METFORMIN EFFICACY AGAINST BREAST CANCER DEPENDS ON ITS CELLULAR UPTAKE VIA CATION TRANSPORTERS AND MODULATION OF INSULIN/IGF1 PATHWAY

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ABSTRACT

Hao Cai: Metformin Efficacy Against Breast Cancer Depends on Its Cellular Uptake via Cation Transporters and Modulation of Insulin/IGF1 Pathway
(Under the direction of Dhiren R. Thakker)

Clinical evidence suggests that metformin is efficacious as an anticancer agent in diabetic patients; however, results about its efficacy are mixed, especially in non-diabetic patients. The goal of this dissertation project was to improve metformin treatment for breast cancer by elucidating molecular and cellular mechanisms that play an important role in its efficacy. Research conducted in this project showed that breast cancer cells exhibit wide variability in the expression of cation transporters, which are required for intracellular uptake and accumulation of metformin. Further, metformin requires a functional intracellular adenosine monophosphate-activated protein kinase (AMPK) pathway to exert its anticancer activity. Interestingly, cancer stem cells, which are more sensitive to the antiproliferative effect of metformin, express higher levels of cation-selective transporters than non-stem cancer cells.

Preclinical dose-response studies showed that estrogen receptor positive breast tumors with low expression of cation transporters required a minimum metformin dose (in combination with 30 mg/kg/day paclitaxel) that is equivalent to the highest current anti-diabetic dose of 2,550 mg/day, suggesting that an even higher metformin dose is needed to optimally treat these patients. The minimum efficacious metformin dose (in combination with 50 mg/kg/day carboplatin) to treat triple negative breast cancer with high expression of cation transporters was equivalent to the 850 mg daily dose of metformin that is typically used in the treatment of type 2 diabetes.

Studies in mice showed that attenuation of the insulin/IGF1 pathway sensitized breast cancer cells to the antiproliferative efficacy of metformin exerted via modulation of the AMPK pathway. These results provide a rationale for lower efficacy of metformin in non-diabetic patients, and suggest that co-administration of metformin with an insulin/IGF1 pathway inhibitor may improve metformin efficacy in non-diabetic breast cancer patients.

In summary, the dissertation research provides valuable insights into cellular and molecular factors that contribute to the variable responses of diabetic and non-diabetic breast cancer patients to metformin therapy. The findings of this research will contribute to improvement in selection of breast cancer patients who would respond to metformin therapy, improved dose selection strategy, and development of new metformin combination therapies for breast cancer.

To my parents, for their unending love, support, and understanding.
To my advisor, I could not make such progress without your support and mentorship.

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ADME	Absorption, distribution, metabolism, and excretion
AKT	Protein kinase B (PKB)
AMPK	Adenosine monophosphate-activated protein kinase
ATM	Ataxia telangiectasia mutated
AUC	Area under the curve
CDC	Cyclin-dependent kinase
CHT	Choline transporter
CSC	Cancer stem cell
CV	Coefficient of variation
DOX	Doxorubicin
EMT	Epithelial-mesenchymal transition
ER+	Estrogen receptor-positive
HMLER	Oncogenic transformed immortalized human mammary epithelial
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
LKB-1	Liver kinase B1
MATE	Multidrug and toxin extrusion protein
MCF-7$^{T\text{-}CTRL}$	MCF-7 cells used as transfection control
MCF-7$^{IRS\text{-}1\ KD}$	MCF-7 cells in which IRS-1 expression is knocked down
NSCC	Non-stem cancer cell
OCT	Organic cation transporter
OCT3-BT20	OCT3-overexpressing BT-20 cells

OCT3-MCF7	OCT3-overexpressing MCF-7 cells
PD	Pharmacodynamics
PI3K	Phosphoinositide 3-kinase
PK	Pharmacokinetics
PMAT	Plasma membrane monoamine transporter
P70S6K	Ribosomal protein S6 kinase
SD	Standard deviation
ST	Serotonin transporter
TGF	Transforming growth factor
TN	Triple-negative
VPC	Visual predictive check

CHAPTER 1

Repurposing Metformin for Breast Cancer Therapy: from Clinical Observations to Molecular Mechanisms to Drug Optimization

1.1 Metformin is A Leading Drug for Type 2 Diabetes

Metformin, also known as 1,1-dimethylbiguanide, is currently the first-line therapeutic agent for type 2 diabetes. Metformin is a type of biguanide compound which originates from goat's rue (*Galega officinalis*), a herbal medicine used for the treatment of diabetes for centuries. Metformin was synthesized in 1922 by Emil Werner and James Bell (1). Compared to other biguanide compounds such as phenformin, metformin is much safer, but also has a relatively lower anti-glycemic efficacy. As a result, metformin was not used in the clinic for treatment of diabetes until the 1970s when other biguanide drugs were withdrawn from the market due to toxicity.

Metformin was approved by the U.S. Food and Drug Administration (FDA) for type 2 diabetes in 1994. Today, it has become the most widely prescribed anti-diabetic agent in the U.S. with 59.2 million prescriptions in 2014 (2). For the treatment of type 2 diabetes, metformin is orally administered as either immediate-release GLUCOPHAGE® Tablets or extended-release GLUCOPHAGE® Tablets (Bristol-Myers-Squibb Inc.). The dose of metformin generally used for anti-diabetic treatment ranges from 500 to 1000 mg, with a maximum recommended daily dose of 2550 mg (for immediate-release formulation) or 2000 mg (for extended-release formulation).

Metformin exerts its anti-glycemic effects in diabetic patients by increasing insulin sensitivity rather than decreasing insulin secretion. The liver is the primary target of the anti-diabetic effect of metformin. Upon being taken up into the hepatocytes, metformin inhibits gluconeogenesis via activation of the intracellular adenosine monophosphate-activated protein kinase (AMPK), and

subsequent downregulation of the genes that drive gluconeogenesis (3). Metformin treatment has also been reported to activate AMPK in the skeletal muscle and recruit glucose transporters to increase glucose uptake into skeletal muscle (4). Studies also suggested that metformin reduces circulating glucose levels by decreasing intestinal glucose absorption, demonstrated by impaired anti-glycemic efficacy of metformin when the drug was delivered through the portal vein (5). The anti-diabetic effect of metformin (i.e. its glucose-lowering effect) in the non-diabetic people has been reported to be insignificant (6). Since metformin has been shown to impact circulating fatty acids (7), growth hormone (8), and transforming growth factor beta (TGFβ) (9), etc., it is also used for the treatment of polycystic ovary syndrome (10), excessive weight (11), insulin resistance (12), and arterial hypertension (13).

1.2 Metformin Pharmacokinetics (PK): The Role of Transporters in Metformin Disposition and Efficacy against Type 2 Diabetes

Metformin is a highly hydrophilic small molecule (logD of -6.13 at pH 6.0) that is positively charged (pka 12.4) at all physiological pH values (structure shown as the insert of **Figure 1.1**). Due to the physicochemical properties of metformin, its intracellular uptake through passive diffusion is very limited (14). Instead, studies have shown that the cellular transport of metformin is primarily mediated by cation-selective transporters (15, 16), as evidenced by observations showing significantly higher permeability of metformin in Caco-2 cells compared to neutral molecules with similar physicochemical properties, such as mannitol (16-18).

The absorption, distribution, metabolism, and excretion (ADME) of metformin have been well studied. The intestinal absorption of metformin is saturable and dose-dependent (6), and its bioavailability from 40%-60%, which is 3-fold higher than the bioavailability of mannitol (6, 19). These observations suggested that the intestinal absorption of metformin is through transporter-mediated processes rather than by passive diffusion. Han et al. identified four cation-selective transporters namely organic cation transporter (OCT)1, plasma membrane monoamine transporter (PMAT), serotonin transporter (ST), and choline transporter (CHT) that are

expressed in human intestinal tissues and are responsible for metformin intestinal absorption, (15). As metformin absorption was also shown to be dependent on intestinal length, a “sponge” mechanism was proposed by Proctor et al., which suggested that the intestinal absorption of metformin is enhanced by an uptake-efflux-reuptake process mediated by cation-selective transporters (16).

Upon being taken up into the intestine, metformin accumulates in the liver and small intestine (17), with a volume of distribution in humans ranging from 60 to 280 L. The absorption of metformin in its primary target organ, the liver, is mediated by OCT1. In the liver, metformin remains unmetabolized (20) and a small proportion of it (<20%)) is secreted into bile by the multidrug and toxin extrusion protein (MATE)1 transporter. The elimination of metformin from the body is achieved primarily through renal clearance. In the kidney, metformin is taken up into the proximal tubules via OCT2, and secreted into the urine via MATE1 and MATE2 (21, 22).

Since cation-selective transporters play a critical role in the ADME of metformin, studies have been conducted to evaluate how mutations in these transporters affect the PK and anti-diabetic efficacy of metformin. Becker et al. reported that OCT1 mutations impaired the anti-glycemic efficacy of metformin in diabetic patients by reducing OCT1-mediated intestinal absorption and decreasing the systemic concentrations of the drug (23). Mutations in transporters, which only reduce accumulation of metformin in its target organ, also affect the efficacy of the drug. A study conducted by Chen, et al. shown that mutations in OCT3, the predominant transporter expressed in skeletal muscles, impaired the uptake and accumulation of metformin in muscle tissues, and led to a reduced anti-diabetic efficacy as metformin-mediated increase in glucose uptake in muscle was affected (24). Similarly, patients carrying mutated MATE1 exhibited improved response to the glucose-lowering effect of metformin, as metformin concentration in liver was elevated due to hampered biliary secretion (25).

1.3 From Leading Anti-diabetic Agent to Potential Anticancer Drug: the Beneficial Effects of Metformin against Breast Cancer in Clinical Studies

Epidemiological studies have shown that patients with type 2 diabetes exhibited an increased risk of developing breast cancer compared to the non-diabetic population (26-28). These clinical observations led to further retrospective analyses on the potential impact of anti-glycemic agents on the incidence of breast cancer. A majority of retrospective studies have shown that diabetic patients on metformin treatment had a significantly lower risk of developing breast cancer compared to those on other therapeutic agents such as insulin and sulfonylurea, although some studies only observed a trend (**Table 1.1**) (29-37). This beneficial effect of metformin against breast cancer occurrence aroused further interest in evaluating the possibility of using metformin as an anticancer agent. In a retrospective study by He, et al., it was concluded that metformin improved the breast cancer-specific survival rate of diabetic patients compared to other anti-glycemic drugs (38). Not only is metformin implicated in cancer prevention, but evidence is mounting for the inhibitory effects of metformin against cancer cell proliferation and tumor growth. In a study of breast cancer patients on neoadjuvant chemotherapy, diabetic patients on metformin therapy showed a significantly improved response to chemotherapy (reflected by higher rates of pathologic complete response) compared to diabetic patients on other anti-glycemic drugs (39). Additionally, treatment of presurgery diabetic breast cancer patients with metformin significantly reduced the proportion of proliferating cells (identified by immunohistochemical (IHC) staining of the proliferation biomarker, Ki-67) in tumor tissues (40).

In addition to breast cancer, metformin has also been reported to exert beneficial effects against lung cancer (41), prostate cancer (42), pancreatic cancer (43), ovarian cancer (4), and colon cancer (45). Also, non-diabetic patients who received short-time metformin treatment showed an improved response to chemotherapy (40, 46, 47) compared to non-metformin users,

although the anticancer efficacy of metformin in non-diabetic cancer patients was not as significant as its efficacy in diabetic patients with cancer.

Since breast cancer is the second leading cause of cancer death among women in the U.S (48), and there is currently no efficacious therapeutic agent for some subtypes of breast cancer (e.g. triple-negative breast cancer), repurposing metformin (a highly cost-effective drug with limited toxicity) for breast cancer therapy would be a significant improvement on the current treatment of this disease. However, metformin has not been used in the clinic for the treatment of breast cancer as some clinical trials report that the drug failed to show significant anticancer efficacy (49, 50). Therefore, there have been an increasing number of clinical studies being conducted on the anticancer efficacy of metformin (**Table 1.2**). At the same time, preclinical studies are also being conducted to identify the anticancer mechanisms of metformin to provide insights into its optimization for future clinical trials.

1.4 Molecular Mechanisms of the Anticancer Effects of Metformin

1.4.1 Reduction of Insulin and Insulin-like Growth Factor (IGF)1

Since metformin has been widely used for the treatment of diabetes, the role of its anti-diabetic effects in its anticancer pharmacology should be evaluated. Studies have shown that the proliferation of cancer cells can be stimulated under hyperglycemic culture conditions *versus* normal glucose levels (51). However, it is likely that the anti-glycemic effect is not the primary contributor to the *in vivo* anticancer effects of metformin, as other anti-glycemic agents such as insulin and sulfonylurea have been reported to increase breast cancer incidence (30-32). The difference in the anti-diabetic pharmacology between metformin and insulin/sulfonylurea is that metformin reduces circulating insulin and IGF1 (secretion suppressed by the reduced insulin level) levels, whereas long-term treatment with insulin/sulfonylurea causes hyperinsulinemia (52). Studies have shown that long-term exposure to high insulin and IGF1 levels increased the risk of breast cancer in diabetic patients (53, 54), suggesting that metformin exerts its anticancer effects in diabetic patients through reduction of insulin and IGF1.

Studies have been conducted to illustrate the role of insulin and IGF1 in breast cancer development (**Figure 1.2**). Both insulin and IGF1 activate the insulin pathway through binding to their receptors, namely insulin receptor (IR) and IGF1 receptor (IGF1R), on cancer cell membranes. Activated IR and IGF1R phosphorylate insulin receptor substrate (IRS)-1 and induce a conformational change in IRS-1, which enables downstream molecules to bind to it (55). Phosphoinositide 3-kinase (PI3K) is one of the major downstream targets of IRS-1. Activation of PI3K and its downstream protein kinase B (Akt) induces protein synthesis, enhances glucose uptake, and inhibits cell apoptosis (56). Compared to somatic cells, cancer cells have a hyperfunctional insulin pathway, which is the result of unregulated expression of IR and IGF1R or mutation of PI3K (57, 58). Several inhibitors of the insulin pathway have been approved for cancer therapy. Clinical studies have shown that co-administration of a PI3K inhibitor and trastuzumab significantly improved progression-free survival of breast cancer patients compared to trastuzumab monotherapy (hazard ratio: 0.78, $p<0.01$) (59), which implies that the insulin pathway plays a critical role in breast cancer development. Besides indirect attenuation of the insulin pathway through modulation of insulin and IGF1 levels, metformin was reported to directly reduce the expression of IR and IGF1R (60). The suppression of the insulin-dependent pathway by metformin was observed only in diabetic patients, which could be the likely cause of a poor response of non-diabetic breast cancer patients to metformin treatment compared to breast cancer patients with type 2 diabetes, as observed in clinical studies.

1.4.2 Activation of the AMPK Pathway

Besides the modulation of circulating insulin and IGF1 levels, attempts have been made to identify the intracellular targets of metformin in breast tumor tissues. As mentioned before, metformin exerts its anti-diabetic effects through suppressing hepatic gluconeogenesis and enhancing glucose uptake in skeletal muscles. Both processes are mediated by activation of intracellular AMPK. Therefore, it is possible that AMPK is the target of metformin anticancer effects if AMPK can modulate the proliferation of cancer cells.

One of the primary roles of AMPK in breast cancer cells is the regulation of energy homeostasis. In somatic cells, energy is produced by two steps: 1) glycolysis in the cytosol to generate pyruvate, and 2) oxidation of pyruvate in mitochondria via Krebs Cycle. In cancer cells, however, pyruvate gets further oxidized into lactate instead of entering into the mitochondria (the Warburg effect) (61). The Warburg effect is critical to cancer cell proliferation as it allows the cancer cells to survive without the need of oxygen, since the microenvironment in tumor tissues is hypoxic. Besides, the Warburg effect not only produces energy but also provides intermediate products that are required for protein and lipid synthesis (61). AMPK is the central regulator of the Warburg effect. Activation of AMPK, on one hand, inhibits multiple enzymes that are involved in Warburg effect, such as phosphofructokinase-1 (62). On the other hand, AMPK activation inhibits the mammalian target of rapamycin (mTOR) and attenuates the phosphorylation of P70S6 kinase (a downstream molecule of mTOR). Suppression of the mTOR pathway attenuates lipid and protein synthesis, inhibits cell proliferation, and induces apoptosis (63).

Studies have shown that metformin can activate AMPK in cancer cells (**Figure 1.1**). After being taken up into cancer cells, metformin blocks Mitochondrial Complex I in the electron transport chain and suppresses ATP synthesis. The elevated AMP/ATP ratio induces a conformational change of AMPK and exposes the site (Thr172) for phosphorylation by liver kinase B1 (LKB-1) (64). The critical role of LKB-1 and AMPK phosphorylation was confirmed in *in vitro* studies by Dowling *et al*., in which the LKB-1 deficient MDA-MB-231 human breast cancer cell line showed a limited response to the anti-proliferative effects of metformin (65).

Studies have implied that the effects of metformin on the AMPK pathway and the insulin pathway are not independent of each other. MCF-7 breast cancer cells cultured in low insulin/IGF1 media exhibited greater metformin-induced activation of the AMPK pathway compared to cells cultured in media with high insulin/IGF concentrations (66). This suggests that inhibition of the insulin pathway may sensitize the AMPK pathway to metformin (**Figure 1.1**).

1.4.3 Regulation of the Cell Cycle

Besides the molecular targets involved in the anti-diabetic effects of metformin, the contributions of other signaling pathways, such as the cell cycle, to the antiproliferative effects of metformin have also been evaluated. Cell proliferation is regulated by the cell cycle regulatory pathway which maintains genomic stability by preventing cells with damaged DNA from proliferating. DNA damage in the cells induces the phosphorylation of Ataxia Telangiectasia Mutated (ATM), which subsequently stabilizes and activates its major downstream modulator, p53 (67). Activation of p53 leads to cell cycle arrest at the G1/S phase or G2/M phase via induction of p21 synthesis, which inhibits a group of cell proliferation initiators including cyclin-dependent kinase 2 (cdc2) (68). Mutations in p53 and ATM are the most frequently observed mutations in tumor tissues, which highlights the critical role of cell cycle regulation in cancer development.

There are some reports to suggest that metformin inhibits cell proliferation through modulation of the cell cycle checkpoint genes (69-72) (**Figure 1.2**). For example, metformin treatment resulted in an increase in the proportion of breast cancer cells that were arrested in the G1 phase (69). Metformin is believed to regulate the cell cycle by enhancing the synthesis of p21 (69). Although some studies also showed that metformin can induce phosphorylation of the two upstream molecules of p21, namely ATM and p53 (70, 71), a functional p53 is not required for metformin-mediated regulation of the cell cycle in cancer cells. Interestingly, studies have shown that metformin exerts better efficacy against p53-deficient tumors rather than p53-competent tumors (72). This suggests that activation of p21 by metformin is likely to be regulated by other signaling pathways such as the AMPK-dependent pathway (71).

1.4.4 Effect on Cancer Stem Cells (CSCs)

The effect of metformin on cancer relapse and metastasis has also been investigated. According to some reports, the preventive effect of metformin against breast cancer reoccurrence and metastasis may be rooted in its antiproliferative efficacy against CSCs. Breast

tumors are believed to be generated from a group of cells named CSCs due to cellular properties that are similar to embryonic stem cells. Several studies showed that CSCs originate primarily from somatic epithelial cells through epithelial-mesenchymal transition (EMT) (73), a process originally discovered in the early development stage by which epithelial cells lose their cell-cell adhesion, acquire the ability to migrate, and become mesenchymal stem cells. Compared to non-stem cancer cells (NSCCs), CSCs are more resistant to chemotherapeutic agents due to higher expression of efflux transporters (ATP-binding cassette transporters or ABC transporter), enhanced DNA damage response system, and increased aldehyde dehydrogenase (ALDH) activity (74, 75). Additionally, CSCs have a lower expression of cell-cell adhesion protein and are more tumorigenic compared to NSCCs (76). It is widely believed that these properties make CSCs major cause of cancer reoccurrence and metastasis.

It has been reported that CSCs are more sensitive to metformin treatment compared to NSCCs (77). Several mechanisms have been proposed to explain this observation (**Figure 1.3**). First, the intracellular concentration of metformin in CSCs is not affected by the upregulation of efflux transporters because metformin is not an ABC transporter substrate under physiological conditions. Second, the inhibitory effect of metformin on ATP synthesis has greater impact on the proliferation of CSCs *versus* NSCCs since CSCs heavily rely on mitochondrial ATP production compared to NSCCs (77). Third, metformin inhibits the generation of CSCs by blocking the EMT process. Metformin reduces the secretion of IGF1 and transforming growth factor (TGF)β, both of which are required to maintain the “stemness” of CSCs and initiate EMT from NSCCs (78, 79). Furthermore, metformin-induced activation of AMPK leads to the inhibition of mTOR. As mTOR is a regulator of E-cadherin expression, its inhibition by metformin increases E-cadherin expression, improves cell-cell adhesion, and halts the EMT process (80).

1.4.5 Other Mechanisms

Recently, new targets of the anticancer effects of metformin have been identified. Several studies have shown that metformin-mediated inhibition of mTOR reduces the

expression of human epidermal growth factor receptor (HER)2 (81) which is critical for the growth of HER+ breast cancer cells. The study conducted by Marini et al. revealed that metformin altered glucose metabolism in breast cancer cells by impairing hexokinase activity through steadily binding to its catalytic pocket (82, 83). These studies imply that, unlike other widely used chemotherapeutic agents, metformin inhibits tumor growth by modulating multiple signaling pathways simultaneously.

1.5 The Role of Transporters in the Efficacy of Metformin as An Anticancer Agent

Although multiple molecular mechanisms/targets have been proposed from preclinical studies to explain the anticancer effects of metformin, their clinical relevance remains unclear. Based on the need for intracellular uptake of metformin for activation of these targets, the molecular targets can be categorized into two types: 1) extracellular targets (e.g. insulin and IGF1) and 2) intracellular targets (e.g. AMPK and p21).

While the activation of extracellular targets of metformin depends on the expression of their receptors on the surface of cancer cells, the activation of intracellular targets is determined by the intracellular concentration of metformin. As mentioned above, the intracellular uptake of metformin is mediated by cation-selective transporters. Previous studies investigating the anti-diabetic effects of metformin showed that mutations in OCT3, the predominant metformin transporter in the skeletal muscle, led to impaired activation of AMPK and reduced anti-diabetic efficacy of metformin (24). Therefore, the expression profiles of cation-selective transporters in tumor tissues should be extensively investigated, as they determine the extent of metformin uptake into tumors and activation of intracellular targets. However, only a few studies have implicated interactions between transporters and the antitumor efficacy of metformin. Patel et al. (84) showed that siRNA-mediated attenuation of OCT3 expression in human head and neck squamous cell carcinoma cells inhibited the attenuation of P70S6K phosphorylation induced by metformin. Despite these preliminary studies, there is little direct evidence that confirms cation-selective transporter expression in human breast tumor tissues.

1.6 Rationale for the Dissertation Project

The overall goal of this dissertation research is to optimize current treatment of metformin against breast cancer. Based on existing knowledge on metformin PK and its molecular mechanisms in cancer therapy, the dissertation research will commence with identifying the molecular targets of metformin that determine its clinical antitumor efficacy, so that selection criteria can be provided for identifying patients who have functional metformin targets and are most suitable for metformin cancer therapy. To evaluate the contributions of extracellular and intracellular targets to the inhibitory efficacy of metformin in breast tumors, cation-selective transporter expression in human breast tumor tissues and breast cancer cell lines will be assessed. The roles of transporter expression and transporter-mediated uptake of metformin in the antitumor efficacy of metformin will be evaluated by comparing metformin efficacy against transporter-overexpressing tumors *versus* tumors with limited transporter expression. In addition, the impact of extracellular insulin and IGF1 levels on the antitumor efficacy of metformin will be evaluated. Based on these results, a therapeutic regimen of metformin, specifically for the treatment of breast cancer, will be developed through optimizing metformin dose and designing a new combination therapy. Therefore, the following **overarching hypotheses** have been proposed to achieve this goal:

- Metformin exerts its antiproliferative effects against both breast cancer stem cells and non-stem cancer cells.
- The anticancer efficacy of metformin is due to 1) transporter-mediated uptake and activation of the intracellular AMPK pathway, and 2) reduction of circulating insulin and IGF1 levels and subsequent attenuation of cell growth stimulus.

Specific Aim 1. Investigate the role of transporter-mediated tumor uptake of metformin and the modulation of intracellular targets (AMPK Pathway) in its anticancer effects against CSCs and NSCCs.

1a. Evaluate the expression of cation-selective transporters in breast cancer cell lines and tumor tissues.

1b. Establish an OCT3-overexpressing MCF-7 (OCT3-MCF7) cell line and confirm increased OCT3 expression in this cell line.

1c. Compare metformin-induced activation of the AMPK-dependent pathway and antiproliferative efficacy between OCT3-MCF7 cells and MCF-7 cells.

1d. Evaluate the importance of transporters in metformin antitumor effects by comparing metformin antitumor efficacy between OCT3-MCF7 tumors and MCF-7 tumors.

1e. Investigate the role of transporters in metformin effects on MCF-7 CSCs.

Specific Aim 2. Optimize metformin doses for the treatment of breast cancer and correlate metformin efficacy with its systemic and intratumoral exposure.

2a. Generate xenograft mice bearing tumors generated from MCF-7 cells and MDA-MB-468 cells.

2b. Optimize metformin dose and establish a relationship between metformin dose-exposure and tumor volumes in mice bearing MCF-7 tumors and MDA-MB-468 tumors.

2c. Relate metformin dose-exposure-response to its effects on intracellular targets.

Specific Aim 3. Investigate the role of metformin-mediated reduction of insulin and IGF1 levels in the anticancer effects of the drug against CSCs and NSCCs.

3a. Generate IRS-1 knockdown MCF-7 (MCF-7$^{\text{IRS-1 KD}}$) cells.

3b. Evaluate the role of insulin and IGF1 in the anticancer effects of metformin using *in vitro* cell models.

3c. Evaluate the role of insulin and IGF1 in the anticancer effects of metformin using food-induced diabetic xenograft mouse models.

Results from the three specific aims will be presented in the following chapters:

Chapter 2. Aim 1a

Chapter 3. Aim 1e

Chapter 4. Aim 1b-d

Chapter 5. Aim 2a-c

Chapter 6. Aim 3a-c

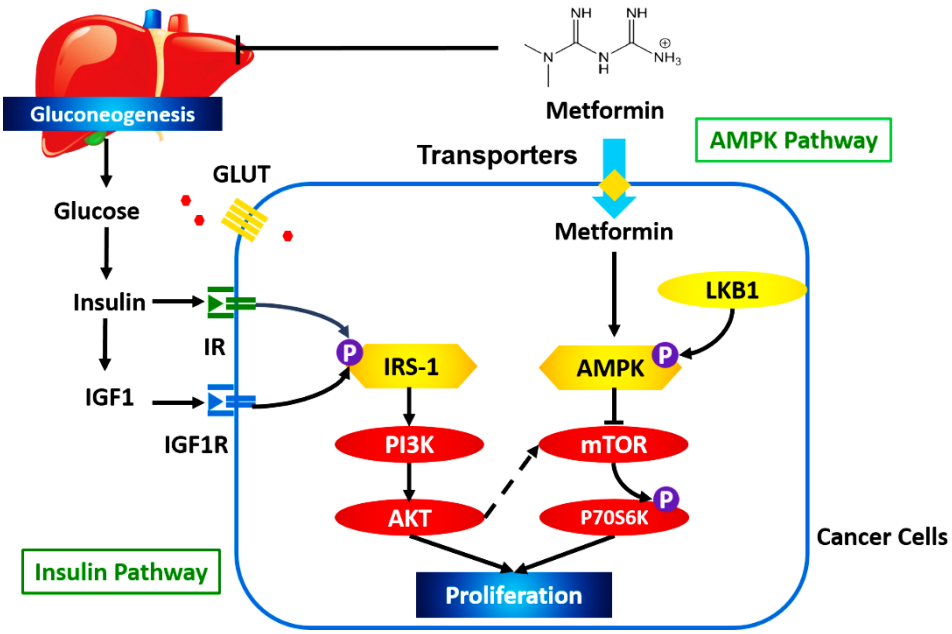

Figure 1.1 Interactions between AMPK Pathway and Insulin Pathway.

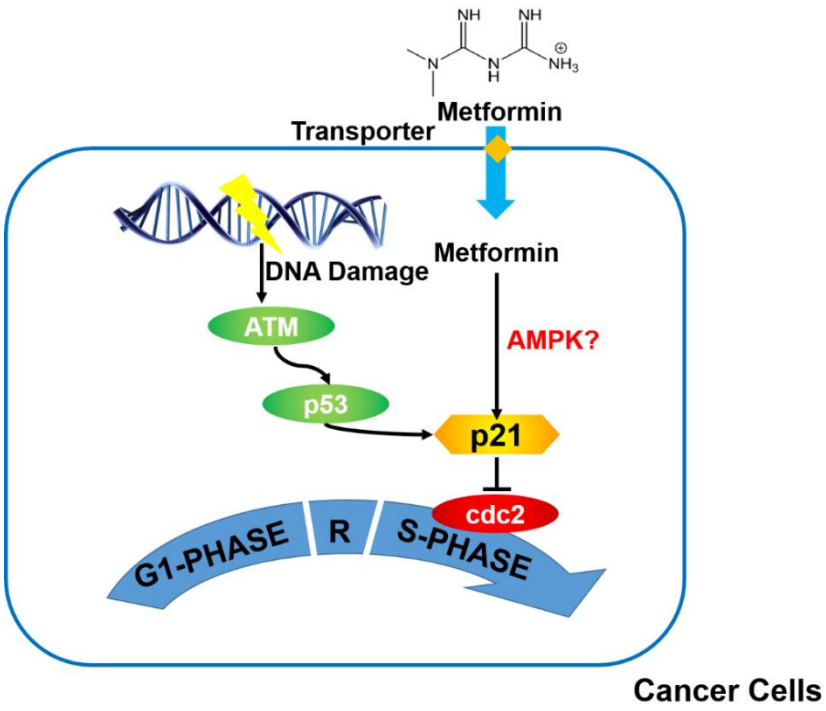

Figure 1.2 The Effect of Metformin on Cell Cycle Check Point Genes

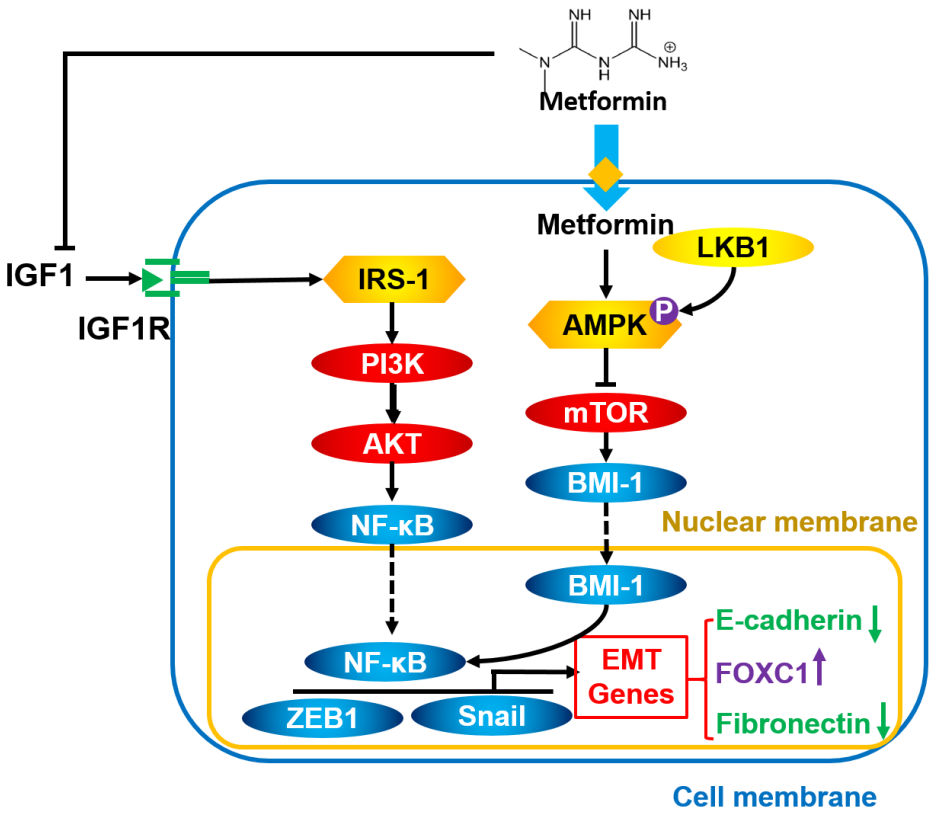

Figure 1.3 The Effect of Metformin on the Generation of CSCs

Year	Study Size	Comparator	Study Population	Hazard Ratio	Ref.
2009	62,809	Sulfonylurea and insulin	Diabetic patients in United Kingdom	0.66-1.21	(29)
2009	8,170	Other anti-diabetic drugs	Diabetic patients in United Kingdom	0.53-0.75	(30)
2010	22,621	Other anti-diabetic drugs	Female diabetic patients in United Kingdom	0.24-0.82	(31)
2011	4,323	Other anti-diabetic drugs	Female diabetic patients in Denmark	0.61-0.99	(32)
2011	85,289	Sulfonylurea derivatives	Diabetic patients in Netherlands	0.91-0.98	(33)
2012	68,019	Other anti-diabetic drugs	Postmenopausal women in United States	0.57-0.99	(34)
2013	1,547	Non-metformin use	Diabetic patients in Australia	0.39-1.00	(35)
2014	476,282	Non-metformin use	Diabetic patients in Taiwan	0.60–0.66	(36)
2015	4,216	Sulfonylureas and insulin	Diabetic patients in United States	0.38-0.79	(37)

Table 1.1 Retrospective Studies on the Effect of Metformin against Breast Cancer Risk

Study Size	Comparator	Study Name (Study Phase)	Outcome	Metformin Dose (mg/day)
40	Before treatment	Pre-surgical trial of the combination of metformin and atorvastatin in newly diagnosed operable breast cancer (0)	Tumor progression	1500
150	Placebo	Phase II study of metformin for reduction of obesity-associated breast cancer risk (II)	Breast cancer risk	850
72	Placebo	A trial of standard chemotherapy with metformin (vs placebo) in women with metastatic breast cancer (II)	Progression-free survival	850
42	Placebo	Metformin for reduction of paclitaxel-related neuropathy in patients with breast cancer (II)	Change in neuropathy	500
46	Placebo	A study of Liposomal Doxorubicin+ Docetaxel + Trastuzumab + Metformin in operable and locally advanced HER2+ breast cancer (II)	Pathologic complete response	1000
96	Placebo or melatoninn	Neoadjuvant FDC with melatonin or metformin for locally advanced breast cancer (II)	Response rate	850
60	Placebo	Metformin Plus Neoadjuvant Chemotherapy in Breast Cancer (II)	Pathologic complete response	500

Table 1.2 Ongoing Clinical Studies Using Metformin for Breast Cancer Treatment. Data from ClinicalTrials.gov (http://clinicaltrials.gov).

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CHAPTER 2[1]

Variability in Cation-selective Transporter Expression in Human Breast Cancer Cell Lines and Breast Tumor Tissues Resulted in Variability in the Antiproliferative Efficacy of Metformin

2.1. OVERVIEW

The antidiabetic drug metformin exerts antineoplastic effects against breast cancer and other cancers. One mechanism by which metformin is believed to exert its anticancer effect involves activation of its intracellular target, adenosine monophosphate-activated protein kinase (AMPK), which is also implicated in the antidiabetic effect of metformin. It is proposed that in cancer cells, AMPK activation leads to inhibition of the mammalian target of rapamycin (mTOR) and the downstream P70S6K that regulates cell proliferation. Due to its hydrophilic and cationic nature, metformin requires cation-selective transporters to enter cells and activate AMPK. This study demonstrates that expression levels of cation-selective transporters correlate with the antiproliferative efficacy of metformin in breast cancer. Metformin uptake and antiproliferative activity were compared between a cation-selective transporter-deficient human breast cancer cell line, BT-20, and a BT-20 cell line that was engineered to overexpress organic cation transporter 3 (OCT3), a representative of cation-selective transporters and a predominant transporter in human breast tumors. Metformin uptake was minimal in BT-20 cells, but increased by >13-fold in OCT3-BT20 cells, and its antiproliferative potency was >4-fold in OCT3-BT20 *versus* BT-20 cells. This increase in antiproliferative activity was associated with greater AMPK phosphorylation and decreased P70S6K phosphorylation in OCT3-BT20

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cells. Collectively, these findings establish a clear relationship between cation-selective transporter expression, the AMPK-mTOR-P70S6K signaling cascade, and the antiproliferative activity of metformin in breast cancer.

