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Metformin: from antidiabetic to cancer therapeutic

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Thesis

METFORMIN: FROM ANTIDIABETIC TO CANCER THERAPEUTIC

by

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METFORMIN: FROM ANTI-DIABETIC TO CANCER THERAPEUTIC

MICHAEL J. JAVORSKI

ABSTRACT

Epidemiology studies have found that type 2 diabetics treated with metformin are at a lower risk for developing cancer. It was speculated that the lowered risk might be attributed metformin’s indirect physiological effect of lowering blood insulin levels, which is the opposite of many other antidiabetic drugs. However, further study of metformin’s mechanism of action at the cellular level helped develop an understanding of its effect on the individual cell. This helped show why, mechanistically, it makes sense to use metformin for the treatment of cancer. As an activator of AMP-activated protein kinase (AMPK) via inhibition of complex 1 of the mitochondrial electron transport chain, metformin causes suppression of tumor growth and cell cycle arrest by acting on the mTOR pathway and cyclin/CDKs, respectively. Metformin has been most extensively studied in breast cancer, showing great efficacy in numerous breast cancer cell lines that include ER positive, HER2 positive, and triple negative breast cancer cell lines. This compilation of data and results of metformin’s efficacy in various cancer subtypes will help push metformin forward as a new chemotherapeutic for breast cancer, and eventually for other cancer types as well.
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<tr>
<td>3-OH</td>
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<td>ADP</td>
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<td>AICAR</td>
<td>5-amino-imidazole carboxamide riboside</td>
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<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
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<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
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<td>ΔΨ</td>
<td>in situ mitochondrial membrane potential</td>
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INTRODUCTION

Diabetes is one of the most prevalent diseases in the United States, affecting an estimated 25.8 million people, 8.3% of the U.S. population; type 2 diabetes mellitus contributes to about 95% of diabetic patients (cdc.gov, 2011). With its increasing prevalence, an association began to emerge between type 2 diabetes mellitus (T2DM) and cancer. This would be an important correlation since cancer is the cause of 8.2 million deaths worldwide, and has placed among heart disease as one of the top causes of death (who.int, 2014). The study of cancer in the context of diabetes is therefore extremely relevant, and is the reason why much focus has been given to this area of interest. Many researchers have found statistical significance showing that T2DM patients, especially if untreated, have a greater chance of developing cancer.

An assessment of the current treatments for T2DM has shown that only one drug, metformin, decreases the risk for cancer relative to the other drugs. Metformin is widely used and a top choice for the treatment of T2DM, and may now receive even greater attention and usage for its potential anti-cancer properties. In order to understand the drug more clearly, researchers have still been trying to elucidate its precise mechanism in the treatment of T2DM. Now that the drug has shown potential for the use in cancer, researchers are reexamining the mechanism of its action. However, this time they are looking at how metformin affects cell growth and proliferation. Knowledge of the precise mechanisms of its antineoplastic effects will help move this drug into clinical trials and maybe some day as a new standard of treatment all other chemotherapeutics for cancer.
Correlation between Diabetes and Cancer

As Giovannucci et al. summarized in a consensus report, the relative risk from diabetes is at least 2-fold or more for liver, pancreatic, and endometrial cancers, while the relative risk is moderate for cancer of the colon, rectum, breast, and bladder (Giovannucci et al., 2010). Luo et al. calculated in a meta-analysis that individuals with T2DM have a significant increase in risk of developing colorectal cancer, with a relative risk of 1.28 (Luo et al., 2012). Another meta-analysis showed that women with T2DM have an increased risk for breast cancer by 27% (Boyle et al., 2012). On the contrary, T2DM patients were actually shown to have a lowered risk of prostate cancer than those without diabetes (Giovannucci et al., 2010). There are a significant number of studies on common cancers; however, one of the areas lacking in the literature is the risk that patients with T2DM have for the less common cancers.

In regards to mortality rate instead of incidence of cancer, T2DM patients diagnosed with cancer also have an increased long term mortality rate (Barone et al., 2008). One meta-analysis showed that T2DM patients had an increased mortality hazard ratio of 1.41, when compared to non diabetic patients across all types of cancer (Barone et al., 2008). The most statistically significant correlations for an increased mortality rate among diabetics compared to non-diabetics were observed in those with cancers of the endometrium, breast, and colorectum, with hazard ratios of 1.76, 1.61, and 1.32, respectively (Barone et al., 2008). Another study found that patients with pre-existing T2DM are associated with higher rates of prostate cancer mortality and all cause mortality, even though the previously mentioned study showed that the incidence of
prostate cancer was lower for T2DM patients (Bensimon et al., 2014; Giovannucci et al., 2010). All cause mortality is referring simply to the death rate from any cause. So an increased all cause mortality rate for T2DM patients means that they have generally an increased death rate when compared to the rest of the population, and are more likely to live a shorter life.

To sum up the data presented so far, patients with preexisting T2DM have increased incidence of liver, pancreatic, endometrial, colon, rectum, breast, and bladder cancer (Giovannucci et al., 2010). T2DM patients with certain cancers are also shown to have an increased mortality rate when compared to non diabetic patients with the same cancer (Barone et al., 2008). These observations show significant correlation between type 2 diabetes mellitus and many types of cancer, so there probably is some physiological or biochemical link(s) between the two. Exposing the links or common risk factors between T2DM and cancer may aid in the development of treatment or preventative measures.

**Diabetic Characteristics Posing a Risk to Cancer**

Patients with T2DM may be at a greater risk of neoplastic growth because of some of the physiologic characteristics of the disease, such as hyperinsulinemia, hyperglycemia, and chronic inflammation (Giovannucci et al., 2010). T2DM, also known as noninsulin-dependent diabetes mellitus, is a disease in which the body has become insulin insensitive (Kemp et al., 2008). Due to consistently high levels of blood glucose, the body constantly demands output of insulin from the pancreas. If this state
becomes chronic, then the body’s cells become desensitized and down-regulate the insulin receptors that are displayed on the surface of cells. These insulin receptors, most importantly on the muscle cells and adipocytes, promote taking up glucose from the blood, thereby lowering the blood glucose levels to normal. If the insulin receptors are down-regulated, as occurs with T2DM, then the mechanism of lowering blood glucose becomes ineffective, and therefore requires more and more insulin to achieve a normal blood glucose level. Eventually the pancreas will reach a limit on the amount of insulin it can produce, and therefore will not be able to lower the glucose levels, leaving the individual in a state termed metabolic syndrome. The chronic hyperglycemia, increased blood pressure, and other conditions of metabolic syndrome can lead to many microvascular and macrovascular disease states, such as atherosclerosis, retinopathy, nephropathy, and peripheral neuropathy (Kemp et al. 2008). The T2DM characteristics, hyperinsulinemia, hyperglycemia, and chronic inflammation, are known to be important factors causing these disease states. However, these characteristics have only been suggested and not yet confirmed to be the reason for the higher risk of cancer incidence and mortality in T2DM patients.

**Hyperinsulinemia**

Firstly, hyperinsulinemia, a state of elevated insulin levels, may induce growth in neoplastic tissues via binding to insulin-like growth factor 1 receptors (IGF1-Rs). The primary ligand for IGF1-R is of course insulin-like growth factor 1 (IGF1); however, insulin is also capable of binding to this receptor, albeit with a lower affinity than IGF1
It has been observed that many cancer cell lines display IGF1-Rs and thus experience potentiated growth when exposed to IGF1 (Pollak, 2008). Recent studies have also shown that the insulin receptor is also commonly expressed, and sometimes overexpressed on human neoplastic cells (Cox et al., 2009; Frasca et al., 2008). The prostate is not usually responsive to insulin since it does not normally express IGF1-R; however, it was found that human prostate cancer cells express such receptors (Cox et al., 2009). Furthermore, subunits of the IGF1-R and the insulin receptor are capable of forming a hybrid receptor, which could in part explain the hypothesis of an insulin mediated IGF1 receptor signaling response (Frasca et al., 2008). Another explanation could simply be that the elevated levels of insulin help overcome the low affinity of insulin for the IGF1-R, causing aberrant activation.

Secondly, hypersinsulinemia may contribute to increased neoplastic growth via insulin receptor activation (Pollak, 2008). The insulin receptor contributes mainly to metabolic signaling, for example by inserting GLUT4 into the plasma membrane so that the cell can uptake more glucose for cell anabolism and for energy production (Frasca et al., 2008). A fact that may be overlooked is that the insulin receptor is also part of a growth signaling pathway, through the activation of Ras and Akt (Frasca et al., 2008). Thus the insulin receptor alone may be a key modulator in cancer progression.

