Targeting cancer stem cells (CSCs) is a key strategy to prevent cancers from developing drug resistance and metastasis. Mitochondria have been reported to be a vulnerability of CSCs by multiple studies. Here, we report that doxycycline, functioning as an inhibitor of mitochondrial biogenesis, can effectively target breast cancer stem cells (BCSCs). Our results revealed that doxycycline significantly decreased the frequency of aldehyde dehydrogenase-positive (ALDH+) BCSCs as well as mammosphere formation efficiency in HER2+ and triple-negative breast cancer (TNBC) subtypes. Doxycycline also ameliorated paclitaxel-induced enrichment of ALDH+ BCSCs in TNBC. Mechanistically, we showed that doxycycline decreased the level of reactive oxygen species and their downstream p38 MAPK pathway. In agreement with the key role for p38 in maintaining BCSCs, a specific inhibitor targeting this MAPK pathway significantly decreased the number of ALDH+ cells. Doxycycline is a FDA-approved drug with minor and limited side-effects. Given doxycycline’s low toxicity and strong effect on BCSC inhibition, we report that doxycycline should be safe to be used concomitantly with chemotherapy drugs to eradicate both CSCs and bulk tumor cells.

Introduction

With an estimated 230,000 new cases and 40,000 deaths in 2013, breast cancer has the highest incidence and is the second leading cause of cancer-related death among women in the United States (1). Four subtypes of breast cancers, namely luminal A, luminal B, HER2+ and basal-like (significantly but not completely overlaps with the triple-negative breast cancer, TNBC), are classified according to the intrinsic gene expression profile (2,3). While the luminal subtypes respond well to hormone therapies, over 50% of patients with HER2+ breast cancer develop trastuzumab-resistance within 1 to 2 years of treatment (4,5). More than 70% of TNBC patients have residual invasive disease after neoadjuvant chemotherapy and are at high risk of disease relapse (6). Recent evidence supports that a small fraction of cancer cells, termed cancer stem cells (CSCs), are capable of self-renewing and differentiating into non-stem cancer cells and are responsible for tumor initiation, drug resistance and metastasis (7-10). Therefore, combining CSC-targeting agents with conventional chemotherapies seems to be a promising strategy for eradicating both CSCs and bulk tumor cells (11,12).

Reprogramming of energy metabolism is one of the hallmarks of cancer (13). Over 80 years ago, Otto Warburg observed that cancer cells favored aerobic glycolytic metabolism in the presence of oxygen (14). Warburg hypothesized that cancer resulted from impaired cellular mitochondrial metabolism. It is clear now that the Warburg effect is not due to the impairment of mitochondrial function in tumors. Indeed, depletion of mitochondrial DNA has been shown to decrease colony formation in soft agar and tumor initiation in mice (15-19), which are the key indicators of CSCs. Recent studies have also demonstrated that mitochondrial features of CSCs differ from those of non-stem cancer cells (20-22), and attenuating mitochondrial metabolism could suspend tumor metastasis and prolong tumor latency in xenograft models (19,23). This phenomenon indicates that mitochondria are functionally indispensable to sustaining CSCs. Therefore, targeting mitochondria is emerging as a new strategy for eradicating CSCs.

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Key words: doxycycline, breast cancer, aldehyde dehydrogenase, cancer stem cells, mitochondria, reactive oxygen species, p38 MAPK
including breast cancer (25-28), indicating the potentiality of using the ‘old’ antibiotic for a new treatment-targeting CSCs.

Although doxycycline-mediated CSC inhibition has been linked to mitochondria (20,24,25), it remains unknown what type of CSCs doxycycline could inhibit. Breast cancer stem cells (BCSCs) can transition between two phenotypic states. One is a more proliferative epithelial-like state characterized by the expression of the CSC marker aldehyde dehydrogenase (ALDH) and the other is a more quiescent mesenchymal-like state characterized by the expression CD44+/CD24− (29). In this study, we selected BT474, SK-BR-3, SUM149, and SUM159 breast cancer cell lines to examine doxycycline’s effects on BCSCs. BT474 and SK-BR-3 are both HER2+ breast cancer cell lines and according to the literature and our previous publication, this subtype has a higher number of ALDH+ epithelial-like BCSCs compared to other subtypes but does not have the CD44+/CD24− mesenchymal-like BCSC population (30,31). In contrast, SUM159 is a Claudin-low TNBC cell line, which has a high percentage of mesenchymal-like bulk tumor cells that are also CD44+/CD24− (30). As a result, the CD44+/CD24− markers cannot be used to define the mesenchymal-like BCSCs in the Claudin-low subtype. SUM149, on the other hand, is a basal-like TNBC and has both ALDH+ and CD44+/CD24− BCSCs. Hence, SUM149 is a suitable cell line for testing the effects of doxycycline on ALDH+ as well as CD44+/CD24− BCSC populations.

