Laidlaw Research Project Report

Investigating the Effects of Cancer Stem Cells on Cancer Treatment Induced Cardiotoxicity

L'Anijae De Leon

Mentored by Dr. Howard Chen, Molecular Cardiology Research Institute, Tufts Medical Center

Co-authors: Howard Chen, Andrew Kung, Dylan Valencia, Purui Xu

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Abstract

This research focused on investigating the impact of cancer stem cells (CSCs) on cardiotoxicity. It also focused on how modulating autophagy can affect CSCs response to a cardiotoxic cancer therapeutic drug and in the end cardiotoxicity. To gain further insights, I tested the hypothesis that CSCs exposed to a cardiotoxic cancer drug will impact cardiotoxicity, and that the cardiotoxicity can be mitigated by modulating autophagy. I investigated whether an autophagy activator (chloroquine) or inhibitor (rapamycin) affected the CSCs response to cardiotoxic cancer therapeutic drug doxorubicin using Fluorescence Activated Cell Sorting (FACS). Using the MTT assay, I investigated the timeline in which continuous doxorubicin treatment on CSCs will lead to resistance to the drug. From FACS ($CD44^+/CD24^-$), we saw a significant increase in the CSC population following prolonged drug exposure. Additionally, using the second-generation autophagy-detecting nanoparticle (ADN2) revealed that these chronically treated cells exhibit impaired autophagy. The MTT assay revealed that the cells exposed to chronic doxorubicin for a few weeks became more sensitive to the drug rather than resistant to it. It also revealed that the MDA cells have a lower survival rate in AC16 media than in MDA media. Our findings support a model in which chronic chemotherapy promotes both CSC expansion and autophagy disruption, creating a state of heightened drug tolerance. Hence, targeting autophagy may represent a viable strategy to re-sensitize resistant populations. In the future, to further investigate the impact of CSCs directly on cardiotoxicity, I plan to co-culture CSCs or non-CSC MDA-MB-231 cells with cardiomyocytes using insert plates and investigate whether CSCs treated with cardiotoxic cancer drug doxorubicin alone or with autophagy activator or inhibitor affects cardiomyocyte viability and apoptosis.

Introduction

Cancer and cancer treatment induced cardiotoxicity are global issues that negatively affect the lives of millions of people. According to Asnani, "over 1.8 million patients in the United States will receive a new diagnosis of cancer within the next year, based on estimates from the National Cancer Institute." [3] Many patients treated with anthracycline-based chemotherapy experience side effects related to the heart such as cardiotoxicity. Approximately 4% of all patients who receive modern-day anthracycline regimens develop symptomatic heart failure, hence researching this would be very beneficial. [3] My proposed study focuses on cancer stem cells (CSCs), a tiny fraction of cancer cells within the tumor bulk tissue that have the ability to self-renew, spread illness, and metastasis (the process that spreads or transports cancer cells to other parts of the body)—all capabilities that are critical for tumor recurrences and resistance to treatment. Recent literature suggests that CSCs can be targeted for new cancer therapeutic strategies that hold the promise to overcome those obstacles and enhance cancer treatment. [1, 2] However the impact of CSCs on cardiotoxicity has not been studied.

CSCs were first identified in human tumors in the late 1990s and early 2000s, beginning with acute myeloid leukemia. Bonnet and Dick (1997) demonstrated that only a small subset of leukemia cells could initiate tumors when transplanted into immunodeficient mice, introducing the concept of tumor-initiating cells. [5] Shortly after, Al-Hajj et al. (2003) identified a similar subpopulation in human breast cancer capable of forming tumors in vivo, establishing CSCs as a feature of solid tumors. [4] CSC-like populations can be induced from established cancer cell lines through exposure to chemotherapy. These models allow researchers to investigate CSC behavior, drug resistance, and differentiation potential, as was being done during this project. CSCs are typically characterized by specific surface markers. In breast cancer, the CD44 phenotype is widely used to distinguish CSCs from the bulk tumor population. [4] CD44 is a cell surface glycoprotein involved in cell adhesion and migration, while CD24 is associated with differentiation; their expression pattern indicates stemness and tumor-initiating capacity. Other markers vary by cancer type and include ALDH1, CD133, EpCAM, and Sox2. Functional assays complement marker-based identification to confirm stem-like properties.