Thirdly, elevated levels of insulin may change the levels of free circulating hormones and growth factors in the blood (Giovannucci et al., 2010; Pasanisi et al., 2006). Hyperinsulinemia has an effect on the liver which reduces the amount of IGF binding protein produced (Giovannucci et al., 2010). A lower level of IGF binding
protein would cause less IGF-1 to be bound in the blood and more to be circulating freely, therefore allowing more IGF-1 to act on its target sites. In a study of 110 postmenopausal women with breast cancer, the women with metabolic syndrome showed significantly higher levels of testosterone and estradiol, and significantly lower levels of the sex hormone-binding globulin (SHBG) when compared to the women without metabolic syndrome (Pasanisi et al., 2006). The increased production and higher levels of circulating sex steroids has been linked to a higher rate of cancer recurrences in these women, and may have important mitogenic factors in other cancers as well (Pasanisi et al., 2006).

**Hyperglycemia**

Hyperglycemia seems like it would be a formidable risk factor for neoplastic growth; however, most cancer cells already have a highly up-regulated insulin-independent uptake of glucose (Giovannucci et al., 2010). This highly up-regulated glucose uptake by cancer cells is exploited by FDG-PET scanning for tumors, which uses a radioactive glucose analogue that should be rapidly taken up the cancer cells (Cornett & Dea, 2014). Chronic elevated blood glucose levels should only give the cancer cells a slight advantage since the cancer cells are already obtaining rapid rates of glucose uptake (Giovannucci et al., 2010). The only major advantage that hyperglycemia provides for neoplastic growth might be that it causes elevated levels of insulin.
**Obesity and Inflammation**

Obesity is a very common characteristic associated with T2DM, and may also be a risk factor for cancer. White adipose tissue is considered to be an endocrine organ, and in the case of obesity, it releases elevated levels of inflammatory cytokines which may have tumorigenic capabilities (Giovannucci et al., 2010). Some of these cytokines, like plasminogen activator inhibitor-1, have been associated with a worse prognosis for women with breast cancer (Giovannucci et al., 2010). Interleukin-6 is another cytokine which is known to promote tumor proliferation and invasion (Giovannucci et al., 2010).

It is clear that there are multiple characteristics of T2DM that may pose a risk to the development and progression of cancer. There are several current drug therapeutics to help control the most dangerous risk of T2DM, which is hyperglycemia. However these drugs have different means of lowering the blood glucose to a safe level. It is further being revealed that these different drugs may have different effects on cancer prevention and risk.

**Current Therapeutics for T2DM**

Most of the treatments for T2DM are not treatments that cures the disease, but rather help manage it. The primary concern with the treatment of T2DM is to control of the blood glucose levels. Some of the classes of antidiabetic drug are the sulfonylureas, biguanides, thiazolidinediones (TZDs), and insulin replacements (Nolte Kennedy, 2012). The sulfonylureas, also known as insulin secretagogues, have a primary action of promoting the release of insulin from the pancreas (Nolte Kennedy, 2012). The
biguanides, like metformin, have a primary effect on the liver by reducing the hepatic glucose production (Nolte Kennedy, 2012). Other minor mechanisms of action of biguanides include stimulation of glycolysis in tissues, decreased absorption of glucose from the intestines, and enhanced removal of glucose from the blood by increasing the sensitivity of tissues to insulin (Nolte Kennedy, 2012). The main mechanism of action of TZDs is to decrease insulin resistance, which would therefore increase the glucose uptake into tissues like adipocytes and muscles, and lower blood glucose levels (Nolte Kennedy, 2012). Lastly, insulin replacement therapy, which is the major treatment for type 1 diabetes mellitus, can be used in T2DM if the patient’s pancreatic cells are burned out from constant secretion of insulin (Nolte Kennedy, 2012). The exogenous insulin would act just like the endogenous insulin by lowering blood glucose levels.

With the increasing prevalence of T2DM and the use of various types of antidiabetic drugs, many groups of researchers have gathered information from larger subject groups in order to compare the outcomes, effectiveness, and side effects of these different drug classes. Many studies have recently found relationships between these drugs and cancer risk. A meta-analysis of primary studies looking at the effects of metformin and sulfonylureas in patients with T2DM concluded that the use of metformin is associated with a reduction of cancer risk, considering all cancer sites (Thakkar et al., 2013). This same meta-analysis also concluded that the use of sulfonylureas in patients with T2DM is associated with an increased cancer risk, considering all cancer sites (Thakkar et al., 2013). A different meta-analysis assessed the risk of hepatocellular carcinoma (HCC) with respect to the use of metformin, TZDs, sulfonylureas, and insulin
in T2DM patients (Singh et al., 2013). Statistically significant data reported that metformin use in T2DM patients confers a 50% reduction of HCC incidence (Singh et al., 2013). Sulfonylurea use in T2DM was shown to increase HCC incidence by 62%, while insulin use in T2DM was shown to increase HCC incidence by 161% (Singh et al., 2013). The use of TZDs in T2DM patients did not show any statistically significant reduction or increase in HCC incidence (Singh et al., 2013). A third meta-analysis looking at the relationship between the various antidiabetic drugs and colorectal cancer observed that metformin use is associated with a statistically significant 11% reduction in colorectal cancer risk (Singh et al., 2013). In this study, TZD use was not shown to affect the risk of colorectal cancer (Singh et al., 2013). Use of sulfonylureas and insulin were observed to confer a higher risk of colorectal cancer; however, these data were not statistically significant (Singh et al., 2013). From these three studies, the biguanide, metformin, is the only antidiabetic drug to show statistically significant associations with a decreased risk of cancer at multiple tissue sites in T2DM patients. Therefore, it is reasonable to assume that metformin may have a cancer protective effect in T2DM patients, and perhaps in non-diabetic patients.

Various other large-scale studies on metformin use in T2DM patients reveal further evidence of its role in reducing cancer risk. A meta-analysis was conducted to determine the previously disputed relationship between metformin use for T2DM and breast cancer incidence (Col et al., 2012). Drawing from four cohort studies and three case control studies, this meta-analysis calculated an odds ratio of 0.83 for metformin use and breast cancer incidence (Col et al., 2012). Due to the large number of patients
assessed in this meta-analysis, the data are statistically significant and suggest that
metformin does indeed have a protective effect on breast cancer in women with T2DM
(Col et al., 2012). To further drive home the point, another meta-analysis focused on the
relationship between metformin use in T2DM patients and the risk of colorectal cancer
(Zhang et al., 2011). After considering three cohort studies and two case-control studies,
patients with T2DM treated with metformin compared to T2DM patients on other
treatment were associated with a relative risk of 0.63 for developing colorectal cancer
(Zhang et al., 2011).

There seems to be an overall association between metformin use and lower
incidence of cancer in T2DM patients. However, before going into the proposed cancer-
protective mechanisms of the drug metformin, its basic role in the treatment of T2DM
and its molecular mechanism of action will be presented.

**Metformin**

Metformin belongs to the biguanide class of antidiabetic drugs and is the first
choice of treatment for T2DM (Nolte Kennedy, 2012). From a clinical standpoint, it
lowers basal and postprandial blood glucose levels; however, it does not stimulate
endogenous insulin secretion or mimic insulin like some of the other antidiabetic drug
classes (Gong et al., 2012). This gives metformin a higher level of safety compared to
some other classes of antidiabetic drugs since it is less likely to cause hyperinsulinemia
and hypoglycemia (Gong et al., 2012). The major mechanism of action, as previously
stated, is the reduction of gluconeogenesis and release of glucose from the liver (Nolte
Kennedy, 2012). Metformin is also known as an insulin sensitizer, which comes from its ability to increase the sensitivity of peripheral tissues to insulin, thus increasing the glucose uptake and lowering blood glucose levels (Gong et al., 2012). Metformin is also thought to have other minor effects such as a reduction of intestinal glucose absorption, reduction of fatty acid and triacylglycerol synthesis, stimulation of glycolysis in peripheral tissues, elevation of the conversion of glucose to lactate in enterocytes, and reduction of glucagon levels (Gong et al., 2012; Nolte Kennedy, 2012). Due to all of the mentioned mechanisms of metformin, it is a very effective drug for lowering blood glucose levels and therefore a top choice of treatment for T2DM.

