TEACHING AN OLD DOG NEW TRICKS: INTRODUCING A HYPOTHETICAL MECHANISM OF SYNERGY BETWEEN METFORMIN AND NEW GENERATION MTOR INHIBITORS IN THE FIGHT AGAINST CANCER

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As members of the Master's Committee, we certify that we have read the thesis prepared by *Anthony Braileanu*, titled *Teaching an Old Dog New Tricks: Introducing a Hypothetical Mechanism of Synergy Between Metformin and New Generation mTOR Inhibitors in the Fight Against Cancer* and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

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I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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Abstract

mTOR is a serine-threonine kinase and the central component of the complexes mTORC1 and mTORC2. The protein mTORC1 is most associated with global regulation of protein synthesis, cell growth, and cell division, with two of its most prominent actions involving the activation of the translational promoter S6K1, and inhibition of the translational suppressor, Eukaryotic Translation Initiation Factor 4E-Binding Protein 1 (4EBP1). The protein mTORC2 is not as well understood as mTORC1, but it has been most directly implicated with regulation of cell proliferation and cytoskeletal function. The most prominent substrates of mTORC2 are Serum- and Glucocorticoid-induced protein Kinase 1 (SGK1) and protein kinase B (Akt), both of which are inhibitors of the Fox0 transcription factor, which promotes apoptosis and inhibits the cell cycle. Cancers attributed to mTOR typically display simultaneous aberrant over activation of both complexes, while down-regulation of the mTOR complexes has been associated with anti-cancer, anti-autoimmune, and even anti-aging effects.

First generation mTOR inhibitors such as rapamycin and its analogs function by allosterically inhibiting mTORC1 through FRB domain binding, but are ineffective at inhibiting mTORC2. This inherent preference for mTORC1 inhibition promotes drug resistance through compensatory PI3K activation via disruption of a negative feedback loop mitigated by the downstream mTORC1 substrate S6k1. In contrast to rapamycin analogs, second generation mTOR kinase inhibitors directly target the catalytic domain of mTOR, consequently inhibiting both complexes and significantly reducing drug resistance. The latest generation of mTOR inhibitors effectively exploit the close physical proximity between the allosteric and catalytic ATP binding site of mTOR by essentially linking a rapamycin analog to an mTOR kinase inhibitor, and have thus far displayed promising results in pre-clinical trials.

Metformin has been prescribed to treat hyperglycemia in type II diabetics since 1995. Numerous retrospective epidemiological studies spanning over two decades show us that patients prescribed metformin have a better prognosis against cancer. The anti-tumor efficacy of metformin has also been demonstrated both in vitro and in vivo on a vast array of cancers including breast, colorectal, endometrial, ovarian, thyroid, lung, and gastric cancers. Metformin's mechanism of action involves inhibition of ETC Complex I, resulting in AMPK activation by virtue of increased AMP:ATP ratio. Activated AMPK has the downstream effect of inhibiting mTORC1 while promoting the activation of the tumor suppressing p53 transcription factor. The activation of AMPK has the additional effect of lowering blood glucose by reducing gluconeogenesis in the liver and promoting upregulation of GLUT4 glucose transporters in skeletal muscle and adipose tissue. The resulting insulin independent glucose uptake circumvents PI3K signaling which in turn inhibits both mTORC1 and mTORC2.

Although synergy between metformin and new generation mTOR inhibitors has not yet been explored, I predict the two will synergize to an even greater extent than what has previously been seen with first generation rapamycin analogs. Metformin's action of effectively lowering intracellular ATP by way of ETC inhibition may be instrumental for increasing the efficiency of mTORKI's competition with ATP for the mTOR catalytic domain.

Metformin's direct and indirect inhibition of PI3K, in addition to its proven clinical safety may warrant further clinical trials for use as a complementary cytostatic agent in supplementing the standard chemotherapeutic treatment of breast cancer. Upregulation of both insulin and IGF receptors is seen in virtually every subtype of breast cancer, and patient prognosis is negatively correlated with the degree of upregulation. Additionally, breast cancer morbidities have been shown to be directly correlated with high serum insulin levels. BRCA-1 mutant breast cancers have been shown to upregulate IGF-1 receptors in several studies and the BRCA-1 gene is in itself a suppressor of IGF-1R transcription. Metformin's potential efficacy towards BRCA-1 breast cancers may impact large populations as genetic testing becomes more of a commonplace in preventative medicine. High risk BRCA-1 mutant carriers may one day be prescribed a safe, low dose prophylactic of the drug. Metformin may additionally be indicated even more so against breast cancers that upregulate human epidermal growth factor receptor 2, as the HER2 receptor is also a PI3K activator. Drug synergy has important clinical implications because it indicates lower therapeutic dosages, thus potentially reducing or counteracting the side effects attributed with individual drugs.