Autophagy is the lysosomal breakdown of cytoplasmic components by sequestering them into double-membraned autophagosomes for degradation and recycling. Autophagy activation elicits tumor suppressing functions during tumor initiation which in turn enhances apoptosis, programmed form of cell death. However, autophagy is required to maintain the stemness of CSCs and therefore autophagy inhibition has shown to be helpful in killing CSCs. Unfortunately, the perfect cure for cancer is yet to be found as solutions like chemotherapy pose threats to the body, especially the heart. While chemotherapy does kill the cancer, it can also cause acute, chronic, or late-onset cardiotoxicity. On the other hand, the Chen lab as well as others in the field have reported that modulating autophagy protects the heart from cardiotoxicity due to cancer therapy.

Cardiotoxicity is a major complication of anthracycline-based cancer therapies like doxorubicin. This treatment could result in structural and functional damage to cardiomyocytes, which can lead to cardiomyopathy, arrhythmias, or heart failure. Emerging evidence suggests that disruptions in autophagy play a central role in chemotherapy-induced cardiotoxicity. Under typical circumstances, autophagy keeps the heart in a state of equilibrium by avoiding the accumulation of reactive oxygen species and malfunctioning mitochondria. However, anticancer drugs have the potential to either inhibit or overactivate autophagy, which might affect heart function. Increased oxidative stress, mitochondrial damage, and decreased cardiomyocyte viability are all caused by this imbalance. This dysregulation contributes to increased oxidative stress, mitochondrial damage, and reduced cardiomyocyte viability. Therefore, it is crucial to comprehend how cancer treatments modify the heart's autophagic pathways in order to create plans to avoid or lessen cardiotoxic consequences.

In addition, I'm interested in exploring the resistance of cancer cells when being treated with increasing levels of doxorubicin over an extended period of time. Doxorubicin is a widely used chemotherapeutic agent, but its long-term effectiveness is often limited by the development of drug resistance in cancer cells. When exposed to increasing concentrations of doxorubicin over an extended period, cancer cells can acquire adaptive mechanisms that enhance their survival. The prolonged exposure of doxorubicin can trigger changes in DNA repair pathways, increase antioxidant defenses, and promote evasion of apoptosis. Cancer stem cells, in particular, may contribute to this resistance through their

inherent quiescence, enhanced efflux capacity, and ability to self-renew after treatment. As resistance accumulates, progressively higher doses of doxorubicin are required to achieve the same therapeutic effect, increasing the risk of systemic toxicity, including cardiotoxicity. Investigating how cancer cells adapt to chronic doxorubicin exposure is therefore critical for improving treatment efficacy and designing strategies to overcome resistance.

Methods

Cell Culture

The cell morphology and confluency of the MDA-MB-231 cells were observed under an inverted microscope. In the hood, all the old media was removed with an aspirator and 1.5mL warm trypsin was added to the 10cm plate. The plate was incubated at 37℃ for 5 minutes and observed under a microscope to ensure that more than 90% of the cells are detached. 9mL DMEM + 10%FBS media was added to the plate. The contents of the 10cm plate was added to three new 10 cm plates and the media in the plates were changed every 2-3 days. In one of the plates, the concentration of doxorubicin was increased by 5nM every time the media was changed. In one of the other plates, the concentration of doxorubicin was increased by 50nM every time the media was changed.

Fluorescence Activated Cell Sorting

A single cell suspension was prepared in the Cell Staining Buffer. 15 mL Cell Staining Buffer was added and the tube was centrifuged at 350xg for 5 minutes. The supernatant was discarded. Blocking was done by pre-incubating cells with 5µL of Human TruStain FcX™ Antibody per 100 cells in a 100µl volume for 5-10 minutes at room temperature. 50µL CD24 Antibody and 50µL CD44 Antibody was added after 2 quick washes with 1mL cell staining buffer by centrifugation at 350xg for 5 minutes. The cells were incubated on ice for 15-20 minutes in the dark and 2 quick washes were done with 1mL cell staining buffer by centrifugation at 350xg for 5 minutes. The cell pellet was filtered into FACS tubes and FACS was performed.