From this information, there are several plausible basic explanations as to how metformin lowers cancer risk in T2DM patients. First of all metformin helps control blood glucose levels, although unlike most other antidiabetic drugs, it does not act by further increasing insulin levels. As previously stated, hyperinsulinemia is one of the potential diabetic characteristics thought to increase the cancer risk in T2DM patients. Metformin increases the responsiveness of peripheral tissues to insulin, which would enable lower levels of insulin to have the same glucose lowering effect compared to the previously higher levels. Hence, metformin should lower the elevated insulin levels which should therefore lower the physiological responses to hyperinsulinemia, which include spill over of insulin onto IGF1-R, overstimulation of cancer cells expressing high levels of insulin receptors, reduction of plasma binding proteins which increases the concentration of free hormones, and elevation of sex steroids (Frasca et al., 2008; Giovannucci et al., 2010; Pollak, 2008). The other antidiabetic drugs, like the
sulfonylureas and the insulin replacement proteins also lower blood glucose levels, but the problem is that they contribute to higher insulin levels, which exacerbates the neoplastic promoting conditions of hyperinsulinemia.

The main mechanism of metformin, which is the reduction of hepatic glucose output, also helps to maintain lower blood glucose levels; however, as previously mentioned, it may not have any significant effect on neoplastic growth, given that cancer cells already have a high uptake of glucose from insulin-independent mechanisms (Giovannucci et al., 2010). The only benefit of reduced hepatic glucose output for cancer protection is that the pancreatic beta cells would receive less stimulation to increase insulin levels. Lastly, metformin is not typically known to increase weight gain, as opposed to the sulfonylureas, and therefore would not exacerbate the inflammatory cytokine signaling from adipose tissue (Nolte Kennedy, 2012).

Metformin may indeed reduce cancer risk through these mechanisms; however, these are only macroscopic mechanisms looking at the big physiological picture. Data on metformin’s precise molecular mechanism of action have been studied extensively over the last 10 to 15 years. A general consensus has been reached on some of the precise mechanisms and pathways that metformin activates; however, new data continue to be published. The following data are some important studies that have led to the elucidation of metformin’s major mechanism of action on a cellular level. The study of metformin’s effects on the molecular level will help determine what other effects it has on cancer prevention besides its influence on insulin levels.
Molecular Mechanism of Action

Previous studies have shown some controversy on the precise mechanism of action of the drug metformin. Metformin’s effect on circulating glucose and lipids was known, but there was no record of a precise mechanism. In 2000, El-Mir et al. and Owen et al. both conducted experiments in rat hepatocytes that showed metformin inhibits complex 1 of the mitochondrial electron transport chain, which causes the levels of ATP to change (El-Mir et al., 2000; Owen et al., 2000). In 2001, Zhou et al. were the first to show that the action of metformin works through an AMPK dependent pathway, which makes sense in light of the evidence that it changes the ATP levels (Zhou et al., 2001). Many studies were published thereafter and have either supported or refuted these proposed mechanisms of action. Some of these studies will be looked at closely in order to determine the true mechanism of action of metformin. Most of these studies revolve around a very important protein called AMP-activated protein kinase (AMPK).

AMPK is a major energy status regulatory protein that detects changes in adenine nucleotide levels, as seen in Figure 1 (Hardie, 2007). It is a heterotrimer composed of α, β, and γ subunits; the α subunit is the catalytic subunit and the β and γ subunits are the regulatory units (Hawley et al., 2010). With lower levels of ATP, and hence a higher ADP/ATP ratio, the AMP/ATP ratio also increases due to the conversion of ADP into ATP and 1AMP (Hawley et al., 2010). AMP levels are usually extremely low so a 1:1 ratio in the increase of ATP and AMP still results in an increased ratio of total AMP/ATP. The rising AMP levels bind AMPK, and stimulate the phosphorylated form of AMPK, which is the active form (Hardie, 2007). The binding of AMP also inhibits the
The AMPK is initially activated via phosphorylation by several upstream kinases; however, the action of AMP is to further stimulate AMPK and keep it activated (Hardie, 2007; Hawley et al., 2010). Once AMPK is activated it phosphorylates key enzymes involved in metabolism that switches the cell from an anabolic to a catabolic state (Hardie, 2007). Therefore, the synthesis of lipids, glucose, proteins, and even cell growth dephosphorylation of AMPK, which keeps it active for a longer period of time (Hardie, 2007). The AMPK is initially activated via phosphorylation by several upstream kinases; however, the action of AMP is to further stimulate AMPK and keep it activated (Hardie, 2007; Hawley et al., 2010). Once AMPK is activated it phosphorylates key enzymes involved in metabolism that switches the cell from an anabolic to a catabolic state (Hardie, 2007). Therefore, the synthesis of lipids, glucose, proteins, and even cell growth

Figure 1: Role of AMPK in cellular catabolism and anabolism. Many stressors, like metformin, cause a reduction in ATP production, which unleash the inhibitor affect on AMPK. The buildup of ADP and AMP act to activate AMPK. AMPK turns off the cellular processes involved with ATP consumption, like cell division and growth.

Figure taken from (Hardie, 2007).
is down-regulated, and fatty acid oxidation and glucose uptake are up-regulated (Hardie, 2007).

**Inhibition of Complex 1 of the ETC**

El-Mir et al. was the first study to show that metformin hinders cellular respiration by its inhibitory effect on complex 1 of the electron transport chain (ETC) of the mitochondria (El-Mir et al., 2000). Complex 1 is one of the multiple units of the ETC in the mitochondria, which helps create an electrochemical gradient between the matrix and interstitial space of the mitochondria. This electrochemical gradient drives protons through an ATPase in the inner mitochondrial membrane, which converts ADP into ATP, a source of energy for the cell. By inhibiting complex 1 of the ETC, the proton pumps are less active and therefore the electrochemical gradient becomes weaker and less capable of driving the synthesis of ATP through the ATPase (Janson, 2014).

El-mir et al. observed no inhibition of complex 1 when isolated mitochondria were treated with metformin, which strongly suggests that metformin has no direct effect on complex 1 (El-Mir et al., 2000). Direct ETC inhibitors like rotenone and cyanide inhibit cellular respiration, and would show decreased cellular respiration when incubated with isolated mitochondria; however, metformin is different from these direct inhibitors (Janson, 2014). Inhibition of complex 1 was observed in intact hepatocytes treated with metformin, which would suggest that metformin works indirectly to inhibit complex 1 via some cellular pathway that has yet to be established (El-Mir et al., 2000). Data showing the inhibition of cellular respiration in rat hepatocytes with treatment of metformin
(dimethylbiguanide) can be seen in Table 1 (El-Mir et al., 2000). After 30 minutes of incubation with metformin, the following parameters were determined: cellular respiratory rate ($JO_2$), in situ mitochondrial membrane potential ($\Delta\Psi$), cytosolic and mitochondrial ATP/ADP ratios, ratio of lactate to pyruvate, and ratio of 3-hydroxybutyrate to acetoacetate (El-Mir et al., 2000). When compared to the control group, the hepatocytes treated with dimethylbiguanide, or metformin, showed a significant decrease in mitochondrial membrane potential ($\Delta\Psi$), which is in accordance with the decrease in cellular respiratory rate ($JO_2$) (El-Mir et al., 2000). The ATP/ADP ratios of the mitochondrial and cytosol lowered significantly, which would mean that the levels of ATP have decreased when compared to ADP levels, explained by the reduced mitochondrial membrane potential and reduced production of ATP from the ATP synthase (El-Mir et al., 2000).

In order to determine whether the inhibition resulted from a reduction of energy substrates for the ETC or from inhibition of ETC function, the lactate/pyruvate (lac/pyr) ratio and 3-hydroxybutyrate/acetoacetate (3-OH/AcAc) ratios were recorded (El-Mir et al., 2000). The lac/pyr ratio reflects cytosolic NADH concentrations, while the 3-OH/AcAc ratio reflects mitochondrial NADH concentrations; higher ratios, as observed in the hepatocytes treated with dimethylbiguanide, are indicative of an increase in energy substrates (El-Mir et al., 2000). This is evidence that the action of dimethylbiguanide, metformin, is indeed through inhibition of the ETC machinery involved in cellular respiration. This study had not yet linked this data to the activation of AMPK though.
Table 1. Effects of dimethylbiguanide, myxothiazol, and rotenone on cellular respiratory function.
Intact rat hepatocytes were incubated with no inhibitors, 10 mM dimethylbiguanide, .15 µM myxothiazol, or .52 µM rotenone. After 30 minutes of incubation, cellular respiratory rate (JO₂), in situ mitochondrial membrane potential (ΔΨ), cytosolic and mitochondrial ATP/ADP ratios, ratio of lactate to pyruvate (lac/pyr), and ratio of 3-hydroxybutyrate to acetoacetate (3-OH/AcAc) were recorded. Table taken from (El-Mir et al, 2000).