2.2. INTRODUCTION

Breast cancer is the second most common cancer and cause of cancer death among women in the United States. The American Cancer Society estimates approximately 231,840 new cases of invasive breast cancer and 40,730 breast cancer deaths in 2016 (1). Epidemiological studies suggest that women with type 2 diabetes are at a greater risk of developing breast cancer. A meta-analysis study showed that type 2 diabetes is associated with 23% increased risk of breast cancer, especially in post-menopausal women (2-5). Evidence indicates that the frontline antidiabetic drug for type 2 diabetes, namely metformin, acts as an anticancer agent in several cancers, including breast cancer (6-11). Studies have also shown that diabetic women on long-term metformin treatment have a lower risk of breast cancer compared to those not on metformin therapy, and that diabetic breast cancer patients on metformin have a lower risk of distant metastases compared to those not receiving metformin (12, 13). Pre-operative metformin treatment of non-diabetic women with operable invasive breast cancer results in down-regulation of Ki-67, a biomarker of cell proliferation and a predictive marker for clinical or pathological response to neoadjuvant therapy (11). Retrospective analyses showed higher pathological complete response rates (24%) in diabetic patients on metformin undergoing neoadjuvant chemotherapy for breast cancer *versus* diabetic patients not on metformin (8%) or non-diabetic patients (16%) (14). An association was observed between survival in diabetic cancer patients and metformin therapy, but not between survival and sulfonylurea or insulin therapy (15). Preclinical studies in xenograft mouse models of breast, prostate and lung cancer showed that metformin and the chemotherapeutic agent doxorubicin (DOX) were more effective in blocking tumor growth and preventing relapse than DOX alone (6). *In vitro* studies also showed that metformin inhibited

growth and colony formation of breast cancer cells, and induced cell cycle arrest and apoptosis (10).

As an anticancer agent, metformin is thought to exert its antiproliferative effects via an extracellular indirect pathway (insulin-dependent) and an intracellular direct pathway (insulin-independent). Insulin can bind to the insulin receptor that is highly expressed in cancer cells, and induce cell proliferation. It is suggested that metformin, by lowering circulating insulin levels, can induce anticancer effects by intercepting insulin-dependent tumor growth (16, 17). The direct antiproliferative effects of metformin in cancer cells are thought to be mediated via activation of intracellular adenosine monophosphate-activated protein kinase (AMPK), which leads to down regulation of the mammalian target of rapamycin (mTOR) and its downstream target, p70S6K (18, 19). In hepatocytes, AMPK and its upstream regulator liver kinase B1 (LKB1) (20) are key mediators in the glucose-lowering effect of metformin. Metformin activates AMPK via LKB1, leading to inhibition of liver gluconeogenesis (21) and lowering of circulating glucose and insulin levels (22). Hence, AMPK appears to be a common intracellular target both for the antidiabetic and anticancer effects of metformin.

Metformin is hydrophilic (logD of -6.13 at pH 6.0) and charged at all physiological pH values (pKa 12.4) (23). Therefore, it cannot enter cells via passive diffusion across the cell membrane (24), and relies on cation-selective transporters to enter the cell where it can activate its intracellular target, AMPK. Transport proteins such as organic cation transporters (OCT 1-3) (*SLC22A1-3*), plasma monoamine transporter (PMAT) (*SLC29A4*), and multidrug and toxin extrusion proteins (MATE1 and 2) (*SLC47A1* and *SLC47A2*) facilitate metformin trafficking in different organs and tissues (25, 26), such as the intestine, liver, and kidney, and thus drive the disposition of metformin. In the liver, metformin is taken up into hepatocytes predominantly via OCT1, and thus this transporter plays a critical role in the antidiabetic effect of metformin. We have recently reported that as many as four cation-selective transporters, namely OCT1, PMAT, serotonin reuptake transporter (SERT), and a high affinity choline transporter (CHT) contribute

to the intestinal uptake and absorption of metformin (27). These four transporters define the systemic exposure to orally administered metformin, and consequently, its pharmacologic behavior.

Emerging literature on the antiproliferative and anticancer efficacy of metformin in cancer cell lines and preclinical models of the disease either ignores the role of transporters or often suggests that a single transporter is responsible for metformin trafficking through tumor cells/tissues (28, 29, 30). Based on our studies on metformin transport in the intestinal tissue, we anticipate that multiple transporters, and interplay among them, may affect the uptake of metformin into tumor cells and tissues, and therefore influence its antiproliferative and antitumor efficacy. Hence, in any preclinical or clinical study in which the anticancer efficacy of metformin is evaluated, one must consider the expression of one or more metformin transporters in tumor cells for appropriate interpretation of the mechanisms underlying the antitumor effect of metformin.

In the present study, we have characterized the expression of cation-selective transporters in human breast cancer tissues and in nine commonly studied human breast cancer cell lines. The nine human breast cancer cell lines analyzed in this study were selected based on the two main subtypes of breast cancer, namely luminal and basal. Luminal breast cancer accounts for >70% of tumors that express estrogen receptors (ER) and/or progesterone receptors (PR), and low or no human epidermal growth factor receptors (EGFR) 1 and 2, the latter also known as HER2 (31). Basal-like breast cancers are generally triple negative as they lack ER, PR, and HER2, but express EGFR1 (32) and exhibit enhanced hypoxia and high tumor grade (33, 34). Further, we have compared transporter expression profiles between breast tissues and breast cancer cell lines, and evaluated an association between transporter expression levels and the antiproliferative efficacy of metformin in human breast cancer cells. The most definitive role of cation-selective transporters in the uptake and antiproliferative efficacy of metformin was obtained in this study by engineering an OCT3-overexpressing cell

line (i.e., OCT3-BT20) from the BT-20 cell line that does not express detectable levels of cation-selective transporter genes. Gene and protein expression of OCT3 in BT-20 and OCT3-BT20 cells are related to metformin uptake, its antiproliferative efficacy, and its modulation of the AMPK-mTOR signaling pathway. It is important to emphasize that OCT3 was chosen for overexpression simply as a representative of cation-selective transporters. Thus, this is the first comprehensive study in which the expression of cation-selective transporter genes and proteins has been characterized in several commonly used human breast cancer cell lines, and an unequivocal relationship has been established between cation transporter expression in human breast cancer cells and the antiproliferative efficacy of metformin.

2.3. MATERIALS AND METHODS

Materials. The human breast cancer cell lines analyzed in this study were obtained from the Tissue Culture Facility (TCF) at the University of North Carolina at Chapel Hill, and were authenticated by TCF through forensic Short Tandem Repeat Analysis techniques. Snap-frozen breast tissues were purchased from the UNC Tissue Procurement Facility with IRB exemption.

Cell Culture. Cells were cultured at 37°C, passaged at 90% confluency, and plated in 75-cm^2 T-flasks. For uptake studies, MDA-MB-231 and BT-20 cells were seeded on 24-well plates at a density of 37,500 cells/cm^2, and MCF-7 cells at 75,000 cells/cm^2.

Generation of OCT3-BT20 Cells. OCT3 from the pSPORT1 vector was cloned into a pcDNA 3.1(+) vector. BT-20 cells (1×10^6) were transfected with 2 µg of vector, and cultured in 6-well plates. Single OCT3-BT20 colonies were isolated in selection medium containing 200 µg/ml Geneticin®, and [14C]metformin (50 µM) uptake (at 5 min) was evaluated in the presence/absence of 50 µM famotidine (OCT3 and MATE1 inhibitor) or 500 µM quinidine (pan transporter inhibitor) to confirm functional activity of OCT3 in OCT3-BT20 cells

Determination of Transporter Gene Expression. Total RNA from cells/tissues was isolated and synthesized into cDNA. Transporter gene expression was determined by real-time

polymerase chain reaction (RT-PCR) using Taqman® assays, and normalized to endogenous 18s rRNA.

Determination of Transporter Protein Expression. Cells were lysed and protein content was measured with a bicinchoninic acid (BCA) protein assay kit. Proteins (20 µg) were subjected to gel electrophoresis, transferred to a nitrocellulose membrane and probed with a primary OCT1, OCT3, PMAT or MATE1 antibody and a secondary goat anti-rabbit IgG-horseradish peroxidase antibody. Protein bands were detected with SuperSignal® West Dura Extended Duration Substrate Kit and imaged. Membranes were stripped and analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

To determine metformin-mediated AMPK activation and P70S6K inhibition (assessed by their phosphorylation status), cells were incubated with culture medium in the presence/absence of 5 mM metformin for 2 days. Protein was extracted and subjected to Western blotting as described above using primary antibodies against p-AMPKα (Thr172) and p-P70S6K. Densitometry of protein bands from three Western blots was performed, and the percent change in p-AMPK and p-P70S6K between metformin-treated and control cells was calculated using the formula:

Cellular Uptake of Metformin. Uptake studies were conducted using methods previously reported with minor deviations (36). Cells were preincubated for 30 min in transport buffer (0.5 ml) which was replaced with transport buffer containing varying concentrations of [^{14}C]metformin or [^{14}C]metformin plus transporter inhibitors (500 µM quinidine, 200 µM MPP^+, or 50 µM famotidine). Metformin uptake was determined over 5 min (within linear uptake range). Cells were lysed with 500 µl of 1 M NaOH-0.1% SDS (3 h with shaking). [^{14}C]metformin in lysates was measured using liquid scintillation spectrometry. Protein content was determined by the BCA protein assay.

Cell Proliferation Assay. MDA-MB-231, BT-20, BT-549 and OCT3-BT20 cells were seeded in 96-well plates. After 24 hours, cells were incubated in medium containing metformin (1 µM to

100 mM) for 5 days and cell viability was assessed by the Alamar Blue® assay. To demonstrate that AMPK activation is required for the antiproliferative activity of metformin, cells were pretreated with 2 μM of the selective AMPK inhibitor, dorsomorphin (Compound C), followed by incubation with 10 mM metformin in the presence/absence of 2 μM Compound C for 48 hours. Cell viability was determined by the Alamar Blue®assay.

Statistical Analyses. All data are expressed as mean ± S.D. Statistical differences in transporter expression between human breast tumor tissues (N=15) and the corresponding adjacent non-malignant tissues (N=15) from the same subject were determined by Wilcoxon Signed-Rank Test. Statistical significance for difference in mean transporter expression in normal human breast tissues from mammoplasty surgeries (N=5) and in human breast tumor tissues (N=15) or non-malignant breast tissues adjacent to tumors (N=15) was determined by Mann-Whitney U test. Tukey's test was used to analyze data from chemical inhibition studies in OCT3-BT20 cells and BT-20 cells, and for percent change in the phosphorylation of AMPK and P70S6K. For chemical inhibition studies in MCF-7, MDA-MB-231, BT-20, OCT3-BT20, and BT-549 cells, an independent t-test was used to compare control and treated groups.

2.4. RESULTS

Gene and Protein Expression of Cation-selective Transporters in Human Breast Cancer Cell Lines. The four luminal human breast cancer cell lines (MCF-7, SK-BR-3, ZR-75-1 and BT-474) and two basal cell lines, BT-20 and MDA-MB-435S, expressed negligible levels of OCT1, OCT2, OCT3, PMAT, MATE1 and MATE2 transporter genes, whereas three basal cell lines, MDA-MB-231, MDA-MB-468 and BT-549 had relatively higher levels of transporter gene expression, with MATE1 being the predominant transporter (**Figure 2.1A**). MATE1 was also the predominant transporter in MCF-7 and MDA-MB-435S cell lines, although its expression was relatively low compared to MDA-MB-231, MDA-MB-468 and BT-549 cells. OCT3, the second most highly expressed transporter gene in MDA-MB-231 cells, showed negligible expression in the other breast cancer cell lines. Expression of OCT1, OCT3, PMAT, and MATE1 transporter

proteins in BT-20, MCF-7, MDA-MB-231, BT-549, MDA-MB-468, and MDA-MB-435S cell lines was assessed by Western blot analyses. Corresponding to gene expression, OCT1, OCT3, PMAT and MATE1 proteins were detected in MDA-MB-468, MDA-MB-435S, and MDA-MB-231 cells (**Figure 2.1B**). No transporter proteins were detected in the BT-20 cell line which had negligible transporter gene expression (**Figure 2.1B**). Thus, transporter protein expression reflected transporter gene expression in the human breast cancer cell lines analyzed. The variability in metformin transporter expression profiles among these cell lines suggests that cells within breast cancer tissues are also likely to show heterogeneity in metformin transporter expression.

Gene Expression of Cation-selective Transporters in Human Breast Tissues. The expression of OCT1, OCT2, OCT3, PMAT, MATE1 and MATE2 genes was assessed by RT-PCR in human breast tumor tissues, their corresponding adjacent non-malignant tissues, and in normal breast tissues obtained from mammoplasty surgeries. OCT3 and PMAT were the predominant transporter genes expressed in all three tissue types (**Figure 2.2**), with lower expression of OCT1 and MATE1 genes, and negligible expression of the OCT2 gene. MATE2 gene expression was negligible in breast tumor tissues, and was low in normal breast tissues and in tissues adjacent to breast tumors. The expression of OCT1, OCT3, PMAT and MATE1 genes was down regulated in all 15 pairs of breast tumor tissues analyzed compared to the corresponding adjacent non-malignant tissues, although this decrease was not statistically significant. No comparison was made between OCT2 and MATE2 gene expression in breast tumor tissues and their corresponding adjacent non-malignant tissues as these transporters were below detectable levels in several tissues examined.

Metformin Uptake in Human Breast Cancer Cell Lines. [^{14}C]Metformin (50 µM) uptake was first assessed in the low transporter-expressing MCF-7 cell line, a widely used *in vitro* model for breast cancer. Uptake was inefficient, and was not inhibited by the pan cation-selective transporter inhibitor MPP^+ (200 µM). Metformin uptake in transporter-deficient BT-20 cells was

also low and comparable to its uptake in MCF-7 cells, and uptake was not inhibited by MPP^+ (**Figure 2.3A**). This result suggests that transporter-mediated metformin uptake in MCF-7 and BT-20 cells is negligible, and corresponds with low transporter expression levels in these cell lines. However, metformin uptake in MDA-MB-231 cells, which express OCT3 and MATE1, was ~12-fold higher compared to BT-20 cells (22.78 *vs.* 1.97 pmol/mg protein/min) (**Figure 2.3A**). Similarly, metformin uptake in BT-549 cells, which express high levels of MATE1, was ~14-fold higher than uptake in BT-20 cells (27.43 *vs*. 1.97 pmol/mg protein/min, respectively; $p<0.001$) (**Figure 2.3A**). Overexpression of OCT3 in BT-20 cells (OCT3-BT20) increased metformin uptake by >13-fold compared to wild-type BT-20 cells (108.38 *vs.* 8.09 pmol/mg protein/min; $p<0.001$) (**Figure 2.3B**). Treatment of OCT3-BT20 cells with the OCT3-selective inhibitor, famotidine (50 µM), and the pan cation-selective transporter inhibitor, quinidine (500 µM), decreased metformin uptake by 88% and 96%, respectively, confirming that almost all metformin uptake in these cells is mediated by OCT3. RT-PCR and Western blot analyses confirmed the overexpression of OCT3 gene and protein in OCT3-BT20 cells. There were no detectable levels of OCT3 mRNA in wild-type BT-20 cells ($p<0.001$) (**Figure 2.3C**).

Role of Cation-selective Transporters in the Antiproliferative Efficacy of Metformin in Human Breast Cancer Cell Lines. Cation-selective transporter-mediated increase in metformin uptake in OCT3-BT20 cells translated into greater potency of the antiproliferative effect of metformin in this cell line compared to BT-20 cells; the extracellular metformin concentrations required to inhibit 50% cell growth (IC_{50}) of OCT3-BT20 cells was 2.13 mM, and IC_{50} of BT-20 cells was 9.06 mM ($p<0.001$) (**Figure 2.4A**). Further, the transporter-competent BT-549 cells, which express high levels of MATE1, were also more sensitive to the antiproliferative activity of metformin compared to transporter-deficient BT-20 cells (IC_{50} 2.93 mM and IC_{50} 9.06 mM, respectively; $p<0.001$) (**Figure 2.4B**). These data establish a direct relationship between cation-selective transporter expression levels and metformin-mediated inhibition of cell growth in human breast cancer cell lines. Interestingly, despite high expression of OCT3 and MATE1 in

MDA-MB-231 cells and a corresponding ~12-fold higher metformin uptake compared to BT-20 cells, the IC_{50} for MDA-MB-231 was comparable to the IC_{50} for BT-20 cells (10.56 mM and 9.06 mM, respectively) (**Figure 2.4B**). This discrepancy between high metformin uptake and its poor antiproliferative efficacy in MDA-MB-231 cells appears to be due to the lack of a functional intracellular AMPK-mTOR-P70S6K signaling cascade that is required for the antiproliferative activity of metformin. It has been hypothesized that metformin suppresses mTOR/P70S6K signaling by activating AMPK in cancer cells (19); since MDA-MB-231 cells do not express LKB1(35) that is required for AMPK activation (36), metformin does not exert antiproliferative effects in MDA-MB-231 cells despite achieving high intracellular concentrations. To further demonstrate that AMPK activation is required for the antiproliferative activity of metformin, breast cancer cell lines with varying transporter expression profiles (i.e., BT-20, OCT3-BT20, MCF-7, BT-549 and MBA-MB-231 cells) were treated with metformin with or without the selective AMPK inhibitor, Compound C. Our results show that Compound C attenuates the antiproliferative activity of metformin in all the breast cancer cell lines tested (**Supplementary Figure 2.1**). As expected, Compound C did not have any effect on the antiproliferative activity of metformin in MDA-MB-231 cells, since the mechanism for AMPK activation is dysfunctional in this cell line.

Interplay of Cation-selective Transporter Expression and AMPK/P70S6K Modulation in BT-20, OCT3-BT20, and MDA-MB-231 Cell Lines. To further test the hypothesis that metformin transporters, as well as AMPK activation by metformin, are essential for its antiproliferative activity in breast cancer cell lines, we utilized a transporter-deficient cell line with a functional AMPK/P70S6K/mTOR signaling cascade (BT-20), an engineered BT-20 cell line that overexpresses a metformin transporter and has a functional AMPK/P70S6K/mTOR pathway (OCT3-BT20), and a cell line that expresses metformin transporters but lacks LKB1 that is required for AMPK activation by metformin (MDA-MB-231). Cells were treated with 5 mM metformin for 48 hr and the phosphorylation status of the two intracellular signaling targets of

metformin, namely AMPK and P70S6K, was evaluated. Additional breast cancer cell lines with varying transporter expression profiles (i.e., low transporter-expressing MCF-7 cells and transporter-competent MDA-MB-468, MDA-MB-435S, and BT-549 cells) were also assayed for comparison.

The increase in AMPK phosphorylation within transporter-deficient BT-20 cells in response to metformin treatment was low, as was the corresponding decrease in P70S6K phosphorylation. In contrast, a noticeably higher AMPK phosphorylation and decreased P70S6K phosphorylation was observed in OCT3-BT20 cells following metformin treatment (**Figure 2.5B**). Metformin treatment had no effect on the phosphorylation status of AMPK and P70S6K in the MDA-MB-231 cell line despite high transporter expression and high metformin cellular uptake, presumably due to a defective AMPK pathway (**Figure 2.5A**). As expected, the transporter-competent MDA-MB-468, MDA-MB-435S, and BT-549 cells showed a metformin-mediated increase in AMPK phosphorylation and decrease in P70S6K phosphorylation (**Figure 2.5A**). The MCF-7 cell line, despite relatively low expression of cation-selective transporters, also showed an increase in AMPK phosphorylation following metformin treatment (**Figure 2.5B**), which could suggest a higher sensitivity of this cell line to metformin as reported in the literature (37), or the presence of an unidentified metformin transporter. Notably, the decrease in P70S6K phosphorylation in MCF-7 cells was relatively small and did not correspond with the extent of AMPK activation (**Figure 2.5B**).

Western blot data on metformin-mediated changes in AMPK and P70S6K phosphorylation in MCF-7 cells (low transporter expression with a functional LKB1), MDA-MB-231 cells (transporter-competent without LKB1), BT-20 cells (transporter-deficient with a functional LKB1) and OCT3-BT20 cells (transporter-competent with a functional LKB1) were subjected to densitometry analyses and the mean p-AMPK and p-P70S6K protein band intensities (normalized to GAPDH) obtained from three Western blots are depicted in Figure 2.5C. In transporter-deficient BT-20 cells, metformin increased AMPK phosphorylation and decreased

P70S6K phosphorylation by about 10%, whereas metformin treatment of OCT3-BT20 cells resulted in ~87% increase in AMPK phosphorylation and ~17% decrease in P70S6K phosphorylation. In the low-transporter expressing MCF-7 cells, metformin increased AMPK phosphorylation by ~50%, with ~9% decrease in P70S6K phosphorylation (**Figure 2.5C**). Metformin had a minimal effect on AMPK and P70S6K phosphorylation (~15% and <1%, respectively) in MDA-MB-231 cells. Collectively, these data clearly establish an association between cation-selective transporter expression, modulation of AMPK/P70S6K phosphorylation, and the antiproliferative potency of metformin.

2.5. DISCUSSION

Initial observations on the unexpected anticancer efficacy of metformin, the leading antidiabetic drug, in diabetic patients with breast cancer (1, 2) have been subsequently supported by other clinical and preclinical studies on the antineoplastic effects of this drug (3-9, 11-15). It is well established that the hepatic and renal disposition of metformin is mediated by cation-selective transporters, and our own data implicate OCT1, PMAT, SERT and CHT in its intestinal absorption (38). Our studies also demonstrated that following metformin uptake into intestinal epithelial cells across the apical membrane, the drug is unable to egress across the basolateral cell membrane as this membrane lacks metformin transporters (38). These data provide strong evidence that metformin trafficking across cell membranes is transporter-dependent. Others have shown a decrease in the glucose-lowering effect of metformin in mOct1-knockout mice, implicating an important role for cation-selective transporters in the antidiabetic efficacy of metformin (39). The interaction of metformin with cation-selective transporters has also been implicated in tumor cells. In a study by Patel et al. (40), siRNA-mediated attenuation of OCT3 expression in human head and neck squamous cell carcinoma cells decreased the effect of metformin on the phosphorylation of P70S6K; however, the study did not investigate a relationship between OCT3 expression and the antiproliferative activity of metformin. In the present study, we have characterized the expression of cation-selective

transporter genes and proteins in human breast cancer tissues, and in human breast cancer cell lines that are commonly used in breast cancer research. We have then correlated transporter expression profiles with the cellular uptake and antiproliferative activity of metformin. We have also provided a mechanistic basis for this correlation by demonstrating that activation of intracellular AMPK and down regulation of P70S6K are associated with transporter-mediated uptake and antiproliferative activity of metformin.

Our results showed that OCT3 and PMAT are the predominant transporters expressed in breast tumor tissues (**Figure 2.2**). Notably, OCT3 gene expression in breast tumor tissues was 13,000-fold higher than its expression in normal breast tissues, suggesting that OCT3 could play an important role in the antitumor efficacy of metformin in breast cancer. Other transporter genes expressed in breast tumors are MATE1, MATE2, and OCT1. Although MATE1 and MATE2 have been reported to predominantly facilitate the egress of compounds from cells, these transporters are bidirectional and can facilitate the uptake of compounds under certain conditions. Our data showing metformin uptake in BT-549 cells, in which MATE1 is the predominant transporter (**Figure 2.1**), provide the first evidence that MATE1 acts as an uptake transporter rather than an efflux transporter in breast cancer cells.

To characterize the expression profiles of OCT1-3, PMAT and MATE1-2 in luminal and basal human breast cancer cell lines, four luminal breast cancer cell lines (MCF-7, SK-BR-3, ZR-75-1 and BT-474) and five basal cell lines (MDA-MB-231, MDA-MB-435S, MDA-MB-468, BT-20 and BT-549) were analyzed. Transporter expression varied among the nine breast cancer cell lines, with negligible or undetectable cation-selective transporter expression in MCF-7, SK-BR-3, ZR-75-1, BT-474 and BT-20 cells and multiple transporters expressed in MDA-MB-231, MDA-MB-435S, and MDA-MB-468 cells (**Figure 2.1**). Generally, there was good correspondence between transporter gene and protein expression (**Figure 2.1**).

The dependence of metformin uptake into human breast cancer cell lines on cation-selective transporters was demonstrated by a head-to-head comparison of metformin uptake in two cell

lines with the same origin (i.e., the BT-20 cell line), and hence the same genetic background, but with one cell line expressing a metformin transporter (OCT3-BT20 cell line) and the other cell line practically devoid of the transporter (BT-20 cell line) (**Figure 2.1**). Metformin uptake in OCT3-BT20 cells was >13-fold higher compared to uptake in BT-20 cells (**Figure 2.3B**). These data from two cells that differ only in the presence or absence of a cation-selective transporter provide the most direct evidence that metformin uptake in breast cancer cell lines is transporter-dependent.

An alternative experimental approach to overexpressing a transporter in a transporter-deficient cell line would be siRNA-mediated knockdown of a transporter in a transporter-competent cell line, and comparison of metformin uptake and its antiproliferative activity between the wild-type and transporter-knockdown cells. However, because metformin uptake into cells is mediated by multiple transporters, this approach would not have been as effective as the one employed in our study. The advantage of our strategy utilizing two cell lines with the same genetic background to demonstrate the critical role of transporters in the uptake and antiproliferative efficacy of metformin is evident from our data comparing these parameters between two cell lines with different genetic backgrounds, namely BT-20 (transporter-deficient) and MDA-MB-231 (transporter-competent) cells. As expected, metformin uptake in MDA-MB-231 cells was ~12-fold higher compared to uptake in BT-20 cells (**Figure 2.3A**), but its antiproliferative activity was similar in both these cell lines (**Figure 2.4B**). This discrepancy between metformin uptake and its antiproliferative activity in the MDA-MB-231 cells is due to an intrinsic deficiency in LKB1 (20), the kinase that is required for the phosphorylation of AMPK, resulting in a defective AMPK-P70S6K-mTOR pathway and subsequent inability of metformin to exert its antiproliferative effect in this cell line. The dependence of the antiproliferative activity of metformin on AMPK activation was confirmed by our results showing attenuation of the antiproliferative effects of metformin in BT-20, OCT3-BT20, BT-549, and MCF-7 cells in the presence of the AMPK inhibitor, Compound C, but not in MDA-MB-231 cells (**Supplementary**

Figure 2.1). Others have reported the involvement of cation-selective transporters, such as OCT1, in the antiproliferative activity of metformin in epithelial ovarian cancer cells, and the contribution of OCT3 to metformin-induced decrease in cell viability in head and neck squamous cell carcinoma cell lines (40, 42). However, our study represents the most comprehensive approach to evaluating the role of transporters in the antiproliferative activity of metformin in cancer cell lines.

Our results underscore the importance of cation-selective transporter expression as a criterion for selecting breast cancer cell lines to investigate the antiproliferative or antitumor activity of metformin. A review of the literature suggests that little or no attention has been paid to cation-selective transporter expression in selecting relevant *in vitro* (cellular) and *in vivo* (xenograft) models of breast cancer for such studies.

Clinical studies have shown that transporter polymorphisms alter the pharmacokinetics (PK) and glucose-lowering efficacy of metformin. Healthy volunteers with reduced function OCT1 alleles had a significantly higher area under the plasma concentration–time curve of glucose (41). It has also been reported that renal clearance and net secretion of metformin were significantly altered in individuals heterozygous for an OCT2 variant allele compared to individuals homozygous for the OCT2 reference allele, and altered metformin disposition and response were observed in patients carrying MATE1 and MATE2 promoter variants (43, 44). Additionally, a wide variability in hepatic expression of OCT1 (113-fold variation) and OCT3 (27-fold variability) in Caucasians has been previously reported (28). Taken together, these data suggest that genetic variants among cation-selective transporters impact the antidiabetic efficacy in metformin, and could explain the sub-therapeutic efficacy of this drug in 36% of diabetic patients on metformin therapy (40, 42, 45, 46). Therefore, it is likely that transporter variants could also affect the efficacy of metformin in breast cancer. Our *in vitro* data showing variability in cation-selective transporter expression profiles in human breast cancer cell lines reflects the degree of variability in transporter expression among cells comprising human

breast tumors, and suggest that treatment outcomes to metformin therapy in breast cancer could be highly variable. Thus, to predict the efficacy of metformin as an anticancer agent in breast cancer or other cancers, screening patients for transporter and LKB1 expression in neoplastic tissues may be necessary.

To demonstrate that low/no cation-selective transporter expression in breast tumors could significantly restrict the antineoplastic effects of metformin, we conducted an *in vivo* proof-of-concept study. Xenograft mouse models of breast cancer were developed by subcutaneous injections of BT-20 and OCT3-BT20 cells into athymic nude mice. However, due to the limited tumorigenicity of BT-20 cells and the inherent slow growth rate of BT-20 tumors (as also reported in the literature (47)) and of OCT3-BT20 tumors, only a small number of mice developed measurable tumors by the end of week 10 post injection. Hence, we used the highly tumorigenic, fast-growing and low transporter-expressing MCF-7 cells to generate an OCT3-overexpressing MCF-7 (OCT3-MCF7) cell line, and used these two cell types to develop xenograft mice bearing MCF-7 and OCT3-MCF7 tumors. A systematic study was conducted on xenograft mice bearing OCT3-MCF7/MCF-7 breast tumors and the correlation between transporter expression in tumor tissues and the response to the antitumor efficacy of metformin was evaluated. The results of this *in vivo* study will be presented in Chapter 4.

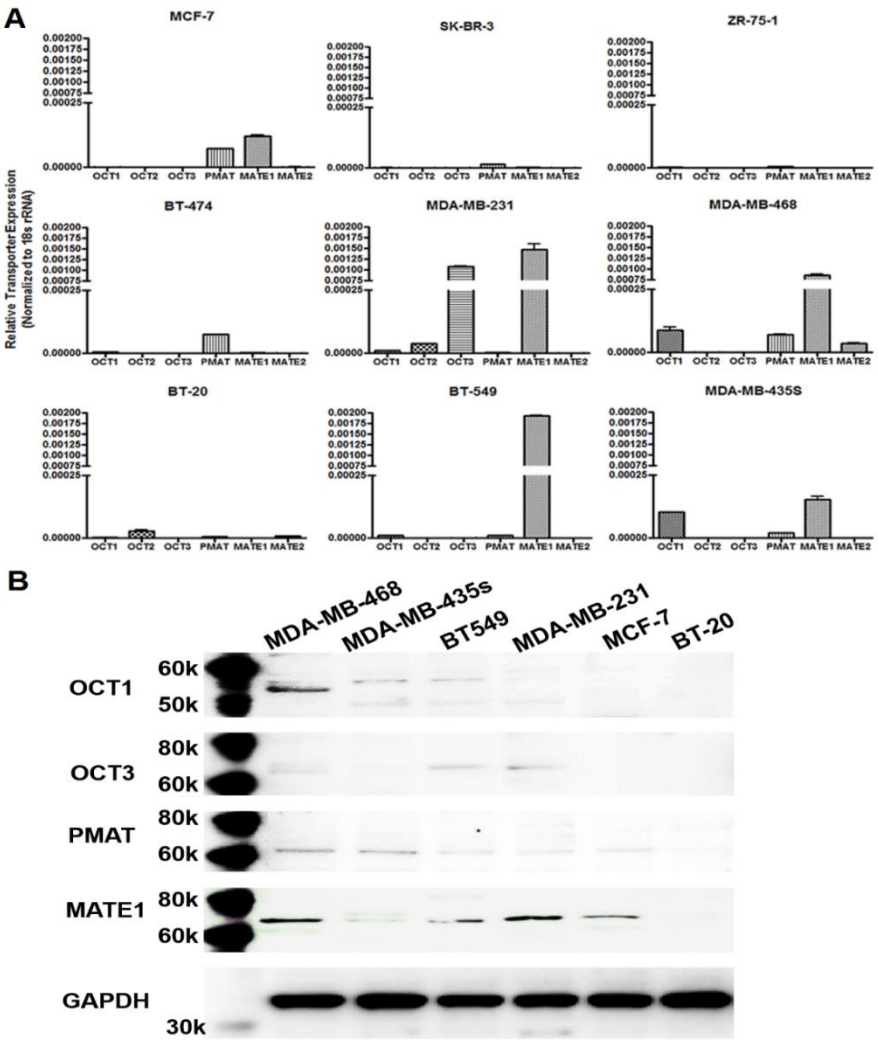


Figure 2.1 Expression of Cation-selective Transporters OCT1-3, PMAT and MATE1-2 in Human Breast Cancer Cell Lines. (**A**) Relative gene expression of known metformin transporters OCT1-3, PMAT and MATE1-2 in four luminal human breast cancer cell lines (MCF-7, SK-BR-3, ZR-75-1 and BT-474) and five basal human breast cancer cell lines (BT-20, MDA-MB-435S, MDA-MB-231, MDA-MB-468 and BT-549) was determined by RT-PCR and normalized to 18s rRNA. (**B**) Expression of OCT1, OCT3, PMAT, and MATE1 transporter proteins in MDA-MB-468, MDA-MB-435S, BT-549, MDA-MB-231, MCF-7, and BT-20 breast cancer cell lines was assessed by Western blot analyses, using primary antibodies specific for human OCT1, OCT3, PMAT and MATE1. GAPDH was used as a loading control.

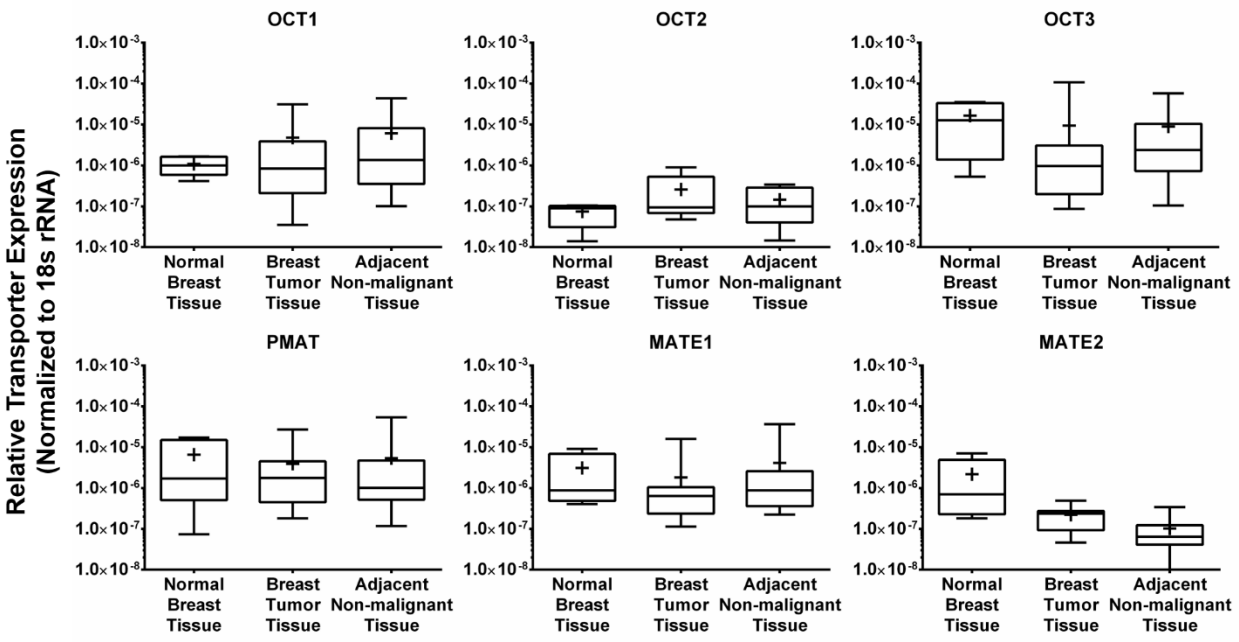


Figure 2.2 Expression of Cation-selective Transporter Genes in Normal Breast Tissues, Breast Tumor Tissues and Their Adjacent Non-malignant Tissues. The variability in relative gene expression of cation-selective transporters among breast tissues is represented as box and whisker plots. The top of each whisker represents the sample maximum, the bottom of the whisker is the sample minimum, the top and bottom of each box are the upper and lower quartiles, the horizontal line in each box represents the sample median, and the plus symbol in each box represents the sample mean.