The Mammalian Target Of Rapamycin

The mammalian target of rapamycin mTOR protein is a serine-threonine kinase whose discovery occurred after the unearthing of the anti-fungal, bacterial derived drug, rapamycin [1]. It is the central component of two very large complexes: mTORC1 and mTORC2. These two complexes have a very intricate relationship with each other; in addition to having direct communication with one another, they are intertwined in many of the same pathways and participate in complicated feedback mechanisms.

mTORC1 is composed of the substrate binding scaffold protein "raptor", the mTORC1 negative regulator, Proline-Rich Akt Substrate 1 (pras40), the DEP domain-containing mTOR-interacting protein (deptor), the mysterious mLST8 protein who's function is unknown, and the tti1 and tel2 scaffold proteins, which primarily function to ensure the stability of the complex. mTORC2 features many of the same proteins, with the exception that raptor and pras40 from mTORC1 are replaced by three proteins: the substrate binding scaffold protein "rictor", the assembly promoting scaffold protein mSin1, and finally the PROTein Observed with Rictor (protor1/2) scaffold protein. Both of these complexes feature the same mTOR kinase enzyme within their central core [2]

When scientific writings colloquially express a "downregulation/upregulation of mTOR", they are typically referring to mTORC1, which is the complex most associated with global regulation of protein synthesis, cell growth, and cell division[1]. The two most prominent actions of mTORC1 include the activation of the translational promoter, S6K1, and inhibition of the translational suppressor, Eukaryotic Translation Initiation Factor 4E-Binding Protein 1 (4EBP1). The mTORC2 complex is not as well understood as mTORC1, but it has been most directly implicated with regulation of cytoskeletal function and cell proliferation[2][3]. mTORC2's most prominent substrates are Serum- and Glucocorticoid-induced protein Kinase 1 (SGK1) and protein kinase B (Akt), both of which are inhibitors of the Fox0 transcription factor, which promotes apoptosis and inhibits the cell cycle[2]. It is important to note that cancers promoted by mTOR typically display simultaneous aberrant over activation of both complexes [4][5][6][7][8], and that novel therapeutic drugs aim to inhibit both complexes in order to mitigate compensatory negative feedback loops that contribute to drug resistance[9].

Down-regulation of the mTOR complexes is associated with anti-cancer, anti-autoimmune, and even anti-aging effects. A vast assortment of cancers have the characteristic feature of aberrant over activation of mTOR, with the most prominent being breast cancer, renal cell carcinoma, and glioblastoma multiform [10][11][12]. Even the infamous tumor suppressing transcription factor, p53, is a negative regulator of mTORC1 [13].

Inhibition of mTOR remains an ongoing subject in precision cancer treatment, and as of today, there are over 500 clinical trials being conducted on mTOR inhibiting drugs [14].

Rapamycin and 1st generation Rapalogs

Rapamycin is a drug with a very interesting historical background. Having first been isolated from soil on Easter Island as a metabolite of Streptomyces hygroscopicus, it was first utilized as an antifungal agent[15]. Soon after, it was discovered to have immunosuppressive properties, so

much so that it made its first appearance clinically in 1999 as an immunomodulator given to kidney transplant recipients in order to prevent acute rejection[16]. However, rapamycin really started gaining attention from the oncology community when the national cancer institute demonstrated that it inhibited the growth of several cancer cell lines when combined with chemotherapeutics [17].