<table>
<thead>
<tr>
<th></th>
<th>JO₂</th>
<th>ΔΨ</th>
<th>ATP/ADP (cyto)</th>
<th>ATP/ADP (mito)</th>
<th>Lac/Pyr</th>
<th>3-OH/AcAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 0</td>
<td>-175 ± 3</td>
<td>7.3 ± 0.5</td>
<td>1.7 ± 0.1</td>
<td>19.8 ± 0.3</td>
<td>0.18 ± 0.001</td>
</tr>
<tr>
<td>Dimethylbiguanide</td>
<td>54 ± 2a</td>
<td>-141 ± 5a</td>
<td>2.5 ± 0.2a</td>
<td>0.5 ± 0.1a</td>
<td>24.4 ± 0.3a</td>
<td>0.38 ± 0.003a</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>56 ± 9a</td>
<td>-154 ± 5a</td>
<td>3.1 ± 0.3ab</td>
<td>0.7 ± 0.1ab</td>
<td>26.0 ± 0.5a</td>
<td>0.25 ± 0.003ab</td>
</tr>
<tr>
<td>Rotenone</td>
<td>50 ± 7a</td>
<td>-149 ± 5a</td>
<td>3.4 ± 0.3ab</td>
<td>0.9 ± 0.1abc</td>
<td>38.3 ± 0.7abc</td>
<td>0.44 ± 0.003abc</td>
</tr>
</tbody>
</table>

* p < 0.001 versus control.

**p < 0.001 versus dimethylbiguanide.

***p < 0.01 versus myxothiazol.
Since the publication of this study by El-Mir et al. in 2000, various other researchers have published studies on metformin and its mechanism of action. Owen et al. published a study also in 2000, which showed further evidence that metformin inhibits complex 1 of the ETC, albeit they showed that metformin acted directly on the mitochondria, instead of indirectly through an undetermined signaling pathway as El-Mir et al. suggested (El-Mir et al., 2000; Owen et al., 2000). Although these two studies differ in the proposed inhibition of complex 1, they still nonetheless gave evidence that complex 1 is indeed inhibited by metformin and results in a reduced ratio of ATP/ADP (El-Mir et al., 2000; Owen et al., 2000). An important finding in the study by Owen et al. was that although the total nucleotide concentrations were the same, the calculated free ATP/ADP ratio was reduced (Owen et al., 2000). Owen et al. went on to explain that the decreased ATP/ADP ratio causes inhibition of pyruvate carboxylase, and the observed increase in the concentration of phosphoenolpyruvate leads to a stimulation of pyruvate kinase; together these effects lead to a decline in gluconeogenesis, a well known characteristic of metformin (Owen et al., 2000).

**Metformin and AMPK**

In 2001, Zhou et al. were the first ones to show that metformin acts through an AMPK dependent pathway (Zhou et al., 2001). They gave metformin to a rat hepatocyte control cell culture, and then to a rat hepatocyte cell culture with an AMPK inhibitor, termed compound C (Zhou et al., 2001). A known AMPK activator, AICAR, was also used in another set of cells in place of metformin in order to compare it to metformin’s
stimulation of AMPK (Zhou et al., 2001). The control group treated with metformin or AICAR showed a decrease in glucose output and acetyl-CoA carboxylase (ACC) activity (Zhou et al., 2001). ACC is a classic AMPK substrate target. The rat hepatocytes given both the AMPK inhibitor and metformin/AICAR showed a reduction of metformin’s effects, which was observed as a lesser reduction of glucose output and less inhibition of ACC (Zhou et al., 2001).

As shown in Figure 2, when metformin or the AMPK activator, AICAR, were given alone there was a significant reduction in ACC activity (Zhou et al., 2001). Once the AMPK inhibitor compound C was added to either cell culture, an increasing ACC activity was associated with an increasing dose of the AMPK inhibitor while the dose of metformin/AICAR was held constant (Zhou et al., 2001). Given that an activated AMPK inhibits ACC activity, a deactivated AMPK should not be able to inhibit ACC, which increases ACC activity. These results therefore showed that metformin requires the activation of AMPK in order to display its effect in lowering hepatic glucose production (Zhou et al., 2001). However, the mechanism of AMPK activation was unclear; it was not known if this was a direct activation or due to a complex signaling pathway.
After these initial studies, it seemed that metformin worked by causing changes in cellular adenine nucleotide levels that affect AMPK. However other researchers postulated that metformin activates AMPK without changing the ratio of AMP to ATP, which refutes both studies performed by El-Mir et al. and Owen et al. (Hawley et al., 2002). Another study observed that an upstream kinase, LKB1, was the major activating kinase of AMPK from metformin use (Shaw et al., 2005). Later, Hardie reported that metformin’s role of reducing hepatic glucose output was not due to direct activation of LKB1 or AMPK by metformin (Hardie, 2006). LKB1 is indeed required for AMPK activation, but it is not altered by metformin (Hardie, 2006) Still more proposed mechanisms were published. Fujita et al. reported that metformin did not increase the AMP/ATP ratio in their mouse hepatocytes; however, it did generate peroxynitrite, a

Figure 2: Use of an AMPK inhibitor to show that metformin works through AMPK dependent pathways. In vitro rat hepatocytes were treated with metformin or AICAR, a direct AMPK activator. Acetyl-CoA carboxylase (ACC) activity was measured at varying concentrations of compound C, the AMPK inhibitor. Figure amended from (Zhou et al, 2001).
reactive nitrogen species, which they suspected to activate AMPK (Fujita et al., 2010). The fact of the matter is that there are multiple ways to activate AMPK, being that it is the center of many metabolic signaling pathways (Hawley et al., 2010). The goal is to determine which are the main pathways involved with the use of metformin.

Stephenne et al. sought out to test the previous studies but in human hepatocytes, and to hopefully make a clear determination as to the correct mechanism of action of the drug metformin (Stephenne et al., 2011). They tested the hypothesis that metformin activates AMPK through an increase in AMP concentration resulting from the inhibition of the mitochondrial complex 1 (Stephenne et al., 2011). In rat hepatocytes, metformin increased AMP levels and AMPK activation (Stephenne et al., 2011). In human hepatocytes, metformin also increased AMP levels and AMPK activation as seen in Figure 3 a & b (Stephenne et al., 2011).

An interesting finding was that the same increase seen in AMP levels in humans caused a greater response of the human AMPK (Stephenne et al., 2011). This led the authors to search for isoforms of AMPK, finding that humans mainly expressed AMPKα1, but rats express both AMPKα1 and AMPKα2 at similar concentrations (Stephenne et al., 2011). A difference in affinity of these isoforms of AMPK most likely accounts for the greater response seen in humans. The difference between metformin’s effect in rats and humans is important to keep in mind when determining potential clinical efficacy.
Next the link between AMPK and the cellular energy levels in metformin treatment was explored using knockouts of liver specific AMPKα1 and 2 from mice (Stephenne et al., 2011). In the wild type mice, metformin increased the level of AMP and increased the activation of AMPK; however, the knockout AMPKα1/2 mice showed increased levels of AMP with no observable expression of AMPKα1/2, shown in Figure 4 (Stephenne et al., 2011). This shows that the inhibition of mitochondrial complex 1 and subsequent increase in AMP was not due to AMPK activation, therefore eliminating the possibility that metformin directly activates AMPK (Stephenne et al., 2011).
With the experimentation on rat and human hepatocytes, Stephenne et al. came to the conclusion that metformin does indeed work through AMPK; however, they ruled out the possibility of direct activation of AMPK by metformin (Stephenne et al., 2011). They showed that AMPK is activated from a decrease in ATP and concurrent increase in AMP which is due to the inhibition of complex 1 of the mitochondrial respiratory complex (Stephenne et al., 2011).

Why was it so important to determine the precise mechanism of action? The target of the drug metformin will help assess whether or not metformin has an effect on neoplastic growth and tumorigenesis. Since it is now generally accepted that metformin leads to the activation of AMPK, and since metformin has been statistically shown to reduce cancer risk in patients with T2DM, it is justified to look at how the activation of

**Figure 4: Measure of AMP/ATP ratios.** Wild type mice and AMPKα1/2 Knockout mice (AMPKα1/2−/−) hepatocytes were treated with metformin and the measured for changes in AMP/ATP ratios. Black is the control and white is the use of metformin. Figure amended from (Stephenne et al., 2011).
AMPK affects tumor cancer progression. Although AMPK may not be the only target for metformin, it is one of the most significant and commonly reported. Therefore, most of the antineoplastic effects of metformin that will be reviewed will involve the activation of AMPK.
Specific Aims/Objectives

The first choice and most prescribed antidiabetic drug, metformin, has statistically been shown to lower risk and improve outcome of cancer in many patients with T2DM in retrospective studies. Extensive research on its possible molecular mechanism of action has helped shed some light on metformin’s role in not only diabetic therapy, but in its role as a potential cancer therapeutic. Metformin’s indirect physiological effect on plasma insulin levels may be a small part of its role as a cancer therapy; however, researchers have dug deeper and explored its effect on cancer cells at the molecular level. This paper will present some of the major pathways by which metformin inhibits cancer growth and tumorigenesis. This paper will also present studies in which metformin has shown potential for use in specific cancer types, with a focus on breast cancer. Lastly, studies of metformin as an adjunctive therapy to current standards of care for specific subtypes of breast cancer will be evaluated for their use in the clinical setting.