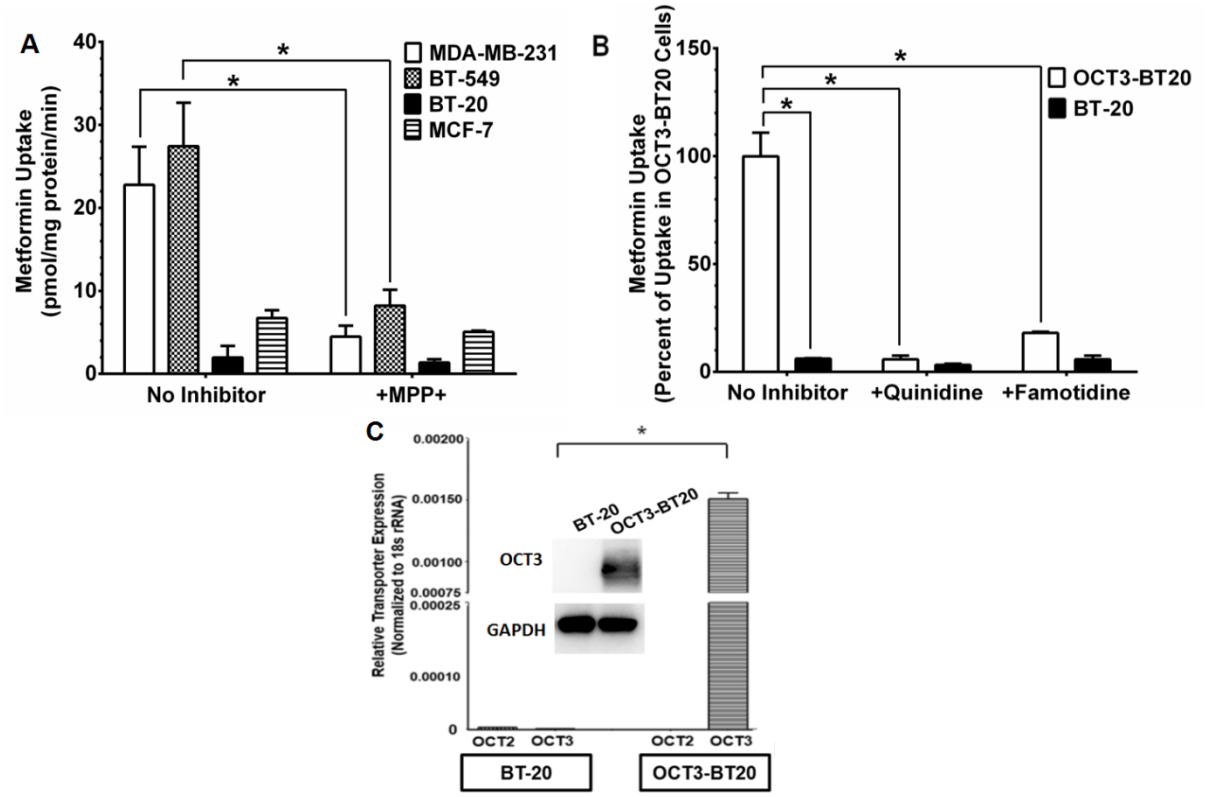


Figure 2.3 Metformin Uptake in Human Breast Cancer Cell Lines with High and Low Cation-selective Transporter Expression. Metformin uptake was assessed in the presence or absence of the pan cation-selective transporter inhibitors MPP^+ (200 µM) and quinidine (500 µM), or the OCT3 and MATE1 inhibitor famotidine (50 µM) in (**A**) low transporter-expressing MCF-7 cells, transporter-competent MDA-MB-231 and BT-549 cells, and transporter-deficient BT-20 cells, and (**B**) transporter-competent OCT3-BT20 and transporter-deficient BT-20 cells. Data represent mean ± SD; N=4. *$p<0.05$ for (A) and *$p<0.001$ for (B). (**C**) Expression of OCT2 and OCT3 genes in OCT3-BT20 and BT-20 cells was analyzed by RT-PCR and normalized to 18s rRNA. OCT3 protein expression in OCT3-BT20 and BT-20 cells, evaluated by Western blot analysis, is shown as an insert. Results are shown as the mean ± SD; N=3. *$p<0.001$.

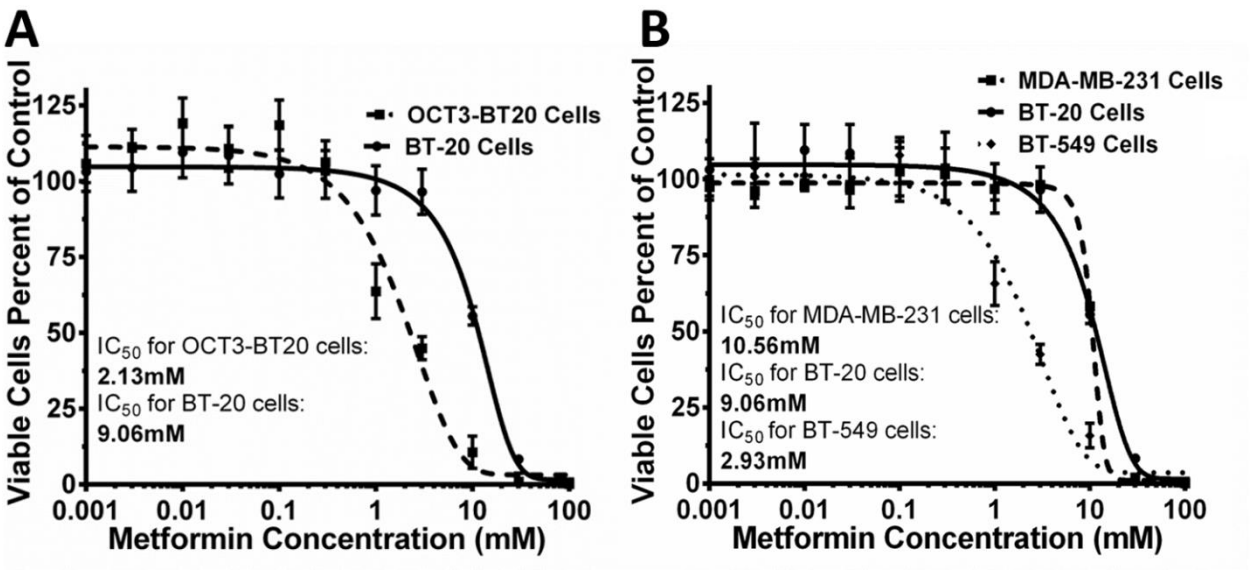


Figure 2.4 Antiproliferative Effects of Metformin in Human Breast Cancer Cell Lines with Varying Transporter Expression Profiles. (**A**) OCT3-BT20 and BT-20 cells and (**B**) MDA-MB-231, BT-20, and BT-549 cells were cultured in the presence of metformin for 5 days. The effect of metformin on cell growth was evaluated, and metformin concentrations that caused 50% growth inhibition (IC_{50}) were calculated. Results are shown as the mean ± SD; N=4.

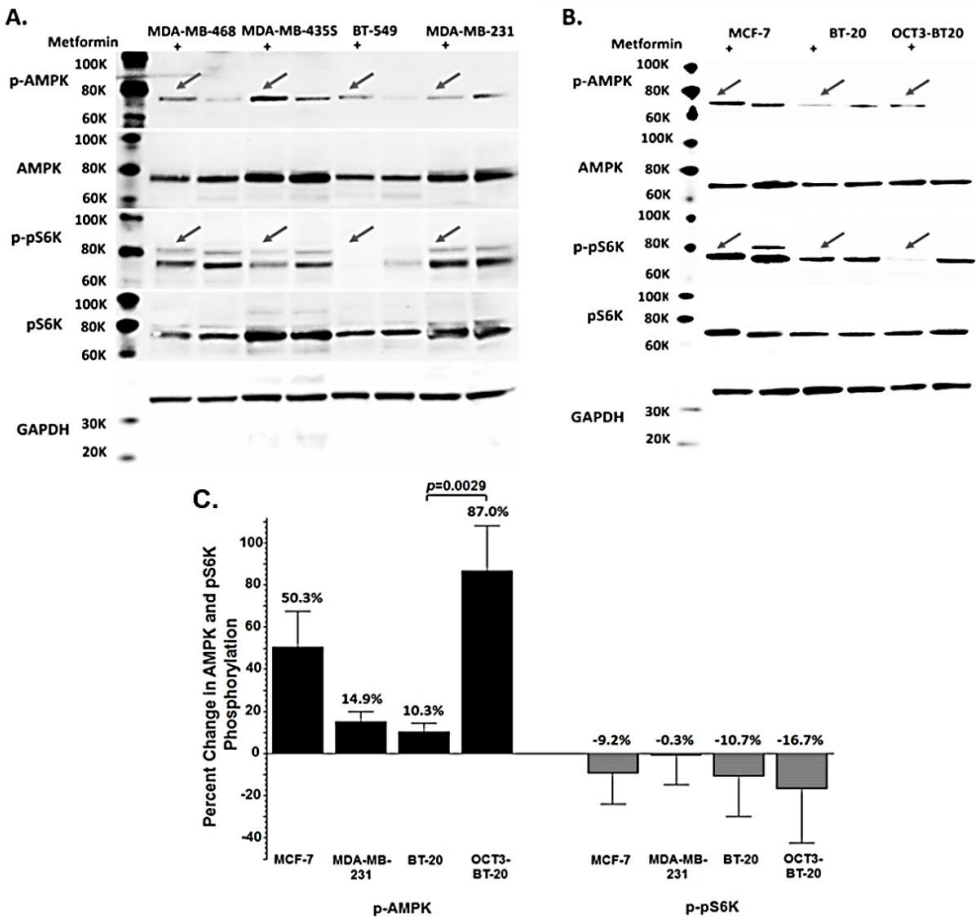
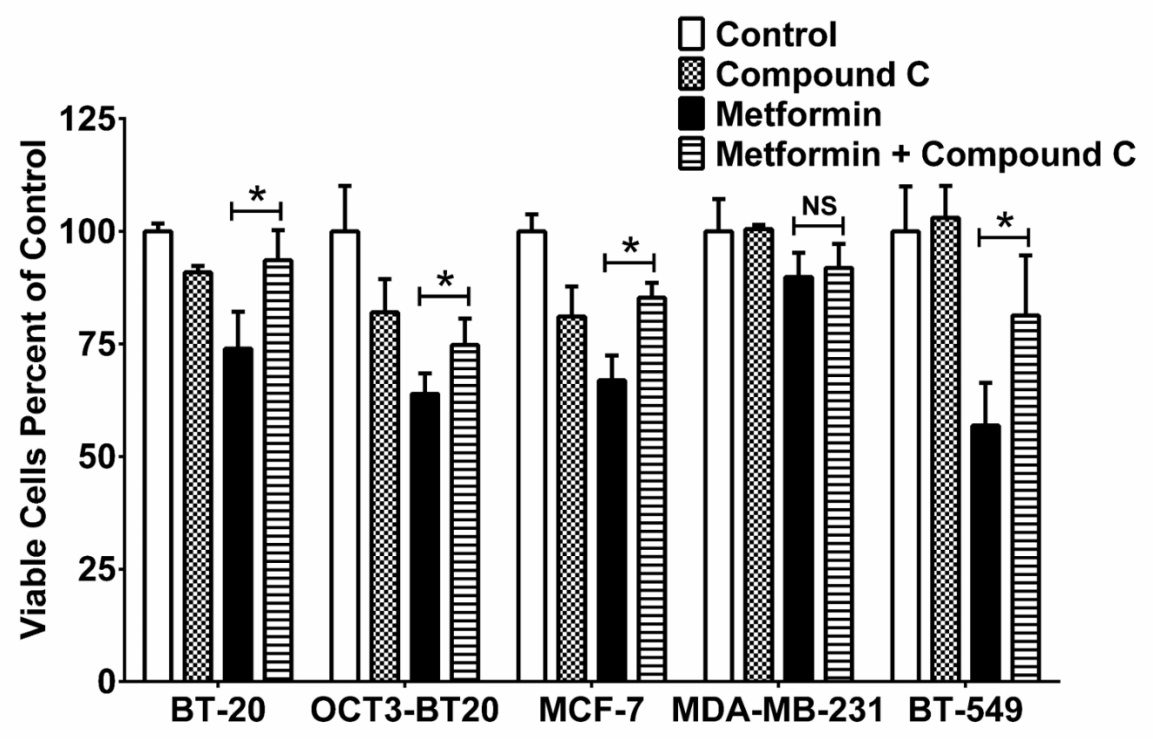


Figure 2.5 Contribution of Cation-selective Transporters to Metformin-mediated AMPK and P70S6K Phosphorylation in Human Breast Cancer Cell Lines. Representative Western blots show metformin-mediated modulation of AMPK and P70S6K phosphorylation in (**A**) high transporter-expressing cell lines (MDA-MB-468, MDA-MB-435S, BT-549, and MDA-MB-231), and (**B**) a low transporter-expressing cell line (MCF-7), a transporter-deficient cell line (BT-20) and a transporter-overexpressing cell line (OCT3-BT20). Metformin-induced changes (or lack thereof) in p-AMPK and p-P70S6K levels are shown with arrows. GAPDH was used as a loading control. (**C**) Graphical representation of the percent change in p-AMPK and p-P70S6K between metformin-treated and control cells using densitometry analyses of the protein bands normalized to GAPDH. Data represent the average percent change from three independent experiments.



Supplementary Figure 2.1 The Interaction of Cation-selective Transporters and the AMPK Signaling Cascade in the Antiproliferative Efficacy of Metformin. BT-20, OCT3-BT20, MDA-MB-231, MCF-7, and BT-549 cells were cultured in media containing 2 µM of the AMPK inhibitor, Compound C, 10 mM metformin, or 2 µM Compound C plus 10 mM metformin for 48 hours, and cell viability was evaluated. Results are shown as the mean ± SD; N=4. *p<0.05.

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CHAPTER 3

Cancer Stem Cells Are More Susceptible to Metformin Due to Enhanced Transporter-mediated Metformin Uptake

3.1 OVERVIEW

Cancer stem cells (CSCs) have been reported to initiate breast tumor development and cause chemoresistance and relapse. Previous studies suggest that CSCs are more susceptible to metformin, and that metformin selectively kills CSCs through modulation of its intracellular targets in CSCs. However, the greater sensitivity of CSCs to metformin, compared to non-stem cancer cells (NSCCs), has not been thoroughly investigated. Since metformin is highly hydrophilic and positively charged under physiological conditions, its uptake into cells must be mediated by cation-selective transporters. Thus we hypothesize that the enhanced sensitivity of CSCs *versus* NSCCs to metformin treatment is due to upregulation of cation-selective transporters in CSCs and a subsequent increase in transporter-mediated metformin uptake. To test this hypothesis, CSCs and NSCCs were isolated from the BT-549 human breast cancer cell line. CSCs from this cell line have >50% higher gene and protein expression of multidrug and toxin extrusion protein (MATE1), the predominant transporter in BT-549 cell line, compared to NSCCs. The higher MATE1 expression resulted in a 40% higher metformin uptake in BT-549 CSCs *versus* NSCCs. We hypothesize that greater expression of cation-selective transporters and consequent greater uptake of metformin in CSCs compared to NSCCs account for greater susceptibility of CSCs to metformin in the treatment of breast cancer.

3.2 INTRODUCTION

Human breast tumor cells are heterogeneous (1). The development of human breast tumors is believed to be initiated by a group of breast tumor cells with high CD44 expression

and low CD24 expression (CD44+/CD24-) on the cell surface (2), and are referred to as cancer stem cells (CSCs) because they exhibit many cellular properties of embryonic stem cells (ESCs). CSCs originate from either ESCs that have mutations in oncogenes or tumor suppressor genes or from somatic cells which are reprogrammed through epithelial-mesenchymal transition (EMT) (3, 4). Compared to non-stem cancer cells (NSCCs), CSCs are more resistant to most chemotherapeutic agents since they have a hyper-functional DNA repair system that enables them to withstand the DNA damage caused by chemotherapeutic agents (5). The efflux transporters (primarily p-glycoprotein and multidrug resistant associated proteins) that are highly expressed on the cell membrane of CSCs also contribute to drug resistance by pumping out chemotherapeutic agents that are mostly lipophilic and charged under physiological conditions (6, 7). Unlike NSCCs, CSCs lack or have reduced expression of cell-cell adhesion proteins, resulting in relatively weak connections with surrounding cells and enabling them to readily migrate to other organs (8). Since CSCs are highly proliferative, tumors can be grown from a small number of these cells (9). These specific cellular properties of CSCs make them the primary contributors to breast cancer chemoresistance, relapse, and metastasis (1, 5, 9).

Studies have shown that low concentrations of metformin can selectively kill CSCs and significantly reduce the proportion of CSCs in several breast cancer cell lines, with limited effects on NSCCs (10, 11). Adenosine monophosphate-activated protein kinase (AMPK) is one of the primary intracellular targets of metformin activity against CSCs (12). Metformin-induced phosphorylation of AMPK leads to the suppression of multiple downstream molecules that are involved in the EMT process, including twist family BHLH transcription factor 1 (TWIST1) and zinc finger e-box binding homeobox 1(ZEB1) (13). A recent study conducted by Gou et al. suggests that, compared to NSCCs, CSCs are more sensitive to metformin as a significantly lower concentration of metformin is required to activate AMPK in CSCs (14). In previous chapters, we demonstrated that metformin uptake in breast cancer cells is mediated by cation-

selective transporters. Therefore, we hypothesize that the higher expression of cation-selective transporters in breast CSCs and subsequent higher metformin uptake into these cells results in higher sensitivity of CSCs to metformin compared to NSCCs. To test this hypothesis, we have selected the BT-549 human breast cancer cell line, which contains both CSCs and NSCCs. These CSCs and NSCCs were isolated using fluorescence-activated cell sorting (FACS), and expression of cation-selective transporters as well as metformin uptake was compared in these cell types.

3.3 MATERIAL AND METHOD

Cell Culture. BT-549 cells were cultured in RPMI-1640 media (11875-093, Invitrogen) with 10% fetal bovine serum (FBS, 12003C, Sigma), penicillin-streptomycin (15140, Invitrogen) and human recombinant insulin (12585, Invitrogen). Cells were cultured in 175 cm^2 cell culture flasks (10-126-39, Corning) and passaged at 80% confluency.

Isolation of CSCs and NSCCs via FACS. The cell sorting buffer was prepared by adding 0.5% FBS to Hank's Balanced Salt Solution (14170112, Gibco). BT-549 cells were trypsinized at 90% confluency and washed once with the sorting buffer. The cells were then filtered through sterile pre-separation filters (30 µm, 130-041-407, Miltenyi Biotech) to remove cell aggregates. Approximately 1×10^7 filtered cells were resuspended and incubated in 1 mL sorting buffer containing 50 µL phycoerythrin-conjugated mouse anti-human CD24 antibody (PE-CD24) (311105, Biolegend) and 25 µL fluorescein isothiocyanate-conjugated mouse anti-human CD44 (FITC-CD44) antibody (338803, Biolegend) at 4ºC for 30 mins. After incubation, cells were washed three times with ice-cold sorting buffer and resuspended in the sorting buffer at a final density of 50,000/mL. AbC™ Total Antibody Compensation Beads (A10513, Life Technologies) incubated with either PE-CD24 or FITC-CD44 antibody were used as compensation controls. Flow cytometry sorting was conducted by the University of North Carolina Flow Cytometry Facility using the Becton Dickinson FACSAria II cell sorter (BioProtect). CD44+/CD24- cells (CSCs) and the remaining cells (NSCCs) were isolated and collected.

Determination of Transporter Gene Expression. BT-549 CSCs and NSCCs were washed with ice-cold phosphate-buffered saline (PBS) and lysed in QiAzol Lysis Reagent (79306, Qiagen). Total RNA was isolated from the lysate, purified using RNeasy® Plus Mini Kit (74134, Qiagen), and synthesized into cDNA with iScript™ cDNA Synthesis Kit (1708891, BioRad). Transporter gene expression was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) by Taqman® assays (4324018, Life Technologies), and normalized to endogenous 18s rRNA.

Evaluation of Metformin Uptake. Uptake studies were conducted using methods similar to those described in Chapter 2. The isolated BT-549 CSCs and NSCCs were seeded on 48-well plates at a density of 100,000 cells/well and cultured in media for two hours to allow the cells to adhere to the bottom of the wells. Cells were incubated for 30 min in transport buffer which was then replaced with transport buffer containing 50 µM [^{14}C]metformin or 50 µM [^{14}C]metformin plus 500 µM pan transporter inhibitor, quinidine. After 10-min incubation, the cells were washed three times with ice-cold transport buffer and lysed in 300 µL of 1 M NaOH-0.1% SDS solution. [^{14}C]Metformin in lysates was measured using liquid scintillation spectrometry (Packard). The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay.

Analyses of Transporter Protein Expression. CSCs and NSCCs from BT-549 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (89900, Thermo Fisher). Protein concentration of each sample was determined by the BCA assay. Protein samples (30 µg) were subjected to Western blot analyses as described in previous chapters. MATE1 expression was probed using a primary antibody against MATE1 (SC-133390, Santa Cruz). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as protein loading control. Densitometry analysis of Western blot bands was performed by Image Lab (BioRad).

Statistical Analyses. All data are presented as mean ± S.D. One-way analysis of variance followed by Tukey's test was performed to determine statistical differences in transporter gene

expression and metformin uptake. All statistical analyses were performed by GraphPad Prism (GraphPad Software).

3.4 RESULTS

Cation-selective Transporters Are Upregulated in CSCs. The expression of six cation-selective transporter genes (i.e. organic cation transporter (OCT)1-3, plasma membrane monoamine transporter (PMAT), multidrug and toxin extrusion protein (MATE)1,2) that are expressed in human breast tumors and breast cancer cell lines (15) (Chapter 2) was assessed in BT-549 CSCs and NSCCs. Among the six transporters studied, MATE1 was the predominant transporter in both cell types (**Figure 3.1**), with negligible levels of OCT1, OCT3, and PMAT gene expression (**Figure 3.1**), which were less than 1% of MATE1 expression (**Figure 3.1**). MATE1 gene expression in CSCs was 87% higher than the expression in NSCCs (1.66×10^{-5} *vs.* 8.99×10^{-6}, $p<0.05$), and this difference translated into similar differences in transporter protein expression. Densitometric analysis of the Western blot data revealed a 69% increase in MATE1 protein expression in CSCs *versus* NSCCs ($p<0.05$) (**Figure 3.2A** and **B**).

CSCs Exhibited Higher Transporter-mediated Metformin Uptake. To assess whether higher MATE1 expression in BT-549 CSCs would lead to higher metformin uptake, [^{14}C]metformin (50 μM) uptake (at 10 mins) into CSCs and NSCCs was measured and compared. Metformin uptake in CSCs was 40% higher compared to its uptake in NSCCs (10.52 *vs.* 7.36 pmol/mg protein/min, $p<0.05$). Coadministration of metformin and quinidine, a pan transporter inhibitor, reduced metformin uptake in both BT-549 CSCs and NSCCs to similar levels (4.48 *vs.* 2.70 pmol/mg protein/min, $p=0.11$), suggesting that the higher metformin uptake in BT-549 CSCs was due to upregulation of metformin transporter(s), mostly upregulation of MATE1 (**Figure 3.3**).

3.5 DISCUSSION

CSCs play a critical role in the development of breast cancer, chemoresistance, cancer relapse, and metastasis. In contrast to several current chemotherapeutic agents, metformin is

implicated in selective killing of CSCs in human breast cancer cell lines and breast tumor tissues, which is consistent with clinical observations that long-term metformin treatment in diabetic patients, in contrast to treatment with other anti-diabetic agents, causes a significant reduction in the risk of developing breast cancer (16, 17), and leads to improved metastasis-free survival after chemotherapy (18). Despite an increasing number of reports on metformin efficacy against CSCs, only a few studies address the differences in sensitivity of breast CSCs and NSCCs to metformin. A study conducted by Lonardo et al. suggested that compared to NSCCs, the highly proliferative CSCs relied heavily on intracellular ATP levels and were subsequently more sensitive to metformin-mediated inhibition of ATP synthesis in mitochondria (19). Qu et al., on the other hand, suggested that AMPK was the primary intracellular target of metformin in both CSCs and NSCCs, but the activation of AMPK in CSCs not only inhibited cell proliferation as it did in NSCCs, but also suppressed EMT-mediated generation of CSCs (12). Although the mechanisms proposed by these two studies were different, both studies suggested that metformin uptake and activation of its intracellular targets were required for its efficacy against CSCs. Therefore, investigating the mechanisms of metformin transport in CSCs will not only contribute to understanding the molecular mechanisms of its efficacy against CSCs, but also provides a rationale for optimizing metformin therapy against CSCs.

The transport of chemotherapeutic agents in CSCs has been relatively well studied. CSCs have a higher expression of multiple ATP-binding cassette (ABC) efflux transporters (e.g. p-glycoprotein and multidrug resistant associated proteins) compared to NSCCs (6, 7). Since many widely used chemotherapeutic agents, such as doxorubicin, paclitaxel, and fluorouracil, are substrates of these ABC transporters (20), the upregulation of ABC transporters is believed to be the primary cause of chemoresistance exhibited by CSCs. Expression of uptake transporters, in contrast to expression of efflux transporters, is seldom studied in CSCs, perhaps because majority of the chemotherapeutic agents are relatively lipophilic and their uptake into CSCs is not dependent on transporters. In contrast to many other chemotherapeutic agents,

metformin is positively charged under physiological conditions and very hydrophilic; thus it requires cation-selective transporters to mediate its cellular uptake. Therefore, evaluating cation-selective transporter expression and transporter-mediated metformin uptake in CSCs is important in elucidating the mechanism(s) underlying greater sensitivity of CSCs over NSCCs to metformin treatment.

In this study, the BT-549 cell line, a triple-negative human breast cancer line, was selected as a representative of human breast cancer cell lines because it contains both CSCs and NSCCs in moderate proportions; thus sufficient numbers of CSCs and NSCCs can be isolated for RNA isolation, uptake studies, and Western blot analyses. Expression of cation-selective transporters was evaluated in CSCs and NSCCs and correlated with intracellular uptake in these two cell types. An increase in the expression of cation-selective transporter genes and proteins, specifically MATE1, was observed in BT-549 CSCs compared to NSCCs. The upregulation of transporters led to higher metformin uptake in CSCs *versus* NSCCs. The results suggest that the increased sensitivity of CSCs to metformin treatment compared to NSCCs could be due to higher transporter-mediated metformin uptake. The role of transporters in metformin-induced AMPK activation and antiproliferative efficacy against CSCs can be elucidated by evaluating the activity of metformin in CSCs in the presence and absence of a transporter inhibitor.

In addition to BT-549 cell line, cation-selective transporter expression in CSCs and NSCCs from oncogenic transformed immortalized human mammary epithelial (HMLER) cells was also investigated; HMLER cells are widely used for breast CSC research. Similar to observations in BT-549 cells, cation-selective transporter gene expression in HMLER CSCs was higher compared to expression in HMLER NSCCs (**Supplementary Figure 3.1**). Similar efforts to investigate cation-selective transporter expression levels in CSCs and NSCCs of other breast cancer cell lines did not succeed because very few CSCs in MCF-7 and BT-20 cells and limited NSCCs in MDA-MB-468 and MDA-MB-231 cells prevented meaningful analyses. Interestingly,

based on the results from Chapter 2, we observe that breast cancer cell lines with a high proportion of CSCs (e.g. MDA-MB-468, MDA-MB-231, and BT-549 cells) are generally transporter-competent cell lines whereas breast cancer cell lines with very few CSCs (e.g. MCF-7, BT-20, and ZR-75-1 cells) are transporter-deficient cell lines. These observations provide indirect evidence that cation-selective transporters are upregulated in CSCs compared to NSCCs.

In this chapter, we provide the first report that cation-selective transporters are upregulated in breast CSCs. However, the physiological role of these transporters in the generation and proliferation of CSCs remains unclear. In contrast to efflux transporters which contribute to chemoresistance of CSCs, we hypothesize that (1) cation-selective transporters play a critical role in the uptake of cationic and hydrophilic nutrients, such as choline, which are required for CSC growth and proliferation, and (2) cation-selective transporters are upregulated to meet the demand for cationic and hydrophilic nutrients due to an increase in protein and lipid synthesis in CSCs compared to NSCCs. Clearly, future studies are needed to elucidate the role of cation-selective transporters in CSC generation and proliferation.

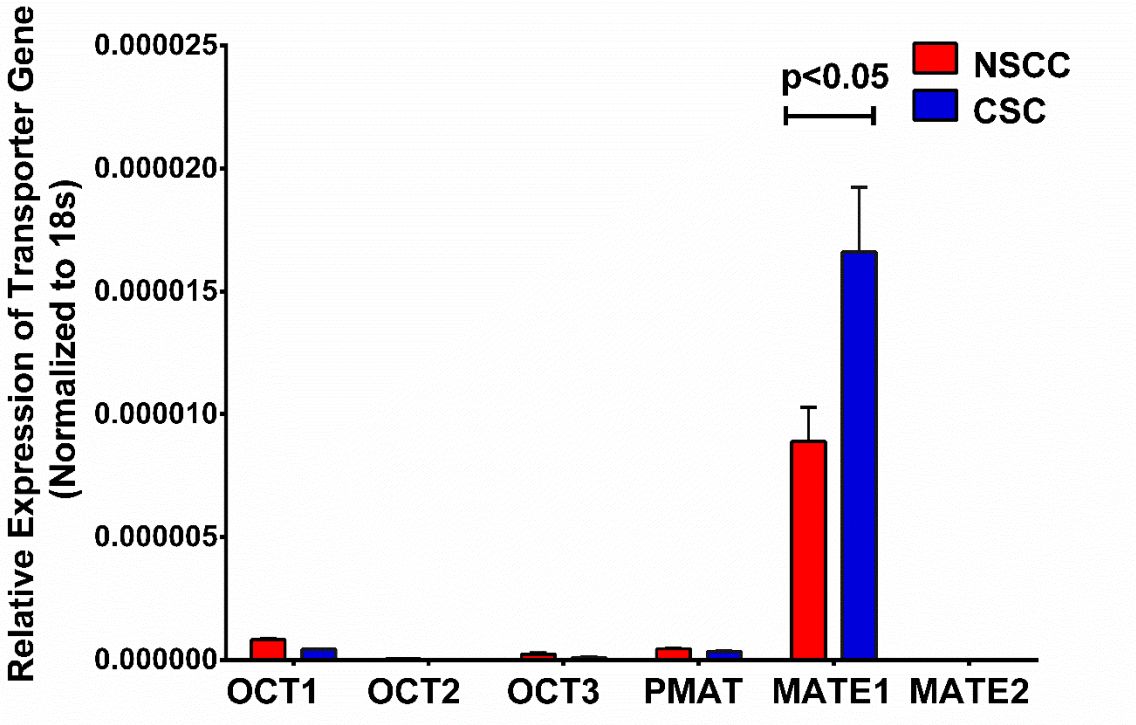


Figure 3.1 Expression of Cation-selective Transporter Genes in BT-549 CSCs and NSCCs. Results are shown as the mean ± SD; N=3.

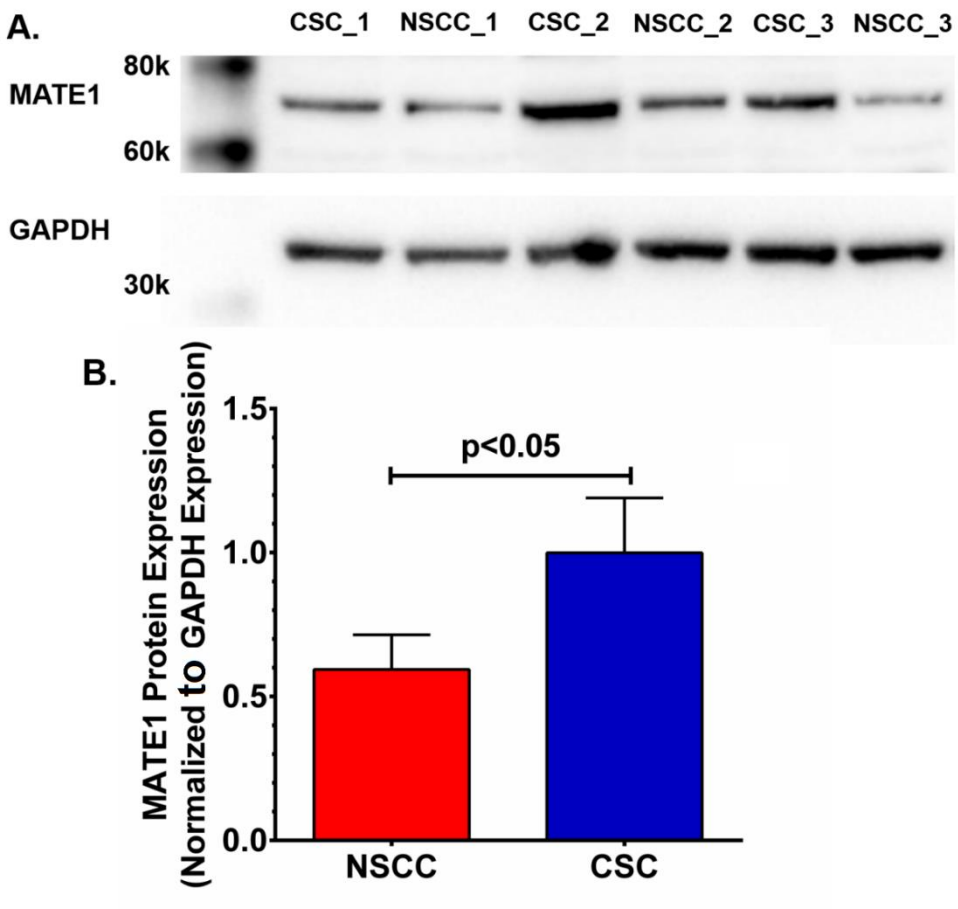


Figure 3.2 Expression of MATE1 Protein in BT-549 CSCs and NSCCs. A) Western blot analysis of MATE1 expression in BT-549 CSCs and NSCCs (isolated from three independent cell sorting experiments) using a primary antibody against MATE1. GAPDH was used as a loading control. **B)** Comparison of MATE1 protein expression in BT-549 CSCs and NSCCs using densitometric analysis of MATE1 bands normalized to GAPDH. Results are shown as the mean ± SD; N=3.

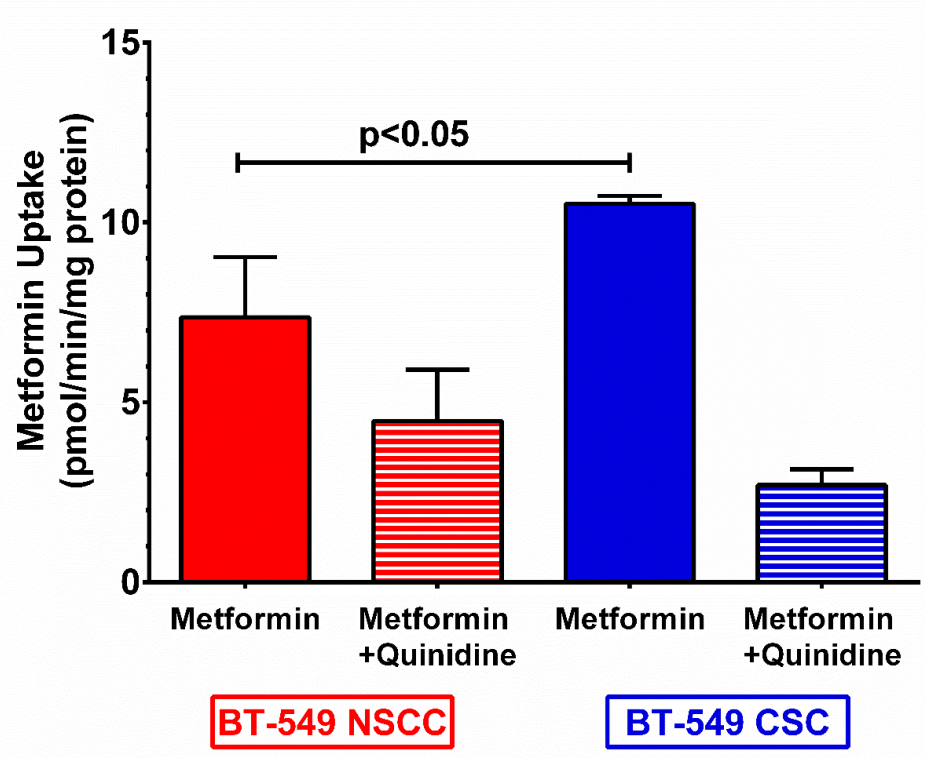
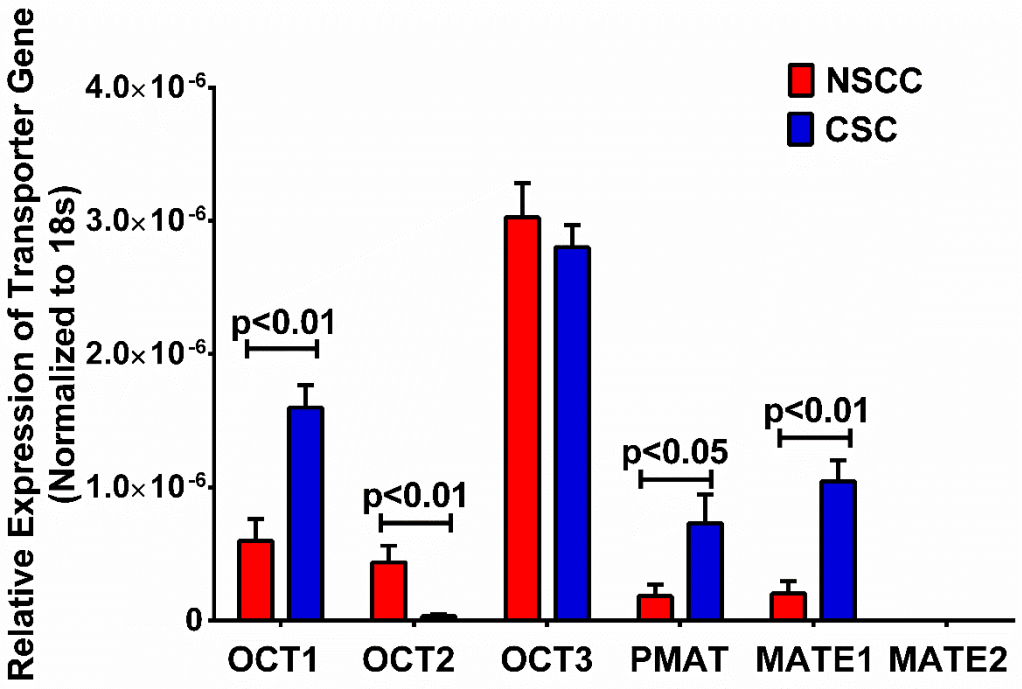


Figure 3.3 Metformin Uptake in BT-549 CSCs and NSCCs. Metformin uptake was assessed in the presence or absence of the pan cation-selective transporter inhibitor quinidine (500 µM). Results are shown as the mean ± SD; N=3.