Resistance Testing - MTT Assay

Cells were seeded into 96-well plates in 100 µL of complete medium and allowed to attach for 24 h at 37 °C with 5% CO_2. After incubation, the medium was replaced with 100 µL of treatment solutions (prepared at 2× concentration to maintain a consistent final volume) and cells were exposed for an additional 24 h. Following treatment, 10 µL of MTT stock solution (5 mg/mL in PBS) was added to each well and plates were incubated for 2 h to allow for the formation of purple formazan crystals. The medium was then carefully removed, and 100 µL of DMSO was added to each well to solubilize the crystals, with gentle shaking at room temperature for 10 min. Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a T13 Microplate Reader. Blank wells (medium + MTT without cells) were subtracted from all readings, and cell viability was calculated relative to vehicle-treated controls.

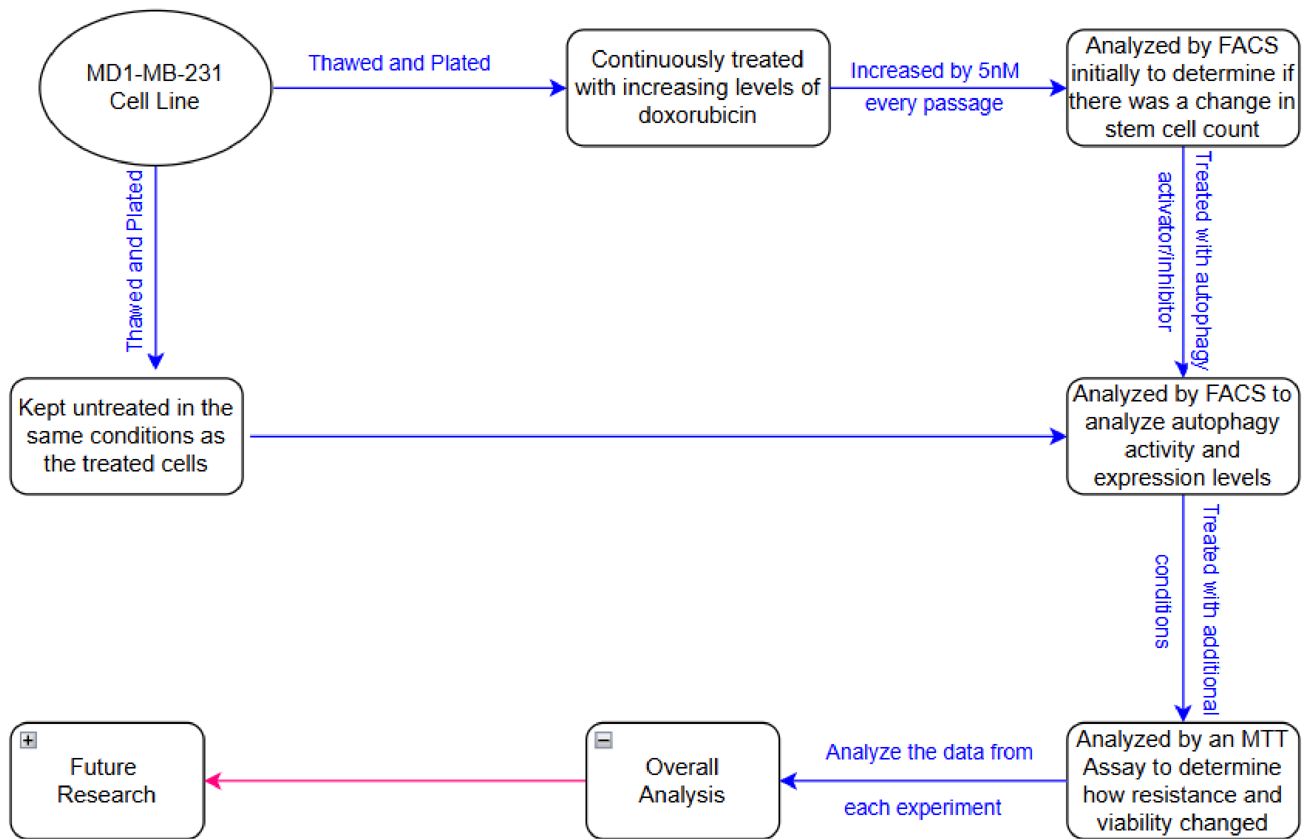

Figure 1. Flowchart Summarizing the General Steps Done During the Research Project