Metformin may be of significant value to the field of cancer. A compilation of publications such as this one will give an understanding of where the field is at the present time in considering metformin as a cancer therapeutic, and what future directions should be explored. If shown efficacious in clinical trials for breast cancer, metformin will be highly sought after for the treatment of other types of cancers as well. It would probably be most useful as an additional therapy to the current regimens of chemotherapy.
PUBLISHED STUDIES

Major mechanisms of action of metformin in cancer cells

There are several well-studied pathways through which AMPK may reduce or halt neoplastic growth. Many of these pathways have been elucidated with the use of breast cancer cell lines, which are currently a major focus for the benefits of metformin. One of the pathways by which metformin induces cell cycle arrest was reported by Zhuang & Miskimins, and it was shown to work downstream from metformin’s activation of AMPK (Zhuang & Miskimins, 2008). The other pathway also works via AMPK and involves the inactivation of the mTOR pathway (Cantrell et al., 2010).

Cell Cycle Arrest via cyclin/CDKs

Zhuang & Miskimins tested the effects of metformin on cell cycle arrest on 6 different breast cancer cell lines and observed growth arrest in 5 of them, which are termed: MCF7, T47D, BT20, MDA-MB-474, and MDA-MB-453 (Zhuang & Miskimins, 2008). The breast cancer cell line termed MDA-MB-231 was the only one out of the six to have unaffected growth from metformin treatment (Zhuang & Miskimins, 2008). The reason why and the importance of this observation will be examined later in this analysis.

After the observation of growth arrest in these 5 breast cancer cell lines, the pathways were looked at further. This study used western blot analysis to examine the cell cycle regulatory proteins, such as the cyclins, CDKs, and CDK inhibitors (Zhuang & Miskimins, 2008). It was observed that the cyclin D1 expression was significantly lower in metformin treated cells than in the control (Zhuang & Miskimins, 2008). The basic
understanding of cyclin D1 is that it complexes with a CDK like CDK4, and this complex is needed for the progression through late G1 phase and into the S phase (Schmid & Carson, 2010). The more complicated version of this is that the cyclin D1/CDK complex contributes to the phosphorylation and inactivation of the tumor suppressor pRB (Schmid & Carson, 2010). This is one mechanism of cyclin D1 that Zhuang & Miskimins did not mention; however, they did mention another one of its mechanisms from the observation that cyclin D1 is downregulated and that the CDK inhibitors p27 and p21 were downregulated (Zhuang & Miskimins, 2008). They proposed that a downregulated cyclin D1 leads to increased levels of CDK inhibitors like p27 and p21 (Zhuang & Miskimins, 2008). Cyclin D1 is involved with the sequestering of CDK inhibitors, and so when cyclin D1 is downregulated via AMPK, the CDK inhibitors become free and available to inhibit one of their targets, the cyclin E/CDK2 complex (Zhuang & Miskimins, 2008). Like the cyclin D/CDK4 complex, this cyclin E/CDK2 complex also contributes to the inactivation of the tumor suppressor pRB (Schmid & Carson, 2010). Therefore, since these cyclin/CDK complexes are downregulated and inhibited, the cell cannot inactivate the tumor suppressor pRB and the cell cycle arrests in G1 phase.

This pathway has enormous significance due to the fact that the cyclin Ds respond to cellular stress levels and mitogenic signals like Ras (Schmid & Carson, 2010). Mitogenic signaling in a cancer cell is often highly upregulated or out of control, but metformin would help to counteract this affect. Theoretically, since cyclin D also
responds to cellular stress levels via AMPK, treatment with metformin would help induce a state of lower energy levels and thus counteract the stimuli of the mitogenic signals.

Another important aspect of this mechanism of inhibition is that it works independent of p53. The protein p21 is downstream of p53, so a mutation in p53 would not affect the inhibition imposed by metformin (Schmid & Carson, 2010). Buzzai et al. showed that a colon cancer cell line deficient in p53 (p53−/−) were susceptible to metformin, and upon treatment showed suppressed tumor growth and increased apoptosis (Buzzai et al., 2007). This is significant and exciting to hear since a mutation in p53 is a common mutation that accumulates during tumorigenesis.

Now getting back to an earlier question, why wasn’t the cell line MDA-MB-231 sensitive to metformin, even though the other five cell lines were? The answer was looked at closely by the experimenters and they found that this cell line expressed significantly lower basal levels of p21 and p27 (Zhuang & Miskimins, 2008) This is an important discovery because it implies that metformin may not be effective in the treatment of other cancers which have a low expression of these CDK inhibitors, which is characteristic of many cancers (Zhuang & Miskimins, 2008).

Kobayashi et al. showed that the amount of phosphorylated pRB was reduced in an esophageal cancer cell line upon treatment with metformin, which supports the mechanism proposed by Zhuang & Miskimins (Kobayashi et al., 2013). Less phosphorylated pRB may indicate increased levels of unphosphorlated pRB, which is the form of pRB that causes cell cycle arrest.
**Growth Inhibition via mTOR**

Another major mechanism by which metformin is proposed to work against cancer cells is by the reduction of the mTOR pathway. The protein mTOR is an effector protein of the PI3K family that responds to growth factors, and therefore is important in transducing pro-tumorigenic signals (Chabner et al., 2011). It is common to find an aberrant mTOR signaling pathway in cancer cells, mostly likely due to gene amplification or activating mutations (Chabner et al., 2011).

Cantrell et al. studied endometrial cancer cell lines and also observed an increased cell cycle arrest in G1 phase and reduced growth upon treatment with metformin (Cantrell et al., 2010). As in the study by Zhuang & Miskimins, they found that after treatment with metformin, AMPK was further activated; however, they focused on the mTOR signaling pathway (Cantrell et al., 2010).

Sixteen hours after treatment with metformin in endometrial cancer cell lines, a Western blot analysis was used to determine the concentration of phosphorylated S6 (P-S6), which is a downstream target of mTOR (Cantrell et al., 2010). Thus the concentration of phosphorylated S6 (the activated form) will indicate whether the mTOR signaling pathway is up-regulated or down-regulated. It was observed that with increasing concentrations of metformin, the concentration of P-S6 was reduced dramatically in both endometrial cancer cell lines (Cantrell et al., 2010). This finding was looked at again by another study, which also was able to show the same decreased phosphorylation of protein S6 upon low dose treatment with metformin (Hanna et al., 2012). This is evidence that metformin may have an anti-proliferative role in cancer by
activation of AMPK and reducing the activation of mTOR. As mTOR has been a common target in many cancer types, metformin may add to the effect of existing mTOR inhibitors and thus have a synergistic anti-proliferative effect.

**Overview of anti-neoplastic effects**

The down-regulation of cyclin D1 and the inhibition of the mTOR pathway are just a few of the effects of activation of AMPK. The time and research spent looking into the precise mechanisms of these pathways has been invaluable in moving the field of cancer therapeutics forward. Metformin for the treatment of cancer is a growing area of interest; however, a lot more time and research will be needed in order to determine its usefulness. Current research is focusing on which types of cancers it is effective in, and part of that should be looking at what cancer genotypes would be most susceptible to metformin. **Table 2** is a compilation of data from some of the previously mentioned studies that proposes the genotypes that may and may not be susceptible to metformin. This table is by no means comprehensive of the entire field of cancer; however, it is a start and will need to be expanded as research continues to better assess the benefits of metformin.
Table 2: Efficacy of metformin in certain cancer genotypes. Efficacy with metformin is categorized as “Yes” only if there is some anti-neoplastic effect observed. The table was made using the data from multiple studies: (Buzzai et al, 2007)(Zhuang & Miskimins, 2008)(Cantrell et al., 2010)

<table>
<thead>
<tr>
<th>Publications</th>
<th>Cancer genotype</th>
<th>Effective w/ metformin</th>
<th>Vehicle of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buzzai et al., 2007</td>
<td>p53 deficient (p53&lt;sup&gt;-/-&lt;/sup&gt;)</td>
<td>Yes</td>
<td>Human colon cancer xenograft in mice</td>
</tr>
<tr>
<td>Zhuang &amp; Miskimins, 2008</td>
<td>P21 or p27 deficient</td>
<td>No</td>
<td>Human breast cancer in vitro</td>
</tr>
<tr>
<td>My speculation from Zhuang &amp; Miskimins, 2008</td>
<td>pRB inactivating mutation</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Cantrell et al., 2010</td>
<td>mTOR up-regulated</td>
<td>Yes</td>
<td>Human endometrial cancer in vitro</td>
</tr>
</tbody>
</table>

Figure 5 from Rattan et al., summarizes some of the main points mentioned thus far regarding the mechanism of action of metformin in cancer cells (Rattan et al., 2012). In the background section of the paper, the glucose and insulin lowering effects of metformin were discussed as one of the probably systemic benefits. The significance of the inhibition of ACC and HMG CoA reductase were not mentioned but play an important role in lipid synthesis needed for cellular growth and division (Rattan et al., 2012). Lastly the effect on the cell cycle and inhibition of cell growth were discussed previously, and these are becoming of increasing importance in future research.