Supplementary Figure 3.1 Expression of Cation-selective Transporter Genes in HMLER CSCs and NSCCs. Results are shown as the mean ± SD; N=3.

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CHAPTER 4

Cation-selective Transporters in Breast Tumors Enhance the Intratumoral Concentration of Metformin and Metformin-Mediated Activation of the AMPK Pathway and Antitumor Efficacy

4.1 OVERVIEW

Our previous *in vitro* studies in human breast cancer cell lines presented in Chapter 2 showed that cation-selective transporters mediate the intracellular uptake of metformin and its subsequent antiproliferative efficacy. The study in this chapter provides evidence that transporter expression is a critical determinant of the anticancer efficacy of metformin via AMPK activation, and is the first to establish a direct *in vivo* relationship between a cation-selective transporter expression (in otherwise matched tumors), metformin intratumoral uptake, and its antitumor efficacy. The low transporter-expressing MCF-7 human breast cancer cells and MCF-7 cells which were engineered to overexpress OCT3, an organic cation transporter that is expressed in breast tumors, were used to generate xenograft mice bearing MCF-7 and OCT3-MCF7 tumors, and the antitumor efficacy of metformin as a monotherapy and in combination with doxorubicin (DOX) in these two tumor types was evaluated. A significantly greater antitumor potency of metformin (alone or in combination with DOX) and greater modulation of the AMPK-mTOR-P70S6 pathway were observed in OCT3-MCF7 tumors *versus* MCF-7 tumors.

4.2 INTRODUCTION

Metformin, the first-line therapy for type 2 diabetes, has beneficial effects against breast cancer as well as other types of cancer. In diabetic cancer patients, metformin appears to exert its anticancer effect indirectly by decreasing circulating insulin levels and reducing the tumor growth stimulus provided by insulin (1, 2). Metformin is also believed to exert direct anticancer effects on tumor cells by inhibiting the respiratory-chain complex 1 (3), leading to elevated

AMP/ATP ratio, increased phosphorylation and activation of 5' adenosine monophosphate-activated protein kinase (AMPK), and subsequent suppression of cell proliferation via down-regulation of the mammalian target of rapamycin (mTOR) and its downstream target, ribosomal kinase P70S6 (P70S6K) (4, 5). According to some reports, metformin selectively kills cancer stem cells (CSC), a small proportion of cells which share characteristics of embryonic stem cells and which confer chemo-resistance and induce cancer metastasis (6, 7).

For metformin to achieve its anticancer effects via the intracellular targets, its cellular uptake and accumulation are critical. Due to the hydrophilic nature (logD -6.13 at pH 6.0) and positive charge of metformin (pKa 12.4) at physiological conditions, its cellular uptake and accumulation in tissues are mediated by cation-selective transporters. In Chapter 2, we showed that multiple cation-selective transporters that play a role in metformin trafficking in other organs are also expressed in breast tumors and in commonly used breast cancer cell lines, and that the expression levels of these transporters is highly variable (8). The study further demonstrated that OCT3, and not OCT1 or OCT2 (the predominant metformin transporters in the liver and kidney, respectively), is one of two predominant transporters in many breast tumors, the plasma membrane monoamine transporter (PMAT) being the other major transporter (8).

In this chapter, we have demonstrated that higher expression levels of a metformin transporter in breast tumor tissue increases the anticancer efficacy of metformin in xenograft mouse models. We selected OCT3 as a representative of all the cation-selective transporters of metformin that are expressed in breast tumors, and overexpressed OCT3 in the low transporter-expressing MCF-7 human breast cancer cell line to generate OCT3-MCF7 cells. The antitumor efficacy of metformin (as a monotherapy and in combination with DOX) was evaluated in xenograft mice bearing tumors generated from OCT3-MCF7 and MCF-7 cells. This is the first study in which the expression level of a metformin transporter in tumor tissue has been directly related to uptake of the drug in tumor cells, exposure of the tumor tissue to metformin, and to

the cellular and pharmacological evidence for the antiproliferative and antitumor effects of the drug in the same *in vivo* experimental model.

4.3 MATERIAL AND METHODS

Cell Culture. MCF-7 cells were cultured in MEM media (11095, Invitrogen) with 10% fetal bovine serum (s-12450, Atlanta), non-essential amino acid (11140, Invitrogen), sodium pyruvate (11360, Invitrogen) and penicillin-streptomycin (15140, Invitrogen). OCT3-MCF7 cells were cultured in media containing 500 µg/ml Geneticin® (10131, Invitrogen).

Generation of OCT3-MCF7 Cells. The OCT3-pSPORT vector was obtained from Dr. Ganapathy at Georgia Regents University. The OCT3 gene was cloned into a pcDNA3.1 (+) vector (V790-20, Life Technologies) which was transfected into MCF-7 cells using the AMAXA Nucleofection™ system (AAB-100, Lonza). Cells were cultured in complete MEM media containing 500 µg/ml Geneticin®. Colonies were isolated and clones with high OCT3 expression were identified by evaluating [^{14}C]metformin uptake at 5 min in the presence or absence of the pan transporter inhibitor, quinidine.

Evaluation of Transporter Gene Expression. Total RNA was isolated from OCT3-MCF7/ MCF-7 cells using QiAzol® (79306, Qiagen) and cDNA was synthesized from RNA using Superscript® III First-Strand Synthesis Supermix kit (18080-400, Life Technologies). Gene expression of PMAT, MATE1 and OCT3 was determined by real-time polymerase chain reaction (RT-PCR) by Taqman® assay (4369016, Life Technologies) and normalized to endogenous 18s rRNA.

Cellular Uptake of Metformin. OCT3-MCF7/MCF-7 cells were cultured until 90% confluent, and incubated in transport buffer (i.e., Hank's Balanced Salt Solution (MT-21-023-CV, Fisher) with 25 mM D-glucose (G8210, Sigma) and 10 mM HEPES (15630-106, Corning)) for one hour. Transport buffer was replaced with 50 µM [^{14}C]metformin in the presence or absence of 500 µM of quinidine (Q-0750, Sigma Aldrich). After 5 min, cells were washed with ice-cold transport buffer and lysed with 500 µl 1M NaOH solution containing 0.1% SDS. [^{14}C]Metformin in cell

lysates was measured by liquid scintillation spectrometry (1600 TR Liquid Scintillation Analyzer; PerkinElmer Life and Analytical Sciences). Metformin uptake was normalized to protein content determined by the bicinchoninic acid (BCA) protein assay (23225, Pierce).

Assessment of the Antiproliferative Effects of Metformin on Breast Cancer Cell Lines. OCT3-MCF7/MCF-7 cells were cultured for 24 hours. Culture medium was replaced with medium containing varying concentrations of metformin (1 nM to 100 mM). After five days, the Alamar Blue® Cell Viability assay (DAL1025, Life Technologies) was performed according to the manufacturer's instructions.

Generation of Xenograft Mouse Models of Breast Cancer. Xenograft mice were generated by subcutaneous injections of 2×10^6 OCT3-MCF7/MCF-7 cells resuspended in 50% Matrigel™ (356234, BD Bioscience) into the right flank of 8-week-old athymic nude mice. Estrogen pellets (SE-121, Innovative Research of America) were subcutaneously implanted into mice to stimulate tumor growth. Tumor size was measured and tumor volume was calculated using the equation below:

$$\text{Tumor Volume} = \frac{1}{2} \times \ (\text{Length}) \ \times (\text{width})^2$$

When tumors were >100 mm^3 in size, mice were intraperitoneally (IP) injected daily with saline, 2 mg/kg DOX (D1515, Sigma Aldrich) every 5 days or 2 mg/kg DOX every 5 days plus 50mg/kg metformin daily for 20 days, and euthanized on day 20. Tumor volumes were measured and plotted against treatment time, and the area under the curve ($AUC_{\text{Tumor Volume}}$) was calculated to assess the effect of the therapy on tumor progression. Tumor weights and $AUC_{\text{Tumor Volume}}$ data are summarized in **Supplementary Table 4.1**.

Evaluation of Metformin-induced Activation of the AMPK Pathway in Xenograft Tumor Tissues. Cells were washed and lysed in a radio-immunoprecipitation assay (RIPA) buffer system. About 10 mg of tumor tissues were isolated from mice following euthanization. Tissues were washed and lysed in RIPA buffer, and protein content was measured by the BCA assay.

Protein from cells/tumors was subjected to Western blot analyses as previously described (23, 26) using primary antibodies against AMPK (5831, Cell Signaling Technology), phospho-AMPK (9205, Cell Signaling Technology), P70S6K (9234, Cell Signaling Technology), phospoh-P70S6K (2535, Cell Signaling Technology) and OCT3 (ab183071, Abcam). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-25778, Santa Cruz) was used as a loading control.

Immunohistochemical (IHC) Staining of Tumor Tissues. Tumor tissues (~20 mg) were fixed in 4% paraformaldehyde (P6148, Sigma Aldrich) in PBS for three days. Tissue blocks were transferred to 70% ethanol, and paraffin-embedded tissue blocks and sections (5 µm thick) were prepared by the Animal Histopathology Core Facility at the UNC Lineberger Cancer Center. Tissue sections were stained in a Bond-Max automated immunostainer (Leica Biosystem) using antibodies against CD44 (3578, Cell Signaling Technology), Ki-67 (VP-RM04, Vector Laboratories) and OCT3. The number of Ki-67-positive cells in OCT3-MCF7 and MCF-7 tumor tissue sections was counted in one field of view from three different tissue sections (i.e., three tissue sections for OCT3-MCF7 tumors and three tissue sections for MCF-7 tumors), and the average of the three values was determined.

Evaluation of Plasma and Intratumoral Concentrations of Metformin. A single dose of 50 mg/kg [^{14}C]metformin was injected IP into mice. Blood was collected from the tail vein at 5 min, 15 min, 50 min, 2 hr, 8 hr and 24 hr post metformin administration and plasma was isolated. Mice were euthanized at 15 min (N=3) and at 24 hr (N=3) post injection. Hepatic, renal and tumor tissues were harvested and lysed. [^{14}C]Metformin in plasma and tissue lysates was measured by liquid scintillation spectrometry. Systemic exposure of metformin (reflected by AUC_{plasma}), maximum plasma concentration of metformin, and metformin clearance were estimated through non-compartmental analysis by Pharsight Phoenix WinNonlin (Certara, NJ).

Statistical Analyses. All data are expressed as mean ± SD. Statistical analyses of transporter expression and IC_{50} values of metformin in OCT3-MCF7/MCF-7 cells were determined by a Student's t-test. Duncan's multiple comparison test was used to determine statistical

significance in metformin uptake between metformin-treated MCF-7 cells and other groups. Analysis of Variance followed by Tukey's test was used to determine statistical significance between the proportions of Ki-67-positive cells and $AUC_{Tumor\ Volume}$ of any two treatment groups. A Student's t-test was used to make comparisons of the effect of metformin plus DOX on tumor weights, tissue concentrations of metformin, AUC_{Plasma}, C_{max} and Cl between OCT3-MCF7 tumors and MCF-7 tumors.

4.4 RESULTS

Generation and Characterization of an OCT3 Overexpressing MCF-7 Cell Line. MCF-7 cells were transfected with a vector containing the OCT3 gene, and a single OCT3-MCF7 clone expressing high levels of the OCT3 gene (3.46×10^{-3} of 18s rRNA) compared to MCF-7 cells (1.61×10^{-7} of 18s rRNA, $p<0.001$) was isolated (**Figure 4.1A**). Higher gene expression of OCT3 in the OCT3-MCF7 clone corresponded with high OCT3 protein levels in comparison to barely detectable OCT3 protein in MCF-7 cells (Western blot insert of **Figure 4.1A**). The expression of PMAT and MATE1 genes, which are expressed at low levels in MCF-7 cells as demonstrated in our previous study in Chapter 2 (8), was evaluated in the OCT3-MCF7 clone to confirm that overexpression of the OCT3 gene did not affect the expression of other metformin transporters. A modest 2.8-fold increase in the expression of MATE1 gene, compared to >1000-fold increase in the expression of OCT3 gene, was observed in the OCT3-MCF7 clone *versus* MCF-7 cells (5.45×10^{-7} *vs.* 1.94×10^{-7} of 18s rRNA, $p<0.05$) (**Figure 4.1A**). No difference in the expression of PMAT gene was observed between OCT3-MCF7 and MCF-7 cells (**Figure 4.1A**). As expected, metformin uptake in OCT3-MCF7 cells was higher compared to uptake in MCF-7 cells (95.1 *vs.* 12.9 pmol/mg protein*min, $p<0.001$); and uptake was attenuated by 85% in the presence of the pan transporter inhibitor, quinidine (14.2 *vs.* 95.1 pmol/mg protein*min, $p<0.001$) (**Figure 4.1B**), confirming that the nearly 7-fold increase in metformin uptake observed in OCT3-MCF7 cells was due to transporter-mediated uptake and not due to unrelated changes in cell membrane integrity or composition. A difference in nearly two orders of magnitude between the increase in

transporter-mediated uptake and the increase in transporter gene expression is not surprising, since the increase in protein expression is often a fraction of the increase in gene expression, and frequently, only a fraction of the transporter protein that is expressed in a cell is localized to the cell membrane where it is functional.

Thus, a head-to-head comparison of OCT3 gene and protein expression and transporter-mediated uptake in two MCF-7 cell lines, one expressing low levels of cation-selective transporters and the other in which one of the metformin transporters is overexpressed, provided reasonable assurance that tumors generated from OCT3-MCF7 cells would undergo greater intratumoral exposure to metformin compared to tumors generated from MCF-7 cells in the xenograft mice.

Metformin is More Efficacious against OCT3-MCF7 *versus* MCF-7 Tumors. Xenograft mice bearing tumors generated from MCF-7 and OCT3-MCF7 cells were developed. To confirm the overexpression of the OCT3 protein in OCT3-MCF7 tumors *versus* MCF-7 tumors, IHC analysis of paraffin-embedded tumor tissue sections was performed. The IHC results showed a noticeably greater staining of OCT3 in OCT3-MCF7 tumors compared to MCF-7 tumors (**Figure 4.2A**).

Metformin monotherapy, at a dose that is equivalent to the clinical dose for T2DM, attenuated the growth of both MCF-7 tumors and OCT3-MCF7 tumors, and compared to DOX alone, metformin was 60% more efficacious in OCT3-MCF7 tumors (**Figure 4.3A**) and 30% less efficacious in MCF-7 tumors (**Figure 4.3B**). In fact, metformin induced complete arrest of tumor growth in OCT3-MCF7 tumors. Metformin, as a combination therapy with DOX (which reflects its potential use for cancer therapy in the clinic), was almost twice as effective in decreasing the size of OCT3-MCF7 tumors *versus* MCF-7 tumors (**Figure 4.3C**). The combined antitumor effects of metformin plus DOX were enhanced by 97% over that of DOX alone against OCT3-MCF7 tumors and by 27% against MCF-7 tumors (**Figure 4.3A-C, Supplementary Table 4.1**). OCT3 overexpression did not alter the inherent responsiveness of MCF-7 cells to DOX

treatment as evidenced by the observation that there was no significant difference between the growth of OCT3-MCF7 and MCF-7 tumors when the animals were treated with DOX alone (**Figure 4.3A and B**).

Metformin, in combination with DOX, was more potent in reducing the number of proliferating cells in OCT3-MCF7 tumors compared to MCF-7 tumors, as evidenced by IHC staining of Ki-67, a biomarker of proliferating cells (26.3% *vs.* 56.3% of Ki-67-positive cells, $p<0.001$) (**Figure 4.2B, C**). Additionally, compared to treatment with DOX alone, metformin plus DOX reduced the number of Ki-67-positive cells in both OCT3-MCF7 tumors (26.3% *vs.* 60.0% of Ki-67-positive cells, $p<0.001$) and MCF-7 tumors (56.3% *vs.* 61.2% of Ki-67-positive cells, $p<0.05$) (**Figure 4.2B, C**).

The greater antitumor efficacy of metformin, both as a monotherapy and in combination with DOX, against OCT3-MCF7 *versus* MCF-7 tumors provides compelling evidence that a higher level of cation-selective transporter expression in breast cancer cells can enhance metformin treatment outcomes in breast cancer.

Metformin Induced Greater Modulation of the AMPK Pathway in OCT3-MCF7 Tumors Compared to MCF-7 Tumors. To elucidate the cellular and molecular basis for the greater antitumor efficacy of metformin in OCT3-MCF7 tumors than in MCF-7 tumors, modulation of the AMPK pathway following metformin treatment was evaluated by Western blot analyses of lysates from these tumors. Consistent with the increased potency of metformin against OCT3-MCF7 tumors, a greater increase in AMPK phosphorylation and a greater decrease in P70S6K phosphorylation was observed in OCT3-MCF7 tumors compared to MCF-7 tumors in both "metformin plus DOX" and "metformin monotherapy" groups (**Figure 4.4**). In contrast, DOX treatment had limited effect on these two kinases in either OCT3-MCF7 or MCF-7 tumors (**Figure 4.4**).

In a pharmacokinetic (PK) study, metformin concentrations in plasma as well as in the liver, kidney and tumor tissues were compared between mice bearing OCT3-MCF7 and MCF-7

tumors (**Figure 4.5**). No difference was observed in the plasma PK of metformin between the two groups of mice; the area under metformin plasma concentration *vs.* time curve ($AUC_{0\text{-}24hr}$), maximum plasma concentration (C_{max}) or clearance (Cl) were comparable in OCT3-MCF7 and MCF-7 mice (**Supplementary Table 4.2**). In contrast, the intratumoral concentration of metformin in OCT3-MCF7 tumors was 5-fold higher than that observed in MCF-7 tumors at 15 min post metformin delivery (403.2 *vs.* 77.4 µM, $p<0.001$), suggesting that metformin is taken up more effectively into tumors that express higher levels of OCT3. Even when metformin is almost cleared from the body at the 24 hr time point, the intratumoral concentration of metformin in OCT3-MCF7 tumors was ~8-fold higher than the concentration in MCF-7 tumors (5.61 *vs.* 0.71 µM, $p<0.001$) (**Figure 4.5**), suggesting that the initial higher exposure of OCT3-overexpressing tumors to metformin persists throughout the 24-hr dosing period. Hepatic and renal tissue concentrations of metformin in OCT3-MCF7 and MCF-7 xenograft mice were comparable (**Figure 4.5**). These PK data clearly showed that overexpression of OCT3 in MCF-7 tumors did not affect the overall systemic exposure of metformin or its concentrations in key organs such as the liver and kidney, while causing an increased exposure of the tumor tissues to metformin.

4.5 DISCUSSION

The anticancer effect of the antidiabetic drug, metformin, was first discovered from retrospective analyses, which showed a decreased risk of breast cancer in diabetic patients who were on metformin treatment. Metformin was initially reported to inhibit tumor growth indirectly via its antidiabetic pharmacology, which involves a decrease in circulating glucose and insulin levels (9). Since insulin enhances tumor growth by binding to its receptors on the surface of cancer cells and activating the downstream insulin pathway (3, 4, 10) that promotes cell proliferation, a decrease in plasma insulin levels would attenuate the proliferation of tumor cells. However, metformin exhibits antitumor effects even in non-diabetic patients in whom the drug has limited insulin and glucose-lowering effects (11), suggesting that the antitumor effects of metformin in non-diabetic breast cancer patients must be mediated via an intracellular

mechanism that is distinct from the extracellular insulin receptor-mediated pathway. Several studies have implicated that metformin suppresses tumor growth by activating intracellular AMPK (via phosphorylation), resulting in attenuation of the activities of the downstream mTOR complex and P70S6 kinase and subsequent suppression of protein synthesis and cancer cell proliferation (12). For breast tumors whose growth is inhibited by metformin via the intracellular AMPK-mTOR pathway, it is reasonable to assume that the intracellular concentration of metformin would affect its antitumor efficacy against breast cancer. Metformin uptake into breast cancer cells and tumor tissues is an obligate step for activation of its intracellular targets. However, since metformin is highly hydrophilic (logD = -6.13 at pH 7) and carries a positive charge at all physiologic pH values, it cannot traverse the cell membrane by passive diffusion, and requires a transporter(s) for cellular entry. Previous studies, including our own, have shown that cation-selective transporters mediate cellular uptake of metformin in the intestine, liver, and kidney (13-16).

The role of one or more cation-selective transporters in the antiproliferative activity of metformin *in vitro* (8, 17) has been reported previously. In Chapter 2 (8), we examined the expression of several known metformin transporters in human breast tumors and in most commonly used human breast cancer cell lines, and have reported that OCT3 and/or PMAT are the major metformin transporters that are overexpressed in breast tumors compared to non-malignant breast tissue. The study further showed that there is a wide variability in the expression of metformin transporters among breast tumors and among the commonly studied breast cancer cell lines. Collectively, current literature reports strongly suggest that metformin transporters in tumor tissues could influence the efficacy of this drug as an anticancer agent. However, we lack the ability to translate this information into the relevance of metformin transporters in clinical outcomes of metformin therapy in breast cancer and other cancers. In part, this is because of a lack of knowledge on how variations in transporter expression among

cells within tumor tissue and among tumor tissues of patient populations could affect responses to metformin treatment for cancer.

This study takes the first step toward understanding a relationship between expression levels of a metformin transporter in breast tumors and the resulting metformin exposure in tumor tissue, the modulation of intracellular mediators of the antiproliferative/antitumor activity of metformin, and the overall potency of the drug against breast cancer, both as a monotherapy and in combination with other chemotherapeutic agents. By necessity, we chose a simple system by artificially overexpressing, in MCF-7 tumors, one of the two major metformin transporters (i.e. OCT3) found in human breast tumors, and measuring the effect of high transporter levels on the anticancer efficacy of metformin in tumor-bearing mice. An alternative approach would have been to compare the antitumor efficacy of metformin in a xenograft mouse model of breast cancer to the antitumor efficacy metformin in the presence of a cation-selective transporter inhibitor. However, evidence is clear that several transporters can mediate metformin disposition in the body, making it challenging to evaluate the contribution of a metformin transporter(s) to the antitumor efficacy of the drug, since the transporter inhibitor would influence the overall disposition of metformin in the body. Another approach would be to attenuate the expression of one or more metformin transporters in a breast cancer cell line that could then be used to generate xenograft tumors, and compare the antitumor efficacy of metformin between transporter-deficient tumors and normal tumors. Since our data show that typically two or more transporters are expressed in most breast cancer cells (and cell lines), we recognized that it would be challenging to achieve complete inhibition of all transporters in a cell line. Hence, we selected a breast cancer cell line that expresses low levels of organic cation transporters (i.e. MCF-7 cells), and overexpressed OCT3, one of the major cation-selective transporters expressed in breast tumor tissues (8). The successful overexpression of OCT3 in OCT3-MCF7 cells was confirmed by increased OCT3 gene and protein expression, as well as

higher metformin uptake in OCT3-MCF7 cells compared to MCF-7 cells. Xenograft mice bearing tumors generated from OCT3-MCF7 cells and MCF-7 cells were then developed.

The MCF-7 human breast cancer cell line was selected for generating OCT3-overexpressing cells because i) MCF-7 cells are widely used to develop xenograft mouse models of breast cancer, ii) overall metformin transporter expression is relatively low in this cell line (8), and iii) MCF-7 cells are highly tumorigenic with a functional AMPK-mTOR-p70S6K signaling pathway (8). The same genetic origin of tumors derived from OCT3-MCF7 and wild-type MCF-7 cell lines enabled a direct comparison of the responsiveness of these tumors to metformin therapy. Our results showing that the OCT3-MCF7 and MCF-7 tumors responded similarly to DOX treatments provide confirmation that overexpression of OCT3 does not affect the intracellular machinery of the MCF-7 cells. This approach is preferable to strategies used by others where the antiproliferative and antitumor efficacies of metformin were compared between cell lines and xenograft tumors from different genetic backgrounds.

It is expected that in clinical settings, metformin is likely to be used as a chemopreventive/chemotherapeutic agent in combination with other cancer drugs; therefore, we evaluated the antitumor efficacy of metformin both as a monotherapy and in combination with DOX. A moderate dose of DOX (2 mg/kg) that had limited toxicity and no effect on animal survival was selected based on our own preliminary studies and those of others as reported in the literature (18). The dose of metformin was determined from our preliminary studies, and was equivalent to the dose commonly used for the treatment of T2DM. In several published reports, metformin was administered to mice orally in drinking water (19, 20). However, to ensure accurate systemic delivery of a defined dose of metformin in our study, the drug was administered intraperitoneally.

Interestingly, metformin as a monotherapy, was efficacious against MCF-7 tumors, but was less efficacious than DOX (**Figure 4.3**). The antitumor efficacy of metformin plus DOX against MCF-7 tumors was slightly better than the efficacy of DOX alone, although this was not

statistically significant. In contrast, metformin as a monotherapy was significantly more efficacious than DOX against OCT3-MCF7 tumors, and in combination with DOX it not only attenuated the growth of OCT3-MCF7 tumors, but reduced tumor size by ~30%. Thus, it is clear that the antitumor efficacy of metformin, as a monotherapy or a combination therapy, was influenced by the expression levels of the OCT3 transporter. The greater antitumor efficacy of metformin against OCT3-MCF7 tumors is due to higher transporter-mediated uptake and higher intratumoral concentrations of the drug, as evidenced by nearly 6 to 8-fold increase in metformin concentration in OCT3-MCF7 tumors compared to MCF-7 tumors (**Figure 4.5**). The overall increase in metformin efficacy against OCT3-overexpressing tumors was accompanied by greater activation of AMPK and inhibition of P70S6K in OCT3-MCF7 tumors compared to MCF-7 tumors, as evidenced by their phosphorylation status (**Figure 4.4**). These results were consistent with data from our *in vitro* studies which showed that OCT3-MCF7 cells were more sensitive to metformin treatment compared to MCF-7 cells (**Supplementary Figure 4.1**).

Metformin is regarded as a useful anticancer agent not only due to its ability to slow or stop tumor growth, but also due to its potential ability to prevent cancer metastasis and relapse, presumably by inhibiting proliferation of CSC within the tumor mass. CSC proliferation is regulated through both the intracellular AMPK-E-cadherin pathway (20) and extracellular growth factors (i.e. TGF-beta) (21), and it is not known which of these factors is the predominant contributor to the antiproliferative efficacy of metformin against CSC. Our results show that metformin, in combination with DOX, achieved a greater reduction in the number of CSC in OCT3-MCF7 tumors compared to MCF-7 tumors, as evidenced by the differential staining intensity of CD44 (a membrane marker of CSC) in tissues from these two tumors (**Supplementary Figure 4.2**). This is the first evidence that suggests that tumors derived from cancer cell lines with different transporter expression levels (OCT3 in this case) may contain CSC that also exhibit different transporter expression profiles, and more importantly, this

difference in cation-selective transporter expression may result in differential effect of metformin in inhibiting tumor growth and cancer metastasis/relapse.

Evidence for the anticancer activity of metformin against various tumor types is mounting, and multiple mechanisms have been implicated, including the activation of AMPK and subsequent modulation of the AMPK-P70S6K-mTOR signaling cascade. Reports that expression of OCT1 is associated with the antidiabetic activity of metformin have been published (17). A recent study by Patel et al. demonstrates that head and neck cancer cells with higher expression of OCT3 are more sensitive to metformin treatment compared to cancer cells with lower OCT3 expression (22). The true significance of our study in light of the prior evidence that suggests that the antitumor efficacy of metformin is associated with OCT1 and/or OCT3, is that in one study we showed a significant relationship between higher gene expression of an organic cation transporter (OCT3 in this case) and (i) higher protein expression of the transporter, (ii) increased uptake of metformin into tumor cells *in vivo*, (iii) increased AMPK phosphorylation and decreased P70S6K phosphorylation in tumors treated with metformin, (iv) greater antitumor efficacy of metformin as a monotherapy and in combination with an established chemotherapeutic agent such as DOX, and (v) greater reduction of CSC in tumors treated with metformin. Clearly, OCT3 is only one of several transporters expressed in tumor cells that can transport metformin into and out of the cells. However, we wish to emphasize that overexpression of OCT3 was employed as a tool to assess the relationship between the expression of metformin transporters and the antitumor efficacy of metformin *in vivo*.

Considering the high variability in transporter expression in tumor tissues among breast cancer patients (8), our study could explain the underlying cause for the sub-therapeutic efficacy of metformin in cancer therapy in some clinical studies (23). The correlation between higher intratumoral metformin concentrations and greater antitumor efficacy of metformin in OCT3-MCF7 tumors compared to MCF-7 tumors can also explain literature reports that phenformin, a more lipophilic biguanide compound, exhibited greater anticancer efficacy compared to

metformin (24) since phenformin does not have an absolute requirement for transporters to enter cells, and can be taken up into cancer cells via passive diffusion through the cell membrane. While further evidence needs to be developed, we believe that cation-selective transporter expression may turn out to be an important biomarker to identify the patient population that would be most responsive to metformin therapy in breast cancer. Further, our study provides a mechanistic rationale for developing metformin prodrugs that mask the charge and add lipophilicity, lipophilic metformin analogs like phenformin, or nanoparticle formulations of metformin that could circumvent the dependence of the success of metformin therapy on cation-selective transporters. Such metformin prodrugs, analogs, or formulations can eliminate the potential variability in response to metformin therapy among patients based on variability in transporter expression profiles and/or polymorphisms in transporters, and improve the utility of this safe, cost-effective and tolerable drug in breast cancer therapy.

In addition to metformin, widely used chemotherapeutic agents such as platinum derivatives or imatinib, a tyrosine kinase inhibitor, have been reported to be substrates of cation-selective transporters (25, 26). Similar to metformin, cellular uptake of these chemotherapeutic agents and activation of their intracellular targets in cancer cells are also required for their anticancer activity. However, the dose of these chemotherapeutic agents was based on their systemic exposure. Although there are a few reports on the relationship between transporter expression and drug-induced toxicity (27, 28), no systematic studies have been conducted to determine the effect of transporters on drug disposition, and the critical role of tumor transporter expression in the antitumor efficacy of these chemotherapeutic agents has been ignored. This study highlights the importance of cation-selective transporters in cancer therapies that use cationic chemotherapeutic agents, and sets the stage for using the transporter expression profiles of tumors as important biomarkers to adjust the therapeutic strategy of such chemotherapeutic drugs.

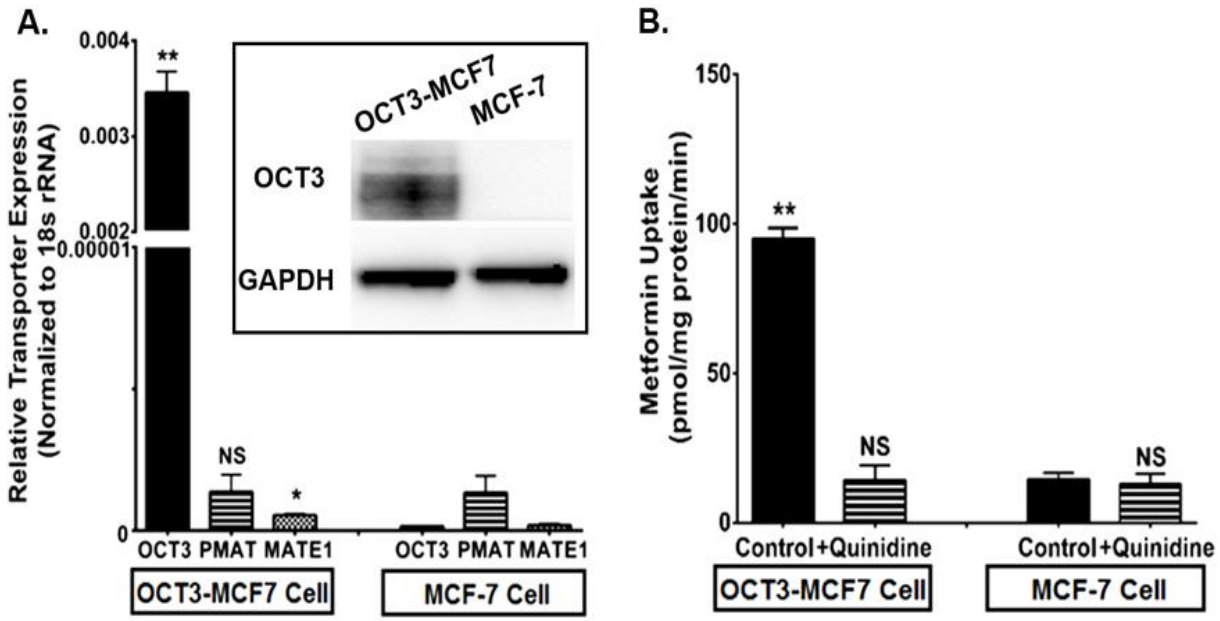


Figure 4.1 Generation and Characterization of OCT3-MCF7 Cells. A) Relative gene expression of three predominant cation-selective transporters (OCT3, PMAT and MATE1) in OCT3-MCF7 cells and MCF-7 cells was determined by RT-PCR and normalized to 18s rRNA. Data represent mean ± SD; N=3. *p<0.05, **p<0.001. NS: not significant. OCT3 protein expression in OCT3-MCF7 and MCF-7 cells was assessed by Western blot analysis and is shown in the insert. **B)** Metformin uptake in OCT3-MCF7 cells and MCF-7 cells was evaluated by incubating cells with [^{14}C]metformin in the presence or absence of the pan transporter inhibitor, quinidine, and uptake was normalized to protein content.**p<0.001, NS: not significant.

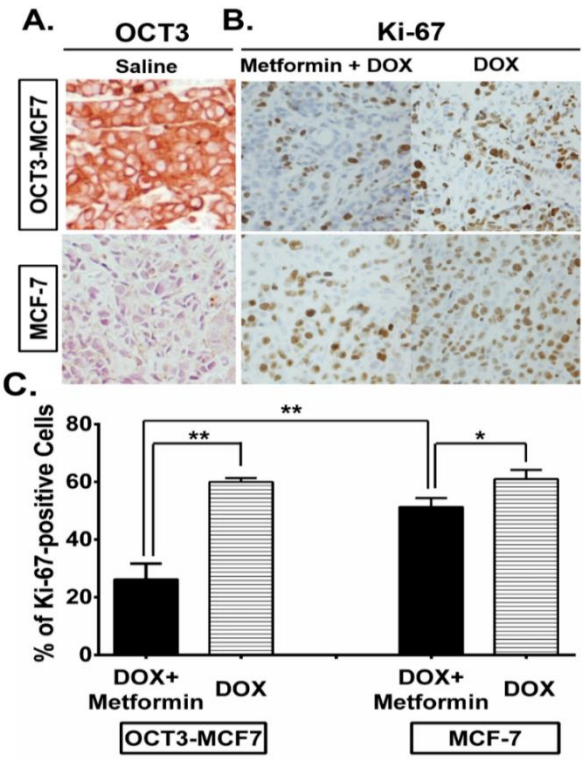


Figure 4.2 IHC Staining of Tissue Sections from OCT3-MCF7 Tumors and MCF-7 Tumors to Evaluate OCT3 Expression and the Antiproliferative Efficacy of Metformin. Images were taken at an amplification of 40X. **A)** OCT3 expression in OCT3-MCF7 tumors and MCF-7 tumors was evaluated using an antibody against OCT3 (staining shown in brown-red). **B)** The antiproliferative efficacy of metformin in OCT3-MCF7 tumors and MCF-7 tumors was determined using an antibody against the cell proliferation biomarker-Ki-67 (staining shown in brown). **C)** The percent of Ki-67-positive cells in OCT3-MCF7 and MCF-7 tumor tissues following metformin plus DOX or DOX treatment. Each bar represents the average of three values obtained from counting the number of Ki-67-positive cells under the microscope in one field of view (at 40X magnification) from three different tissue sections (i.e., three tissue sections for OCT3-MCF7 tumors and three tissue sections for MCF-7 tumors). Data represent mean ± SD; N=3. *$p<0.05$.