Results

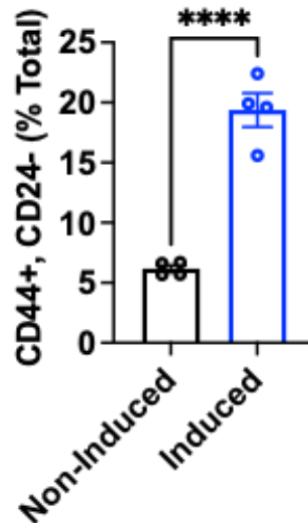

Figure 2. Comparison of the *% Increase in CSCs in the MDA-MB-231 Human Breast Cancer Cells*

The data show that long-term exposure to low-dose doxorubicin (1.5 months) induces a significantly higher proportion of CSCs in MDA-MB-231 human breast cancer cells compared to cells treated with a standard acute dose of doxorubicin. This suggests that chronic chemotherapy may promote the enrichment or selection of CSCs rather than eliminating them. CSCs possess self-renewal capabilities and are known to initiate new tumor growth even after bulk tumor reduction. Therefore, the observed enrichment of CSCs implies that although low-dose chemotherapy may shrink tumors initially, it could unintentionally prime the cancer for recurrence or metastasis. So rather than eliminating the tumor entirely, sustained low-dose exposure may apply selective pressure that eliminates the weaker cells and leaves behind the strongest, stem-like ones. These surviving CSCs are typically more drug-resistant, more invasive, and more capable of re-establishing tumors after treatment. This finding has important clinical implications — it suggests that treatment strategies focused solely on reducing tumor size may be insufficient if they do not also specifically target the CSC population. Future therapies may need to combine conventional chemotherapy with CSC-targeted agents or autophagy inhibitors to prevent this adaptive survival response. This highlights the importance of treatment scheduling and dosage strategy in preventing CSC expansion during chemotherapy.

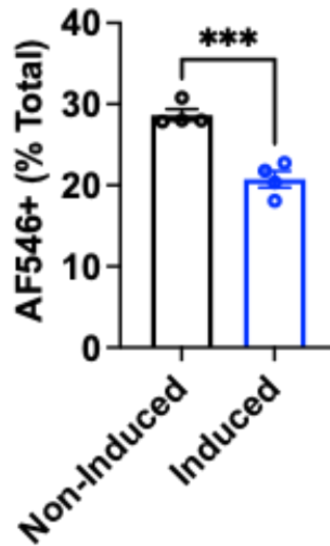

Figure 3. ***Comparison of the Total % of ADN2 Uptake in the MDA-MB-231 Human Breast Cancer Cells***

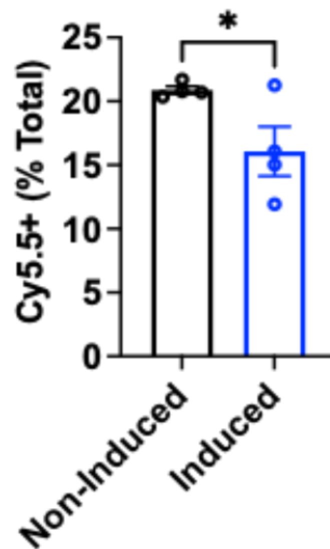

Figure 4. ***Comparison of the Total % of Autophagy Activation in the MDA-MB-231 Human Breast Cancer Cells***

The MDA-MB-231 cells exposed to chronic low-dose doxorubicin for 1.5 months exhibited a marked impairment in autophagy, as detected using the second-generation autophagy-detecting nanoparticle (ADN2). Both ADN2 uptake (AF546 fluorescence) and autophagic activation (Cy5.5 fluorescence) were significantly reduced when compared to untreated controls. These findings suggest that prolonged exposure to sublethal concentrations of doxorubicin disrupts normal autophagic flux, potentially suppressing cellular recycling and stress response mechanisms. This impairment in autophagy may represent an adaptive response in which cancer cells downregulate autophagic activity to escape autophagy-mediated cell death. Importantly, reduced autophagic activity has been linked to the acquisition of drug resistance and cancer stem cell enrichment, as cells shift toward metabolic states that favor survival under therapeutic stress. Therefore, the observed decrease in ADN2 uptake and activation confirms autophagy dysregulation. Together, these results highlight autophagy impairment as a key feature of chemotherapy-adapted cancer cells. This raises the possibility that restoring or further targeting autophagy in this context—either through autophagy inducers or inhibitors, depending on pathway status—could resensitize cells to treatment.