These are just some of the main and most recognized mechanisms for metformin’s effect on cancer. However, there many other proposed mechanisms, like the reduction of hTERT (telomerase reverse transcriptase) mRNA, which is one of the mechanisms of keeping cancer cells immortal (Hanna et al., 2012).
Preclinical/Clinical Studies

Many of the mechanisms presented are through AMPK in individual cells, and not the result of metformin’s insulin and glucose lowering effects. Therefore, most of metformin’s effects are through insulin independent pathways, and it has potential for use in non-diabetic patients. Since metformin does not raise insulin levels like other antidiabetic drugs, it is safe to use in patients with normal levels of insulin because it will not usually cause hypoglycemia (Gong et al., 2012). Metformin is currently being studied alone for its effect on some cancers in non-diabetic patients, and is also being...
studied as either a sensitizer or an adjuvant to the current standard of treatment for specific cancers in non-diabetic patients.

Since there has been a greater focus on metformin in breast cancer than any other cancer so far, the current theories and proposed treatment involving metformin for breast cancer will be presented, along with any conflicting viewpoints or observations in which metformin has shown no benefit.

Most of the current research in specific types of cancers is in the preclinical phase. Breast cancer, however, has been given a much greater focus and has some clinical trials that are in motion (Litzenburger & Brown, 2014). Metformin as an adjunct to chemotherapy is currently in some early stages of clinical trials for the treatment of breast cancer (Esteva et al., 2013). The first clinical trial for metformin in the treatment of breast cancer was actually in 2011, and was a placebo vs. metformin neoadjuvant study for non-diabetic women during the preoperative period (Hadad et al., 2011). Metformin was also used in a couple other small scale neoadjuvant studies where it was given prior to surgery in women with breast cancer (Bonanni et al., 2012; Niraula et al., 2012). Some of the results from these studies found that metformin treatment in non-diabetic patients supported the theory that it has an anti-neoplastic property, whereas one study showed that the effect of metformin treatment was not statistically significant overall in non-diabetic patients with breast cancer, but it was more effective for those that showed metabolic characteristics of a pre-diabetic (Bonanni et al., 2012; Niraula et al., 2012). The sample sizes in these studies were small, so a larger scale study is needed in order to determine the efficacy of metformin with breast cancer. Litzenburger & Brown
reported that there have been a few phase 1 and 2 clinical trials in recent years, and that phase 3 trials, which usually involve a greater subject population, are currently underway (Litzenburger & Brown, 2014).

Even though it will take some time for these clinical trials to run their course, the preclinical trials can still be looked at in order to determine which types of breast cancer metformin would be most effective in. Some of the common characteristics that are looked at in describing the breast cancer type are the overexpression or lack thereof: estrogen receptor (ER), progesterone receptor (PR), or HER2 (also called erbB2) (Litzenburger & Brown, 2014). When breast cancer lacks the expression of all three of these, then it is called a triple-negative breast cancer, which is more difficult to treat and occurs 15 – 20 % of the time (Litzenburger & Brown, 2014).

**ER Positive**

Breast cancer subtypes that overexpress ER and PR are more likely to have a better outcome with treatment and are at a lesser risk of tumor recurrence (Lippman, 2012). In addition to a better prognosis with current standard of care, Ma et al. and other studies have shown support of metformin’s efficacy in reducing cancer growth in ER positive cell lines (Alimova et al., 2009; Ma et al., 2014; Zhuang & Miskimins, 2008). An even more significant finding from Ma et al., however, was that metformin increased the efficacy of the drug tamoxifen (Ma et al., 2014). A growing issue in the field of breast cancer is that some ER positive breast cancers cells are developing low sensitivity to tamoxifen treatment (Ma et al., 2014). This study has recently shown that tamoxifen
treatment in breast cancer xenograft mice acts synergistically with metformin to cause a more powerful inhibition of tumor growth in ER positive breast cancer, especially the ones with acquired resistance (Ma et al., 2014). This shows that metformin either increases the sensitization of the cancer cell to tamoxifen and/or it has its own anti-neoplastic properties. Therefore the search for combination therapy has been of great interest to help increase efficacy of tamoxifen, and should be further explored in the case of other current standards of treatment.

Endocrine therapies, like tamoxifen, are important in ER and PR positive types of breast cancer; however, they show little effect in ER negative breast cancers (Lippman, 2012). Instead, cytotoxic chemotherapy like the topoisomerase II inhibitor, doxorubicin, can be used for ER negative breast cancer cells, and will be discussed more in the triple negative breast cancer section (Chabner et al., 2011).

**HER2 Positive**

The overexpression of human epidermal growth factor receptor 2 (HER2) is found in 30% of breast cancer cases and is due to gene amplification on chromosome 17 (Chabner et al., 2011b). Alimova et al. observed reduced cell growth and increased cell cycle arrest with metformin treatment in many breast cancer cell lines, including ER positive and negative, and HER2 normal and overexpressing cell lines; however, this study focused more on the HER2 characteristic (Alimova et al., 2009). Upon dosage with metformin in the HER2 overexpressing cell line, lower concentrations of phosphorylated erbB2/HER2 were observed using western blot analysis (Alimova et al.,
Since the phosphorylated form of HER2 is the active form, these data indicate that metformin reduces the activity of HER2 (Alimova et al., 2009).

A similar study also observed inhibition of HER2 upon treatment with metformin, but took it a step further by giving evidence that the inhibition of HER2 was due to direct inhibition of an mTOR effector protein (Vazquez-Martin et al., 2009). This is great support for metformin’s role in treating HER2 cancers. On the other hand, the authors in this study indicated that the molecular mechanism that produces this effect is independent of AMPK, which contradicts the previously mentioned mechanism of metformin. This is a good example of the fact that the mechanism of action of metformin is still not entirely understood, despite the amount of research and time that has been put into this drug.

Resistance to drugs targeting HER2 overexpressing cancer cell lines has also unfortunately become increasingly prevalent. Trastuzumab, an antibody for the HER2 external domain, is a common chemotherapeutic for HER2 positive breast cancer cells; however, trastuzumab resistant cancers have created a problem for treatment (Chabner et al., 2011b). Searching for a drug to target these cells, researchers once again turned to metformin to determine if it proved to be efficacious here as well. Liu et al. observed that trastuzumab resistant cancer cells undergo cellular changes that alter the normal molecular mechanisms of the cell, and that because of this, metformin is more effective in inhibiting tumorigenesis in these specific cells through the reduction of interaction between erbB2/HER2 and IGF1 (Liu et al., 2011, p. 2). As complex as this may seem, it is further support for a potential use of metformin in yet another type of drug resistant breast cancer.
Lastly, metformin was also studied for its effect on breast cancer stem cells, which display high expression of erbB2/HER2, and it is being increasingly shown that these are the types of cells that are responsible for breast cancer initiation and development (Zhu et al., 2014). It was found that metformin inhibited tumor formation of these breast cancer stem cells by interfering with the HER2, Akt, and mTOR pathways (Zhu et al., 2014).

**Triple Negative Breast Cancer**

The type of breast cancer that is the most difficult to treat, with the poorest prognosis, is triple negative breast cancer, which lacks the ER, PR and HER2 receptors (Giuliano & Hurvitz, 2014). Since hormone therapy proves to be unsuccessful for the treatment of this type, cytotoxic therapy is used instead, which is a toxic therapy with the purpose of killing the cancer cell (Giuliano & Hurvitz, 2014). However, cytotoxic therapy also leads to the death of many normal human cells, usually the ones that undergo higher turnover. Due to the problems associated with this type of breast cancer, a new drug that is effective and non-toxic for the treatment of triple negative breast cancer would be groundbreaking.