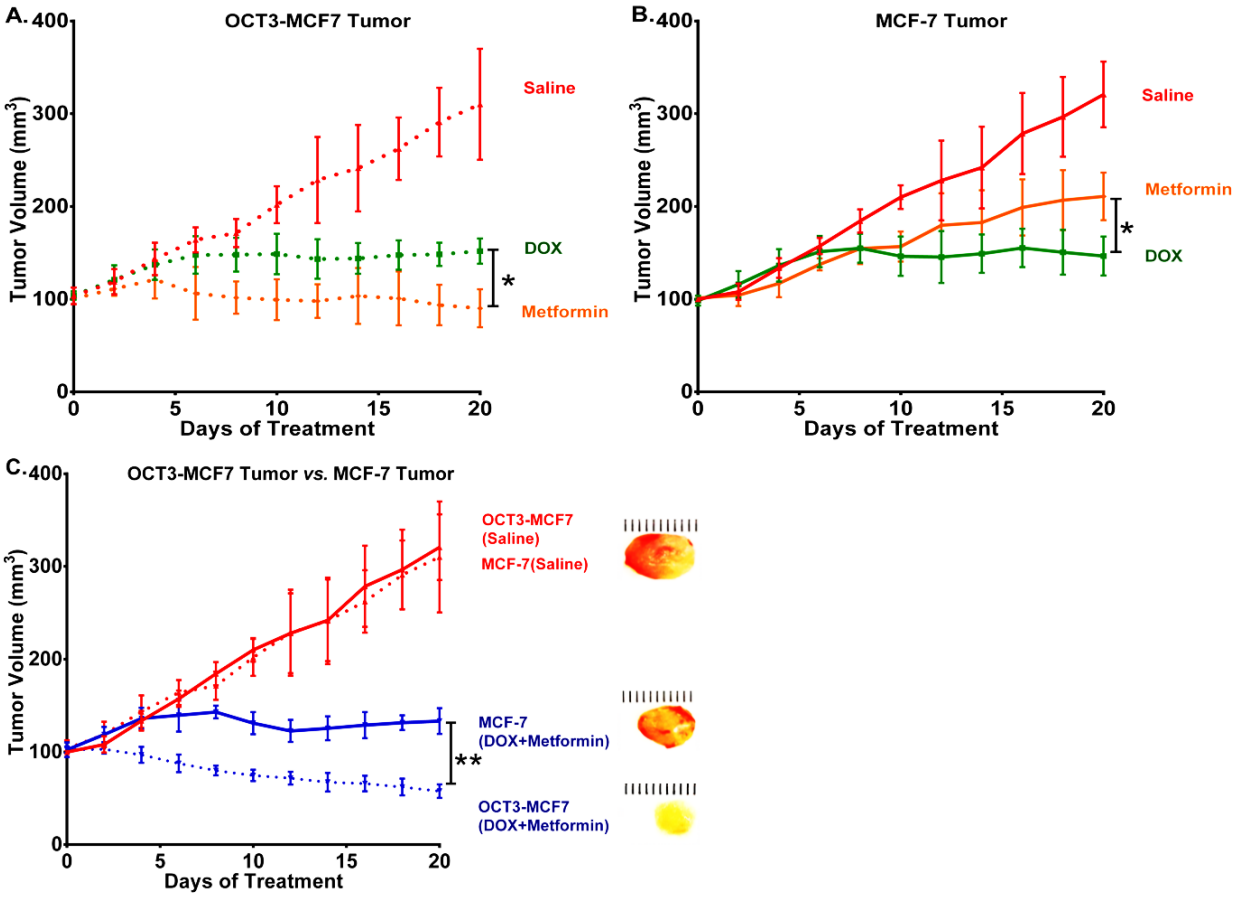


Figure 4.3 The Effects of Saline, DOX Alone, Metformin Alone and Metformin Plus DOX on Breast Tumors. Change in volumes of (**A**) OCT3-MCF7 tumors and (**B**) MCF-7 tumors over a 20-day treatment period. **C)** Head-to-head comparison of the effect of metformin plus DOX on OCT3-MCF7 tumors *vs.* MCF-7 tumors. N=8. *p<0.05, **p<0.001; NS: not significant. Data are summarized and shown in Supplementary Table 4.1.

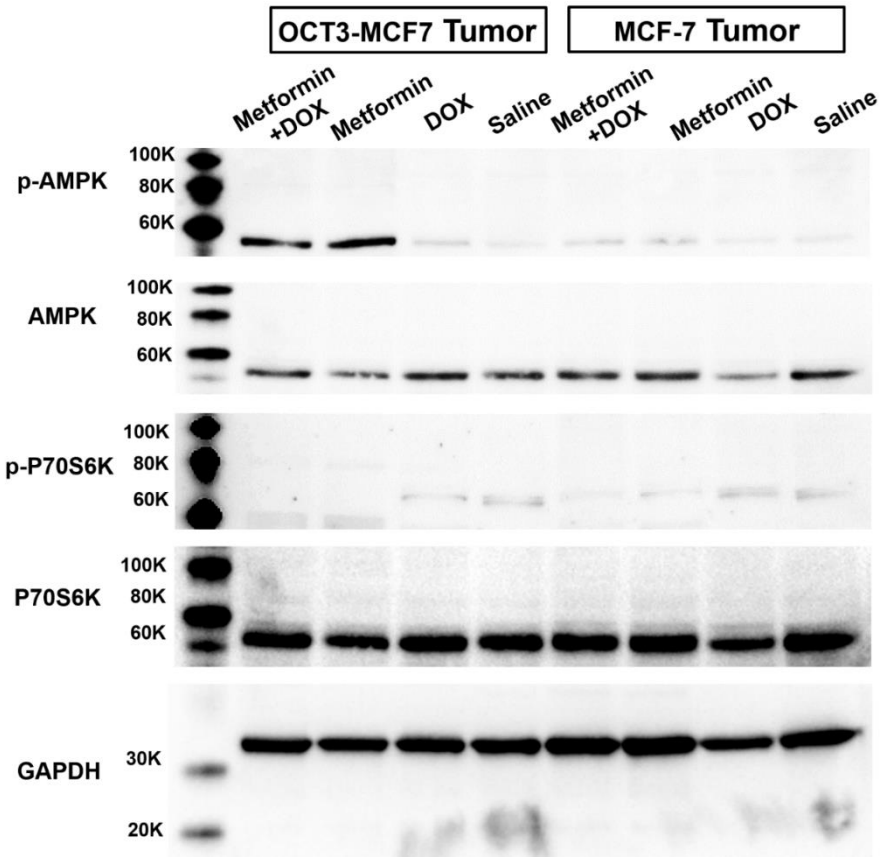


Figure 4.4 Western Blot Analyses Showing the Effect of Metformin Treatment on AMPK Phosphorylation and P70S6K Phosphorylation in OCT3-MCF7 Tumors and MCF-7 Tumors. GAPDH was used as a loading control. Mice were euthanized on Day 20, tumor tissues were harvested, and proteins from tissue lysates were subjected to Western blot analyses.

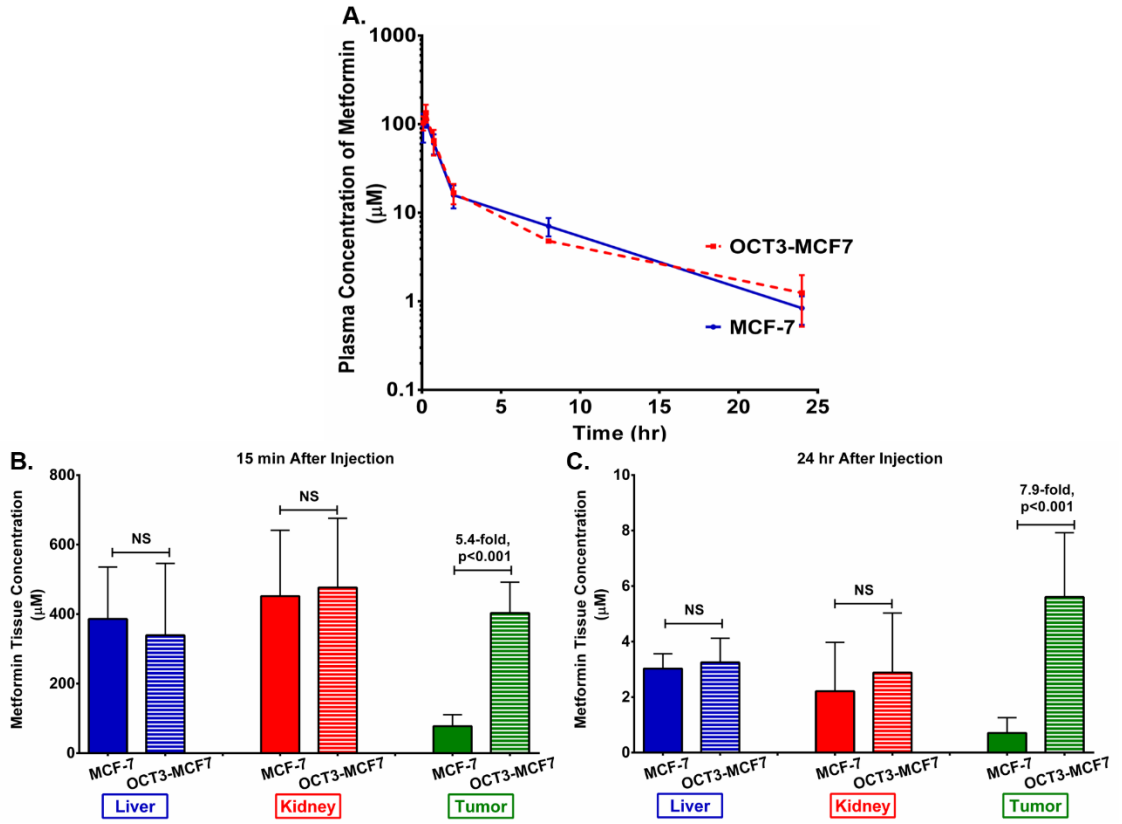
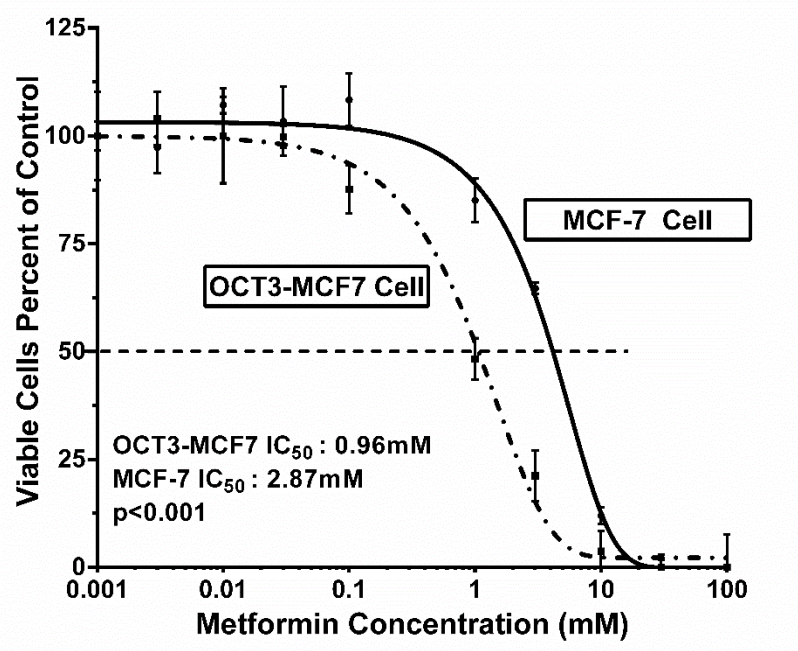
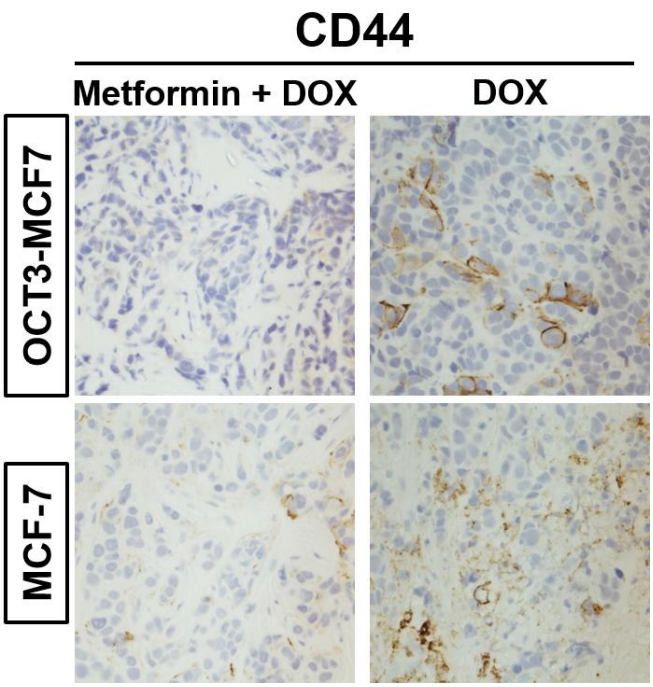


Figure 4.5 Plasma and Tissue Concentrations of Metformin in Xenograft Mice Bearing OCT3-MCF7 Tumors and MCF-7 Tumors. (**A**) Metformin plasma concentration-time profiles. Metformin concentrations in the liver, kidney and tumor tissues at (**B**) 15 min and (**C**) 24 hr post metformin administration. Data represent mean ± SD. N=3. ** signifies p<0.001; NS: not significant. Pharmacokinetic data are summarized and shown in Supplementary Table 4.2.



Supplementary Figure 4.1 The Antiproliferative Efficacy of Metformin in OCT3-MCF7 Cells and MCF-7 Cells. The efficacy of metformin was assessed by incubating cells for five days in culture media containing varying concentrations of metformin. Data represent mean ± SD; N=5



Supplementary Figure 4.2 Metformin-induced Decrease in the Proportion of CSC in OCT3-MCF7 Tumors and MCF-7 Tumors. CSC was probed using an antibody against CD44, a membrane surface marker of CSC (staining shown in brown).

Summary of Metformin and DOX Efficacy against the Progression of OCT3-MCF7 Tumors and MCF-7 Tumors					
		Saline	**DOX**	**Metformin**	**Metformin+DOX**
Tumor Progression (mm^3*d)	OCT3-MCF7	4058 (624)	2828 (353)	2063 (433)	1578 (158)
	MCF-7	4097 (488)	2855 (410)	3188 (452)	2589 (249)
Tumor Weight (mg)	OCT3-MCF7	252 (2)	96 (7)	62 (13)	35 (13)
	MCF-7	245 (44)	115 (27)	182 (70)	102 (38)

Supplementary Table 4.1 The Effect of Metformin Plus DOX on Tumor Volumes and Tumor Weights of OCT3-MCF7 Tumors and MCF-7 Tumors. Data are represented as mean (SD). N=3.

Metformin PK in Mice Bearing OCT3-MCF7 Tumors and MCF-7 Tumors			
	AUC (μM*hr)	C_{max} (μM)	Cl (mg/(hr*uM)/kg)
OCT3-MCF7	249.01 (37.09)	128.16 (23.14)	0.20 (0.03)
MCF-7	245.63 (61.13)	102.42 (19.97)	0.21 (0.05)
p-value	0.94	0.22	0.83

Supplementary Table 4.2 AUC_{plasma}, C_{max} and Cl of Metformin in Xenograft Mice Bearing OCT3-MCF7 and MCF-7 Tumors. Data are represented as mean (SD). N=3.

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CHAPTER 5

Relationship between Metformin Dose and Efficacy against Estrogen Receptor-positive and Triple-negative Breast Cancer

5.1 OVERVIEW

The ability of the antidiabetic drug, metformin, to reduce breast cancer incidence was discovered from retrospective analyses of clinical studies in diabetic cancer patients. Since then, numerous clinical trials have been conducted with the goal to repurpose metformin for cancer therapy. However, results from these studies are mixed, with some studies reporting efficacy of metformin as an anticancer agent in combination chemotherapy regimens while other studies show that the anticancer efficacy of metformin is marginal. One explanation for these mixed results is that current metformin treatment for cancer is not optimized. The dose of metformin used in a majority of these clinical studies was based on the doses used for treatment of type 2 diabetes. Since the results in Chapters 2 and 4 showed that transporter-mediated uptake and accumulation of metformin in tumor cells is related to its antitumor efficacy against breast cancer, it was hypothesized that increasing the metformin dose over that used for treatment of type 2 diabetes would achieve higher intratumoral exposure and would increase the antitumor efficacy of metformin against breast cancer. Therefore, efficacy of metformin, used in combination with paclitaxel (for estrogen-receptor positive breast tumors) and carboplatin (for triple-negative breast tumors), was evaluated in relation to its dose in mouse models of breast cancer. Xenograft mice bearing MCF-7 estrogen receptor-positive breast tumors or MDA-MB-468 triple-negative breast tumors were administered four escalating doses of metformin (12, 36, 120, 360 mg/kg/day for the duration of the study) in combination with a fixed dose of paclitaxel (30 mg/kg/day for 5 days) (for MCF-7 tumors) and carboplatin (50

mg/kg/day for 5 days) (for MDA-MB-468 tumors), and the antitumor efficacy was evaluated by measuring tumor volume. The four metformin doses were calculated from human doses used in the treatment of type 2 diabetes mellitus, and ranged from a dose lower than the initial dose to the maximum recommended daily dose. Our results showed an increase in the antitumor efficacy of metformin with increasing dose. A combination of carboplatin and a metformin dose of 120 mg/kg/day (equivalent to the most common anti-diabetic daily dose of 850 mg in humans) was required to observe a significant improvement in antitumor efficacy compared to carboplatin alone in MDA-MB-468 tumors, whereas a 3-fold higher metformin dose of 360 mg/kg/day (equivalent to the 2,550 mg maximum anti-diabetic daily dose in humans) in combination with paclitaxel was needed to observe a significant increase in antitumor efficacy compared to paclitaxel alone in MCF-7 tumors. The lower metformin dose required for efficacy against MDA-MB-468 tumors compared to MCF-7 tumors is consistent with high expression of cation-selective transporters in MDA-MB-468 tumor cells and relatively modest expression of these transporters in MCF-7 tumor cells. These results suggest that efficacious metformin dose for breast cancer therapy may vary depending on the tumor type, and that relative expression levels of cation-selective transporters in the tumor cells may provide a useful guide for selection of efficacious metformin doses. Because the metformin dose-response curve did not achieve a plateau in this study, it is recommended that metformin doses that are higher than the maximum daily dose of 2,550 mg may be considered for combination chemotherapy of both estrogen-receptor positive and triple negative breast cancer.

5.2 INTRODUCTION

Breast cancer is the second most frequently diagnosed cancer and cause of death due to cancer among women in the United States. The American Cancer Society estimates that there will be over 307,000 newly diagnosed cases and over 40,000 deaths in 2016 (1). Breast cancer is categorized into multiple types based on the expression of growth factor receptors on the cell membrane. Due to significant differences in the patterns of gene mutation and

expression of oncogenes and tumor suppressors, the mechanism of cancer development, tumor growth rate, and clinical outcomes vary among different types of breast cancer (1). Therefore, the type of chemotherapeutic agent to be used in clinical cancer therapy is also determined based on the type of breast cancer. Estrogen receptor-positive (ER+) breast cancer and triple-negative (TN) breast cancer are the two most commonly diagnosed types of breast cancer, which account for more than 85% of all breast cancer cases (1). Compared to ER+ breast cancer, TN breast cancer is more aggressive and relatively poorly controlled by the chemotherapeutic agents that are currently used for breast cancer (2, 3). It is clear that effective chemotherapy of TN breast cancer is an unmet medical need, and new agents that can improve the outcomes of other forms of breast cancer are also desired.

Recently there has been a surprising discovery that the leading drug for type 2 diabetes, metformin, contributes to reduced incidence of breast cancer among women with diabetes and better outcome of their treatment with conventional chemotherapeutic agents (4-11). Retrospective studies showed that type 2 diabetes patients have an increased risk of developing breast cancer compared to the non-diabetic population (6, 7), which led to further investigations on the impact of different anti-glycemic agents on breast cancer incidence. Compared to other widely administered anti-diabetic agents such as insulin and sulfonylurea, metformin significantly reduced the risk of breast cancer in diabetic patients in most retrospective studies (6-8). This led to clinical studies in type 2 diabetes and non-diabetic breast cancer patients aimed at evaluating metformin as an anticancer agent, either as a monotherapy or in combination with chemotherapeutic agents. Some studies showed that, unlike other anti-diabetic drugs, metformin improved clinical outcomes to neoadjuvant chemotherapy (9), inhibited cell proliferation in tumor tissues (10), and increased metastasis-free survival rates (11) in diabetic breast cancer patients. However, others have reported that no beneficial effects of metformin against breast cancer were observed (12, 13). It is worth noting that in a majority of clinical studies, metformin did not produce significant anticancer effects in non-diabetic breast

cancer patients, although a trend for anticancer effect was generally observed (9, 11, 14). It is likely that the anticancer efficacy of metformin did not reach significance due to inter-individual variability in response to metformin treatment among breast cancer patients. Although the response to anticancer agents generally varies significantly among different types of breast cancer, breast cancer type was not considered to stratify patients in many of these clinical studies in which metformin was evaluated for its anticancer efficacy. Further, the dose and administration frequency of metformin were not optimized in these clinical trials; rather the doses that are generally considered efficacious for treatment of diabetes were used for treatment of cancer. A typical dosing regimen for treatment of diabetes starts with a daily dose of 850 mg and the dose is titrated up by 500 mg weekly until a minimum dose for adequate anti-glycemic effect is achieved (15). In short-term studies of non-diabetic breast cancer patients, the daily dose of metformin ranged from 500 mg twice a day to its maximum daily dose for anti-diabetic treatment, namely 2550 mg (12, 16, 17).

Because the anticancer effect of metformin in diabetic patients with breast cancer is believed to be achieved, at least in part, by lowering circulating glucose, insulin, and IGF1 levels (18)), the assumption that the doses of metformin that are efficacious in the treatment of diabetes would also be efficacious against breast cancer may be considered reasonable. However, studies clearly showed that unlike metformin, other anti-diabetic drugs were not effective as anticancer agents, suggesting that the anticancer effects of metformin may be mediated by mechanisms that are distinct from and /or in addition to its effect on lowering circulating levels of glucose, insulin, and IGF1. Interestingly, some studies have shown that the key molecular target that is implicated in the inhibition of gluconeogenesis in the liver, i.e. adenosine monophosphate-activated protein kinase (AMPK), may also mediate the antiproliferative effects of metformin in cancer cells because it is an important modulator of energy homeostasis (19). Studies show that upon being taken up into the cancer cells, metformin induces the phosphorylation of AMPK, and subsequently attenuates protein

synthesis, inhibits cancer cell proliferation, and induces cell apoptosis through modulation of the downstream signaling molecules including mammalian target of rapamycin (mTOR) and phosphoprotein 70 ribosomal protein S6 kinase (P70S6K) (19, 20). If this is true, then sufficient intratumoral concentration of metformin must be achieved to modulate its intracellular targets. Unlike other chemotherapeutic agents, metformin is not capable of passing through the cell membrane via passive diffusion (21) since it is hydrophilic (logD -6.13 at pH 6.0) and positively charged (pKa 12.4) under physiological conditions (22). Instead, cellular uptake of metformin is mediated via cation-selective transporters (23-25). Cai et al. (26) have shown that the expression of cation-selective transporters in breast tumor tissues is relatively low and varies significantly among breast cancer patients. Therefore, it is hypothesized that metformin is not efficacious in those breast cancer patients because the doses of metformin used for the treatment of type 2 diabetes are not sufficient to achieve adequate intratumoral exposure of metformin that is required for activating its intracellular targets and exerting its antitumor efficacy.

In this study, a dose-response relationship for anticancer efficacy of metformin is evaluated against ER+ and TN human breast cancer in xenograft mouse models. The metformin doses used in this preclinical study were calculated such that the lowest dose would be equivalent to the human dose that is lower than the starting dose used in the treatment of type 2 diabetes, and the highest dose would be equivalent to the maximum daily dose administered to humans thus far (26). This is the first study that systematically investigates the relationship between metformin dose, systemic and tumor exposure, effect on intracellular targets, and antitumor efficacy against two most commonly encountered (ER+ and TN) breast cancers.

5.3 MATERIAL AND METHODS

Cell Culture. MCF-7 and MDA-MB-468 cells were cultured in DMEM media (11965, Invitrogen) with 10% fetal bovine serum (12003C, Sigma) and penicillin-streptomycin (15140, Invitrogen).

Human recombinant insulin (12585, Invitrogen) was added to the culture media to stimulate the growth of MCF-7 cells. Cells were plated in 175 cm^2 cell culture flasks (10-126-39, Corning) and passaged at 80% confluency.

Evaluation of antitumor Efficacy of Metformin as Monotherapy or Combination Therapy in Orthotopic Xenograft Mice Bearing MCF-7 and MDA-MB-468 Tumors. Athymic nude mice were purchased from the Animal Studies Core at the University of North Carolina at Chapel Hill. Two million MCF-7 cells or MDA-MB-468 cells were resuspended in 50% Matrigel™ (356234, BD Bioscience) and orthotopically injected into the breast region of 8-week-old nude mice. To stimulate the growth of MCF-7 tumors, estrogen pellets (SE-121, Innovative Research of America) were subcutaneously implanted into mice. Tumor size was measured externally with a caliper and tumor volume was calculated using the equation below:

$$\text{Tumor Volume} = \frac{1}{2} \times \ (\text{Length}) \ \times (\text{width})^2$$

When tumor size was larger than 100 mm^3, mice were assigned to seven groups (N=8 per group) which received a two-month treatment with saline (02049-0, Hospira), paclitaxel (760350, APP Pharmaceuticals) or carboplatin (C177500, Toronto Research Chemicals) alone, metformin (M258815, Toronto Research Chemicals) alone, or paclitaxel or carboplatin plus different doses of metformin (**Figure 5.1A**). Paclitaxel and carboplatin were administered via intravenous injections every week, and metformin was administered by oral gavage every day. During the treatment period, tumor volumes were measured and plotted against treatment time. In addition to tumor volume, body weights of the xenograft mice were monitored during the treatment period. Kaplan-Meier survival curves were plotted to assess the outcome of the different dosing regimens on overall survival of the animals.

Evaluation of Plasma and Intratumoral Concentrations of Metformin. On the last day of treatment, unlabeled metformin was replaced with the same dose of [^{14}C]metformin (50 µCi, MC 2043, Moravek) to assess the concentrations of metformin in plasma and tumor tissues. Blood

was collected from the tail vein at 5 min, 15 min, 1 hr, 2 hr, 8 hr, and 24 hr post administration and plasma was isolated. Mice were euthanized at 1 hr or 24 hr post metformin administration. Tumor tissues were isolated, weighed and lysed, and [^{14}C]metformin in plasma and tumor tissues was assessed by liquid scintillation spectrometry (1900 TR, Packard Tri-Carb).

Analysis of the Effect of Treatment Regimens on the AMPK Pathway. Tumor tissues (approximately 20 mg) were isolated from euthanized mice and lysed in radio-immunoprecipitation assay (RIPA) buffer system (89900, Thermo Fisher). Protein concentration in the lysed sample was measured by bicinchoninic acid assay (23225, Thermo Fisher). Tumor lysates were subjected to Western blot analyses as described in previous chapters (26) using primary antibody against p-AMPK (9205, Cell Signaling Technology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-25778, Santa Cruz) was used as a loading control. Densitometric analyses of p-AMPK and GAPDH bands were performed using Image Lab (BioRad).

PK Model Development. A two-compartment PK model was considered to represent the distribution phase and systemic clearance phase of metformin PK based on the metformin plasma concentration-time profiles (**Figure 5.5A**). Since the systemic exposure of metformin increased linearly as the dose was escalated within the dose range that was used in this study, a first-order absorption kinetics was applied in this PK model. It was also assumed that only the central compartment contributed to the clearance of metformin and the disposition of metformin in tumor tissues. Differential equations were established based on the modeling scheme shown in **Figure 5.1B**. Phoenix WinNonlin (Certara) was applied to solve the differential equations shown below and fit the plasma and intratumoral concentrations to the model and to estimate the PK parameters.

$$\frac{\mathrm{dA0}}{\mathrm{dt}} = -\mathrm{Ka} * \mathrm{A0}$$

$$\frac{\mathrm{dAc}}{\mathrm{dt}} = \mathrm{Ka} * \mathrm{A0} - \mathrm{Ke} * \mathrm{Ac} - \mathrm{K12} * \mathrm{Ac} + \mathrm{K21} * \mathrm{Ap} - \mathrm{Q} * \mathrm{Ac/Vc} + \mathrm{Q} * \mathrm{At/Vt}$$

$$\frac{\mathrm{dAp}}{\mathrm{dt}} = \mathrm{K12} * \mathrm{Ac} - \mathrm{K21} * \mathrm{Ap}$$

$$\frac{\mathrm{dAt}}{\mathrm{dt}} = \mathrm{Q} * \mathrm{Ac/Vc} - \mathrm{Q} * \mathrm{At/Vt}$$

The inter-individual variability was estimated via an exponential model and the intra-individual error was estimated using a proportional-additive mixed model. The property of the model was evaluated by the assessment of the goodness-of-fit (reflected by Akaike's Information Criterion) as well as visual predictive checks by comparing the simulated mean and 90% confidence interval with the experimental data. The time profile of plasma and intratumoral concentrations of metformin was simulated. Plasma/intratumoral exposure of metformin (reflected by the area under the metformin plasma/intratumoral concentration-time curve, namely AUC_{plasma} or AUC_{tumor}) was also estimated by the model and compared among different treatment groups.

Statistical Analyses. All data are expressed as mean ± S.D. Analysis of variance (ANOVA), and Tukey's test was performed to determine the statistical significance for the difference in tumor weight, $AUC_{tumor\ volume}$, AUC_{plasma}, and AUC_{tumor} among different treatment groups. Statistical significance for differences in survival rates among different treatment groups was assessed by Log-rank (Mantel-Cox) test. Kruskal-Wallis test was performed to determine the statistical significance in AMPK phosphorylation induced by different treatments. All statistical analyses were conducted with GraphPad Prism (GraphPad Software Inc.).

5.4 RESULTS

A Different Minimum Dose of Metformin is Required to Significantly Improve the Efficacy of Chemotherapeutic Agents against ER+ and TN Breast Cancer. The antitumor efficacy of metformin alone, paclitaxel or carboplatin alone, and the combination of metformin and paclitaxel or carboplatin was assessed and compared in xenograft mice bearing MCF-7 tumors or MDA-MB-468 tumors, respectively. **Figure 5.2A and B** show the percent change in tumor volume over time as the mice bearing tumors are treated daily with the indicated agents; **Figures 5.2C and D** show the tumor weights at the end of the therapy with each regimen

tested. As a monotherapy, metformin caused significant tumor reduction at 360 mg/kg/day dose, but not at lower doses (results are shown for metformin monotherapy only at this highest dose tested). Compared to saline, paclitaxel (MCF-7), carboplatin (MDA-MB-468), and metformin monotherapy significantly inhibited the growth of MCF-7 tumors and MDA-MB-468 tumors (**Figure 5.2**). Among the four combination therapy treatment groups, antitumor efficacy against MDA-MB-468 tumors was enhanced by metformin at 120 and 360 mg/kg/day doses. For the two lower doses of metformin (i.e. 12 and 36 mg/kg/day) used in combination therapy, difference in efficacy of the combination over monotherapy was not statistically significant (**Figure 5.2A and C**). Carboplatin plus two high doses of metformin (120 and 360 mg/kg/day) not only inhibited MDA-MB-468 tumor growth, but also caused tumor shrinkage (**Figure 5.2A and C**). Analysis of the anticancer efficacy of paclitaxel with metformin against MCF-7 tumors revealed that only the highest dose of metformin (i.e. 360 mg/kg/day) significantly improved antitumor efficacy of paclitaxel (**Figure 5.2B and D**). Thus, different minimum doses of metformin are required to enhance the antitumor efficacies of chemotherapeutic agents against MDA-MB-468 and MCF-7 tumors. For the low transporter expressing MCF-7 tumors, the minimum dose of metformin that enhances anticancer efficacy of paclitaxel is 3-fold higher than that needed for enhancing anticancer efficacy of carboplatin against the high transporter-expressing MDA-MB-468 tumors.

Metformin Dose that Improves Survival by Chemotherapeutic Treatment of Tumor-bearing Mice is Lower Than that Improves Anticancer Efficacy. Animal survival rates during treatment were also assessed, as the survival rate is generally regarded as the most important and direct indicator of cancer therapy outcome. In general, the tumor-bearing mice treated with combination therapy had higher survival rates during the treatment period compared to those treated with chemotherapeutic agents alone (**Figure 5.3A and B**). Metformin at 120 mg/kg/day in combination with paclitaxel or carboplatin was most effective in prolonging survival of tumor bearing animals. Interestingly, although paclitaxel/carboplatin in combination with 360 mg/kg/day metformin had the highest antitumor efficacy against MCF-7/MDA-MB-468 tumors

among all treatment groups, the efficacy of this combination on survival rate was lower than that of other doses of metformin (**Figure 5.3A and B**). Even the doses of metformin that did not cause significant improvement in the anticancer efficacy of paclitaxel and carboplatin were effective in improving survival of tumor bearing mice being treated by these agents.

Metformin-mediated Activation of the AMPK Pathway in Tumor Tissues is Dose-dependent. Since AMPK is regarded as the primary target of the antitumor efficacy of metformin, the activation of AMPK (reflected by its phosphorylation) in tumors from different treatment groups was evaluated and compared. Activation of AMPK in both MDA-MB-468 and MCF-7 tumors increased as the dose of metformin was escalated (**Figure 5.4**). With the same metformin dose, greater AMPK activation was observed in MDA-MB-468 tumors compared to MCF-7 tumors. In MDA-MB-468 tumors, 120 mg/kg/day and 360 mg/kg/day of metformin, in combination with carboplatin, caused a significantly greater AMPK activation compared to saline treatment, whereas in MCF-7 tumors, only the 360 mg/kg/day dose of metformin in combination with paclitaxel caused significantly greater AMPK phosphorylation compared to saline treatment. Carboplatin and paclitaxel as monotherapies did not activate AMPK nor did they interfere with the effect of metformin on AMPK activation. These results establish a clear association between activation of intracellular AMPK in tumor cells, metformin dose, and its antitumor efficacy, either as a monotherapy or in combination with other chemotherapeutic agents

Metformin PK in MCF-7 and MDA-MB-468 Tumor-bearing Mice. A PK study was conducted to evaluate and compare the systemic and intratumoral concentrations of metformin in tumor bearing mice during treatment of these mice with metformin in combination with paclitaxel or carboplatin. Metformin plasma concentrations as a function of time were plotted (**Figure 5.5A and B**) and the systemic exposure of metformin (reflected by the area under the plasma concentration-time curves, AUC_{plasma}) was calculated using non-compartmental analysis. The metformin AUC_{plasma} was comparable in mice bearing MDA-MB-468 and MCF-7 tumors, and

increased linearly as the metformin dose was elevated. In contrast, a 70%~80% higher intratumoral metformin concentration was observed at 1 hr and 24 hr post administration in MDA-MB-468 tumors compared to MCF-7 tumors (**Figure 5.5C and D**), which correlates with high cation-selective transporter expression in MDA-MB-468 tumors *versus* low transporter expression in MCF-7 tumors (Chapter 2). No difference in systemic and intratumoral concentrations of metformin was observed in mice on metformin monotherapy and in mice on metformin (same dose) plus carboplatin/paclitaxel combination therapy (**Supplementary Table 5.1**), suggesting that metformin PK was not affected by either paclitaxel or carboplatin. These results show that the different sensitivities of MDA-MB-468 and MCF-7 tumors to metformin treatment are due to differences in intratumoral exposure to metformin rather than systemic exposure.