In the present study, we report that doxycycline can reduce the ALDH+ BCSC population. Mechanistically, our results suggest that doxycycline inhibits ALDH+ BCSCs via inhibiting reactive oxygen species (ROS) production and their downstream p38 MAPK signaling pathway.

Materials and methods

Cell lines and chemicals. BT474 (ATCC, Manassas, VA, USA) and SK-BR-3 (ATCC) cells were grown in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS and 1X antibiotic-antimycotic (Invitrogen; Thermo Fisher Scientific, Inc.). SUM149 and SUM159 cell lines were kindly provided by Dr Max Wicha (University of Michigan, Ann Arbor, MI, USA). SUM149 and SUM159 cells were grown in F12 (Invitrogen; Thermo Fisher Scientific, Inc.). SUM149 and SUM159 cell lines were grown in F12 (Invitrogen; Thermo Fisher Scientific, Inc.) containing 5% FBS, 1X antibiotic-antimycotic, 5 µg/ml of insulin (Invitrogen; Thermo Fisher Scientific, Inc.) and 1 µg/ml of hydrocortison (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a 5% CO2 incubator at 37°C. p38 MAPK inhibitor SB203580 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Doxycycline hyclate was purchased from Sigma-Aldrich.

Mammosphere formation assay. Mammosphere formation was performed as previously described (32). Single cells were seeded in low-attachment 6-well plates (Corning Inc., Corning, NY, USA) at a density of 5,000 cells/well. Cells were cultured in 2 ml of MammoCult® (Stemcell Technologies, Inc., Vancouver, BC, Canada) with doxycycline from 0-10 µM. Doxycycline was replenished every 2 days, and mammospheres were counted on day 6. To test the self-renewal ability of CSCs, secondary mammosphere formation was performed in the absence of doxycycline. Briefly, primary spheres were dissociated to single cells enzymatically (trypsin) and mechanically (23G needle). Secondary mammosphere formation was performed by plating 5,000 cells/well of the dissociated single cells from the primary mammospheres.

Aldefluor assay. Cells were treated with 0, 0.1, 1 or 10 µg/ml of doxycycline for 7 days. The aldehyde dehydrogenase (ALDH) activity was then determined by Aldefluor assay (StemCell Technologies Inc., Cambridge, MA, USA) according to the manufacturer's instructions. Diethylaminobenzaldehyde (DEAB) was used as a negative control for gating. To test the importance of the p38 pathway in maintaining ALDH+ BCSCs, BT474 cells were treated with SB203580, a p38-specific inhibitor, for 2 days and then Aldefluor assay was conducted. To inhibit chemotherapy-induced Aldefluor-positive CSCs, cells were pretreated with 10 µM of doxycycline for 3 days, and then were treated with a combination of doxycycline and 10 nM of paclitaxel for another 4 days.

CD44 and CD24 analysis. Cells were treated with 0, 0.1, 1 or 10 µg/ml of doxycycline for 7 days, and then were harvested for CD44 (BD Biosciences, San Jose, CA, USA) and CD24 (BioLegend, Inc., San Diego, CA, USA) antibody staining. The cells were then analyzed by flow cytometry.

Analysis of reactive oxygen species (ROS). Cells were treated with 10 µg/ml of doxycycline for 7 days and then ROS were determined by a 2',7'-dichlorofluorescin diacetate (DCFDA)-based kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Briefly, the cells were incubated with 20 µM of DCFDA at 37°C for 30 min. Samples were then spiked with 300 µl of ice-cold 1X buffer containing DAPI and kept on ice before the ROS level was measured by flow cytometry.

Immunoblotting. Breast cancer cells were treated with 0, 0.1, 1 or 10 µg/ml of doxycycline for 7 days. Cells were then lysed using RIPA buffer containing proteinase inhibitor cocktail (Thermo Fisher Scientific, Inc.) and Calbiochem® phosphatase inhibitors (MilliporeSigma, Burlington, MA, USA). Proteins were separated by SDS-PAGE and probed with antibodies. Phosphorylated p38 MAPK (1:1,000; cat. no. 4511; rabbit mAb), p38 MAPK (1:1,000; cat. no. 8690; rabbit mAb) and vinculin (1:2,000; cat. no. 13901; rabbit mAb) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Statistical analysis. Two-tailed Student's t-test was used to compare the statistical difference between two groups. One-way ANOVA was used if the comparison involved more than two groups. A P-value <0.05 was considered to indicate statistical significance.

