

Alternative therapies for metastatic breast cancer: multimodal approach targeting tumor cell heterogeneity

Manpreet Sambi¹
Sabah Haq¹
Vanessa Samuel¹
Bessi Qorri¹
Fiona Haxho¹
Kelli Hill^{1,2}
William Harless²
Myron R Szewczuk¹

¹Department of Biomedical and Medical Sciences, Queen's University, Kingston, ON, Canada; ²ENCYT Technologies, Inc., Membertou, NS, Canada

Correspondence: Myron R Szewczuk
Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON K7L 3N6, Canada
Tel +1 613 533 2457
Fax +1 613 533 6796
Email szewczuk@queensu.ca

William Harless
ENCYT Technologies, Inc., 201 Churchill Dr, Suite 205, Membertou, NS B1S 0H1, Canada
Tel +1 902 539 3365
Fax +1 902 539 3363
Email williamharless@ymail.com

Abstract: One of the primary challenges in developing effective therapies for malignant tumors is the specific targeting of a heterogeneous cancer cell population within the tumor. The cancerous tumor is made up of a variety of distinct cells with specialized receptors and proteins that could potentially be viable targets for drugs. In addition, the diverse signals from the local microenvironment may also contribute to the induction of tumor growth and metastasis. Collectively, these factors must be strategically studied and targeted in order to develop an effective treatment protocol. Targeted multimodal approaches need to be strategically studied in order to develop a treatment protocol that is successful in controlling tumor growth and preventing metastatic burden. Breast cancer, in particular, presents a unique problem because of the variety of subtypes of cancer that can arise and the multiple drug targets that could be exploited. For example, the tumor stage and subtypes often dictate the appropriate treatment regimen. Alternate multimodal therapies should consider the importance of time-dependent drug administration, as well as targeting the local and systemic tumor environment. Many reviews and papers have briefly touched on the clinical implications of this cellular heterogeneity; however, there has been very little discussion on the development of study models that reflect this diversity and on multimodal therapies that could target these subpopulations. Here, we summarize the current understanding of the origins of intratumoral heterogeneity in breast cancer subtypes, and its implications for tumor progression, metastatic potential, and treatment regimens. We also discuss the advantages and disadvantages of utilizing specific breast cancer models for research, including in vitro monolayer systems and three-dimensional mammospheres, as well as in vivo murine models that may have the capacity to encompass this heterogeneity. Lastly, we summarize some of the current advancements in the development of multitarget therapeutics that have shown promising results in clinical and preclinical studies when used alone or in combination with traditional regimens of surgery, chemotherapy, and/or radiation.

Keywords: breast cancer, alternative therapy, drug resistance, cancer stem cells

Introduction

Breast cancer is the most diagnosed form of cancer in women and accounts for 14% of cancer-related deaths.¹ Current therapies in treating breast cancer include one or a combination of surgery, chemotherapy, and/or radiotherapy. Surgery can often cure localized breast tumors. Both chemotherapy and radiotherapy can show efficacy in shrinking tumors while chemotherapy can occasionally eradicate micrometastatic disease. However, the challenge that oncologists face continues to be cancer cell recurrence after these treatments and metastatic spread. In general, clinically evident metastatic breast cancer remains an incurable malignancy. It is now important

to develop novel therapies that disrupt specific molecular pathways that are critical to cellular proliferation and those that confer metastatic ability.²

Metastatic disease occurs when cancer cells disseminate from the primary tumor, they enter the systemic circulation, and colonize other organs. Breast cancer cells generally metastasize and colonize the lung, liver, bones, and brain to generate secondary tumors.³ Recent findings have shown that not all cancer cells are equal in their ability to metastasize to novel sites and form new tumors. Instead, a distinct cancer cell subpopulation, often referred to as cancer stem cells (CSC) or tumor initiating cells, may uniquely possess the requisite genetic repertoire to accomplish this task.⁴ Their identity and complex behavior remains an intensely studied area. A better understanding of these cells may yield enormous opportunities to improve the care of patients with cancer.

Traditional treatment options were designed to target a tumor assumed to have a homogenous phenotype in that all cells in a solid tumor had the same proliferative capability.⁴ Moreover, the assumption was that these cells were equally susceptible to cytotoxic therapies and radiation. These therapies targeted the tumor as a whole rather than the CSC subpopulation that permits continuous tumor growth and metastatic capability. Furthermore, there is strong evidence to suggest that the CSC subpopulation may be particularly resistant to conventional anticancer therapies such as chemotherapy and radiation.⁵ In order to treat the disease, it is critical to identify and selectively eradicate this cancer cell subpopulation.