Establish A PK Model to Estimate Metformin Intratumoral Exposure. To determine intratumoral metformin concentration-time profiles in tumor bearing mice treated with different doses of metformin, a population-based two-compartment PK model was developed (**Figure 5.1B**). The PK Parameters were estimated from the model and are listed in **Table 5.1**. A reasonable coefficient of variation (CV%) for all the estimated PK parameters suggested that the model appropriately described the variability in PK parameters among individual animals. The robustness of the model was further confirmed by visual predictive checks, which showed that most of the metformin plasma and intratumoral concentration data points fell within the simulated 90% confidence interval (**Figure 5.6**). The intratumoral exposures of metformin were simulated using the PK model. The intratumoral exposures of metformin in MDA-MB-468 tumors were more than 2-fold higher than the exposures in MCF-7 tumors following treatment with the same dose of metformin (**Table 5.2**).

5.5 DISCUSSION

Breast cancer is the second leading cause of cancer death among women in the United States. The current challenge in treating breast cancer is believed to be largely due to genetic

variability among different types of breast cancer, and a lack of efficacious chemotherapeutic agents for some types of breast cancer, such as TN breast cancer (3). Metformin, the most widely administered anti-diabetic agent, reduces breast cancer incidence (8), improves outcomes to chemotherapy (9), and prevents breast cancer metastasis and reoccurrence (10) in both type 2 diabetes and non-diabetic cancer patients. At the same time, other studies question the efficacy of metformin as an anticancer agent (12, 13). Because most of the evidence for anticancer efficacy of metformin, particularly for its efficacy against breast cancer, is derived from studies in which metformin was administered as an anti-diabetic agent, it is not surprising that the outcomes of such studies are variable and ambiguous since the doses used in these studies were not optimized for the anticancer efficacy of metformin. Hence, studies were performed to provide unambiguous evidence to confirm the anticancer efficacy of metformin against breast cancer, and to assess the dose-response relationship for metformin when it is used in combination with the established chemotherapeutic agents for ER+ and TN cancers.

Orthotopic xenograft mouse models of breast cancer were employed to evaluate the dose-response of metformin in breast cancer. ER+ tumors were produced with MCF-7 cells and TN tumors were produced with MDA-MB-468 cells. Of note, MCF-7 cells express low levels of cation-selective transporters whereas MDA-MB-468 cells exhibit high expression of multiple cation-selective transporters (Chapter 2.1). To simulate clinical situations, metformin was co-administered with paclitaxel to mice with ER+ (MCF-7) tumors and with carboplatin ER+ to mice with TN (MDA-MB-468) tumors. Metformin doses used in this preclinical study (360, 120, 36, and 12 mg/kg/day) were calculated from the maximum recommended human daily dose (2,550 mg) and two commonly administered doses (850 mg/day and 250 mg/day) for the treatment of patients with type 2 diabetes, as well as a sub-therapeutic dose (85 mg/day). Metformin was administered orally, and the two chemotherapeutic agents were administered intravenously, as is the case in the clinic. A metformin monotherapy group was included in the study (360 mg/kg/day) to enable interpretation of the combination chemotherapy results. Mice with MDA-

MB-468 tumors were treated for two months, whereas mice with MCF-7 tumors were treated for one month as MCF-7 tumors grow more aggressively when stimulated with estrogen pellets.

Metformin (360 mg/kg/day) inhibited both MCF-7 and MDA-MB-468 tumor growth, but to a lower extent than the inhibition of tumor growth by either paclitaxel or carboplatin alone. At the same dose, metformin was more effective in inhibiting the growth of MDA-MB-468 tumors than MCF-7 tumors. This is consistent with the greater efficacy of monotherapy and combination therapy against MDA-MB-468 tumors than against MCF-7 tumors. Metformin was also found to be efficacious at 120 mg/kg/day (given intraperitoneally) against MCF-7 tumors in studies reported in Chapter 4 (**Figure 4.2**). Metformin at a dose of 360 mg/kg/day improved the efficacy of paclitaxel and carboplatin against MCF-7 and MDA-MB-468 tumors (**Figure 5.2**), suggesting that metformin may prove to be useful in enhancing efficacy of currently accepted therapeutic agents for breast cancer. This could translate into improved control of the disease with combination therapy with metformin or reduction in the doses of the chemotherapeutic agents and less severe side effects. A minimum metformin dose of 120 mg/kg/day (equivalent to the 850 mg human daily dose generally used for anti-diabetic treatment) in combination with carboplatin (50 mg/kg/day) was required to observe significant improvement in antitumor efficacy in MDA-MB-468 tumors, whereas a metformin dose of 360 mg/kg/day (equivalent to the 2,550 mg maximum recommended daily dose for human anti-diabetic treatment) in combination with paclitaxel (30 mg/kg/day) was needed to observe significant increase in efficacy against MCF-7 tumors. Considering that an 850 mg daily dose of metformin is most frequently used in metformin cancer clinical trials, these results provide a rationale for the lack of significant improvement in treatment outcomes in some clinical studies where metformin was co-administered with chemotherapeutic agents.

Although chemotherapy in combination with a 360 mg/kg/day metformin dose exhibited greatest antitumor efficacy in all treatment groups, only a limited improvement in survival rates was observed when compared to survival rates with paclitaxel or carboplatin monotherapy

(**Figure 5.3**). This may be due to increased toxicity when a high metformin dose of 360 mg/kg/day is used in combination with a chemotherapeutic agent compared to the effect of the chemotherapeutic agent alone. Interestingly, metformin at lower doses (e.g. 120 mg/kg/day) in combination with paclitaxel or carboplatin improved survival of the animals over that observed with these chemotherapeutic agents alone.

This study revealed that differences in metformin PK contributed to the differences in the sensitivity of MDA-MB-231 tumors and MCF-7 tumors to metformin treatment. Metformin plasma concentration-time profiles were comparable between mice bearing MDA-MB-468 tumors and MCF-7 tumors (**Figure 5.5**), but the tumor levels of metformin at 1 hr time point at different doses were nearly 2-fold higher in the MDA-MB-468 tumors than in the MCF-7 tumors; further, the difference in the metformin levels between the two tumor types at 24 hr was several fold. The two-compartment PK model developed in this study predicts that the exposure of both type of tumors to metformin is dose-dependent, and that exposure of MDA-MB-468 tumors to metformin over a 24 hr period is expected to be nearly 2-fold greater than exposure of MCF-7 tumors to the drug (**Figure 5.6 and Table 5.2**). The greater exposure of the MDA-MB-468 tumors to metformin can be attributed to higher expression of cation-selective transporters and subsequent greater transporter-mediated metformin uptake in these tumors compared to the MCF-7 tumors (Chapter 2). These results suggest that response to metformin therapy may be predicted based on transporter expression profiles in tumor tissues. Further, the level of AMPK phosphorylation in tumor tissues increased with increased dose of metformin, and as expected, the level of phosphor-AMPK was greater in the MDR-MB-468 tumors than in MCF-7 tumors corresponding to higher cation-selective transporter expression in MDR-MD-468 tumors and greater efficacy of metformin against this tumor (**Figure 5.4**). Thus AMPK, in addition to the expression of cation-selective transporters, in the tumor tissue may prove to be a good biomarker to assess the antitumor efficacy of metformin. AMPK is widely believed to be the primary intracellular target of the anticancer efficacy of metformin and it has been evaluated as

a biomarker to predict success of metformin cancer therapy previously, although without a definitive conclusion (28). The present study provides support to using AMPK as a biomarker for metformin therapy.

The simulated intratumoral concentration data from the population-based two-compartment PK model developed in this study can be used to create the PK-pharmacodynamic (PD) model. Such a PK-PD model can be used to estimate the efficacy of combination therapy of paclitaxel or carboplatin and metformin at doses that are higher than the highest dose used in this study, i.e. 360 mg/kg/day. In conclusion, this study systematically evaluated the antitumor efficacy of metformin as a monotherapy and in combination with paclitaxel or carboplatin for the treatment of ER+ or TN breast cancer, respectively. The results clearly suggest that metformin would be beneficial as a combination therapy for breast cancer, and provide the first direct evidence that a different minimum dose of metformin is required to achieve sufficient intratumoral exposure and antitumor efficacy of chemotherapeutic agents in different tumor types. The study also showed that cation-selective transporter expression levels and transporter-mediated metformin uptake into tumors directly affect the sensitivity of breast tumors to metformin treatment. Thus, screening transporter expression profiles in tumor biopsies from breast cancer patients would be beneficial in determining the initial metformin dose required for cancer therapy. Since chemotherapeutic agents are generally more toxic than metformin, investigating whether coadministration of metformin reduces the current dose of chemotherapeutic agents, without affecting treatment outcomes, could be an area of further investigation.

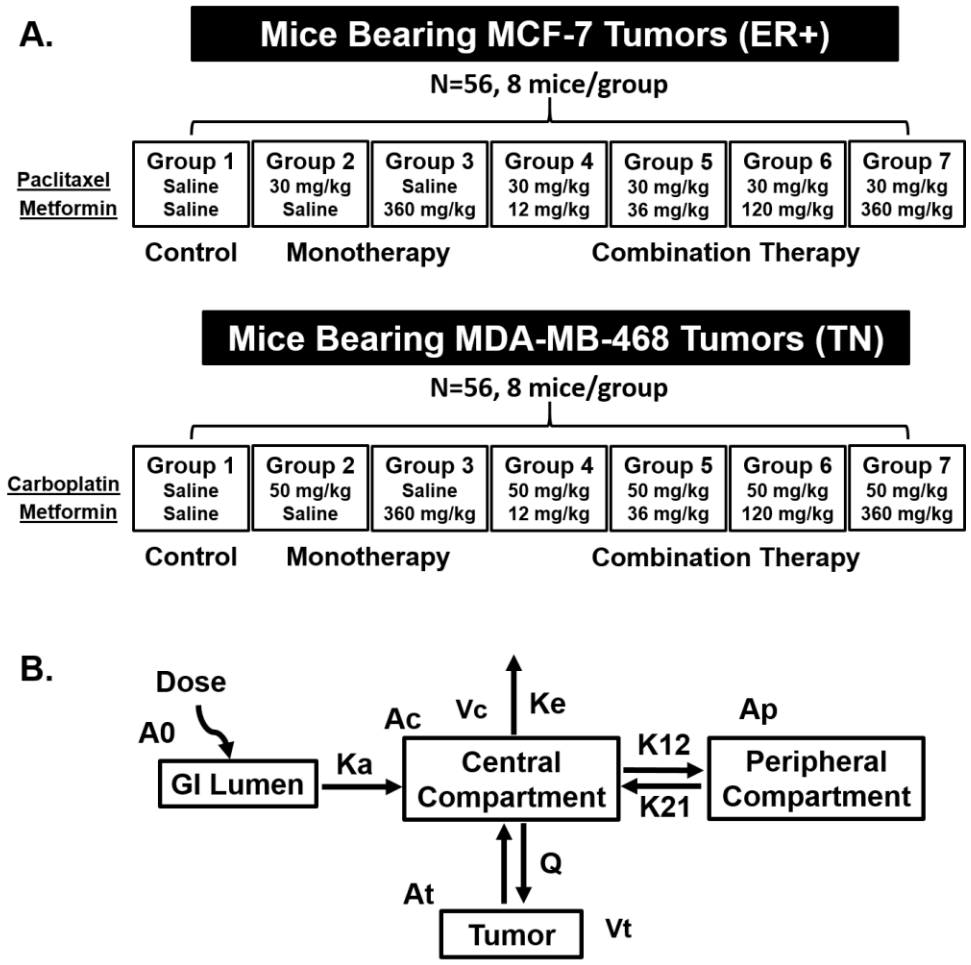

Figure 5.1 Design of the *In Vivo* Study to Evaluate the Anticancer Efficacy of Metformin as a Monotherapy and in Combination with Chemotherapeutic Agents Paclitaxel (for MCF-7 Tumors) and Carboplatin (for MDA-MB-468 Tumors) (**A**) Treatment group assignment for mice bearing MCF-7 tumors and MDA-MB-468 tumors. (**B**) Schematic of the two-compartment PK model used for estimating time profiles of intratumoral concentrations of metformin. V: Volume of distribution in each compartment. Q: Intercompartmental clearance. K: Rate constant. A: Amount of metformin in each compartment.

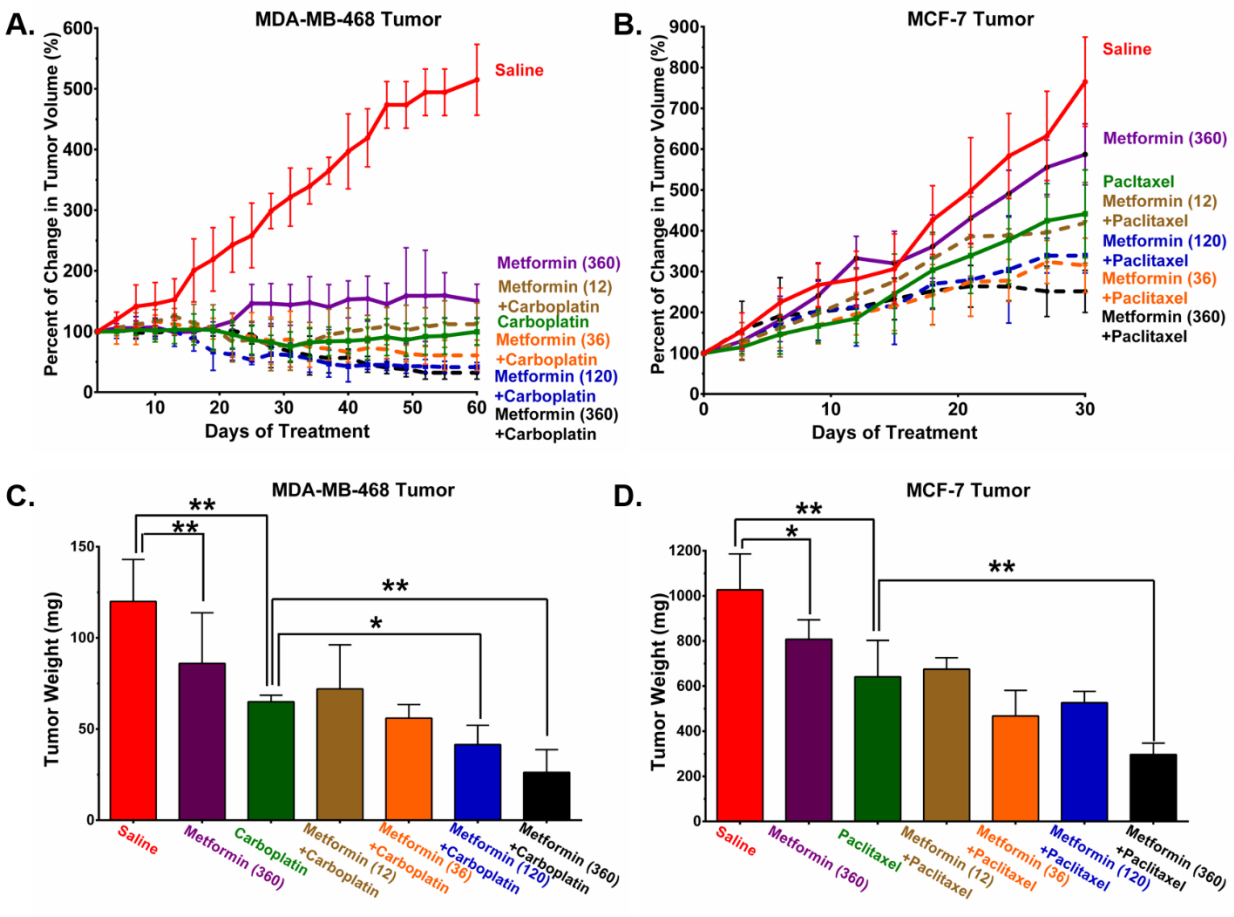

Figure 5.2 Antitumor Efficacy of Metformin as a Monotherapy and in Combination with Carboplatin (for MDA-MB-468 Tumors) and Paclitaxel (for MCF-7 Tumors). The progression of MDA-MB-468 tumors (**A**) and MCF-7 tumors (**B**) under different treatments was assessed by measuring tumor volume. Monotherapy is represented as solid lines and combination therapy as dashed lines. Weights of MDA-MB-468 tumors (**C**) and MCF-7 tumors (**D**) isolated from euthanized mice at the end of the treatment period were compared among different treatment groups. Data represent mean ± SD. N=6. * signifies $p<0.05$. ** signifies $p<0.01$.

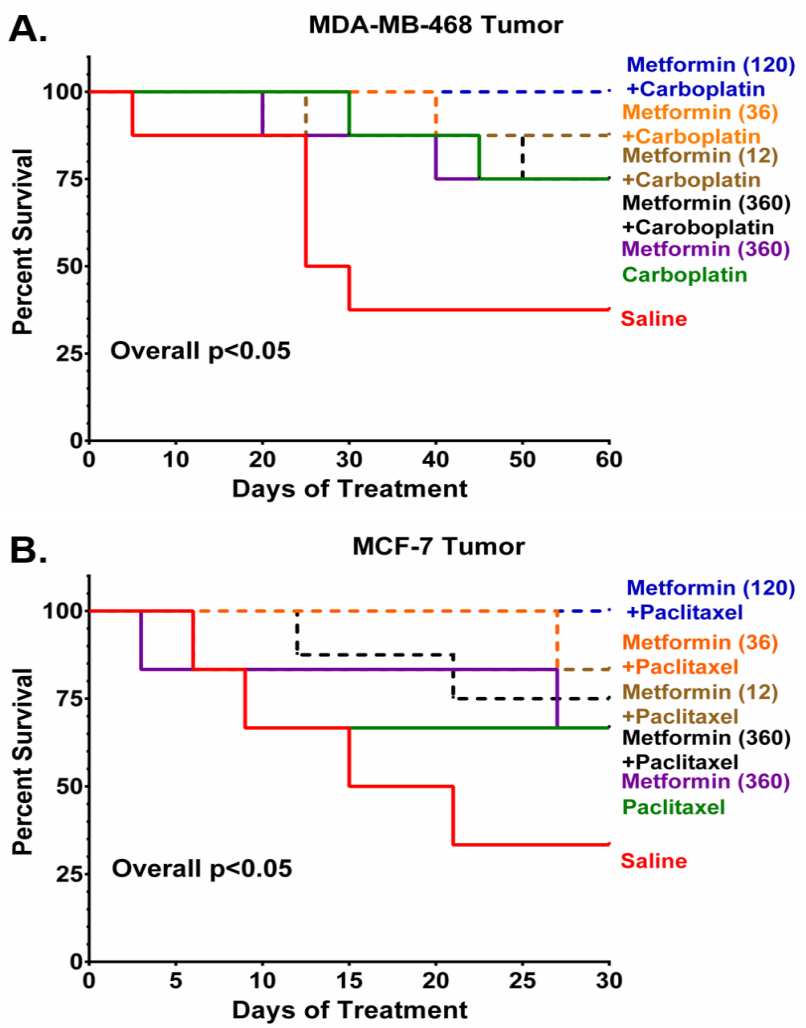

Figure 5.3 Kaplan–Meier Survival Curves Showing the Effect of Metformin Treatment as a Monotherapy and in Combination with (A) Carboplatin or (B) Paclitaxel on the Survival of Mice Bearing MDA-MB-468 or MCF-7 Tumors, Respectively. Monotherapy is represented as solid lines and combination therapy as dashed lines. Mice on a combination therapy of carboplatin or paclitaxel and metformin (12 mg/kg/day, 36 mg/kg/day and 120 mg/kg/day) had higher survival rates compared to carboplatin and paclitaxel monotherapy, but combination with 360 mg/kg/day metformin reduced the survival rate of mice. N=8 mice per treatment group.

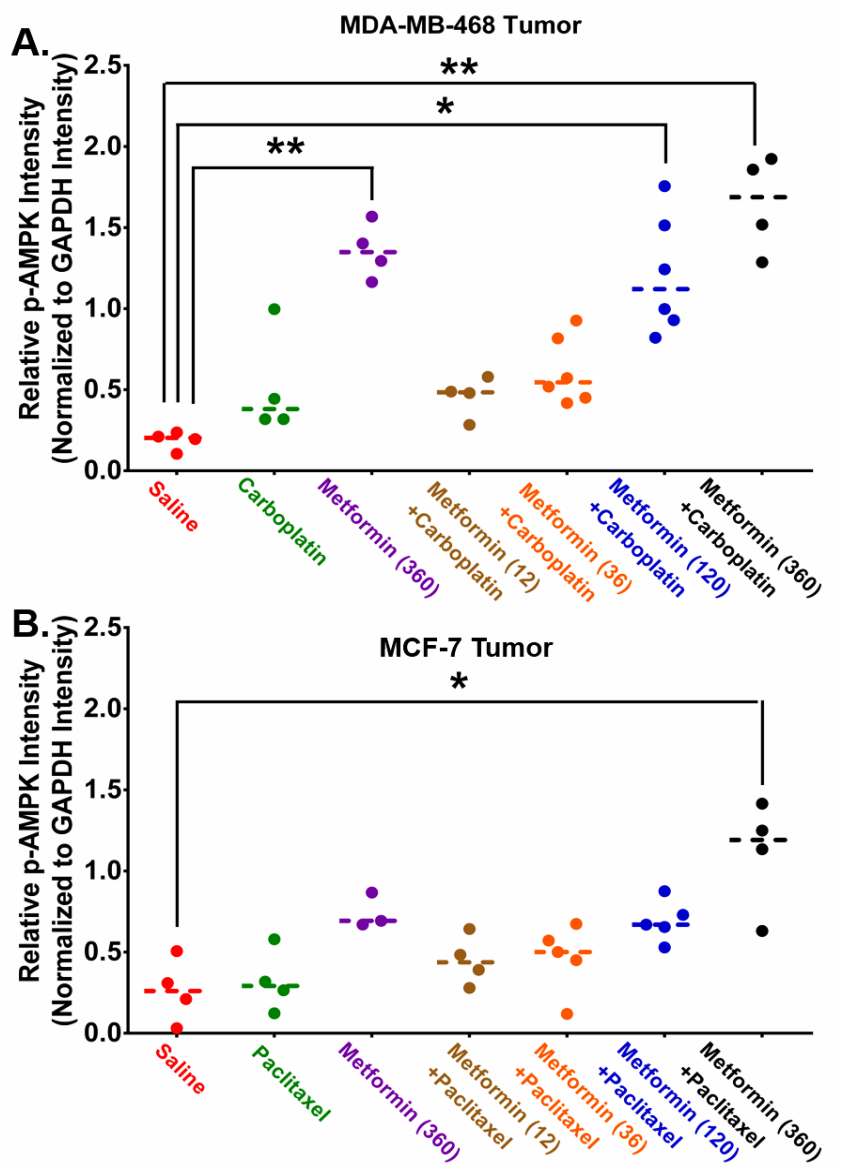

Figure 5.4 Activation of AMPK Caused by Metformin and/or Paclitaxel and Carboplatin in MDA-MB-468 Tumors (A) and MCF-7 Tumors (B). Tumor tissues from mice euthanized at the end of treatment were lysed and analyzed by Western blot analyses for the extent of AMPK phosphorylation, using GAPDH as a loading control. Data are represented as scatter points and the median value of each group is represented as a dashed line. * signifies $p<0.05$. ** signifies $p<0.01$

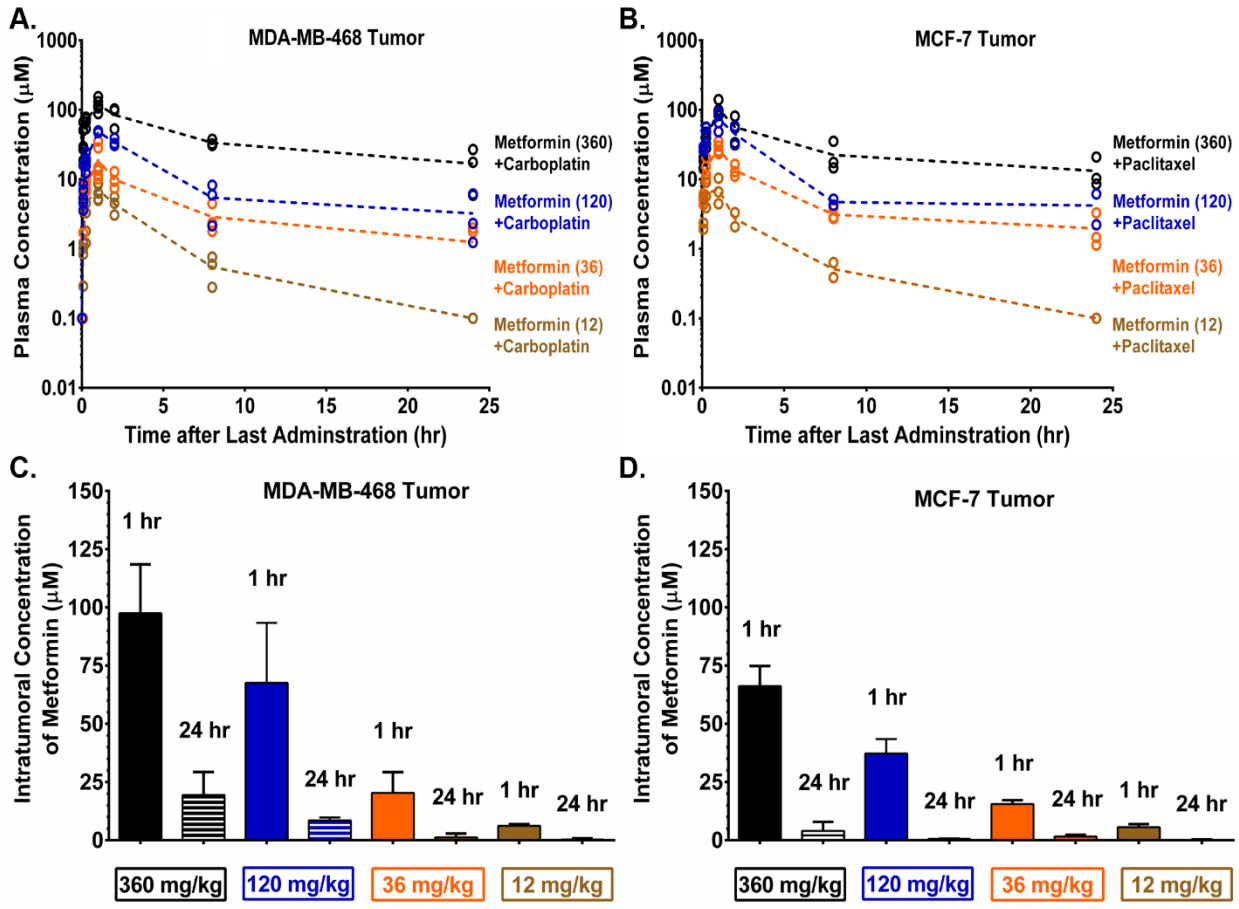

Figure 5.5 Plasma and Intratumoral Concentrations of Metformin in Mice Treated with Different Doses of Metformin. Metformin plasma concentrations *versus* time profiles in mice bearing MDA-MB-468 tumors (**A**) and MCF-7 tumors (**B**). Plasma concentration data points are represented as open circles and time profiles of the mean plasma concentration of metformin are represented as dashed line. Tumor tissues were isolated at 1 hr or 24 hr after administration of [^{14}C]metformin. Intratumoral concentrations of metformin in MDA-MB-468 tumors (**C**) and MCF-7 tumors (**D**) are represented as mean± SD. N=3.

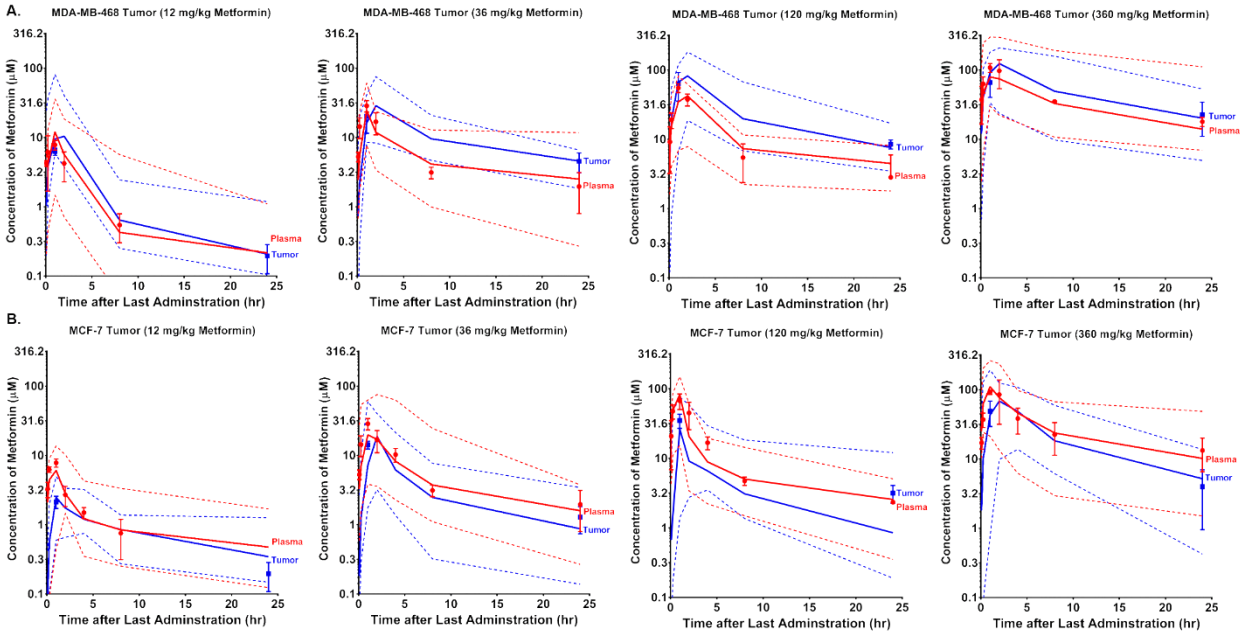

Figure 5.6 Visual Predictive Checks of the PK Model to Predict Plasma and Tumor Metformin Concentrations as a Function of Time in Tumor-bearing Mice. Simulated profiles of metformin plasma (red) and intratumoral (blue) concentrations as a function of time in mice bearing MDA-MB-468 tumors (**A**) and MCF-7 tumors (**B**). Simulated mean concentrations are represented as solid lines and the simulated 90% confidence interval is reflected by dashed lines.

MDA-MB-468					MCF-7				
Parameter	Estimate	Units	Stderr	CV%	Parameter	Estimate	Units	Stderr	CV%
tvVc	3.10	L/kg	0.40	13.02	***tvVc***	3.03	L/kg	1.09	36.10
tvVt	1.29	L/kg	0.44	34.17	***tvVt***	1.18	L/kg	0.45	37.92
tvka	0.58	1/hr	0.06	9.65	***tvka***	0.75	1/hr	0.23	30.45
tvk12	1.38	1/hr	0.28	20.55	***tvk12***	0.10	1/hr	0.32	32.24
tvk21	0.31	1/hr	0.10	31.27	***tvk21***	0.12	1/hr	0.02	18.18
tvke	0.59	1/hr	0.07	11.91	***tvke***	0.58	1/hr	0.19	32.49
tvQ	10.34	L/(kg*hr)	5.18	50.09	***tvQ***	1.23	L/(kg*hr)	0.57	46.19
stdev0	0.39		0.06	14.04	***stdev0***	0.28		0.05	18.33
stdev1	0.72		0.11	15.11	***stdev1***	0.69		0.14	20.55

Table 5.1 Estimates of the PK Parameters Calculated Using the Model Described in Figure 5.1 for Mice Bearing MDA-MB-468 Tumors and MCF-7 Tumors.

Metformin Dose (mg/kg)	MDA-MB-468 AUC_{Tumor} (μM*hr)	MCF-7 AUC_{Tumor} (μM*hr)	Ratio ($AUC_{MDA\text{-}MB\text{-}468}/AUC_{MCF\text{-}7}$)
12	56 (22-269)	20 (8-44)	2.8
36	158 (103-594)	66 (15-286)	2.4
120	634 (174-1645)	195 (66-994)	3.2
360	1236 (261-3101)	516 (122-1449)	2.4

Table 5.2 Summary of the Simulated Intratumoral Exposures of Metformin in Tumor-bearing Mice Treated with Varying Doses of Metformin. Data are represented as mean (90% confidence interval).

Treatment	MDA-MB-468 AUC_{Tumor} (μM*hr)	MCF-7 AUC_{Tumor} (μM*hr)
Metformin (360 mg/kg)	945 (202)	892 (61)
Metformin (360 mg/kg) + Carboplatin/Paclitaxel	917 (133)	865 (123)
P Value	0.59	0.75

Supplementary Table 5.1 Systemic Exposures of Metformin in Tumor-bearing Mice Treated with 360 mg/kg/day Metformin *versus* 360 mg/kg/day Metformin and Carboplatin or Paclitaxel. Data are represented as mean (SD).

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CHAPTER 6

Antiproliferative Efficacy of Metformin against Breast Cancer Cells is Enhanced by Inhibition of the Insulin/IGF1 Pathway

6.1 OVERVIEW

The effect of metformin on reducing breast cancer occurrence has been observed in several retrospective clinical studies. However, in several prospective clinical trials of metformin as an anticancer agent, breast cancer patients with type 2 diabetes generally had a better response than non-diabetic breast cancer patients to the antitumor efficacy of metformin. Since metformin suppresses the secretion of insulin and insulin-like growth factor (IGF)1 in breast cancer patients with type 2 diabetes, but not in non-diabetic cancer patients, and since insulin and IGF1 promote breast cancer cell proliferation, it was hypothesized that the modulation of insulin/IGF1 through the anti-diabetic pharmacology of metformin sensitizes breast cancer cells to its antiproliferative activity. *In vitro* studies using cell culture media devoid of insulin and IGF1 increased the sensitivity of MCF-7 cells to the antiproliferative efficacy of metformin. Further analysis suggested that sensitization of MCF-7 cells to metformin was mediated by the intracellular target of metformin, namely the adenosine monophosphate-activated protein kinase (AMPK) pathway, rather than through modulation of intracellular uptake of the drug via increased expression of cation-selective transporters. Inhibition of the insulin pathway via knockdown of the insulin receptor substrate (IRS)1, a key mediator in the insulin pathway, improved sensitivity of MCF-7 cells to metformin beyond that expected by attenuation of the effect of insulin and IGF1 on promoting cell growth. These results suggest that metformin can be used as a combination therapy with current chemotherapeutic agents such as inhibitors of

phosphoinositide 3-kinase (PI3K), a signaling molecule downstream to IRS1 in the insulin pathway, so as to enhance efficacy in non-diabetic breast cancer patients.

6.2 INTRODUCTION

Retrospective analyses show that type 2 diabetes patients on long-term metformin treatment exhibit a lower risk of developing breast cancer compared to patients on other types of anti-glycemic therapy, such as insulin and sulfonylurea (1-3). These data led to prospective clinical studies to evaluate the efficacy of metformin in breast cancer therapy; the patient population in these studies included both diabetic and non-diabetic breast cancer patients. In the diabetic patients, metformin improved response to chemotherapy (4), inhibited cancer cell proliferation in breast tumor tissues (5), and improved metastasis-free survival following chemotherapy (6). In contrast, no significant beneficial effects of metformin were observed in non-diabetic patients with breast cancer (5-9), although a trend towards better outcomes was reported in some studies. These data suggest that current metformin therapy for non-diabetic breast cancer patients may be inadequate and needs to be optimized.

In previous chapters of this dissertation, two strategies were proposed to optimize metformin treatment for breast cancer: 1) identify breast cancer patients who are most suitable for metformin cancer therapy using cation-selective transporter expression in tumor tissues as a biomarker, and 2) increase the dose of metformin in patients with breast tumors that exhibit low cation-selective transporter expression levels, so as to overcome insufficient uptake of metformin into tumor cells. This chapter details a new strategy to enhance the sensitivity of non-diabetic breast cancer patients to metformin treatment. This strategy involves exploiting differences in the underlying molecular mechanisms of the antitumor activity of metformin in diabetic and non-diabetic breast cancer patients.

The molecular mechanisms involved in the anticancer effects of metformin have been widely studied. The adenosine monophosphate-activated protein kinase (AMPK) pathway is the primary target of metformin in the tumor cells of diabetic and non-diabetic breast cancer

patients. Upon cation-selective transporter-mediated uptake into tumor cells, metformin activates AMPK, attenuates phosphorylation of P70S6K, a downstream molecule of AMPK, and subsequently inhibits breast cancer cell proliferation and induces apoptosis (10-11). In diabetic breast cancer patients, the pharmacology of metformin manifested in the treatment of the disease also contributes to the attenuation of tumor cell proliferation by lowering circulating insulin and insulin-like growth factor (IGF)1 levels. This results in suppression of insulin-mediated tumor growth. Insulin and IGF1 bind to their receptors on the cell membrane and stimulate phosphorylation of the insulin-receptor substrate (IRS)-1, and thus activate the insulin pathway and promote cancer cell proliferation (12). Activation of the insulin pathway also modulates multiple signaling molecules including phosphoinositide 3-kinase (PI3K) and Protein kinase B (PKB, Akt) (13). The literature reports an upregulation of insulin and IGF1 receptors on breast cancer cell membranes (14), which enhances the sensitivity of these cells to changes in insulin and IGF1 levels affected by metformin therapy in diabetes.