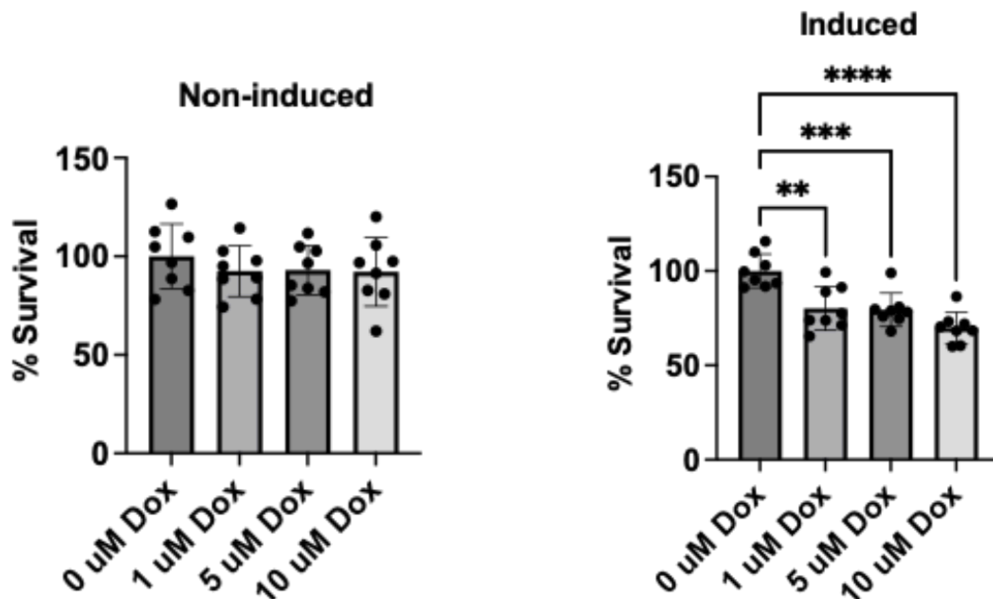

Figure 5. ***Comparison of the Total % of Survival in the MDA-MB-231 Human Breast Cancer Cells as the Concentration of Doxorubicin Increases***

Treatment of MDA-MB-231 cells with doxorubicin at concentrations ranging from 1–10 μM for 24 hours did not significantly reduce cell viability. However, cells that had been chronically induced with low-dose doxorubicin showed a significant decrease in viability when re-exposed to doxorubicin. This indicates that the induced population did not develop doxorubicin resistance as expected. Interestingly, this finding contradicts the established literature, which suggests that cancer stem cells (CSCs)—known to increase in the induced population—are typically more resistant to doxorubicin. Instead, our data suggest that the induced cells may have become more sensitive to doxorubicin treatment, implying that chronic low-dose exposure may alter the cellular response mechanisms typically associated with drug resistance.