The CSC hypothesis may hold enormous promise to improve the care of patients with a wide variety of cancers. Dick⁶ described the heterogeneity of a leukemic tumor and elucidated the possibility of a core CSC subpopulation that conferred the uncontrolled and indefinite growth seen in most forms of tumors.⁶ This idea put into question the assumption that all cancer cells were equal in their ability to grow indefinitely and form secondary tumors, and by the same logic, were equally viable targets for drug therapies.

Previous reviews and papers published on breast tumor heterogeneity and intratumoral heterogeneity as a whole have primarily focused on the origins,⁷ characterization,⁸ identification,^{9,10} and confirmation¹¹ of a tumor cell initiating population within breast and other tumors. These reviews have briefly touched on the genetic diversity between cells in a primary tumor that possess varying proliferative capacity, chemoresistance, and metastatic ability and have continued to build on the theory that was first proposed by Nowell¹²

in 1976. Although more advanced experimental techniques since then have confirmed this intratumor variation and characterized and identified unique markers that could potentially differentiate subpopulations within tumors, they have not established a strong link between this heterogeneity and its clinical implications. Furthermore, there is very little discussion on developing more accurate study models with the capacity to encompass this heterogenic framework on which efficacy of alternative treatment regimens can be tested. This is particularly important when modifying current treatment protocols and options for patients with cancer as the treatment must be able to target a diverse population of cancer cells, and the study model must reflect this. Therefore, this review will highlight the clinical relevance of the unique heterogeneous framework of the primary breast tumor and will focus primarily on the specialized cells within the tumor that have the capacity to metastasize and allow for recurrence and regrowth after chemotherapy. We will also review the experimental use of multicellular tumor spheroids as a tool for studying the penetrance and effectiveness of anticancer agents. Such an *in vitro* system provides insight into the complex organization of primary breast cancer cells and the formation of compact tumor spheroids. Furthermore, three-dimensional (3D) tumor modeling can equip us to characterize the initial onset of heterogeneity among cancer cells *in vitro*, as well as elucidate the underlying mechanisms responsible for the development of a nutrient gradient and hypoxia in tumors *in vivo*.

Intratumoral heterogeneity of breast cancer: origins and clinical implications

One of the most significant challenges in the successful treatment of breast cancer is the selective yet potent killing of tumor cells and micrometastases. This hurdle is primarily due to the genetic diversity of cells within a primary tumor as well as its secondary and distant metastatic growths.

Breast cancer cell heterogeneity

There are currently two different models that may explain the advent of breast cancer cell heterogeneity: the clonal expansion model¹² and the CSC model.^{6,7,13} The clonal expansion model incorporates the theory of natural selection as it applies to tumor growth and development.¹² It postulates that cancer cells mutate as they undergo mitosis, with some cells acquiring traits that confer resistance against chemotherapy, stem-like proliferative potential, and the ability to metastasize.⁷ These cells are then selected for their ability to

survive in environments exposed to chemotherapy or other assaults, and thus clonally expand until they are untreatable.

In contrast, the CSC model suggests that all tumors arise from a central tumor initiating population or a CSC population that will give rise to a more differentiated and heterogeneous cell types that comprise the bulk of the tumor. It is the more differentiated cancer cell population that is thought to be more sensitive to chemotherapy and radiation, while the CSC subpopulation remains relatively resistant to these therapies. This may account for the observation that chemotherapy can often shrink metastatic tumors down to a size that may even be undetectable with the most sophisticated imaging devices; however, these tumors invariably return. In the stem cell model, the premise is that the stem cell is the root of the cancer, and without killing the stem cell, the malignancy will invariably return, regardless of how many of the more differentiated cancer cells are targeted by the therapy.