The potential interaction between extracellular insulin/IGF1 and the intracellular AMPK pathway in response to metformin treatment has been implied in some studies. Zakikhani et al., showed a greater inhibition of cell proliferation by metformin with the removal of both insulin and IGF1 from the culture media (11); however, this study did not investigate the underlying mechanisms involved. Others have demonstrated a potential role of metformin-mediated activation of the AMPK pathway in the expression of insulin and IGF1 receptors on breast cancer cell membranes (15). A systematic investigation was undertaken to elucidate a possible interaction between inhibition of the insulin pathway and activation of the AMPK pathway by metformin.

6.3 MATERIAL AND METHODS

Generation of IRS-1 Knockdown MCF-7 (MCF-7$^{IRS\text{-}1\ KD}$) Cells. The MCF-7 breast cancer cell line, which has a high expression level of insulin and IGF1 receptors compared to other breast cancer cell lines, was employed for these studies and cultured under the same conditions

described in Chapters 2 and 4. A TRC2 plasmid containing a puromycin-resistant gene and shRNA sequence that specifically targets human IRS-1 (SHCLNG-NM_010570, Sigma Aldrich) was transfected into MCF-7 cells using the AMAXA Nucleofection^{TM} system (AAB-100, Lonza). The transfected cells were seeded on a 100mm cell culture dish (100-62-878, VWR) at a low density, and cultured for three weeks in selection media containing 0.5 µg/mL puromycin (A1113802, Gibco) until puromycin-resistant colonies were observed. A single MCF-7$^{IRS\text{-}1KD}$ clone with low IRS-1 expression was identified by real-time polymerase chain reaction (RT-PCR) analysis of IRS-1 gene expression as described below. MCF-7 cells stably transfected with a non-target shRNA sequence (MCF-7$^{T\text{-}CTRL}$) were generated using the same method described above and used as a transfection control.

Determination of Expression of IRS-1 Genes and Cation-selective Transporter Genes. MCF-7 cells**,** MCF-7$^{T\text{-}CTRL}$ cells, and MCF-7$^{IRS\text{-}1\ KD}$ cells were cultured in serum-deprived media in the presence or absence of insulin (5 µg/mL) and IGF1 (40 ng/mL) for 48 hours and lysed in QIAzol Lysis Reagent (79306, Qiagen) to isolate total RNA. cDNA was synthesized from total RNA and subjected to RT-PCR as described in Chapters 2 and 4 to determine the expression of IRS-1, plasma membrane monoamine transporter(PMAT) and multidrug and toxin extrusion protein (MATE)1 genes. The expression of endogenous 18s rRNA was used as a loading control.

Evaluation of the Inhibition of Cell Proliferation by Metformin. MCF-7 cells**,** MCF-7$^{T\text{-}CTRL}$ cells, and MCF-7$^{IRS\text{-}1\ KD}$ cells cultured in serum-deprived media in the presence or absence of insulin (5 µg/mL) and IGF1 (40 ng/mL) were exposed to 10 mM metformin for 48 hours. The antiproliferative efficacy of metformin in each treatment group was assessed by the Promega CellTiter 96® Cell Proliferation Assay (G3582, Promega).

Measurement of Metformin Intracellular Uptake. MCF-7 cells**,** MCF-7$^{T\text{-}CTRL}$ cells, and MCF-7$^{IRS\text{-}1\ KD}$ cells were seeded on 24-well plates at a density of 75,000 cells/cm^2. After reaching 90% confluency, the cell culture media was replaced with serum-deprived media with or without

insulin (5 µg/mL) and IGF1 (40 ng/m), and cells were pre-incubated for 48 hours. The intracellular uptake of metformin was evaluated by incubating 50 µM [^{14}C]metformin for 5 min in the presence or absence of 500 µM of the pan transporter inhibitor, quinidine. Details of experimental procedure are described in Chapters 2 and 4.

Assessment of Intracellular AMPK Activation. Western blot analysis was performed to evaluate activation of the AMPK pathway (reflected by increased AMPK phosphorylation and attenuated P70S6K phosphorylation) following metformin (10 mM) treatment for 48 hours in serum-deprived media with or without insulin (5 µg/mL) and IGF1 (40 ng/mL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Details of the Western blot assay are provided in Chapters 2 and 4.

Statistical Analyses. One-way analysis of variance (ANOVA) followed by Tukey's test was applied to determine statistical significance in the differences between experimental groups in IRS-1 gene expression, cell proliferation, and intracellular uptake of metformin. Statistical analyses were conducted by GraphPad® (GraphPad Inc.,CA).

6.4 RESULTS

Generation of MCF-7$^{IRS\text{-}1KD}$ and MCF-7$^{T\text{-}CTRL}$ Cell Lines. MCF-7$^{IRS\text{-}1\ KD}$ cells, in which IRS-1 was stably knocked down, exhibited greater than 70% reduction in IRS-1 gene expression compared to MCF-7 cells ($p<0.001$, **Figure 6.1**). To ensure that any difference in cellular behavior and response to metformin treatment in MCF-7$^{IRS\text{-}1\ KD}$ cells, compared to wildtype MCF-7 cells, was only due to IRS-1 knock-down and not the transfection process, MCF-7 cells stably expressing a non-target shRNA sequence (namely MCF-7$^{T\text{-}CTRL}$ cells) were used as a transfection control. No difference in IRS-1 gene expression was observed between MCF-7 cells and MCF-7$^{T\text{-}CTRL}$ cells (**Figure 6.1**) suggesting that transfection did not alter IRS-1 gene expression. Therefore, in subsequent *in vitro* studies, the role of the insulin pathway in response to metformin treatment was evaluated and compared between MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells.

Inhibition of the Insulin Pathway Enhanced Sensitivity to the Antiproliferative Efficacy of Metformin. Metformin at a concentration of 10 mM inhibited proliferation of MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells in the absence of insulin/IGF1 (**Figure 6.2A and B**). In MCF-7$^{T\text{-}CTRL}$ cells, the antiproliferative effect of metformin was reversed by the addition of insulin/IGF1 to the culture media (**Figure 6.2A**). In contrast, addition of insulin/IGF1 to the culture media did not reverse the antiproliferative activity of metformin in MCF-7$^{IRS\text{-}1\ KD}$ cells (**Figure 6.2B**), suggesting that the antiproliferative effect of metformin is countered by the active insulin pathway; stated alternatively, the sensitivity of breast cancer cells to the antiproliferative effects of metformin is enhanced when the insulin pathway is attenuated either by removing extracellular insulin/IGF1 or knocking down IRS-1, which is a modulator of the insulin pathway (**Figure 1.1**). In the absence of metformin, the removal of insulin/IGF1 from the culture media had no impact on the proliferation of MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells (**Figure 6.2A and B**), suggesting that a 48-hour exposure of breast cancer cells to insulin/IGF1 does not enhance cell proliferation.

Inhibition of the Insulin Pathway Increased Activation of the AMPK Pathway by Metformin. Western blot analysis showed that, in both MCF-7$^{IRS\text{-}1\ KD}$ and MCF-7$^{T\text{-}CTRL}$ cells, metformin caused activation of the AMPK pathway (reflected by an increase in AMPK phosphorylation and decrease in P70S6K phosphorylation) whether insulin/IGF1 was present or absent in the culture media (**Figure 6.3, lanes 1, 3, 5 and 7**). However, modulation of the AMPK pathway by metformin was less pronounced in the presence of insulin/IGF1 (**Figure 6.3, lanes 1 and 2**) than in the absence of insulin/IGF1 (**Figure 6.3, lanes 3 and 4**). As would be expected, modulation of the AMPK pathway by metformin in MCF7$^{IRS\text{-}1\ KD}$ cells was not sensitive to the presence or absence of insulin/IGF1 (**Figure 6.3, lanes 5 and 6**), and was comparable under both conditions; further, the modulation in the AMPK pathway by metformin in these IRS-knocked down cells was comparable to that in MCF-7$^{T\text{-}CTRL}$ observed in the absence of insulin/IGF1 (**Figure 6.3, lanes 3 and 4**). These data suggest that insulin/IGF1 suppresses the

modulation of the AMPK pathway and this could lead to suppression of antiproliferative activity of metformin by insulin/IGF1 (**Figure 6.2**).

Inhibition of the Insulin Pathway Had Limited Effects on Transporter-mediated Metformin Uptake. The results reported in Chapter 2 showed that transporter-mediated metformin uptake was required for modulation of the AMPK pathway. Therefore, it is important to determine if insulin/IGF1 sensitized the metformin-mediated modulation of AMPK pathway by simply increasing the expression of metformin transporters and thus cellular uptake of metformin. This was achieved by evaluating expression of the two predominant cation-selective transporters in MCF-7 cells, namely PMAT and MATE1 (Chapter 2), in the transfected MCF-7$^{IRS\text{-}1\ KD}$ and MCF-7$^{T\text{-}CTRL}$ cells upon treatment of the cells with insulin/IGF1, and correlating changes in the transporter expression to changes in intracellular metformin uptake. Insulin/IGF1 caused approximately 100% increase in PMAT gene expression in MCF-7$^{T\text{-}CTRL}$ cells but had no effect on the expression of MATE1 gene (**Figure 6.4**). In contrast, insulin/IGF1 had no effect on the expression of both PMAT and MATE1 gene in MCF-7$^{IRS\text{-}1\ KD}$ cells (**Figure 6.4**). Interestingly, expression of both PMAT and MATE1 gene was significantly lower in the MCF-7$^{IRS\text{-}1\ KD}$ cells than in the MCF-7$^{T\text{-}CTRL}$ cells regardless of treatment with insulin/IGF1 (**Figure 6.4**).

Metformin uptake over 5 min was assessed in MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells to determine if there is any association between the differences in expression of PMAT and MATE1 genes and intracellular uptake of metformin. Although cells with intact insulin pathway and cells fed with insulin/IGF1 had higher expression of PMAT and MATE1 genes, this did not result in an increased uptake of metformin (**Figure 6.5**). These results suggest that the higher sensitivity of the AMPK pathway to metformin treatment was not due to an increase in transporter-mediated metformin uptake.

6.5 DISCUSSION

The number of clinical studies focusing on repurposing the anti-diabetic drug metformin as a therapeutic agent for breast cancer has been increasing in recent years. Clinical trials have

shown that breast cancer patients with type 2 diabetes generally had a better response to the anticancer efficacy of metformin compared to non-diabetic breast cancer patients (4-9). Understanding the mechanisms responsible for this difference in treatment outcomes to metformin between the two patient populations will be critical to determining whether metformin would be suitable as a therapeutic agent in non-diabetic breast cancer patients, and will provide insights into improving current metformin therapy for breast cancer. The pharmacology of metformin in breast cancer patients with diabetes, as in other diabetic patients, involves inhibition of hepatic gluconeogenesis that results in a reduction in circulating levels of glucose, insulin, and growth factors like IGF1. It is important to note that these nutrients and growth factors contribute to the generation and growth of breast tumors (16-17). Although breast cancer cells are known to grow faster under hyperglycemic conditions *versus* under normal glucose levels (18), clinical observations suggest that the simple reduction of circulating glucose level by metformin does not improve its anticancer efficacy as other anti-glycemic agents such as insulin and sulfonylurea increase breast cancer incidence (1-3). Unlike insulin and sulfonylurea, metformin enhances insulin sensitivity (19, 20) and subsequently suppresses the secretion of insulin and IGF1 (21). Since insulin and IGF1 have been reported to stimulate breast cancer cell proliferation through activating the insulin pathway (12-14), it is possible that the inhibitory effects of metformin on insulin and IGF1 secretion reduces the stimulus for tumor growth and/or improves the sensitivity of breast tumors to its antitumor efficacy of metformin via other mechanisms, such as modulation of the AMPK-mTOR-P70S6K pathway.

In this chapter, a strategy was developed to evaluate the interplay between the anti-diabetic and anticancer effects of metformin in an *in vitro* system. The anti-diabetic effect of metformin that is observed in diabetic patients was simulated *in vitro* by regulating insulin/IGF1 levels in cell culture media. MCF-7 human breast cancer cells with high insulin and IGF1 receptor expression were used in this study as they are more sensitive to changes in insulin/IGF1 levels in the culture media compared to other human breast cancer cell lines. MCF-

7 cells were allowed to grow in cell media containing metformin but with low levels of insulin/IGF1 to simulate the condition of diabetes patients on metformin treatment. In an alternative approach, the insulin signaling pathway was attenuated/disabled by knocking down one of the key modulator of this pathway, namely, IRS-1. Since IRS-1 is a signaling molecule downstream to insulin and IGF1 receptors, reducing its expression should reduce any effect that insulin/IGF1 may have on metformin-mediated effects on cell proliferation.

These *in vitro* studies showed that the antiproliferative efficacy of metformin in breast cancer cells was enhanced when the insulin pathway was inhibited either by deprivation of insulin/IGF1 or knockdown of IRS-1 (**Figure 6.2**). Removal of insulin/IGF1 from the culture media by itself had no effect on cell proliferation, ruling out that this enhancement in metformin antiproliferative activity was due to a metformin-independent effect. It is postulated that attenuating the insulin pathway increases the antiproliferative efficacy of metformin by causing a greater modulation of the AMPK pathway in breast cancer cells by the drug (**Figure 6.3**). The results further showed that the higher sensitivity of AMPK to metformin treatment caused by inhibition of the insulin pathway was not due to an increase in transporter-mediated intracellular metformin uptake (**Figure 6.4**). Instead, Western blot analysis showed that inhibition of the insulin pathway modulated the baseline phosphorylation levels of AMPK and P70S6K prior to metformin treatment (**Figure 6.3**). Since several studies have reported that activation of Akt1 and 2, which are downstream molecules of IRS1, modulates the activation of AMPK and P70S6K in skeletal muscle cells (21) and hepatocytes (22), it is possible that the insulin pathway also impacts the AMPK pathway in human breast cancer cells through the insulin/IGF1 receptor-IRS-1-Akt signaling cascade.

Attempts were made to elucidate the interplay between the insulin and AMPK pathways *in vivo* by developing diabetic xenograft mice bearing MCF-$7^{T\text{-}CTRL}$ tumors and MCF-$7^{IRS\text{-}1\ KD}$ tumors. However, only the mice injected with MCF-$7^{IRS\text{-}1\ KD}$ cells were able to develop sizeable tumors, whereas mice injected with MCF-$7^{T\text{-}CTRL}$ cells developed very small tumors that failed to

grow (**Supplementary Figure 6.1A**). Although *in vitro* studies showed that MCF-7$^{T\text{-}CTRL}$ cells and wildtype MCF-7 cells had comparable levels of IRS-1 gene expression (**Figure 6.1**) as well as similar responses to the antiproliferative activity of metformin and similar intracellular metformin uptake (**Supplementary Figure 6.2**), the growth of MCF-7$^{T\text{-}CTRL}$ tumors was unexpectedly slower than that of wildtype MCF-7 tumors (**Supplementary Figure 6.1A**). Thus the MCF-7$^{T\text{-}CTRL}$ clone selected in this study was only suitable for *in vitro* studies. Interestingly, analysis of tumor tissues showed noticeably fewer blood vessels in MCF-7$^{T\text{-}CTRL}$ tumors compared to MCF-7$^{IRS\text{-}1\ KD}$ and wildtype MCF-7 tumors (**Supplementary Figure 6.1B**), suggesting impaired angiogenesis in MCF-7$^{T\text{-}CTRL}$ tumors.

Despite these unsuccessful attempts at generating xenograft mice bearing MCF-7$^{T\text{-}CTRL}$ tumors, preliminary studies were conducted in which results from the xenograft mice bearing MCF-7$^{IRS\text{-}1\ KD}$ tumors (data obtained in this study) were compared with the results from the wildtype MCF-7 tumors (data from Chapter 5). Compared to wildtype MCF-7 tumors, MCF-7$^{IRS\text{-}1\ KD}$ tumors had a slower endogenous growth rate and were more sensitive to the antitumor effects of a high dose of metformin (360 mg/kg/day) monotherapy (**Supplementary Figure 6.3**). These results indirectly demonstrate that, similar to the results from the *in vitro* studies, inhibition of the insulin pathway in tumor tissues improved the sensitivity of tumors to metformin treatment. In future studies, an MCF-7$^{T\text{-}CTRL}$ clone will be identified that has an endogenous growth rate and response to metformin treatment similar to wildtype MCF-7 cells so as to successfully develop xenograft mice bearing MCF-7$^{T\text{-}CTRL}$ tumors. Diabetes will be induced in xenograft mice bearing MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ tumors by feeding the animals a high-fat high-carbohydrate diet, and the interplay between the anti-diabetic and anticancer effects of metformin in these mice will be evaluated.

The results in this study suggest that the antitumor efficacy of metformin in diabetic patients is enhanced by the decrease in insulin/IFG1 levels caused by the drug therapy. Clearly, altering circulating insulin and IGF1 levels in non-diabetic patients is not a good option as insulin

and IGF1 also impact the physiology of other organs such as liver, pancreas, intestine, and muscles (23- 25). Hence, a more appropriate strategy would be to attenuate one or more signaling molecules in the insulin pathway of cancer cells. PI3K inhibitors are a category of cancer drugs that exert their anticancer effects by targeting the insulin pathway. However, several clinical studies using PI3K inhibitors reported a substantial patient dropout rate in the treatment group *versus* the placebo group, which was due to the adverse effects possibly caused by insulin resistance or hyperinsulinemia (26, 27). Based on this report and the results from the present study, it is proposed that a combination therapy of metformin and a PI3K inhibitor will improve the treatment for breast cancer by metformin alone or metformin with other chemotherapeutic agents. The PI3K inhibitor will inhibit the insulin pathway, subsequently increasing the antitumor efficacy of metformin in non-diabetic breast cancer patients, and metformin could improve insulin sensitivity and alleviate hyperinsulinemia caused by the PI3K inhibitor. The efficacy of this combination therapy can be assessed in preclinical *in vivo* studies by evaluating and comparing the antitumor efficacy of metformin alone, PI3K inhibitor alone, and metformin plus PI3K inhibitor in xenograft mouse models of breast cancer.

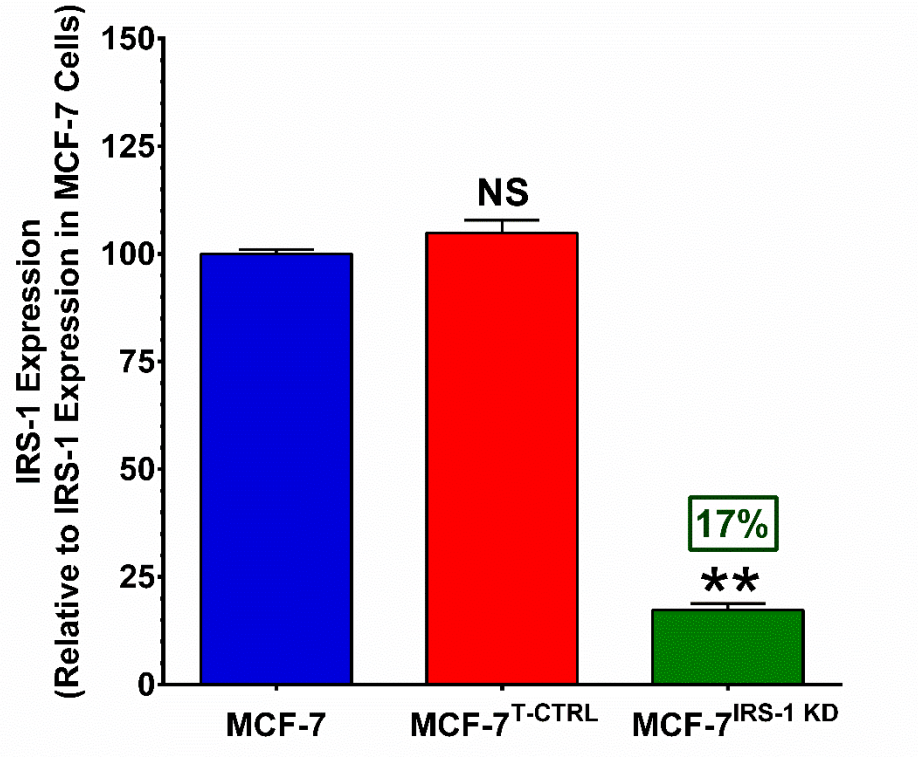


Figure 6.1 Generation of MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ Cell Lines. Expression of the IRS-1 gene in MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells was normalized to IRS-1 expression in MCF-7 cells and compared to MCF-7 cells. Data represent mean ± SD, N=3. ** signifies $p<0.001$. NS: not significant.

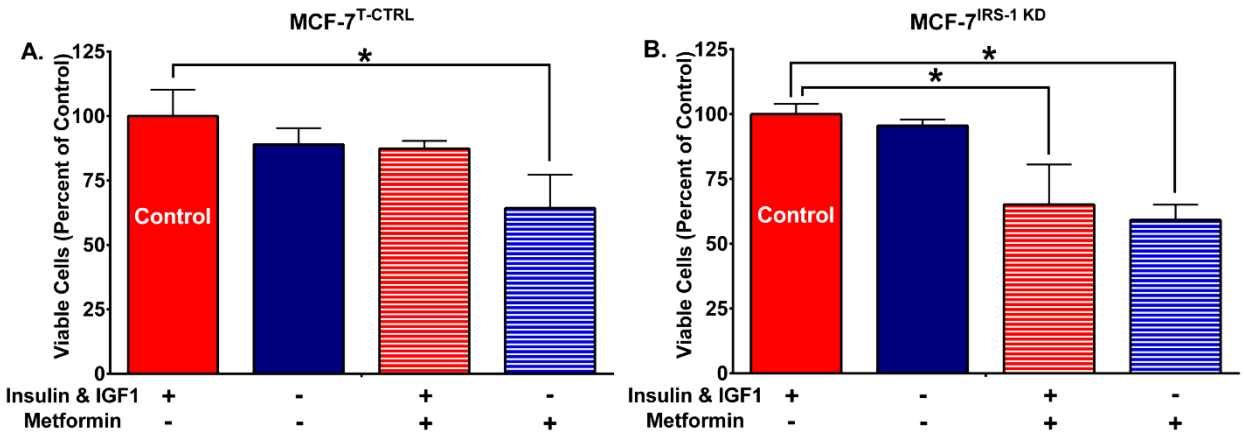


Figure 6.2 Comparison of the Antiproliferative Efficacy of Metformin against MCF7$^{T\text{-}CTRL}$ Cells and MCF-7$^{IRS\text{-}1\ KD}$ Cells. The impact of insulin/IGF1 on the antiproliferative efficacy of metformin was assessed in MCF-7$^{T\text{-}CTRL}$ cells (**A**) and MCF-7$^{IRS\text{-}1\ KD}$ cells (**B**). Metformin and insulin/IFG1 treatments are shown below each bar. MCF-7 or MCF-7$^{T\text{-}CTRL}$ cells cultured in media containing insulin/IGF1 were set as the control group to which the cell proliferation of the other three groups were normalized and compared. Data represent mean ± SD, N=3. * signifies $p<0.05$. NS: not significant.

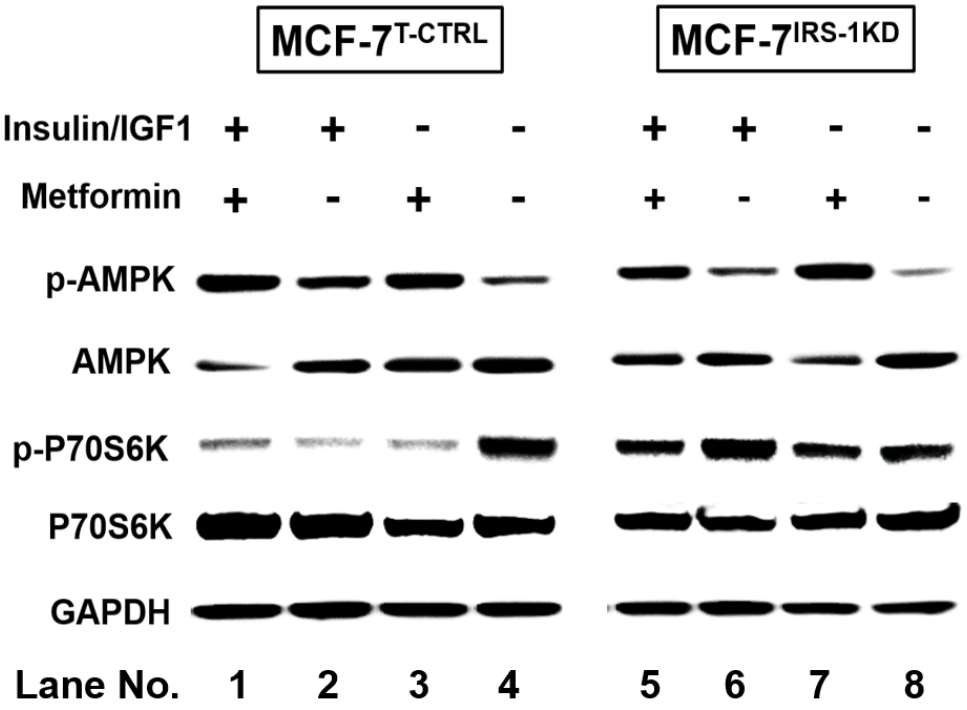


Figure 6.3 Impact of Insulin/IGF-1 on Metformin-mediated Modulation of the AMPK Pathway in MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ Cells. Metformin-mediated increase in AMPK phosphorylation and attenuation of P70S6K phosphorylation in MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells was assessed by Western blot analyses. Metformin and insulin/IFG1 treatments are shown for each lane. Total AMPK and P70S6K expression was also assessed. GAPDH was used as a loading control.

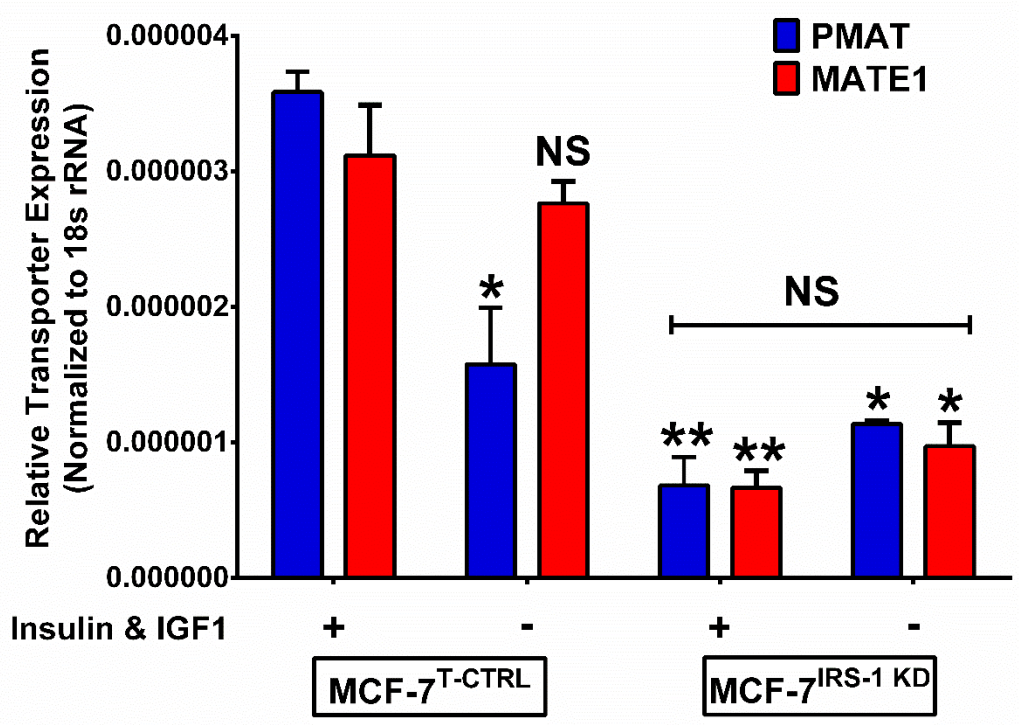


Figure 6.4 Effect of Insulin/IGF1 Treatment on the Expression of PMAT and MATE1 Genes in MCF-7 Breast Cancer Cells with Attenuated Insulin/IGF1 Signaling Pathway. Expression of PMAT and MATE1 genes was assessed by RT-PCR. PMAT and MATE1 expression in MCF-$7^{T\text{-}CTRL}$ cells cultured in media containing insulin/IGF1 was used as a control to which transporter expression in the other three groups (i.e. MCF-$7^{T\text{-}CTRL}$ cells deprived of insulin/IFG1, MCF-$7^{IRS\text{-}1\ KD}$ with or without insulin/IFG1 in the media) was compared. Data represent mean ± SD, N=3. * signifies $p<0.05$, ** signifies $p<0.01$. NS: not significant.

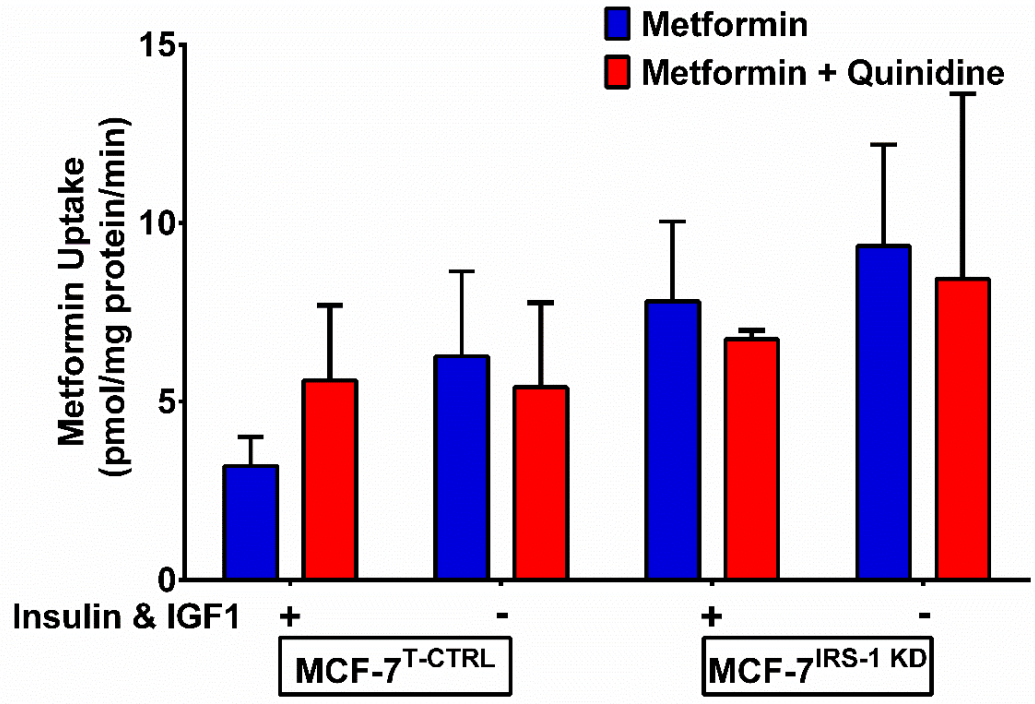
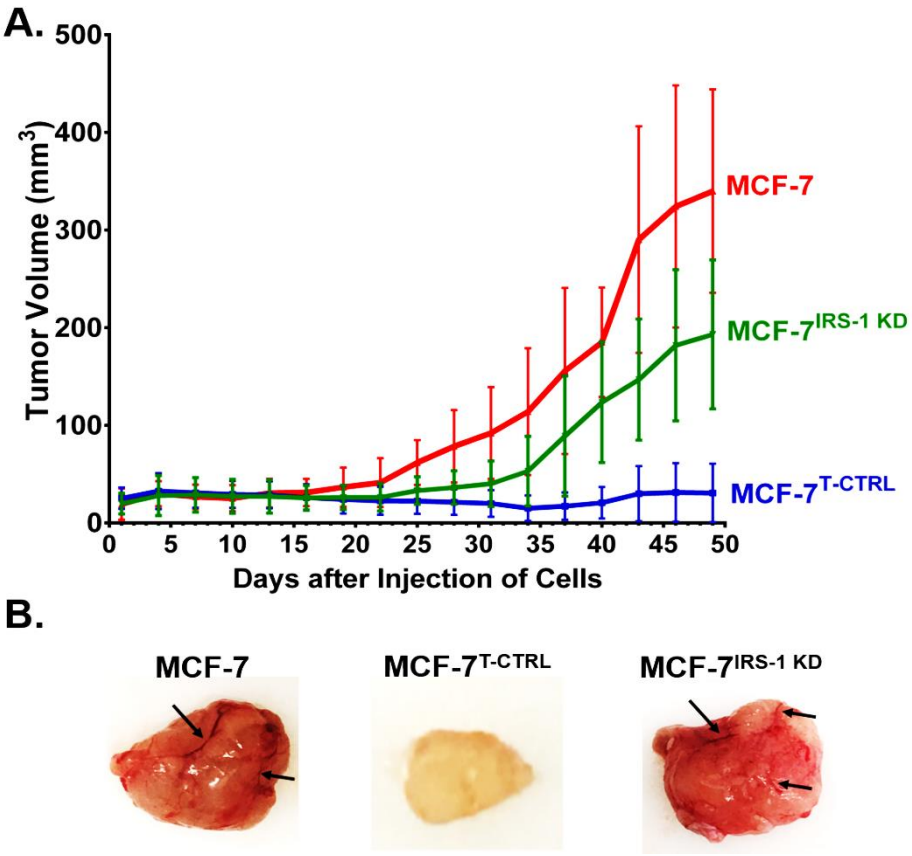
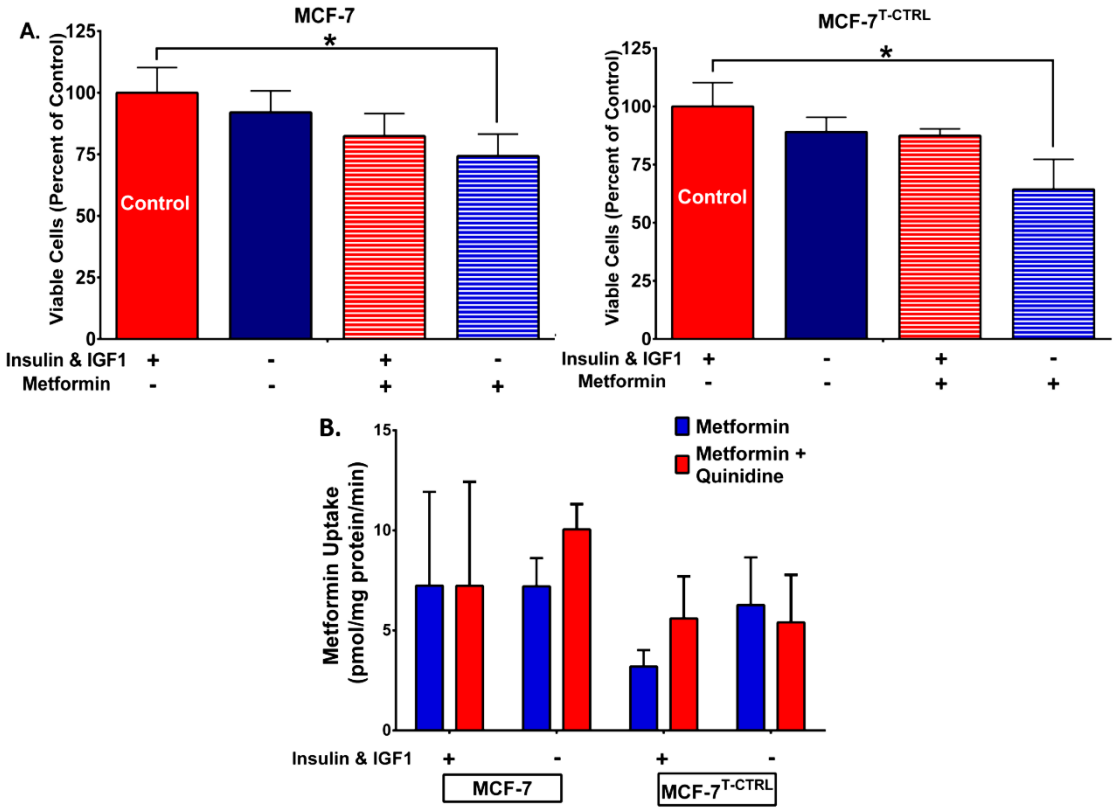


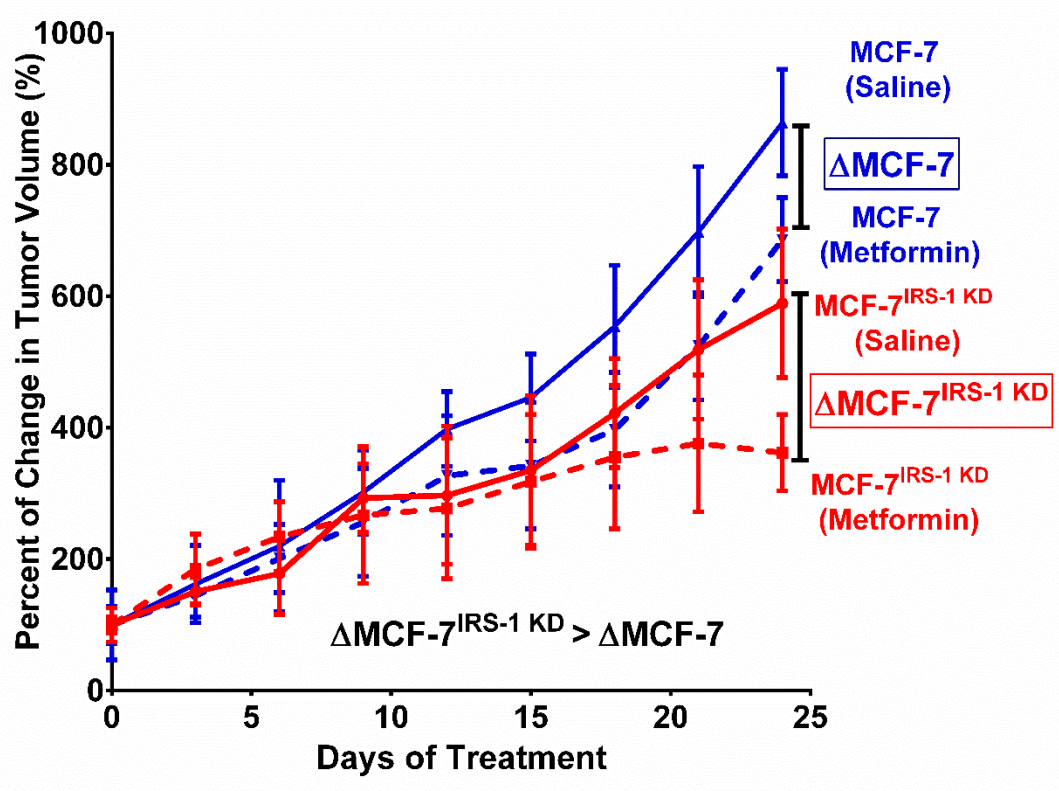
Figure 6.5 Effect of Insulin/IFG1 Treatment on Intracellular Uptake of Metformin in Intact and Attenuated Insulin/IGF1 Signaling Pathway. Uptake of [^{14}C]metformin was assessed in the presence or absence of the pan cation-selective transporter inhibitor quinidine in MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ cells cultured in media containing insulin/IGF1 or in media deprived of these growth factors. Data represent mean ± SD**,** N=3.