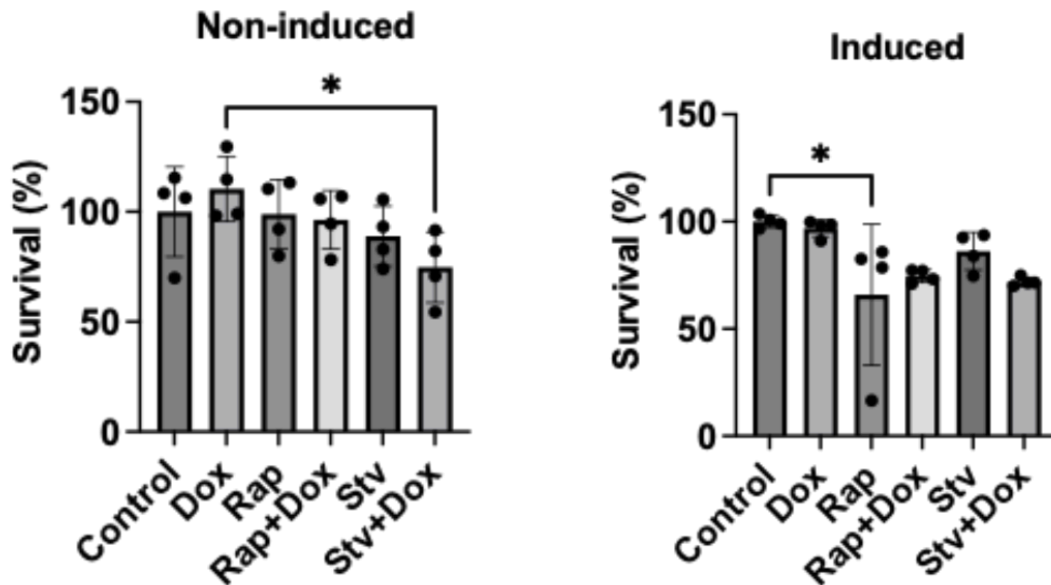

Figure 6. ***Comparison of the Total % of Survival in the MDA-MB-231 Human Breast Cancer Cells Under Various Conditions***

No significant reduction in cell viability was observed in either the non-induced or induced MDA-MB-231 populations following treatment with doxorubicin (Dox) for 24 hours. In the non-induced cells, however, treatment initiated with Dox resulted in a significant decrease in survival, while a similar but statistically non-significant reduction was observed in the induced population. Notably, treatment with rapamycin (Rap) caused a significant reduction in survival in the induced cells, an effect that was not observed in the non-induced group (this finding should be interpreted cautiously, as one data point appeared to be an outlier and warrants replication of the experiment for confirmation). Statistical analysis was performed using one-way ANOVA with Tukey's post-test, comparing all treatment conditions; only statistically significant differences are indicated in the figures.

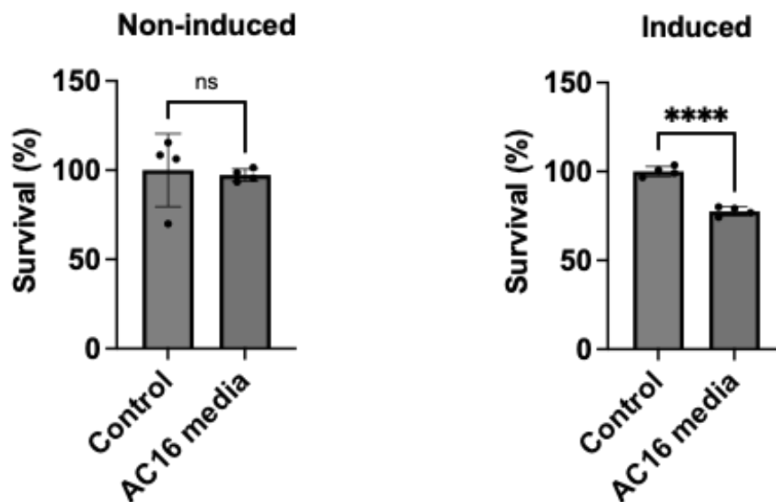

Figure 7. ***Comparison of the Total % of Survival in the MDA-MB-231 Human Breast Cancer Cells in MDA Media vs AC16 Media***

Cell survival was significantly reduced in the doxorubicin-induced population when cultured in AC16 media compared to standard culture conditions. This suggests that the change in media composition negatively affected the viability of the induced cells. Statistical analysis was performed using an unpaired, two-tailed Student's t-test.

Discussion

In this study, we investigated how chronic exposure to low-dose doxorubicin influences cancer stem cell (CSC) enrichment and autophagy regulation in MDA-MB-231 human breast cancer cells. Using flow cytometry-based CSC marker analysis ($CD44^+/CD24^-$), we observed a significant increase in the CSC population following prolonged drug exposure, suggesting that chemotherapy—rather than eliminating all tumor cells—may inadvertently promote the survival and expansion of stem-like, therapy-resistant subpopulations. Complementary assessment using the second-generation autophagy-detecting nanoparticle (ADN2) revealed that these chronically treated cells exhibit impaired autophagy, demonstrated by reduced nanoparticle uptake and activation. This autophagic dysfunction likely contributes to the acquisition of resistance by allowing cells to evade autophagy-mediated cell death or by shifting toward alternative metabolic survival pathways.