Mechanisms of primary tumor invasion and secondary metastasis

The molecular mechanisms that regulate the onset of primary tumor metastasis are perceived to be highly dependent upon the CSC subpopulation or the cancer cell populations that have undergone metastatic capability via a partial epithelial–mesenchymal transition (EMT). The mechanism and genetic events involved with EMT are not well understood; however, Hanahan and Wienberg¹⁴ detailed the current theories of EMT, and Creighton et al¹⁵ have eloquently reviewed EMT programming in cancer cell invasion and metastasis. In general, EMT is a two-step process. First, tumor cells must detach from the primary tumor and migrate to distant organs by entering systemic circulation. Second, they must undergo reverse transition and colonize distant organs in order to establish micrometastases, which later may form macrometastases in the form of secondary tumors. This process is highly inefficient, and not all cells are able to undergo partial EMT. Another difficult aspect to successful metastatic colonization of tissue is reverting back to an epithelial phenotype after undergoing the partial EMT or dedifferentiation genetic program characteristic of a mesenchymal or migratory phenotype that permits anchorage independent survival while in the circulation. This molecular change, called MET, may be critical to allowing circulating cancer cells to colonize tissue. In work done in squamous cell cancer cells that may also have relevance to breast cancer cells, Tsai et al^{16,17} showed that the upregulation of an EMT inducer like Twist was essential to allowing cancer cells to invade the circulation and migrate effectively, while its downregulation was important in the

subsequent colonization of the novel tissue¹⁷ and the formation of a micrometastasis. This important work demonstrated the distinct molecular changes involved in allowing a cancer cell to migrate effectively in the circulation and to colonize novel tissue and form new tumors.

Micrometastatic colonization does not always lead to a macrometastasis of secondary growth. In some forms of cancer, micrometastases are suppressed by the primary tumor and remain dormant until the primary tumor is excised and eventually lead to secondary tumors at distant sites.¹⁴ The clinical implication of disseminated breast cancer cells that have undergone partial EMT expressing N-cadherins is that they are particularly resistant to chemotherapy and are capable of forming secondary lesions that are difficult to detect. Furthermore, breast cancer metastases appear to remain dormant and undetectable at distant sites for years until an optimal number of disseminated cells are able to successfully colonize the tissue and form a macrometastatic tumor.¹⁴

Therefore, only a select number of cells are able to successfully metastasize, and this subset of cells are thought to be cancer cells that have stem-like characteristics.¹⁸ The process of metastasis is very similar to organogenesis during embryonic development. For example, migrating neurons and mesenchymal cells express N-cadherin, a marker which is also expressed by cancer cells that have the capacity to invade distant organs and have undergone partial EMT.¹⁴ The unique and difficult aspect of metastasis is reverting back to an epithelial phenotype in order to colonize a new microenvironment at a distant location and develop micro- and macrometastases. This two-stage metastatic process is thought to be a result of a partial EMT or a dedifferentiation process, which allows for enhanced migration and survival under anchorage independent conditions, while these cells simultaneously retain the ability to revert back to an epithelial phenotype and colonize tissue via mesenchymal-epithelial transition (MET).

With regard to breast cancer as a whole, one of the key hurdles is its initiation and the heterogeneity of its pathology.¹³ Studies have shown that breast cancer progresses in a very unique manner when compared with other cancers of epithelial origin. There are currently 18 different subtypes of breast tumors classified into the various categories based on a number of parameters including lesion size, cell arrangement, and necrosis.¹³ Recently, Al-Hajj et al⁹ presented evidence of a “stem cell population” in breast tumors and elucidated the phenotype being cells that expressed CD44⁺CD24^{-low} and termed them as tumor initiating cells. This population of cells was shown to have the capacity to produce tumors

in mice with as little as 100 cells injected. In addition, this tumor initiating cell population displayed characteristics that were similar to those of normal stem cells in that they were able to produce secondary tumors with similar heterogeneous phenotypes with differentiated tumor cells and reduced proliferative capacity. This discovery was important as these cells are hypothesized to be the population that allows breast tumors to grow indefinitely, even after administration of chemotherapy. Therefore, in the clinic, it is important to consider a therapy that is cytotoxic to both nontumorigenic and tumorigenic cells. This latter population of tumorigenic cells is left intact after chemotherapy and will reestablish itself as the original tumor.