Supplementary Figure 6.1 Endogenous Growth Rates of Wildtype MCF-7 Tumors, MCF-7$^{T\text{-}CTRL}$ Tumors, and MCF-7$^{IRS\text{-}1\ KD}$ Tumors. (**A**) The change in tumor volumes (over 50 days) of xenograft mice bearing wildtype MCF-7, MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ tumors. Data represent mean ± SD**,** N=20. (**B**) Images of the cross sections of wildtype MCF-7, MCF-7$^{T\text{-}CTRL}$ and MCF-7$^{IRS\text{-}1\ KD}$ tumor tissues; arrows point to blood vessels.



Supplementary Figure 6.2 Cell Proliferation (A), and Metformin Intracellular Uptake (B) in Wildtype MCF-7 Cells and MCF-7$^{T\text{-}CTRL}$ Cells. The treatment of various groups (insulin/IGF1, metformin) in the cell proliferation assay (A) is shown. MCF-7 or MCF-7$^{T\text{-}CTRL}$ cells cultured in media containing insulin/IGF1 were set as the control group to which cell proliferation of the other three groups were normalized and compared. (B) Metformin uptake in each treatment group was measured in the presence or absence of quinidine to assess transporter-mediated uptake. Data represent mean ± SD**,** N=3.



Supplementary Figure 6.3 The Effect of Metformin Treatment (360 mg/kg/day) in Relation to Saline Treatment (Control) on Wildtype MCF-7 Tumors and MCF-7$^{IRS\text{-}1\ KD}$ Tumors. The change in volumes of MCF-7 and MCF-7$^{IRS\text{-}1\ KD}$ tumors over a 25-day treatment period was plotted. Metformin efficacy was reflected by the difference in the volumes of MCF-7$^{IRS\text{-}1\ KD}$/MCF-7 tumors between metformin group *vs.* saline group (i.e. ΔMCF-7$^{IRS\text{-}1\ KD}$ or ΔMCF-7), and pointed out by the vertical bars. Data represent mean ± SD**,** N=8.

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CHAPTER 7

Conclusions

Breast cancer is the second leading cause of cancer death among women in United States, with an estimated 40,730 breast cancer deaths in 2016 (1). Based on different patterns of cell surface receptor expression, breast cancer can be categorized in to different subtypes such as estrogen receptor-positive (ER+) breast cancer, triple-negative (TN) breast cancer, and human epidermal growth factor receptor 2-posiitve (HER2+) breast cancer (2). A significant challenge in the successful treatment of breast cancer is the wide variability in the expression of and mutations in oncogenes and tumor suppressor genes among different subtypes of breast cancer (2,3). Therefore, the selection of appropriate chemotherapeutic agents and the therapeutic dose depends on the breast cancer subtype. For luminal breast cancer, such as ER+ breast cancer (3), the tumors are relatively well-controlled by current chemotherapy. In contrast, patients with basal breast cancer, such as TN breast cancer, generally have a poor response to chemotherapy (2). Therefore, a new chemotherapeutic agent that is efficacious in all subtypes of breast cancer will represent a major advance in breast cancer therapy.

The length of survival in breast cancer patients following chemotherapy (generally expressed as a 5- or 10-year survival rate) is one of the primary standards of evaluating treatment outcomes, which is not only determined by the tumor inhibitory efficacy of the treatment, but also by the extent to which risk of cancer relapse and metastasis exists. Although the number of approved chemotherapeutic agents has been increasing in recent years, these agents have not contributed to cure of breast cancer and are generally too toxic for long-term therapy that is required to prevent cancer metastasis and relapse. Therefore, discovery of

metformin as a therapeutic agent that is safe, cost-effective, and efficacious in preventing cancer metastasis and relapse can be hailed as an important advance in cancer therapy that can improve survival outcomes in breast cancer patients.

Since type 2 diabetes has been reported to increase the risk of developing breast cancer (4), the impact of anti-diabetic agents on breast cancer incidence was investigated by retrospective analyses. Compared to insulin and sulfonylurea, diabetic patients on metformin exhibited significantly lower breast cancer incidence in a majority of clinical trials (**Table 1.1**). Since metformin is a highly cost-effective drug with excellent safety profile that has been used for decades to treat type 2 diabetes, these retrospective observations led to follow-up prospective clinical studies to evaluate the antitumor efficacy of metformin, either as a monotherapy or in combination with chemotherapeutic agents. Several studies showed that metformin treatment was able to suppress tumor growth (5), prevent cancer relapse (6), and inhibit metastasis (6). However, in some clinical trials, particularly in those involving non-diabetic cancer patients, these beneficial effects of metformin were reported to be insignificant (6, 7). These contradictory observations from different clinical studies suggest that there are cellular and molecular factors in anticancer effects of metformin against breast cancer that are not well understood.

As has been discussed in **Chapter 1**, because the anticancer effects of metformin were observed in the patient populations who were on metformin therapy for type 2 diabetes. Thus the two major mechanisms that have been considered for anticancer effect of metformin are those that seem to play a key role in metformin's antidiabetic effects; namely, reduction of the circulating insulin and insulin-like growth factor 1 (IGF1) levels, and activation of AMP-activated protein kinase (AMPK) that leads to inhibition of gluconeogenesis in the liver cells. There is growing consensus that these mechanisms may play a critical role in the anticancer effects of cancer. High levels of circulating insulin and IGF1, a common physiologic feature found in type 2 diabetic patients, are likely to provide the stimulus for cell proliferation and tumor growth. Thus

it is reasonable to propose that metformin therapy in type 2 diabetes that causes these levels to come down can reduce the stimulus for cell proliferation in the breast cancer tissue. At the cellular level, metformin appears to regulate the mobilization of glucose from stored glycogen in the liver cell by activation of AMPK, which is an important cellular signal for modulating metabolism at the cellular level according to the energy needs of the cell. It is important to note that AMPK activation can also trigger changes in the downstream signals, such as mammalian target for rapamycin (mTOR) and P70S6K, that play a critical role in programming a cell toward apoptosis (8, 9). Thus metformin's known ability to activate AMPK in the liver cells would suggest that it can activate AMPK in breast cancer cells and contribute to reprogramming proliferating breast cancer cells toward apoptosis by modulation of the downstream signaling molecules mTOR and P70S6K.

An important question that was unanswered at the time this dissertation project was being conceived was: how does metformin get into the tumor cells to cause activation of the intracellular target AMPK? Metformin is a small and very hydrophilic drug; in fact it is one of very few drugs that have more nitrogen atoms in its structure than carbon atoms. In addition, metformin carries a positive at all physiologic pHs. Hence, there was a strong consensus within the Thakker Group that metformin could not cross cell membranes or physiologic barriers such as intestinal epithelium or blood-brain barrier by passive diffusion, and that it requires cation-selective transporters to carry it across the cell membranes and physiologic barriers. The previous and ongoing work on the treatment of type 2 diabetes with metformin had provided evidence that cation-selective transporters, such as organic cation transporters (OCT)s play an important role in its therapeutic efficacy (10) and renal elimination (11-13), respectively. Studies in the Thakker Group had produced new evidence that four different transporters, namely OCT1, plasma monoamine transporter (PMAT), serotonin reuptake transporter (ST) and high affinity choline transporter (CHT), played a significant role in metformin uptake into and transport across the intestinal epithelium (14, 15). Thus the major goal of this dissertation

project was to elucidate the expression and function of key cation-selective transporters in breast cancer tissues and breast cancer cell lines that are extensively used as surrogates of breast cancer cells in research, and to investigate the role of these transporters in the antiproliferative and antitumor activity of metformin.

The studies in **Chapter 2** provided the first evidence that OCT3, PMAT, and OCT1 are the predominant cation-selective transporters in human breast tumor tissues, and that variability in the expression of these transporters is greater than 1000-fold among breast cancer patients (**Figure 2.2**). This result implies that sufficient metformin uptake into breast tumor tissues is likely achieved only in a subpopulation of breast cancer patients. These studies also revealed a high variability in cation-selective transporter expression among different types of breast cancer cell lines (**Figure 2.1**). For breast cancer cell lines such as MDA-MB-468, MDA-MB-231, and BT-549 cells, multiple types of transporters are expressed at high levels, whereas no or very limited transporter expression is observed in breast cancer cell lines such as BT-20, MCF-7, and SK-BR-3 cells (**Figure 2.1**). As the activation of intracellular targets by metformin depends on its intracellular concentrations, it is possible that using breast cancer cell lines with different transporter expression profiles may lead to contradictory observations on metformin efficacy against breast cancer.

These studies established an association between high cation-selective transporter expression in breast cancer cells and enhanced metformin uptake, enhanced activation of the AMPK pathway, and greater sensitivity to the antiproliferative activity of metformin (**Figure 2.3-5**). A compelling evidence for these concepts was provided by designing studies in which the antiproliferative efficacy of metformin was compared between a transporter-deficient human breast cancer cell line (BT-20 cells) and an engineered OCT3-overexpressing cell line from the same genetic background (i.e. OCT3-BT20 cells) (**Figure 2.3-5**). Here, OCT3 was selected as a representative of all cation-selective transporters because it is most highly expressed in breast tumor tissues (**Figure 2.1**). As these cation-selective transporters share similar transportation

mechanisms, it is assumed the conclusion obtained from the study on OCT3 can also be applied to other cation-selective transporters. As both BT-20 and OCT3-BT20 have same genetic background, we can conclude that the increased metformin uptake, enhanced AMPK activation by metformin, and improved response to the antiproliferative efficacy of metformin observed in OCT3-BT20 cells compared BT-20 cells are only due to the OCT3 overexpression. These results suggest that transporter expression and transporter-mediated metformin uptake are both required for the antiproliferative efficacy of metformin.

Besides overexpressing transporters in a transporter-deficient cell lines, the critical role of transporters in metformin anticancer effects can be illustrated through knocking down a transporter in transporter-competent cells, and comparing the antiproliferative activity between the wildtype and the transporter-knockdown cells. However, this approach would not have been as effective as the one employed in this research as the reduction of metformin uptake caused by the knockdown of one transporter may be compensated by other transporters that are also expressed in the cancer cells.

Besides *in vitro* studies, attempt was made to use BT-20 and OCT3-BT20 cells to generate a pair of breast tumors which have similar genetic background and are only different in OCT3 expression, and compare metformin antitumor efficacy against both types of tumors. However, due to the endogenous slow growth of BT-20 tumors, there were not enough continuous growing BT-20 tumors generated for the *in vivo* studies. To overcome this difficulty, OCT3 was overexpressed in MCF-7 cells (OCT3-MCF7 cells), which are highly tumorigenic, fast-growing, and have low expression of transporters. *In vivo* studies were conducted using xenograft mice bearing low transporter expressing MCF-7 tumors or transporter-competent OCT3-MCF7 tumors. As in clinical conditions, metformin is used as monotherapy or in combination with chemotherapeutic agent, the antitumor efficacy of metformin as well as metformin plus doxorubicin (DOX) were evaluated. DOX monotherapy was also included into the study to evaluate any possible interactions between DOX and metformin. The *in vivo* studies

demonstrated higher intratumoral concentrations of metformin in OCT3-MCF7 tumors than MCF-7 tumors (**Figure 4.5**), which suggested that the intratumoral accumulation of metformin is also mediated by cation-selective transporters. Consistent with the elevated accumulation of metformin, a greater increase in AMPK phosphorylation by metformin was observed in OCT3-MCF7 tumors *versus* MCF-7 tumors (**Figure 4.4**). This greater activation of metformin led to an increased sensitivity to the antitumor efficacy of metformin monotherapy and DOX plus metformin treatment in OCT3-MCF7 tumors compared to MCF-7 tumors, which demonstrates a correlation among transporter expression, metformin accumulation in tumor tissues, activation of intracellular targets, and the response to metformin treatment. Collectively, these data clearly showed that high cation-selective transporter expression is required for adequate intracellular uptake and accumulation of metformin in breast cancer cells and tumors that can then activate its intracellular targets.

The studies also demonstrated that a functional AMPK pathway is required for metformin to exert its antiproliferative efficacy, as inhibition of this pathway by Compound C, an AMPK-specific inhibitor, reversed the antiproliferative activity of metformin in breast cancer cells (**Supplementary Figure 2.1**). This finding was also supported by comparing the antiproliferative efficacy between MDA-MB-231 cells and BT-20 cells. Due to the mutation in LKB-1, the kinase which is responsible for AMPK phosphorylation (**Table 1.1**), metformin treatment was not able to induce the AMPK phosphorylation in MDA-MB-231 cells (**Figure 2.5**). Therefore, even though the transporter-competent MDA-MB-231 cells exhibited more than 5-fold higher metformin uptake compared to BT-20 cells (**Figure 2.3**), both cell lines showed similar sensitivity to metformin treatment compared to BT-20 cells (reflected by comparable values of metformin IC_{50}) (**Figure 2.4**).

In summary: (1) these studies provided important information on cation-selective transporter expression in human breast tumor tissues and breast cancer cell lines and, that in turn, yielded key rationale for the *in vitro* and *in vivo* studies designed in this work to explore the

role of cellular mechanisms underlying antiproliferative and anticancer efficacy of metformin. These studies will prove to be critical in future basic and clinical research on the role of metformin in breast cancer. (2) The studies showed the importance of transporters in metformin uptake and accumulation in breast tumor cells/breast cancer cell lines and subsequent activation of the intracellular AMPK pathway, and the role of the AMPK pathway in the antitumor and antiproliferative activity of metformin. This correlation between cation-selective transporters and the antitumor efficacy of metformin suggest that at least one reason for poor outcomes of metformin treatment in breast cancer patients may be low transporter expression levels or a dysfunctional AMPK pathway in breast tumors.

Future studies: *1. Clinical studies will be designed by stratifying patients with respect to metformin transporter expression (not only in breast cancer but in other cancers). This will provide a more definitive evidence for the efficacy of metformin in breast cancer and other cancers, and provide more clarity about its intracellular mechanism underlying its anticancer effect. 2. These future studies can lead to exploration of metformin transporters and AMPK as biomarkers for predicting clinical outcome for patients to be treated with metformin as a monotherapy or in combination therapy.*

Additionally, metformin prodrugs with high lipophilicity can be developed to enhance the intracellular uptake of metformin by passive diffusion, so as to circumvent the need for cation-selective transporters in intracellular/intratumoral uptake and accumulation of the drug. This approach would enable all breast cancer patients, regardless of transporter expression levels or transporter polymorphisms, to avail of this efficacious and cost-effective drug.

Another set of important questions about metformin therapy against breast cancer that remains unanswered to date are: In the retrospective studies in diabetic patients that provided evidence for efficacy of metformin as an anticancer or cancer-preventive agent, were the doses of metformin adequate or optimum for its anticancer efficacy? Second, in the prospective preclinical and clinical studies that have been conducted to evaluate anticancer efficacy of

metformin, what was the rationale for selection of the doses used and was there a systematic assessment of dose-effect relationship? These questions clearly articulate the need to investigate dose-response relationship for treatment of breast cancer with metformin. Hence, studies were designed in mouse models of breast cancer to systematically evaluate the relationship between metformin dose, exposure of tumor tissues to the drug, and the antitumor efficacy **(Chapter 5).**

To simulate clinical situations and provide important insights into dose selection/adjustment for future clinical trials, a comprehensive preclinical study was designed (**Figure 5.1A**). The potential impact of breast cancer subtypes and tumor transporter expression profiles on the efficacy of metformin doses was evaluated in orthotopic xenograft mice bearing ER+ MCF-7 tumors (low transporter expression) and TN MDA-MB-468 tumors (high transporter expression) (**Figure 2.1**). Since most clinical trials investigating metformin for cancer therapy use the drug as a combination therapy with chemotherapeutic agents (6, 7), the preclinical study was designed to include varying doses of metformin as a combination therapy with paclitaxel (for MCF-7 tumors) or carboplatin (for MDA-MB-468 tumors), the two widely used chemotherapeutic agents for ER+ and TN breast cancer. The different doses of metformin were calculated (using body surface area) from human anti-diabetic doses, namely, the maximum recommended daily dose, the most commonly prescribed daily dose, and the two lower doses. In addition, the feasibility of using metformin as a monotherapy in breast cancer therapy was also evaluated in this preclinical study, as it is challenging to do so in clinical trials due to concerns that metformin monotherapy may not be potent enough to inhibit tumor growth. Such a study would play a critical role in interpreting the results of the studies involving combination of metformin with other chemotherapeutic agents. The antitumor efficacy was compared among the different treatment groups through comprehensive analysis of tumor progression, tumor weights at end points, and overall survival rates.

The results showed that metformin, in combination with chemotherapeutic agents like paclitaxel or carboplatin, produced demonstrated superior antitumor efficacy and better clinical outcomes than when metformin, paclitaxel or carboplatin was used as a single agent (**Figure 5.2-3**). The data also revealed that in order to achieve significant improvement in antitumor efficacy by adding metformin to either carboplatin or paclitaxel, a minimum metformin dose of 120 mg/kg/day (equivalent to 850 mg daily dose used for the treatment of type 2 diabetes) is required for the treatment of TN MDA-MB-468 tumors by carboplatin, and a 3-fold higher metformin dose of 360 mg/kg/day (equivalent to the 2550 mg maximum recommended daily dose for diabetes) is required for treatment of ER+ MCF-7 tumors with paclitaxel (**Figure 5.2**). These results could explain the ambiguous treatment outcomes observed in clinical studies that used a metformin daily dose of 850 mg.

The doses of metformin in these studies were related to the systemic and intratumoral exposure to metformin, and to the efficacy of metformin. This is the first study in which the dose-response and exposure-response relationships for metformin have been investigated in breast cancer therapy. While the intratumoral concentrations of metformin were only be determined at two time points (at Tmax and 24 hr time points), a two-compartment pharmacokinetic (PK) model was developed to simulate and predict intratumoral exposure over the entire 24 hour dosing period. The measured metformin plasma and intratumoral concentrations and the simulated intratumoral concentrations/exposure showed that while there was no difference in plasma concentrations of metformin between xenograft mice bearing the ER+ MCF-7 and TN MDA-MB-468 tumors when the same metformin dose was administered, intratumoral exposure of metformin in MDA-MB-468 tumors was approximately 2.5-fold higher than the exposure in MCF-7 tumors. Further, to achieve comparable exposure of MDA-MB-468 and MCF-7 tumors to metformin, 3-fold higher metformin dose (360 mg/kg/day) to mice bearing MCF-7 tumors was required over the metformin dose (120 mg/kg/day) to mice bearing MDA-MB-468 mice (**Table 5.2**). These results are consistent with higher cation-selective transport expression in the MDA-

MB-468 tumor cells than in the MCF-7 tumor cells, and suggest that the minimum required dose of metformin for combination therapy is primarily determined by intratumoral exposure, which argues for screening breast tumors for transporter expression levels to guide dose selection.

The study described in **Chapter 5** provides another important insight into breast cancer therapy. The results suggest that by adding metformin to the breast cancer treatment regimen that comprise conventional chemotherapeutic agents, it may be possible to reduce the doses of the chemotherapeutic agents and thus reduce their adverse effects. The survival data were very interesting, and showed that metformin improves survival of animals with breast cancer on chemotherapy even at doses that are sub-therapeutic in metformin monotherapy. Also, the high dose of 360 mg/kg/day that proved to be more efficacious than 120 mg/kg/day in inhibiting cancer growth, the lower dose proved to be more effective in improving survival.

Future studies: *Thus, future preclinical and clinical studies should evaluate efficacy and adverse effects of treatments in which a high dose of metformin (e.g. 360 mg/kg/day) is titrated with different doses of chemotherapeutic agents. Also, future studies should evaluate metformin doses higher than 360 mg/kg/day in monotherapy and combination therapy regimens to evaluate the maximum effective dose of the drug in ER+ and TN breast cancers.*

In the studies discussed so far, the factors that impact the antitumor efficacy of metformin were investigated, and strategies were proposed to select breast cancer patients with good prognosis for metformin therapy and to select metformin doses for the best treatment outcomes. Metformin is also reported to suppress breast cancer relapse and metastasis, and this effect is thought to be mediated by suppression of breast cancer stem cells (CSCs). Breast CSCs are a subpopulation of cancer cells in breast tumors that are generally identified by a high expression of CD44 and low expression of CD24 on the cell surface (16). CSCs are widely believed to cause cancer relapse and metastasis as they are more resistant to chemotherapeutic agents (17), can readily migrate to other organs (18), and are highly tumorigenic (19) compared to non-stem cells cancer cells (NSCCs). Analyses of human breast

tumor tissues have revealed that basal breast tumors with a high proportion of CSCs are more aggressive and enhance the risk of cancer relapse and metastasis compared to luminal breast tumors that have a smaller proportion of CSCs (20). This finding suggests that CSCs play a central role in cancer relapse and metastasis.

Breast CSCs are reported to be more sensitive than non-stem cancer cells (NSCCs) to metformin treatment, as metformin treatment has been shown to reduce the number of CSCs in breast tumors and breast cancer cell lines (21). Several studies have reported that metformin suppressed the proliferation of CSCs through activation of the AMPK pathway, and caused AMPK activation at a significantly lower concentration compared to NSCCs (22). However, the mechanisms involved in the higher sensitivity of CSCs *versus* NSCCs to metformin treatment have not been investigated.

Chapter 3 of this dissertation described the studies conducted to elucidate the mechanistic differences in the sensitivities of CSCs and NSCCs to metformin. These studies used the BT-549 TN breast cancer cell line as a representative cell line, as it has moderate proportions of CSCs and NSCCs. Multidrug and toxin extrusion protein 1 (MATE1) transporter is the only predominant transporter expressed in BT-549 cells. A greater than 50% increase in MATE1 expression (**Figure 3.1 and 2**), (**Figure 2.1**), was observed in CSCs compared to NSCCs. This upregulation in MATE1 resulted in an increase in transporter-mediated metformin uptake in BT-549 CSCs *versus* NSCCs (**Figure 3.3**), and provided a rationale for the activation of intracellular AMPK and subsequent antiproliferative activity in CSCs at lower metformin concentrations compared to NSCCs. Since CSCs are believed to play a role in cancer relapse and metastasis, the greater sensitivity of CSCs to metformin treatment suggests that a lower metformin dose may be adequate to prevent cancer metastasis and relapse compared to the dose required for tumor inhibition.

Although there are several published reports on the upregulation of the ATP-binding cassette transporters (ABC transporters) and ABC transporter-mediated chemoresistance in

CSCs (17), there are no reports on the expression of uptake transporters in CSCs or on a correlation between uptake transporter expression and the cellular behavior of CSCs. This study provided the first evidence that cation-selective transporter expression is upregulated in CSCs and could be one of the factors that contributes to increased sensitivity of this subpopulation of cancer cells to metformin treatment. However, it remains to be determined whether the antiproliferative efficacy of metformin in CSCs is dependent on activation of intracellular AMPK.

Future studies*: Clearly, these findings open up an important area of research to investigate the distinct properties of CSCs that make them uniquely susceptible to metformin. This is an important area of research as few drugs have shown selectivity for CSCs that play such an important role in recurrence and metastases of breast cancer and other cancers.*

It is believed that in non-diabetic breast cancer patients, metformin exerts its anticancer activity predominantly via modulation of its intracellular targets in tumor cells, whereas in diabetic breast cancer patients, the drug can also exert its anticancer effect indirectly through its anti-diabetic effects (5). Studies have shown that diabetic breast cancer patients generally respond better to metformin treatment compared to non-diabetic breast cancer patients; hence, understanding the interactions between the anti-diabetic and anticancer effects of metformin will not only help explain differences in clinical outcomes to metformin therapy among these two patient populations, but will also provide insights for the development of new combination therapies that can synergistically improve the anticancer efficacy in non-diabetic breast cancer patients. Therefore, the focus of the studies in Chapter 6, was to evaluate the impact of the anti-diabetic effects of metformin on its anticancer activity at the cellular and molecular level. Similar to other agents used for the treatment of type 2 diabetes, the primary anti-diabetic effect of metformin is its glucose-lowering activity. However, the reduction of circulating glucose levels alone appears to have a very limited impact on the growth of breast tumors, as the long-term administration of other widely used glucose-lowering agents such as insulin and sulfonylurea enhances breast cancer incidence (23, 24). In contrast to insulin and sulfonylurea, metformin

improves insulin sensitivity and therefore, suppresses insulin and insulin-like growth factor (IGF1) secretion. Since both insulin and IGF1 have been reported to promote cancer cell proliferation by activating the insulin pathway through the modulation of the cell signaling cascade involving insulin/IGF1 receptor, insulin receptor substrate (IRS1)1, and phosphoinositide 3-kinase (PI3K) (25, 26), it was hypothesized that the insulin/IGF1-lowering effects of metformin inhibits breast cancer proliferation through regulation of the insulin pathway. To test this hypothesis, the dual effects of metformin (namely, its anticancer effects and its insulin/IGF1-lowering effect) in diabetic breast cancer patients were simulated by simultaneously adding metformin to MCF-7 cell culture media and removing insulin/IGF1 from the culture media. The results showed that the antiproliferative efficacy of metformin was enhanced in the absence of insulin/IGF1, whereas removal of insulin/IGF1 from the culture media without metformin treatment had limited effect on MCF-7 proliferation (**Supplementary Figure 6.2**). Further analyses showed that the increase in the antiproliferative effect of metformin in the absence of insulin/IGF1 from the culture media was through activation of the AMPK pathway (**Figure 6.3**) without increasing transporter-mediated intracellular uptake of the drug (**Figure 6.4 and Supplementary Figure 6.3**).

In MCF-7 cells where the insulin pathway was inhibited by knocking down IRS-1 (the key regulator of this pathway) to generate MCF-7$^{IRS\text{-}1\ KD}$ cells, metformin caused a greater activation of AMPK and greater antiproliferative activity regardless of insulin/IGF1 levels in the culture media compared to MCF-7 cells (**Figure 6.2 and 3**). This result suggested that the effects of extracellular insulin/IGF1 levels on the antiproliferative efficacy of metformin was mediated by the insulin pathway, and implied that inhibition of the insulin pathway by the insulin/IGF1-lowering effects of metformin sensitizes breast cancer cells to its antiproliferative activity through modulation of the AMPK pathway. The results from studies described in Chapter 6, for the first time, provide clear evidence of interplay between the antidiabetic and anticancer effects of metformin.

Future studies: *The significance of these results is that not only do they provide a rationale for differences in clinical outcomes to metformin treatment between diabetic and non-diabetic breast cancer patient populations, but also they also suggest new strategies for improving metformin therapy in non-diabetic breast cancer patients through a combination of metformin and an insulin pathway inhibitor. Currently, several PI3K inhibitors are used in breast cancer therapy. Although they have shown significant efficacy against multiple types of breast tumors, there are concerns about the induction of hyperinsulinemia. Therefore, it is possible that a combination of metformin and a PI3K inhibitor can exert greater antitumor efficacy in non-diabetic breast cancer patients, as the PI3K inhibitor will suppress tumor growth and sensitize tumor cells to the antiproliferative efficacy of metformin with reduced adverse effects, as metformin can alleviate hyperinsulinemia caused by the PI3K inhibitor.*

Studies in Chapter 6 investigated the insulin-lowering effects of metformin in vitro by removing insulin/IGF1 from cell culture media. These in vitro results need to be confirmed in in vivo studies. To do so, the anticancer and anti-diabetic effects of metformin can be evaluated in xenograft mice bearing MCF-7 tumors or MCF-7$^{IRS\text{-}1\ KD}$ tumors that are fed a high-fat high-carbohydrate diet to induce diabetes. The impact of the insulin/lGF1-lowering effect of metformin on AMPK activation in tumor tissues, as well as intratumoral concentrations of metformin and treatment outcomes can be evaluated. To test the feasibility of using a combination therapy of metformin and a PI3K inhibitor to improve antitumor efficacy and reduce adverse effects, tumor growth and circulating insulin/IGF1 levels following treatment with saline, metformin alone, PI3K inhibitor alone, or metformin and a PI3K inhibitor can be evaluated

In summary, the purpose of this dissertation research project was to identify important gaps in knowledge about metformin use in breast cancer (**Chapter 1**) and address some of the gaps, questions and limitations of metformin treatment (**Figure 7.1**). Studies in Chapter 2 confirmed that the AMPK pathway is the primary intracellular target of the anticancer activity of metformin, and that intracellular uptake of metformin is mediated by cation-selective

transporters. Results from **Chapters 2 and 4** demonstrated that transporter-mediated uptake and activation of the AMPK pathway are required for the anticancer activity of metformin, and suggested that metformin would be effective in breast cancer patients with a functional AMPK pathway and high transporter expression in tumors. Studies in **Chapter 3** showed that the antiproliferative activity of metformin in CSCs is also achieved via transporter-mediated uptake of the drug and activation of intracellular targets, and that upregulation of cation-selective transporters in CSCs confers greater sensitivity to metformin treatment compared to NSCCs. Data generated from studies described in **Chapters 1-4** led to the formulation of two strategies to optimize metformin for breast cancer therapy (**Chapters 5 and 6**). The PK studies in **Chapter 5** showed that metformin exerts greater antitumor efficacy in combination with a chemotherapeutic agent compared to monotherapy, and suggested that a minimum dose of metformin and intratumoral exposure of metformin are required to enhance antitumor efficacy compared to chemotherapy. Studies in **Chapter 6** investigated interactions between the anti-diabetic and anticancer effects of metformin *in vitro* and demonstrated that inhibition of the insulin pathway by the anti-diabetic effect of metformin sensitizes the AMPK pathway to metformin and improves antiproliferative efficacy in human breast cancer cells.

Results from this research provide important insights and point to future directions for optimizing clinical use of metformin in cancer therapy.

1. Selection criteria can be used to determine the most suitable breast cancer patients for metformin therapy by screening transporter expression and AMPK mutations in breast tumor biopsies.
2. These selection criteria can be used as inclusion criteria in future clinical studies to reduce variability in metformin treatment outcomes in breast cancer patients.
3. Metformin prodrugs with increased lipophilicity can be designed to enhance cellular uptake via passive diffusion and improve the antitumor efficacy of this drug in breast cancer patients with tumors that have limited transporter expression.

4. The current anti-diabetic metformin dose (850 mg/day) used in most clinical studies for breast cancer needs to be increased to the maximum recommended anti-diabetic dose of 2,550 mg/day, and even higher doses of metformin should be evaluated for use in combination therapy of breast cancer.
5. Clinical studies should be initiated in which metformin is administered in combination with an insulin pathway inhibitor, such as PI3K inhibitor, so as to improve antitumor efficacy of the combination and reduce the adverse effects of the PI3K inhibitors.

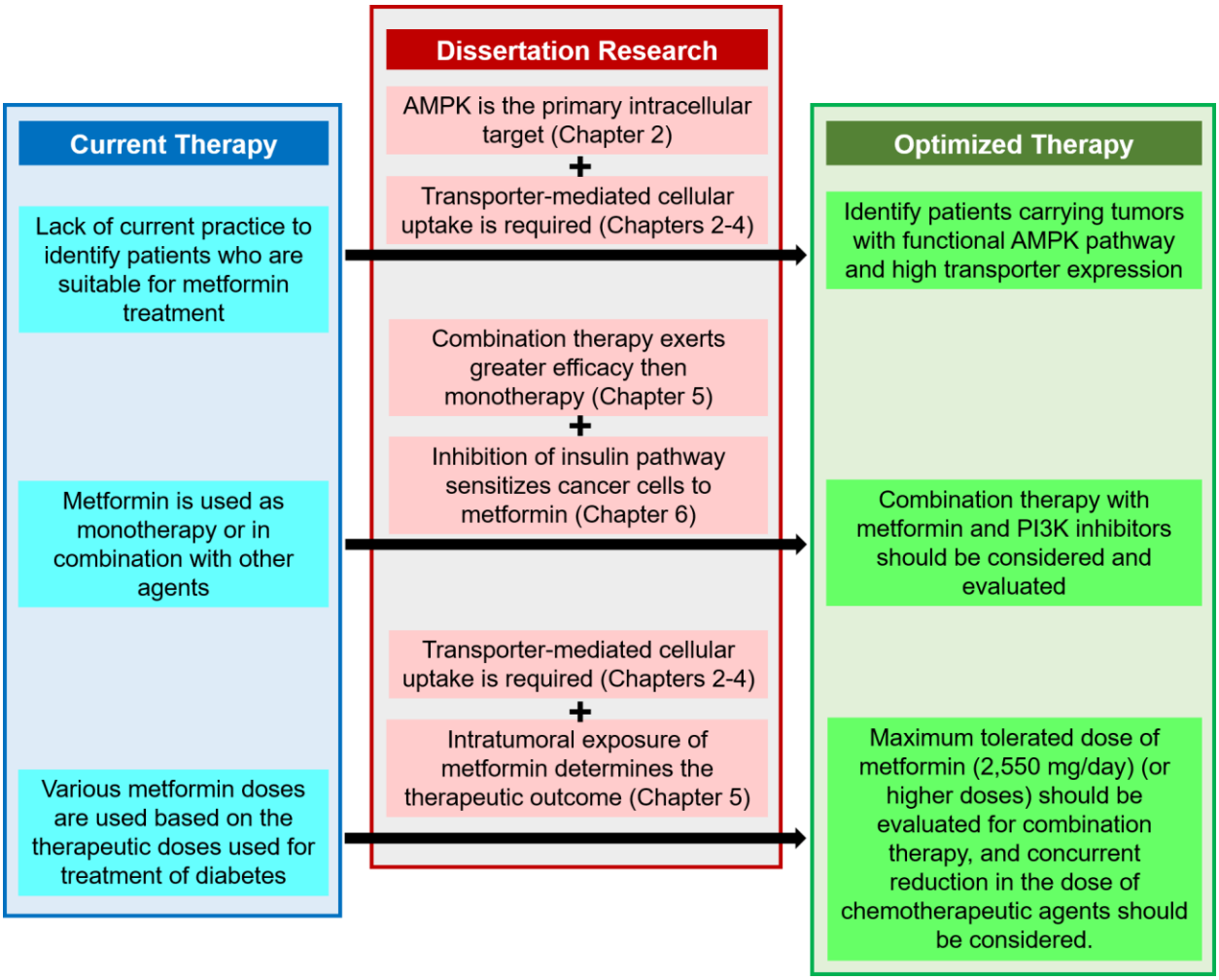

Figure 7.1 Summary of the Dissertation Research and Future Directions

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