Pharmacological research demonstrated that rapamycin had much more preference for mTORC1, with little to no inhibition of mTORC2 [18]. Indeed, this is the reasoning behind the nomenclature of the different substrate binding subunits of the two complexes: Regulatory Associated Protein of mammalian Target Of Rapamycin (raptor) on mTORC1, and Rapamycin Insensitive Companion of mTOR (rictor) on mTORC2. After much further investigation, scientists revealed that rapamycin was in fact allosterically inhibiting mTOR by forming a dimer with the intracellular immunophilin protein FKBP-12 (which promotes immunosuppression in T-lymphocytes, and is the basis for rapamycin's initial use as a prophylactic to organ transplant rejection) before binding to the FRB (allosteric) domain of mTOR. However, the same allosteric domain is much less accessible on mTORC2, owing to the shape and location of the surrounding rictor protein [19]. The ability of rapamycin to inhibit cancer cell growth prompted pharmacologists to develop analogs with superior solubility and pharmacokinetic properties. Eventually the FDA approved two rapamycin analogs for cancer treatment, these were Temsirolimus and Everolimus (both initially approved for renal cell carcinoma in 2009, but have now extended their indications.)[20]. To date, these are still the only two FDA approved rapamycin analogs for the treatment of cancer, and as such, they are collectively referred to as "rapalogs"[21].

The greatest challenges faced by 1st generation FDA approved rapalogs

Although the rapamycin analogs Temsirolimus and Everolimus showed efficacy inhibiting cancer growth, the main challenge faced by this generation of therapeutics is the eventual acquisition of therapeutic resistance in most patients. Mechanistically, the greatest contribution to rapalog drug resistance is the compensatory activation of Phosphoinositide 3-kinase (PI3K) resulting from the inhibition of mTORC1[22][23][24]. This occurs via disruption of a negative feedback loop controlled by the mTRORC1 substrate S6k1: a kinase which phosphorylates and activates the S6 ribosomal protein, which in itself is a component of the ribosomal 40S (small) subunit [25][26]. It is important to note that in addition to promoting translation, S6K1 phosphorylates and inhibits insulin receptor substrate (IRS)-1, a signaling protein that activates phosphatidylinositol 3-kinase (PI3K) in response to ligand binding at insulin receptors and/or insulin-like growth factor-1 (IGF-1) receptors [27].

Activation of PI3K results in the phosphorylation and activation of both mTORC2 and Akt/PKB, a protein kinase which activates mTORC1 by phosphorylating and deactivating the mTOR inhibitor: tuberous sclerosis complex 2 [28] [29]; this signaling cascade is commonly referred to as the PI3k/Akt/mTOR pathway.

It is very important to note that mTORC2 is in itself an mTORC1 activator by means of its downstream target: Akt/PKB (which it shares with PI3K) [2]. Thus, inhibiting only mTORC1 essentially reactivates itself by interrupting its own negative feedback loop, and in addition,

mTORC2 can activate mTORC1. The complexity of this signaling pathway makes it is easy to appreciate the limitations of rapamycin analogs for clinical use.

In an effort to counteract the compensatory activation of PI3K that occurs with the preferential inhibition of mTORC1, a characteristic of first generation rapalogs, pharmacologists set out to develop a drug that would directly block the active kinase site of mTOR [30]. It is important to note that the catalytic domain of mTOR is so similar in sequence to the catalytic domain of PI3K that mTOR itself is classified as a member of the PI3K-related kinase family [31] The high similarity between the PI3K and mTOR active domains encouraged drug developers to initially produce dual acting PI3K-mTOR inhibitors [32]. These drugs are essentially small molecules that competitively block ATP binding to the active sites of both PI3K and mTOR. Although the concept was extremely promising, clinical trials showed no improvement over conventional rapalogs, with one of the possible culprits being compensatory activation of the epidermal growth factor receptor (EGFR) induced MAPK/ERK pathway leading to S6K activation [33][34][35][36]. Even worse, the treatments were poorly tolerated and produced extremely toxic and unpleasant side effects which included: nausea, diarrhea, vomiting, decreased appetite, hyperglycemia, mucositis, cutaneous rash, elevated liver enzyme levels, renal failure, and hypertension [37][38][39][40].

Second Generation mTOR-KIs

In an effort to eliminate the catastrophic side effects of dual PI3K-mTOR inhibitors, drug developers went back to the drawing board and came up with what are now known as Second Generation mTOR kinase inhibitors: “mTOR-KIs”. This class of drugs applies the same approach of small molecule competitive inhibition as dual PI3K-mTOR inhibitors, but this time with exclusive specificity for mTOR’s catalytic domain[41].