Pharmacological manipulation of autophagy provided additional insight into the functional role of this pathway. Chloroquine was used to inhibit autophagy by preventing lysosomal acidification and blocking autophagosome degradation, allowing us to determine whether further autophagy disruption sensitizes CSC-enriched populations. Rapamycin was used to activate autophagy by inhibiting the mTOR pathway, enabling us to test whether restoring autophagic flux could reverse drug-induced resistance. To further evaluate how autophagy modulation and CSC enrichment affect therapeutic sensitivity, an MTT assay was conducted to assess cell viability under various treatment conditions.

Across all the experiments, doxorubicin-induced MDA cells demonstrated altered sensitivity to treatment and environmental conditions, revealing a potential link between cardiotoxicity and the modulation of autophagy. The doxorubicin-induced population showed impaired survival under conditions that activate or inhibit autophagy, suggesting that chronic exposure to the drug may disrupt the balance of autophagic processes that normally support cellular homeostasis. Since cardiotoxicity from doxorubicin in cardiac tissue has also been associated with dysregulated autophagy, these findings suggest that the same mechanisms influencing survival in cancer cells may contribute to damage in cardiomyocytes. Therefore, modulating autophagy—either pharmacologically with agents like rapamycin

or chloroquine, or through metabolic control—may not only affect cancer cell resistance but could also influence how cardiac cells respond to stress and toxicity. Further investigation, particularly through co-culture studies of cancer stem cells and cardiomyocytes, would clarify whether the observed changes in autophagy and survival directly contribute to doxorubicin-induced cardiotoxicity.

Collectively, our findings support a model in which chronic chemotherapy promotes both CSC expansion and autophagy disruption, creating a state of heightened drug tolerance. Targeting autophagy—either through restoration or further inhibition—may therefore represent a viable strategy to re-sensitize resistant populations. Future studies using co-culture systems with cardiomyocytes, as well as sorted CSC versus non-CSC populations, will be essential for fully dissecting the therapeutic vulnerabilities of these drug-adapted cells.

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Future directions

While our findings provide valuable insight into how cancer stem cells (CSCs) respond to autophagy activation and inhibition, the implications of these responses on cardiomyocytes remain unclear. To better understand the mechanistic crosstalk between CSCs and cardiac cells — especially in the context of treatment-induced cardiotoxicity — future studies should involve co-culturing CSCs with cardiomyocytes and replicating the autophagy modulation experiments under these more physiologically relevant conditions. This approach would help determine whether CSC-derived autophagic signals exert protective or deleterious effects on cardiomyocyte survival and function.

Additionally, further investigation is needed to determine whether autophagy levels differ between stem-like ($CD44^{+}/CD24^{-}$) and non–stem-like subpopulations. Fluorescence-activated cell sorting (FACS) could be employed to isolate CSC-enriched fractions, allowing direct assessment of their autophagic activity under treatment with autophagy modulators, with or without chemotherapeutic agents such as doxorubicin. Comparing these responses between sorted CSCs and bulk tumor cells may reveal whether autophagy contributes to the enhanced drug resistance typically observed in CSCs.

We can also dive deeper and expand the scope of this research. Exosome profiling from autophagy-modulated CSCs could be done to determine whether they release cardiotoxic or cardioprotective factors that may influence cardiomyocyte health. We could do transcriptomic or proteomic analyses to identify autophagy-associated survival pathways uniquely activated in CSCs under therapeutic stress. We could do a CRISPR-based autophagy gene knockdown to establish causal links between autophagy dependence and treatment resistance in CSCs. Lastly, in vivo validation using tumor–heart interaction models could be used to observe systemic effects of CSC-derived autophagy during chemotherapy. Together, these future studies would not only clarify the role of autophagy in CSC survival but also determine whether targeting this pathway could simultaneously enhance cancer treatment efficacy while mitigating cardiotoxicity.

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