Proponents of these original breast cancer models suggest further study in order to advance drug development and alternative treatment options. Regardless of which model is correct, the current treatment options are inadequate in controlling and even treating breast cancer because of the diversity of the cells found within the tumor. In fact, breast cancer tumor samples taken from a single patient from distant metastases showed phenotypic variability from the original tumor,¹⁹ suggesting that the tumor evolves separately from secondary tumors and would therefore require a more tailored treatment approach. One possibility is to use small inhibitor molecules²⁰ that target key biological processes of cells that display stem-like characteristics, an important avenue worth considering. Ideally administering these drugs at the time of chemotherapy would not only allow the tumor to shrink, but the core cells that give rise to the tumor or cells that display stem-like characteristics as an artifact of clonal expansion, might also be eliminated and prevented from regrowth. However, the characterization of breast tumor cell subpopulations is an important avenue to explore in order to elucidate viable targets. Whether it is a receptor that is found on metastatic cancer cells or a gene that is conserved in all cancer cells exhibiting stem-like characteristics, determining the unique cell populations within a breast tumor that will allow for more specific and targeted therapies need to be developed. Multimodal approach is to target tumor cell heterogeneity found within a tumor as well as to determine strategic drug targets during critical therapeutic time points either in conjunction with chemotherapy or after chemotherapy to prevent metastasis and chemoresistance.

Biological models for breast cancer research

The development of viable treatments to target heterogeneous breast cancer cell populations and prevent metastasis is only

one aspect of current breast cancer research. Current efforts also aim to evaluate the efficacy of drug treatment protocols and determine the time points necessary to administer these therapies in a safe and cost-effective manner. While many studies use animal models and *in vitro* assays on monolayer cancer cells, the interaction between the tumor and its microenvironment is difficult to study in both of these models. This microenvironment is a key player in the establishment and progression of tumors.²¹ The development of an *in vitro* model that can mimic this interaction may serve as a significant platform for drug testing and drug discovery efforts in current breast cancer research.

3D multicellular tumor spheroid: an emerging research model

The multicellular tumor spheroid (MCTS) is an optimal 3D *in vitro* tumor model that can be used to achieve an improved understanding of breast cancer properties and characteristics.²² The MCTS model overcomes the deficits of 2D monolayer cultures which do not accurately represent the tumor microenvironment.^{23–25} 2D monolayers cannot support complex cell–cell interactions, lack extracellular matrix (ECM) components, do not allow for quiescent and heterogeneous cell populations (ie, stromal cells), and cannot develop the nutrient, oxygen, and catabolite gradients that are present in solid *in vivo* tumors.²⁶ On the other hand, MCTS models have a well-defined and controlled microenvironment that serves as a more accurate representation of the complex *in vivo* host environment.²² MCTS also exhibits increased chemoresistance compared to 2D monolayer cultures as they have extensive cell–cell and cell–matrix interactions, realistic drug penetrance gradients, and diverse chemoresistant gene expression.^{27,28} MCTS would thus serve as an ideal model for the screening of novel anticancer drugs, especially for tumors that have demonstrated chemoresistance to past treatments.^{29,30} Furthermore, MCTS in matrigel or in ECM-based matrixes is a valuable tool for the study of cancer cell processes and chemotherapeutic responses *in vitro*.^{28,31} Recently, matrix-free spheroids have been developed to investigate cell growth, mutations, invasion, cell motility, and metastasis that contribute to the pathophysiology of tumors.^{32–36} MCTS is a powerful 3D *in vitro* model to study tumor cell propagation, phenotypes, genotypes, cell invasion, metastasis, angiogenesis, and chemoresistance.^{27,32,37,38}

Multicellular tumor spheroids have been mechanically developed using diverse cell-aggregation techniques, including rotating culture, spinner flasks, gravity-based techniques (ie, hanging drop), and ultra-low attachment plates.²⁷ How-

ever, these methods are either labor-intensive, expensive, difficult to replicate, or produce a limited number of spheroids per well.³⁹ As a result of these practical drawbacks, MCTS models have not been fully embraced in cancer research, despite their recognized advantages.⁴⁰

To overcome current limitations, Akasov et al²³ recently developed a simple, cost-effective, and reproducible technique for MCTS formation. Instead of using mechanical force or gravity to induce MCTS formation, their novel method takes advantage of inherent biochemical properties of cell adhesion. They developed synthetic arginine–glycine–aspartic acid (RGD) peptides to mimic the natural RGD motif in fibronectin (FN), as FN typically binds the $\alpha 5\beta 1$ integrin *in vivo* as a key step of cell–cell adhesion.^{23,41} Akasov et al²³ determined that a cyclic version of the RGD peptide conjugated with triphenyl phosphonium cation (TPP) was the most efficient form of their synthetic cell adhesion protein. The decreased entropy and rigidity imposed by the cyclic shape improved target specificity,⁴² and the TPP cation covalently binds to aspartic acid within the RGD motif to increase electrostatic interactions and facilitate RGD- $\alpha 5\beta 1$ integrin binding.²³ The cyclo-RGDfK(TPP) peptide was shown to induce extensive spheroid formation (~100 spheroids/well) without any cytotoxic effects on the cells.²³ The spheroids were also more resistant to chemotherapy drugs than their 2D monolayer equivalents, which provides additional support for the biochemical MCTS model as a platform for antitumor drug testing.²³