Along with the other various breast cancer subtypes, metformin is also being studied in the triple negative phenotype. When studying 6 different breast cancer cell lines, Zhuang & Miskimins observed significant inhibition of growth in all the cell lines except for MDA-MD-231, which is a triple negative breast cancer cell line (Zhuang & Miskimins, 2008). A different study further investigated the use of metformin in this
subtype of cancer cells by studying its affect on growth and proliferation in 4 different triple negative breast cancer cell lines, one of them being MDA-MD-231 (Liu et al., 2009). In vitro, all four cell lines showed tumor growth inhibition, but some of the lines required more of the drug that others to reach significant values (Liu et al., 2009). Then an in vivo study using xenograft mice transplanted with MDA-MD-231 was performed and showed slower tumor growth and increased survival in the mice given metformin (Liu et al., 2009). So, Zhuang & Miskimins presented data showing that metformin does not induce an antineoplastic effect in the MDA-MD-231 cell line, whereas Liu et al. did observe an antineoplastic effect in the MDA-MD-231 cell line (Liu et al., 2009; Zhuang & Miskimins, 2008). The differing results from these two studies may be due to the difference in incubation time with metformin. Zhuang & Miskimins measured growth after 3 days, whereas Liu et al. measured growth after 2-3 weeks (Liu et al., 2009; Zhuang & Miskimins, 2008).

An article published within the past year also studied the triple negative breast cancer cell line, MDA-MD-231, and reported data indicating that this cell line is sensitive to metformin treatment only at normal glycemic condition, and not at high glycemic conditions (Zordoky et al., 2014). This article suggests that something special about the molecular pathways in MDA-MD-231 enables normal glycemic levels to sensitize the cells to metformin treatment and inhibit growth (Zordoky et al., 2014). The theory is that the metformin shows a significantly higher activation of AMPK at normal rather than high glucose levels (Zordoky et al., 2014). This is supported by the observation that high glucose levels downregulate AMPK in normal cells (Saha et al., 2011). Lower AMPK
levels would allow a smaller quantity of AMPK to respond to metformin. These data suggest that normoglycemic patients with triple negative breast cancer similar to the MDA-MD-231 cell line would show better results with metformin treatment than patients with hyperglycemia.

Even though cytotoxic therapy like doxorubicin is a toxic treatment, it is still effective sometimes in the treatment of triple negative breast cancer. However, like other drugs, the cancer cells can acquire resistance to doxorubicin. Hirsch et al. investigated this area of interest and observed that metformin worked synergistically with doxorubicin to kill the cancer cells, even in the triple negative breast cancer cell line (Hirsch et al., 2009). They found that the cancer stem cells were more resistant to doxorubicin than the non-stem cell cancers (Hirsch et al., 2009). An important finding in this study, that is similar to findings of Zhu et al., is that metformin worked to preferentially kill the cancer stem cells, which the doxorubicin treatment struggled to kill (Hirsch et al., 2009). As will be touched on in the discussion section, the concentrations required to observe an effect were crucial here. Low concentrations of metformin showed an effect on the cancer stem cell populations; however, a significantly greater concentration was needed to observe an effect in the other cancer cells, which make up the majority of the cancerous tumor (Hirsch et al., 2009).

Given that there have been numerous articles investigating the potential of metformin in treating breast cancer, Table 3 displays some of the recently published findings of observed effects of metformin in various breast cancer subtypes and cell lines.
It is important to note which breast cancer subtypes have and have not been extensively studied in order to assess where it is most effective.

Combination therapy is also important for cancer therapy due to the resiliency of cancer cells. Finding drugs with different mechanisms of action against a cancer cell give the best chance for an effective treatment. The issue of overcoming drug resistant cancer cells is also becoming of utmost importance, and so the search for potential new antineoplastic agents is a major field of investigation. Some of the current and recent publications involving metformin and an additive chemotherapeutic are displayed in Table 4.
Table 3: Antineoplastic effects of metformin observed in various breast cancer cell lines. Some of the significant publications in this area of study that are leading the way for further study of metformin in breast cancer.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Publication</th>
<th>Breast Cancer cell lines</th>
<th>Vehicle of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective in ER+ breast cancer cell lines</td>
<td>Alimova et al., 2009</td>
<td>ER+, HER2- (MCF7)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>Ma et al., 2014</td>
<td>ER+, HER2- (MCF7)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>Zhuang &amp; Miskimins, 2008</td>
<td>ER+, HER2- (ZR-75-1)</td>
<td>In vitro</td>
</tr>
<tr>
<td>Effective in HER2+ breast cancer cell lines</td>
<td>Alimova et al., 2009</td>
<td>ER-, HER2 + (MCF7/713)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER+, HER2 + (Bt-474)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>Vazques-Martin et al., 2009</td>
<td>ER-, HER2 + (SKBR-3)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>Zhu et al., 2014</td>
<td>HER2+ (78617)</td>
<td>In vitro and vivo</td>
</tr>
<tr>
<td>Effective in stem cell breast cancer cells</td>
<td>Hirsch et al., 2009</td>
<td>Transformed Stem cells (MCF10A ER-Src)</td>
<td>In vitro</td>
</tr>
<tr>
<td>Not effective in Triple Negative Breast Cancer cell line</td>
<td>Zhuang &amp; Miskimins, 2008</td>
<td>MDA-MD-231</td>
<td>In vitro</td>
</tr>
<tr>
<td>Effective in Triple Negative Breast cancer cell lines</td>
<td>B. Liu et al., 2009</td>
<td>MDA-MB-468</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BT20</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BT549</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>In vitro and vivo</td>
</tr>
<tr>
<td></td>
<td>Zordoky et al., 2014</td>
<td>MDA-MB-231</td>
<td>In vitro</td>
</tr>
</tbody>
</table>
Table 4: Synergistic antineoplastic effect of metformin with current cancer therapeutics in breast cancer cell lines.

<table>
<thead>
<tr>
<th>Findings/Publication</th>
<th>Breast Cancer cell line</th>
<th>Vehicle of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synergistic effect of metformin with doxorubicin Hirsch et al., 2009</td>
<td>ER+, HER2- (MCF7)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>ER-, HER2+ (SKBR3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triple Negative (MDA-MB-486)</td>
<td></td>
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<tr>
<td></td>
<td>Transformed Stem cells (MCF10A ER-Src)</td>
<td>In vitro and vivo</td>
</tr>
<tr>
<td>Synergistic effect of metformin with tamoxifen Ma et al., 2014</td>
<td>ER+, HER2- (MCF7)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>ER+, HER2- (ZR-75-1)</td>
<td></td>
</tr>
<tr>
<td>Synergistic effect of metformin with trastuzumab B. Liu et al., 2011</td>
<td>BT474-HR20 Trastuzumab resistant</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>SKBR3-pool3 Trastuzumab resistant</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Metformin is one of the main choices of treatments for T2DM and has recently been proposed as a potential antineoplastic agent. This was first suggested after epidemiology studies showed that metformin confers a significant reduction in cancer risk and mortality when compared to the other drugs for the treatment of T2DM. Further investigation into the molecular mechanism of action allowed for an analysis of its effect on a cancer cell. The knowledge of its indirect activation of AMP-activated protein kinase was very important in elucidating the proposed link between metformin and inhibition of cell cycle and proliferation. However even after all these years of research on metformin’s mechanism of action, it is still not fully understood due to the fact that many publications show contradictions or new unexplored mechanisms.

Some of the major mechanisms of metformin’s antineoplastic effects are mediated through the cyclin/CDKS and mTOR pathways. Metformin causes AMPK activation, which then proceeds to reduce cyclin D1 levels and allow the CDK inhibitors, p27 and p21 to inhibit cyclin E/CDK2 (Zhuang & Miskimins, 2008). The reduced activity from this cyclin/CDK complex leads to the inability to inactivate the tumor suppressor pRB, and therefore pRB causes arrest in G1 phase. A major implication that can be taken from this study is that metformin may not be effective if the cancer cell expresses low or depleted levels of p21 and p27. Also it important to understand that metformin halts the cell cycle by a p53 independent pathway, which was confirmed by a study that showed that metformin suppressed tumor growth in a colon cancer cell line deficient in p53.
This is a significant finding since p53 is usually one of the mutations that a cell needs to acquire in order to become cancerous.