The ability of mTOR kinase inhibitors to inhibit both mTORC1 and mTORC2 eliminated much of the compensatory PI3K activation seen in first generation rapalogs[42], while giving much milder side effects (neutropenia, diarrhea, and hyperglycemia) compared to dual PI3K-mTOR inhibitors [43]. These new drugs however still suffered the acquisition of tolerance by cancer cells.

Rodrik-Outmezguine et al [44] treated MCF-7 breast cancer cells with either rapamycin or the mTOR kinase inhibitor, AZD8055, for three months until they conferred drug resistant mutations. Their studies demonstrated that cells treated with rapamycin acquired resistance by mutating the FRB (allosteric) domain of mTOR, while the cells treated with mTOR kinase inhibitors acquired resistance due to mutations in the catalytic domain of the mTOR subunit. The mutations acquired on MCF-7 cells were then exogenously replicated in MDA-MB-468 breast cancer cells, and these cells became resistant to AZD8055 as well, confirming that these mutations impart resistance [44].These findings were deemed clinically relevant because a previous case study of a thyroid cancer patient had acquired the same mutation as the rapamycin resistant MCF-7 cells in the FRB domain (F2108L) after being treated with the rapamycin analog, everolimus [45]. Interestingly, the M2327I mTOR kinase mutation displayed by MCF-7 cells with acquired resistance to mTOR-KIs did not hinder drug binding affinity compared to wild type MCF-7 cells, instead the mutation increased the kinetic properties of the kinase by three fold, hyperactivating mTOR [44].

Third Generation mTOR Inhibitors

In an effort to counteract the resistance-associated mutations seen with second generation mTOR inhibitors, Rodrik-Outmezguine et al [44]engineered the latest class of inhibitors as part of the same aforementioned study. These third generation mTOR inhibitors were designed to exploit the extremely close physical proximity between the allosteric and catalytic ATP binding site of mTOR. As such, third generation mTOR inhibitors function as both rapalogs and mTOR-KIs by targeting both the FRB (allosteric) and catalytic domain of mTOR at the same time. Essentially, they are a rapalog linked to an mTOR Kinase Inhibitor, utilizing a linker that approximates the physical distance between the two binding sites [44].

The reasoning behind this combination is to remain one step ahead of mutations in mTOR that could promote drug resistance. If the allosteric site were to mutate, the drug would still have its effects on the catalytic domain and vice versa. It would not matter which of the two sites mutate first, because the drug hits dual targets at the same time. The most prominent third generation mTOR inhibitor is called Rapalink-1, and it has shown promising effects in its fight against therapeutic resistance [44].

Rapalink-1 has much better efficacy in vitro compared to the first two generations of mTOR inhibitors. In addition to its increased potency, the ability of Rapalink-1 to cross the blood brain barrier has proved invaluable, as it has demonstrated the ability to counteract the highly aggressive brain cancer, glioblastoma, using in vivo mouse models[12].

Although third generation mTOR inhibitors like Rapalink-1 show much promise in the fight against highly aggressive cancers, it is still too early to reach any conclusions, as this new generation of inhibitors are still infantile and have not yet reached phase I clinical trials.

Metformin and its potency as a cancer drug

The drug Metformin has been prescribed to safely treat hyperglycemia in type II diabetics since 1995 [46]. With a similar history to the unexpected clinical utility of Rapamycin, significant data over the last decade has pointed to the anti-tumor characteristics of metformin, and it has been proven to have these effects both *in vitro* and *in vivo* on a vast array of cancers including breast [47], colorectal [48], endometrial [49], ovarian [50], thyroid [51], lung [52], and gastric [53] cancers.

Metformin's mechanism of action is complex and not completely understood. Unlike most modern drugs, metformin was not designed to target a particular pathway or disease mechanism. In the context of regulating blood glucose, metformin is thought to act on cells of the liver, skeletal muscles, and adipose tissue by inhibiting complex I of the electron transport chain (ETC), causing a consequential reduction in ATP output that leads to the activation of adenosine monophosphate kinase (AMKP) by virtue of increased AMP: ATP ratio [54][55]. Activated AMPK promotes a reduction in gluconeogenesis in the liver, while promoting upregulation of GLUT4 glucose transporters in skeletal muscle and adipose tissue. Together, these effects ultimately allow type II diabetics to lower their blood glucose levels without the use of insulin [54][55][56][57].