Results

Doxycycline inhibits ALDH+ BCSCs. To test doxycycline’s ability to inhibit ALDH+ BCSCs, we treated breast cancer cell lines with doxycycline and then measured ALDH activity in these cells. ALDH is an important biomarker of CSCs in many types of cancer (33). In breast cancer, cells with high ALDH
activity have self-renewal ability to regenerate tumors that recapitulate the heterogeneity of the parental tumors (8). Our results demonstrated that doxycycline at 10 µM significantly decreased the percentage of cells with high ALDH activity in the BT474, SK-BR-3 and SUM159 cells (Fig. 1). These results suggested that doxycycline could be used to target ALDH+ BCSCs. However, in SUM149 cells, doxycycline did not decrease ALDH+ (Fig. 1) or CD44+/CD24- (Fig. 2) BCSCs. This result may be due to the characteristics of the SUM149 cell line (see Discussion).

To further confirm whether doxycycline could functionally inhibit BCSCs, we treated BT474, SUM149 and SUM159 cells with various concentrations of doxycycline in the primary mammosphere culture. Mammosphere formation is an in vitro surrogate assay to evaluate self-renewal ability of BCSCs. In BT474 and SUM159, primary mammosphere formation was significantly inhibited by doxycycline in a concentration-dependent manner, whereas in SUM149, it was inhibited only at 10 µM (Fig. 3). The primary mammospheres were then dissociated and reseeded to form secondary mammospheres in the absence of doxycycline. In the secondary mammosphere culture, a 50% decrease in mammospheres was observed in the 10 µM doxycycline-pretreated BT474 and SUM159 cells (Fig. 3), indicating that doxycycline could inhibit the self-renewal ability of BCSCs in these cell lines.

Doxycycline inhibits reactive oxygen species and their downstream p38 signaling. In mammalian cells, doxycycline inhibits mitochondrial biogenesis by binding to 28S small mitochondrial ribosome (24,34). Mitochondria is the main organelle of ROS generation. High mitochondrial mass (20) and elevated ROS levels (35) have been reported to sustain ALDH+ CSCs. We demonstrated that doxycycline treatment significantly inhibited ALDH+ BCSCs (Fig. 1). Therefore, we hypothesized that doxycycline inhibited ALDH+ BCSCs via ROS attenuation. To test if doxycycline could decrease cellular ROS levels, we performed DCFDA assays after doxycycline treatment and analyzed samples by flow cytometry. As expected, a significant decrease in ROS levels was observed in the doxycycline-treated cells (Fig. 4).

Next, we examined whether the p38 MAPK signaling downstream of ROS was affected by doxycycline. We found that doxycycline treatment resulted in a decrease in p38 phosphorylation in a dose-dependent manner in the BT474 and SUM159 cell lines (Fig. 5A and B). To further test the correlation between p38 MAPK signaling and ALDH+ BCSCs, we treated BT474 cells with a p38 MAPK-specific inhibitor SB203580 and then performed the Aldefluor assay. The result showed that SB203580 abolished ALDH activity (Fig. 5C), indicating that p38 MAPK plays a key role in ALDH+ BCSC maintenance, which is targeted by doxycycline treatment.
Doxycycline attenuates paclitaxel-induced enrichment of ALDH+ BCSCs. Paclitaxel has been reported to kill the bulk of tumor cells, yet enriching ALDH+ CSCs via elevating the ROS level (35). To ascertain whether doxycycline could ameliorate paclitaxel-induced enrichment of ALDH+ BCSCs, SUM159 cells were pre-treated with doxycycline and then in combination with paclitaxel. In agreement with the previous report, paclitaxel treatment resulted in approximately 4 times more ALDH+ BCSCs as compared to the vehicle control. However, this enrichment of ALDH+ BCSCs induced by paclitaxel was significantly inhibited when cells were pre-treated and later co-treated with doxycycline (Fig. 6).

Discussion
Recent studies have demonstrated that metastasis and drug resistance of cancer are driven by small subpopulations of cells...
termed cancer stem cells (CSCs). CSCs are therefore emerging as important therapeutic targets for cancer treatment. In contrast to conventional cytotoxic chemotherapy which aims to kill the bulk of the tumor, CSC targeting therapy focuses on blocking specific signaling pathways which CSCs rely on. Thus, combining chemotherapy and CSC targeting therapy could help reach the goal of eradicating the entire tumor. In the present study, we found that doxycycline significantly decreased ALDH + BCSCs by inhibiting MAPK signaling, the downstream pathway of ROS. While applied in combination with paclitaxel, doxycycline also attenuated paclitaxel-induced enrichment of ALDH + BCSCs, implying the potentiality of combining the two drugs for removing both the bulk of cancer cells and CSCs.