The MCTS model could be further employed in breast cancer research to develop an improved understanding of tumor metastasis through gene expression profiles that characterize periods of metastatic disease. Breast cancer mammospheres exhibit realistic profiles of oncogenes and tumor biomarkers, which could then be used to identify therapeutic gene targets for metastatic breast cancer.⁴³ A recent study by Pacheco-Marín et al⁴⁴ found that 3D breast cancer mammospheres displayed a “metastatic signature” involving the downregulation of several cell-adhesion molecules (ie, EPCAM, E-cadherin, integrins, etc), suggesting that the tumor cells were more likely to separate, migrate, and metastasize. Another report has shown that mammospheres express biomarkers (eg, vimentin) that are involved in EMT, which is known to promote tumor metastasis.⁴⁵ Another MCTS breast cancer study found high levels of gene expression in all three stages of metastasis (initiation, progression, and colonization), which were significantly amplified compared to the respective findings in the 2D monolayer model.⁴⁶

Furthermore, our group has shown that tumor cell sialylation promotes MCTS formation in both parental

and tamoxifen-resistant MCF-7 breast cancer cell lines using the cyclo-RGD peptide platform.⁴⁷ We also showed that sialylation of triple-negative breast cancer (MDA-MB-231) facilitates cell aggregation and compaction in mouse models and validated the key role of sialylation in MCTS formation. This triple-negative breast mammosphere model accurately mimics α -2,3-sialic acid interactions between adjacent cells within the tumor. When *in vivo* mouse tumors generated from MDA-MB-231 breast cancers cells were treated with oseltamivir phosphate, we showed an increase in α -2,3-sialic acid when compared with α -2,6-sialic acid expression.⁴⁷ When extrapolating this experiment to our MCTS model, we also observed higher levels of α -2,3-sialic acid when compared with α -2,6-sialic acid. The MCTS model has thus demonstrated the ability to precisely mimic *in vivo* conditions. Furthermore, our results confirmed that sialylation allows cells to remain tightly bound in a spheroid and maintain an epithelial phenotype characterized by E-cadherin expression on immunofluorescence-stained tissues. This is particularly relevant to breast cancer as its metastatic ability is higher than other cancers due to overexpression of HER-1 in some breast cancer subtypes.⁴⁸ Cancer cells with metastatic potential tend to have aberrant sialic acid expression and a mesenchymal phenotype (indicated by N-cadherin expression) as they disseminate from the original tumor and enter systemic circulation.⁴⁹ Therefore, an increase in α -2,3-sialic acids on the surface of cancer cells could increase cell–cell adhesion, allow cells to maintain an epithelial phenotype, and prevent metastasis.⁴⁷

Collectively, these findings support the premise that 3D MCTS can compensate for many of the deficiencies observed in monolayer cultures. They can display many morphological and functional similarities to tumors and develop chemical gradients of oxygen, nutrients, and catabolites that mimic *in vivo* tumor growth.

Combination therapy: targeted multimodality approach to breast cancer

Current treatments for cancer patients involve one or a combination of the following three options: surgery to excise a tumor or mastectomy, chemotherapy, or radiation therapy.⁵⁰ These treatment options initially provide long-term survival or allow the patients to go into remission.⁵¹ Sometimes patients live for years before the cancer is detected again, and unfortunately, more often than not, this is a fatal outcome.⁵² Therefore, one aspect of cancer research that is currently

being considered is why this occurs and how to prevent it from reoccurring even when it appears that the therapy was successful and the patient is in remission.⁵² Developing a treatment protocol that encompasses the ability to target multiple stages of breast cancer development such as initiation, progression, and development of metastasis as a treatment is very important in increasing patient survival and reducing metastatic burden.