The other critical consequence of AMPK activation is downstream inhibition of mTORC1, which is one of the main traits contributing to the anti-tumor properties of metformin. Activated AMPK initiates the phosphorylation of both tuberous sclerosis complex 2 [28] and the binding subunit of mTORC1: raptor; both of these actions leading to mTORC1 inhibition [58]. In addition to the above mentioned effects, AMPK also promotes activation of the tumor suppressing P53 transcription factor [59].

If we re-explore anti-diabetic action of metformin, it is clear that there is much more involved in the mechanism by which metformin exerts its anti-proliferative properties than simply inhibiting mTORC1. It is important to reiterate that the insulin receptor is a mitogenic pathway, since it's stimulation directly activates PI3K and the resulting downstream targets mTORC1 and mTORC2[27][29][60]. Keeping in mind that blood-insulin levels are positively correlated with blood-glucose levels, the activation of AMPK via metformin has the effect of decreasing hepatic gluconeogenesis while upregulating muscle and adipose GLUT4 expression, thus the lowered blood glucose levels translate to lowered PI3K signaling [56][57][59][60][61].

There is yet even more anti-PI3K activity when we consider that metformin has been demonstrated to reduce mRNA expression levels of the PI3K protein. Liu Y et al. [62] not only demonstrated that metformin inhibits the proliferation of A431 human squamous cell carcinoma cells in a time and dose dependent manner, but also proved that it inhibited mRNA transcription of PI3K within the same cell line. Nozhat et al. [51] showed that in addition to reducing cell viability and migration, metformin inhibited the mRNA expression levels of both PI3K and AKT in various anaplastic thyroid cancer cell lines. In another study, using endometrial stromal cells, DiasFerreira et al. [63] showed that metformin reduced mRNA expression of PI3K and concentrations of phosphorylated (activated) AKT in the presence of androgen and insulin.

Taking all of these things into consideration, it is a fair statement to say that metformin is essentially functioning in the manner of a dual PI3K-mTOR inhibitor, only without any of the toxic side effects that plague this novel class of drugs.

Metfomin's synergy with rapalogs

Metformin's anti-cancer synergism with first generation rapalogs has been demonstrated both *in vitro* and *in vivo*. Using MTT assays which measure the concentration of viable cells using a spectrophotometer that quantifies the electrochemical reduction of a tetrazole (MTT) incubation solution to formazan by mitochondrial reductase enzyme, Jia-Wei Zhang et al. [64] were able to demonstrate that metformin and rapamycin synergistically inhibited the growth of SW1990 pancreatic cancer cells compared to individual administration of either drug. Interestingly, it was observed that metformin treatment alone was more effective than an equivalent dose of rapamycin. In an attempt to explore a mechanism of synergy of the two drugs, western blot assays were used to test the expression of various effector proteins at differing concentrations of either drug alone, or in combination. Metformin treatment alone not only significantly increased phosphorylated (activated) AMPK concentrations while decreasing phosphorylated (activated) mTOR concentrations, but it also reduced the expression of vascular endothelial growth factor (VEGF) and B-cell lymphoma 2 (BCL-2) protein expression. VEGF overexpression leads to aberrant angiogenesis and metastasis, while over expression of the anti-apoptotic BCL-2 protein contributes to inhibition of cell proliferation control mechanisms. Rapamycin treatment alone

significantly reduced phosphorylated mTOR concentrations, but did not have any significant effect on the expression of the aforementioned proteins. Combination treatment amplified the increase of p-AMPK and decrease of p-mTOR, VEGF, and BCL-2 seen with metformin alone, however the greatest indicator of synergism was the drastically reduced concentration of phosphorylated mTOR compared to either drug alone [64].

In the same study, Zhang et al. [64] used a mouse model to demonstrate the *in vivo* antitumor efficacy of metformin and rapamycin alone, or in combination. Interestingly, mono-drug therapies produced the opposite effect observed in vitro; administration of metformin had a small inhibitory effect, while rapamycin injection produced a moderate inhibitory effect. Inhibition of tumor growth was significantly greater than observed with either of the drugs alone, demonstrating synergy between the two drugs.

