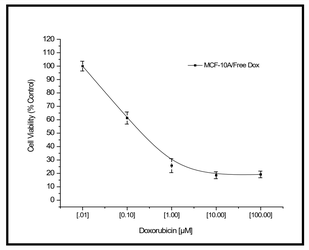

Fig. 4: Cytotoxicity graph of non-cancerous breast cells (MCF-10A) treated with free doxorubicin over 48 hours. The resulting IC-50 value was approximately 0.3 µM ± 0.01.

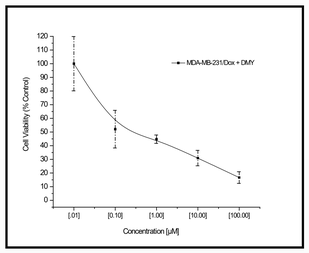

Fig. 5: Cytotoxicity graph of metastatic breast cancer cells (MDA-MB-231) treated with doxorubicin and DMY over 48 hours. The resulting IC-50 value was approximately 0.3 µM ± 0.04.

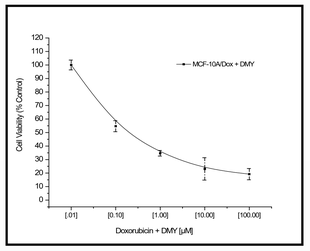

Fig. 6: Cytotoxicity graph of non-cancerous breast cells (MCF-10A) treated with doxorubicin and DMY over 48 hours. The resulting IC-50 value was approximately 0.2 µM ± 0.01.

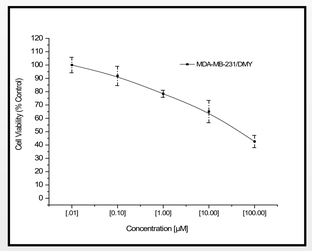

Fig. 7: Cytotoxicity graph of metastatic breast cancer cells (MDA-MB-231) treated with DMY over 48 hours. The resulting IC-50 value was approximately 67.1 µM ± 2.9.

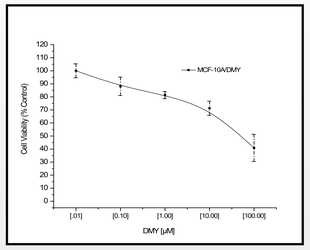

Fig. 8: Cytotoxicity graph of non-cancerous breast cells (MCF-10A) treated with DMY over 48 hours. The resulting IC-50 value was approximately 70.0 µM ± 2.8

Cell Line	Free Dox	Dox + DMY	Free DMY
MCF-10A	0.3µM ± 0.01	0.2µM ± 0.01	70.0µM ± 2.8
MDA-MB-231	2.4µM ± 0.2	0.3µM ± 0.04	67.1µM ± 2.9

Table 1.1: Summary of IC-50 values for the various cell line/ treatment combinations.

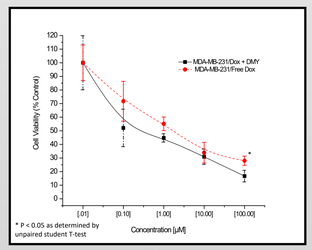

Fig. 9: Cytotoxicity graph of metastatic breast cancer cells (MDA-MB-231) treated with doxorubicin and DMY (black) and free doxorubicin (red) over 48 hours. The resulting IC-50 values were approximately 0.3 µM ± 0.04 and 2.4 µM ± 0.2 respectively. Statistical analysis of the values using an unpaired student T-test indicates a P value of less than 0.05 indicating a significant difference in the IC-50 values.

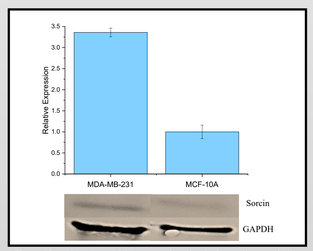

Fig. 10: Western blot graph depicting the relative expression of sorcin in metastatic breast cancer cells (MDA-MB-231) and non cancerous breast cells (MCF-10A).

Conclusions and Future Work

The results of this study indicate an overall increase in the efficacy of doxorubicin in the presence of the sorcin inhibitor, DMY, when used to treat metastatic breast cancer, while no observable difference was observed when treating non-cancerous cells with either free doxorubicin or doxorubicin in the presence of the inhibitor. As well, western blot analysis indicates roughly a three-fold increase in sorcin expression in MDA-MB-231 cells versus the non-cancerous MCF-10A cells. These data may support a correlation between decreased sorcin expression and increased cytotoxic capability of doxorubicin. As previously stated, future work will aim to include a targeted-liposomal formulation (fig. 11) in which DMY and doxorubicin will be co-encapsulated in a liposome decorated with a unique targeting peptide (fig. 12) specifically designed to target the α3β1 integrin known to be overexpressed in metastatic breast cancer (fig. 13) [10-13] . In fact, previous work involved incorporation of this unique peptide construct (synthesized by, and purchased from, ChinaPeptides Co., Ltd) into a liposome, which was validated via absorbance spectroscopy (fig. 14). Furthermore, the relative expression of the α3β1 integrin in the MDA-MB-231 cell line versus the MCF-10A cell line was analyzed using a whole cell ELISA (fig. 15).

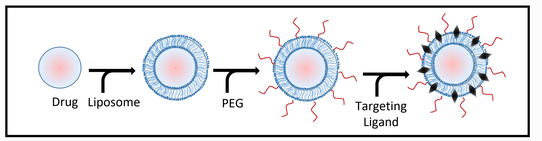

Fig. 11 : Depiction of innovations in drug delivery for chemotherapeutic agents.

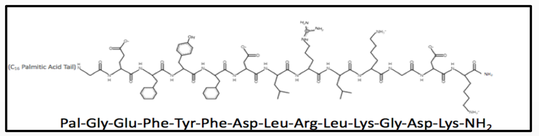

Fig. 12: Structure and sequence of the peptide generated using software from Tulane University "PepDraw" http://www.tulane.edu/~biochem/WW/PepDraw/1022. A palmitic acid tail has been added to the N-terminus region of the peptide.

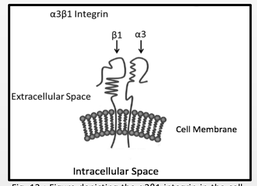

Fig. 13 : Figure depicting the α3β1 integrin in the cell membrane adapted from Fields, G. (2007). "Peptide Characterization and Application Protocols." Methods in Molecular Biology 10 1-58829-550-8: 274-289.

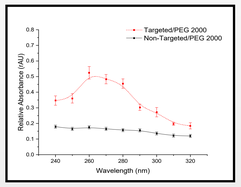

Fig. 14 : Graph depicting the relative absorbance values of both targeted/PEG 2000 (red) as well as non-targeted/PEG 2000 (black) liposomes.

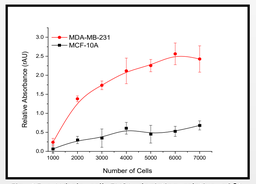

Fig. 15 : Whole cell ELISA depicting relative α3β1 integrin expression levels on human metastatic breast cancer cells (red) and normal human mammary epithelial cells (black).

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