Anti-inflammatory agents and their therapeutic role in breast cancer

Inflammation, for example, has been shown to have a role in the development of tumor progression in breast cancer, suggesting that it may involve an inflammatory component. The expression of inflammatory chemokines CCL2 and CCL5, and inflammatory cytokines TNF- α and IL-1 β was investigated during the course of the disease. Elevated expression of TNF- α and IL-1 β in patients diagnosed with invasive ductal carcinoma with relapse (IDC-with-relapse) group suggests that these two cytokines support disease progression and recurrence by promoting EMT. To demonstrate the contribution of EMT, it was found that TNF- α potently induced a reduction in E-cadherin expression at the cell membrane of tumor cells in a dose-dependent manner.⁵³

NF- κ B functions as a tumor promoter in inflammation-associated cancer. TNF- α was shown to activate NF- κ B. Frequent NF- κ B activation has been shown to induce drug resistance in cancer cells. This frequent activation suggested that inflammation-associated NF- κ B activation promotes neoplastic growth.⁵⁴ NF- κ B is triggered in response to infectious agents and proinflammatory cytokines via the I κ B kinase (IKK) complex. It was found that the deletion of IKKB in myeloid cells resulted in a significant decrease in tumor size, which diminished the expression of proinflammatory cytokines that may have served as tumor growth factors. Inactivation of this pathway can attenuate the formation of inflammation-associated tumors.⁵⁵

Nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated the ability to suppress transcription factor NF- κ B, which ultimately controls the expression of cyclooxygenase 2 (COX-2) and cyclin D1, leading to the inhibition of proliferation of tumor cells. In several NSAIDs tested, aspirin and ibuprofen were the least potent, with celecoxib and tamoxifen being the most potent anti-inflammatory and antiproliferative agents.⁵⁶ Whether NSAIDs like aspirin mediate their effects by a COX-1- or COX-2-dependent manner has not yet been elucidated, due to contradicting results from various studies.

Furthermore, it has been demonstrated that COX-2, the inducible form of the COX enzyme, is overexpressed in breast tumors and also linked to metastasis.⁵⁷ NSAIDs are known to inhibit cyclooxygenases, suggesting that their therapeutic effect is related to inhibition of COX overexpression. The COX-1 and COX-2 expression profile of patients with breast cancer who had undergone a lumpectomy or mastectomy was analyzed by immunoblot and immunohistochemical analyses. It was found that COX-1 was localized in stromal cells adjacent to the tumor but not in the tumor cells, and COX-2 was found primarily in the tumor cells, but also appeared in stromal cells.⁵⁸ COX-2 inhibitors were found to produce a significant (71%) decrease in the risk of breast cancer.⁵⁹ This approach strongly suggests that COX-2 inhibitors play a role in the treatment of breast cancer due to their prevalence in breast cancer tumors and their chemoprotective role in mitigating the risk of breast cancer. Furthermore, Holmes et al⁵⁷ showed that when patients were treated with aspirin following breast cancer diagnosis and treatment, there was an association between aspirin and the reduction in metastasis as well as breast cancer-related death. It has been shown the COX-2 is overexpressed in animal and human breast cancers that metastasize. Therefore, they postulated that the decrease in metastatic burden in breast cancer patients after being treated with aspirin could be attributed to the inhibition of COX-2. In addition, they found that endothelial cell proliferation was inhibited with a reduction in cell viability of human endothelial cell line HMEC1 upon treatment with 2 and 5 mM aspirin. A TUNEL assay further demonstrated that 5 mM of aspirin triggers an extensive apoptotic response in these endothelial cells. Aspirin was also found to reduce levels of angiogenesis at all concentrations. Sixty percent reduction of angiogenesis was seen with 0.5 mM of aspirin, which had not resulted in any detectable decrease in cell viability or proliferation. Both COX-1 and COX-2 are inhibited by 0.5 mM aspirin, but it remains to be determined if aspirin exerts its effect through a COX-1-dependent mechanism or a COX-2-dependent mechanism. The COX-1 inhibitor SC-560 had no effect on cell proliferation, cell viability, or angiogenesis at 50 nM and 1 μ M, even a dose 5,000 times greater than the half maximal inhibitory concentration (IC₅₀) for COX-1 led only to a small decrease in branch formation. Celecoxib, a selective COX-2 inhibitor had no effect on cell proliferation, cell viability, or angiogenesis at a dose 50 times higher than the selective dose. Celecoxib did demonstrate an 85% decreased in branch formation, but only at 400 times the selective dose, in which the toxic effects and targets of celecoxib are not yet known. Doses of SC-560 and celecoxib were added

simultaneously to determine if there is a combined effect on COX-1 and COX-2 inhibition. There was no detectable effect on angiogenesis.⁶⁰ These data suggest that aspirin exerts its antiangiogenic effects through a COX-independent pathway.