The other major mechanism discussed was the inhibition of mTOR via activation of AMPK. This was determined from the observation of decreased phosphorylated S6 after metformin treatment. An activated mTOR will phosphorylate S6, so their findings show that metformin acts to inhibit mTOR (Cantrell et al., 2010). The mTOR pathway is a commonly up-regulated in cancer cells and it is involved in promoting proliferation, growth, angiogenesis, and metabolism (Lake et al., 2012). Therefore the finding that metformin decreases mTOR signaling is in strong favor of its antineoplastic capabilities.

As the antineoplastic properties of metformin became increasingly evident, researchers started to look for its efficacy in specific cancer types. Initially, metformin was studied the most for its efficacy in breast cancer. Various breast cancer cell lines were tested and helped elucidate some further support of its mechanism of action. Of the breast cancer subtypes, metformin was shown to have some efficacy in reducing cancer growth in the ER positive, HER2 positive, and even the triple negative phenotypes. Its efficacy on the HER2 positive cancer cells, which have an overexpression of HER2, was found to be due to metformin’s ability to reduce the mTOR signaling pathway. However, as mentioned previously, researchers studying the same cells and pathways sometimes receive different results. One study reported that metformin’s inhibition of the HER2 positive cancer cells was due to an AMPK independent mechanism (Vazquez-Martin et al., 2009). Thus it is important to realize that the cellular pathways involved in growth,
proliferation, and metabolism are extremely complex, and that one drug may have numerous effects on the cell.

It is more important to realize, however, that a drug at extremely high concentration will begin to show effects on many more pathways than seen from in vivo, where the concentration is usually significantly lower. A criticism of one metformin study described that the concentrations used to study metformin’s effects on cancer cells in vitro were 100 to 1000 times the blood plasma concentration seen in humans at a normal dosage (Stambolic et al., 2009). This criticism of one study can be applied to many of the studies that have published results in support of metformin’s effect on inhibiting tumorigenesis. One study that used concentrations of metformin closer to the clinically accepted levels did not find any effect of metformin on multiple subtypes of breast cancer (Sadighi et al., 2014). However, some studies have shown efficacy with metformin at a lower concentration (Ma et al., 2014; Zordoky et al., 2014). Thus this issue should not destroy the initiative for metformin usage in cancer, but it suggests that more attention should be paid to clinically relevant dosage in preclinical studies. It also suggests that metformin may best play a part in cancer treatment as an additional therapy to the current standards of treatment. Due to its non-toxic nature, it would not add to the gruesome side effects of the toxic chemotherapy. Therefore preclinical studies may find greater success when studying metformin at low doses in combination with currently prescribed chemotherapeutics for their synergistic effect on tumor growth. On the other hand, since the dosage of metformin used for the treatment of T2DM results in a plasma level that is relatively safe and nontoxic, it may be beneficial to raise the accepted plasma
level that is allowed for use in humans. This will require pharmacokinetics studies in
order to determine the point at which metformin starts showing adverse side effects.

As metformin has shown to be of some usefulness in breast cancer, it has recently
passed phase 1 and 2 clinical trials and is currently in the 3rd stage of testing
(Litzenburger & Brown, 2014). Due to the large push for metformin use in breast cancer,
many researchers have thus decided to look at metformin in other types of cancer. Li et
al. are looking at the use of metformin as an adjunctive treatment in resistant subtypes of
lung cancer (Li et al., 2014). Honjo et al. has observed that metformin sensitizes
esophageal tumors to 5-fluorouracil treatment, improving outcome (Honjo et al., 2014).
Metformin was also shown to act synergistically with 5-fluorouracil and oxaliplatin for
the treatment of recurrent colorectal cancer (Nangia-Makker et al., 2014). Lastly, due to
metformin’s independence of the p53 pathway, it is currently under consideration for the
use as a cancer therapeutic in patients with Li-Fraumeni syndrome, which is caused by
germline mutations in the p53 gene (Sorrell et al., 2013). The role of metformin as an
adjunctive therapy to the current standard of treatments is a growing area of research,
which may prove to be an effective combination therapy for a diverse set of cancer types.

Another area of interest that has recently developed is the use of a metformin
derivative in the treatment of cancer (Koh et al., 2013). Koh et al. synthesized a
derivative of metformin that showed greater efficacy in the treatment of a triple negative
breast cancer cell line than the original metformin formulation (Koh et al., 2013). Often
new drug formulations are made in order to increase efficacy or decrease toxicity. Koh et
al. may have discovered a better formulation than the original substance, metformin,
which has proven to be of great potential for cancer treatment. However, until either of these two drugs’ potentials can be fulfilled, many years of ongoing research will still be needed. Preclinical trials in various types of cancer should be performed in order to maximize the usage of metformin, and perhaps the new derivative. Lastly, at the end of all the meticulous research, only large scale clinical trials will be the determining factor for whether or not metformin will be shown to be efficacious for the treatment of cancer, whether as a single or combination therapy.
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EDUCATION

Boston College - Chestnut Hill, Massachusetts - 2013
Bachelor of Science in Biology, GPA: 3.6, cum laude

Boston University School of Medicine – Boston, MA – Expected 2014
Master of Science in Medical Sciences, GPA: 4.0
Recipient of the Robert F. Troxler Award in Biochemistry - 2014

RESEARCH

The Framingham Heart Study – Boston University 2013
Worked on a study with Dr. Michael Pencina, using biostatistical analysis to show how great the risk for developing CVD is for those who are obese, and also the risk for those with diabetes. The analysis has shown that a non-obese person with unhealthy biomarkers for CVD has the same risk for CVD and/or diabetes as an obese person with healthy biomarkers for CVD.

Heart Research at The University of Chicago Summer 2011
Participated in a pilot laboratory study to confirm the use of drug conditioning strategies to protect the heart during both pediatric open-heart surgery and the postoperative period. Used western blots to look for the expression of apoptotic markers like Bcl-2 and LDH in rat myocytes that were and were not treated with milrinone and isoflurane.

Advocate Christ Hospital & Medical Center Summer 2011
Helped develop and organize a new thyroid cancer registry, to monitor quality of care and outcome, for general surgery patients.

EXPERIENCE

Christ Hospital and Medical Center, Oak Lawn, Illinois March 2012
Shadowed, Frank Zimmerman, M.D., Pediatric Cardiologist, Electrophysiologist and Director Pediatric Electrophysiology. Attended electrophysiology studies and radiofrequency ablation procedures in children with supraventricular cardiac arrhythmias.
Observed and rounded on children with pacemakers, automatic intracardiac defibrillators, and cardiac rhythm disturbances.

**The University of Illinois, Department of Neurosurgery** \hspace{1cm} Summer 2011
Shadowed Dr. Demetrios Nikas and Dr. Ziad Hage: observed neurosurgery, patient clinical examination, discussions, rounds, and patient management.

**Midwest Orthopedics, Orland Park, Illinois** \hspace{1cm} Summer 2009
Shadowed Dr. Anton Fakhouri: observed patient clinic follow-up visits, patient clinical examination, cortisone injections, and patient management.

**The University of Chicago** \hspace{1cm} Summer 2008
Shadowed Drs. Kapustiak and Albanis: observed eye surgery, patient clinical examination, discussions, and patient management.

**VOLUNTEER**

**Rosie’s Place - Boston** \hspace{1cm} January 2014 – April 2014
Volunteered at Rosie’s place, a shelter on the south side of Boston that provides meals, shelter, and other opportunities to poor and homeless women. Helped prepare meals, serve food, clean up for about 100 woman each time.

**Plymouth Place - La Grange, Illinois** \hspace{1cm} Summer 2013
Helped with special events, arts and crafts, serving food, and other personal activities at this retirement home. Worked with residents with mild to severe dementia, giving them company and comfort.

**St. Anthony’s Foundation Dining Room - San Francisco, CA** \hspace{1cm} Dec. 2009
Served Christmas Dinner as part of the Boston College Football team to approximately 1000 of San Francisco’s hungriest residents who found solace in a hot Christmas meal.

**ATHLETIC INVOLVEMENT**

**Member of the Boston College Football Team** \hspace{1cm} Aug. 2009- Dec. 2012
+40 hours a week in team meetings, practices, weight training, and games.

Earned full athletic scholarship for senior year \hspace{1cm} August 22, 2012
Athletic Director’s Award for Academic Achievement \hspace{1cm} 2009, ʼ10, ʼ11, ʼ12
Atlantic Coast Conference Academic Honor Roll \hspace{1cm} 2009–2010, ʼ10-ʼ11, ʼ11-ʼ12
Atlantic Coast Conference Academic First Honors \hspace{1cm} Fall 2012