High mitochondrial mass is associated with the ALDH + CSC population (20). Since doxycycline has been shown to interrupt mitochondrial biogenesis in eukaryotic systems (24), we hypothesized that doxycycline can be used as an inhibitor for ALDH + CSCs. The hypothesis is supported by our results of aldefluor and mammosphere formation assays. However, we also found that doxycycline failed to decrease the CD44+/CD24 - BCSC population (Fig. 2). CD44+/CD24- are cell-surface markers acquired by epithelial cancer cells when they undergo epithelial-to-mesenchymal transition (EMT), a developmental program that enriches CSCs (36). CD44+/CD24- EMT CSCs have characteristics that are distinct from those of ALDH + CSCs. Unlike proliferative and epithelial-like ALDH + CSCs, CD44+/CD24- EMT CSCs are quiescent and mesenchymal-like (29,37). Recent studies have reported that doxycycline can inhibit the propagation of mitochondrial-related hypoxic CSCs (27), whereas doxycycline-resistance may occur when cancer cells switch to a purely glycolytic phenotype (28). The relationship between CD44+/CD24- EMT CSCs and the glycolytic phenotype is yet to be determined. Nonetheless, it is likely that only mitochondrial-driven ALDH + CSCs but not CD44+/CD24- EMT CSCs are sensitive to doxycycline.

Mitochondria are an important source of ROS generation in most mammalian cells (38). ROS play an important role in stabilizing hypoxia-induced factor 1α (HIF-1α), which is known to induce ALDH + CSCs (35,39,40). Studies have shown that the p38 MAPK pathway, a downstream pathway of ROS, is required for HIF-1α signaling (41,42). Knockdown of p38 MAPK in the HER2-overexpressing MCF-7 cell line can inhibit ALDH + CSCs, cancer cell migration and invasion (43,44). In the present study, we demonstrated that doxycycline significantly decreased intracellular ROS levels, p38 MAPK phosphorylation and ALDH + CSCs. Cancer cells treated with a p38 MAPK-specific inhibitor also exhibited a significant reduction in ALDH + CSCs, indicating that doxycycline inhibited ALDH + CSCs potentially via blocking the
p38 MAPK signaling pathway. However, more evidence is needed to further support this hypothesis. Future studies will focus on directly investigating the involvement of p38 MAPK in doxycycline-mediated inhibition of ALDH+ CSCs. First, knockdown of p38 MAPK could be carried out in HER2+ and TNBC cell lines to ascertain whether ALDH+ CSC population is affected. Second, a constitutively active p38 MAPK could be overexpressed to examine its ability to prevent or decrease doxycycline's effect on ALDH+ CSCs.

It is worth noting that doxycycline failed to inhibit ALDH+ CSC population and secondary mammosphere formation in SUM149 cells (Figs. 1C and 3B). The number of mammospheres formed is mainly determined by the number of stem cells seeded in the culture. The results, however, can be affected if the treatment changes the proliferation of cells. Therefore, to evaluate whether doxycycline can really affect CSCs, we performed the secondary mammosphere formation assays in the absence of doxycycline. Hence, the effect of doxycycline on proliferation was avoided, and the mammospheres should be decreased if the number of CSCs has been reduced by doxycycline in the primary assays. In SUM149 cells, we found that doxycycline significantly decreased primary but not secondary mammosphere formation. The reason may be that doxycycline inhibits cell proliferation (data not shown) instead of decreasing CSCs in SUM149 cells. In addition, SUM149 has been reported as an inflammatory breast cancer cell line that constitutively adapts to hypoxia (45,46). Therefore, SUM149 can behave as if it is continuously hypoxic even under normoxia (45). This may explain why doxycycline decreases the ROS level but fails to inhibit ALDH+ BCSCs in SUM149 cells.

Recent studies and our results indicate the potentiality of repurposing doxycycline, an old drug as a new treatment to target CSCs. Doxycycline is an FDA-approved antibiotic since 1960s. With limited toxicity to cells, doxycycline is relatively safe to be used concomitantly with chemotherapy drugs in patients (25). A recent clinical trial demonstrated that pathogenic bacteria-negative patients with lymphoma still benefit from doxycycline (47). More phase II clinical trials are ongoing to test the use of doxycycline as a CSC-targeting agent. In addition to targeting CSCs, doxycycline was also found to ameliorate tumor metastasis via inhibition of matrix metallopeptidases (48,49). As such, we propose that doxycycline is an ideal drug that can be used in combination with cytotoxic chemotherapy drugs to eradicate both CSCs and bulk tumor cells.

Acknowledgements

The authors thank the Flow Cytometry Core of University of Michigan, supported by the NCI Grant P30CA046592 from the National Institutes of Health, for the technical support on flow cytometry work in this study.

Funding

No funding was received.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CCL conceived and designed the study and wrote the paper. CCL, RRM, NOS and SLT performed the experiments. MCL reviewed and edited the manuscript. MCL and DS supervised the research and manuscript preparation. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