The therapeutic effects of aspirin are not limited to its mitigation of inflammation and decreasing COX expression, but also to its potential sensitizing effects. Aspirin has been shown to sensitize chemoresistant pancreatic cells to gemcitabine and increase the efficacy of chemotherapy treatment.⁶¹

It has already been demonstrated that aspirin use initiated during diagnosis and continued postdiagnosis of colon cancer is associated with a lower risk of mortality.⁶² This same therapeutic effect may be expanded to other cancers including breast cancer. Aspirin was found to display synergy with doxorubicin in HepG2 human hepatocellular carcinoma cells *in vitro* and in a xenograft model in nude mice. In combination, aspirin and doxorubicin resulted in enhanced effects in inhibiting tumor growth, arresting cell cycle and causing apoptosis *in vitro* when compared to single treatments. Combination therapy also resulted in synergistic antitumor activity in the xenograft model in nude mice.⁶³ Others have shown that varied concentrations of aspirin,⁶⁴ metformin,⁶⁵ and heparin⁶⁶ have shown efficacy in treating cancer when given in conjunction with chemotherapy. This provides further evidence that aspirin, metformin, and heparin work synergistically with chemotherapy treatments to increase efficacy. The use of aspirin and metformin in sensitizing breast cancer cells to chemotherapeutic treatments is a promising area of research.

Neu1 sialidase: therapeutic multimodal targeting in breast cancer

Our group is currently working on developing a new treatment regimen that we believe targets both the primary tumor as well as the tumorigenic population that sustains cancer in an effort to reduce the possibility of patients developing metastatic disease. This treatment regimen is based on our findings on a novel signaling paradigm that is linked to receptors known to play a critical role in tumorigenesis including epidermal growth factor receptor,⁶⁷ nerve growth factor receptor,⁶⁸ insulin receptor,^{69,70} cell surface TOLL-like receptor-4,⁷¹⁻⁷⁴ and intracellular TOLL-like receptor-7 and -9,⁷⁵ and this is reported in detail by Abdulkhalek et al.⁷⁶ This new therapeutic multimodal approach is designed to target the neuraminidase-1 enzyme that plays a central role in chemoresistance, angiogenesis, metastasis, and tumorigenesis, and this is reported in detail by Haxho et

al.⁴⁹ Another report by Haxho et al⁷⁷ has shown that targeting neuraminidase-1 with oseltamivir phosphate in MDA-MB-231 triple-negative breast tumor-bearing mice showed a decrease in tumor volume of up to 80% when compared to the tumor volume of untreated cohorts with no relapse and no metastatic burden. Oseltamivir phosphate treatment strategies are proposed here to take the form of a horizontal approach, of which different oncogenic signaling pathways as well as macrophage-mediated tumorigenesis are targeted with promising therapeutic intent. Based on these promising findings and those of others, it is important to develop strategic treatments that are not only cytotoxic to tumor cells, but also combine these treatments in order to prevent metastasis and tumor regrowth.

Conclusion

Resistance to chemotherapy, tumor recurrence, and metastatic disease remain major obstacles in the development of effective treatment strategies for breast cancer. They are currently the most difficult aspects of breast cancer to treat. As such, breast tumors cannot be viewed as isolated units, but rather as dynamic growths composed of multiple cell types that orchestrate self-sustaining signaling and invasion. The CSC hypothesis presents an exciting and promising avenue for targeting molecular markers associated with aggressive cancer cell subpopulation. Indeed, knowledge is increasing rapidly, and it is reasonable to think that we are entering a highly productive period for the discovery of novel anticancer agents for the multistage treatment of breast cancer. Research models, such as the *in vitro* 3D multicellular tumor spheroids and animal models, are invaluable for assessing the safety, selectivity, and efficacy of new therapeutic agents. Future studies should implement these multimodal techniques to advance our understanding of the complex processes involved in breast cancer development, malignant progression, and response to therapy.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

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