In a separate study, Yunshan Wang et al. [65] used MTT assays to demonstrate that the combination of metformin and rapamycin synergistically inhibited the growth of HCC1428, MDA-MB-549, and BT549 breast cancer cells to a much greater extent than either of the drugs alone. In vivo study using xenograft mouse models demonstrated that combination therapy suppressed tumor formation significantly more than either metformin or rapamycin alone. Western blotting of tumor tissue demonstrated that monotherapies of both metformin and rapamycin decreased concentrations of phosphorylated 4EBP-1 and phosphorylated S6 Ribosomal Protein (S6) thus indicating decreased translation. Combination therapy of metformin and rapamycin decreased the phosphorylation of both proteins to a significantly greater extent than either of the drugs alone.

Rationale behind more clinical trials of combined metformin and rapalog therapy

Temsirolimus and Everolimus are thus far the only FDA approved rapalogs for cancer treatment [20], and as such they are prone to the classic compensatory PI3K activation seen with rapalogs that preferentially inhibit mTORC1. As previously mentioned, the mTORC1 substrate S6K1 inhibits insulin receptor substrate (IRS)-1 and this is the mechanism by which it exerts a negative feedback loop to the PI3K pathway downstream of S6K1 [27]. Metformin's action of promoting insulin independent glucose uptake via AMPK mediated GLUT4 upregulation, together with AMPK mediated inhibition of both mTOR complexes is likely a significant factor leading to its synergism with rapalogs.

An additionally important attribute that deserves consideration is metformin's indirect inhibition of mTOR. This is an important point when we consider that resistance mechanisms to rapalogs manifest as mutations on the FRB (allosteric) domain of mTOR in many cancers [44]. I suspect that this type of mutation may be reduced when metformin is present since its inhibition of mTOR occurs through AMPK activation. Keeping clinical side effects in mind, it is also worth noting that metformin is prescribed to treat hyperglycemia, and this is one of the most common side effects associated with rapalog treatment [66][67][68][69].

The correlation of metformin and better cancer treatment outcomes has been demonstrated with countless epidemiological studies demonstrating that type II diabetics prescribed metformin respond better to cancer treatment than patients without metformin in their system

[70][71][72][73][74]. There is in fact already evidence pointing to the clinical safety and efficacy of metformin's synergy with rapalogs. A retrospective analysis [75] of 455 cancer patients spanning from 1999 to 2015 demonstrated that diabetic patients using metformin were associated with longer progression free survival of pancreatic neuroendocrine tumors when prescribed everolimus and/or analogs of the pancreatic growth hormone-inhibiting hormone somatostatin. In addition to showing that metformin treatment was associated with a better prognosis, the longitudinal study demonstrated the synergy of rapalogs and metformin in human patients. Patients prescribed everolimus +/- somatostatin alone (n=111) had a progression free survival of 12.1 months, while patients prescribed metformin and everolimus +/- somatostatin (n=68) had a significantly longer progression free survival of 43.7 months [75].

Lastly, both metformin and rapalogs have side effects that can only become worse with dosage. Side effects of metformin include abdominal pain, diarrhea, muscle pain, and fever or chills [76]. Rapalogs on the other hand have side effects that include hyperglycemia, hyperlipidemia, sepsis, pneumonia [77]. It has been reported that 12-50% of patients receiving rapalogs for a variety of cancers in phase 3 trials experienced hyperglycemia as a side effect [28][69][78][79][80][81][82][83][84][85]

The indication of utilizing an antidiabetic drug to mitigate the side effect of hyperglycemia due to rapalog treatment is straightforward in its own right. However, two different studies of metformin and rapamycin monotherapies on the same cell type may shed some light on the details of their opposing influence on glucose uptake. Alain Veilleux et al. [86] demonstrated that chronic inhibition of mTORC1/S6K1 via rapamycin alone on 3T3-L1 adipocytes inhibited glucose uptake by reducing expression levels of GLUT4, all the while hyperactivating PI3K activity. In a different study Jung Ok Lee et al. [87] demonstrated that 3T3-L1 adipocytes respond to metformin by upregulating GLUT4 expression through the activation of AMPK. It is worth reminding that GLUT4 is normally an insulin dependent glucose transporter, which in addition to being expressed in adipocytes, is prevalent in muscle tissue. In another similar study, Jing Yang et al. [56] demonstrated that cardiomyocytes upregulate GLUT4 in response to metformin treatment by virtue of AMPK activation. These studies not only provide evidence that metformin counteracts rapalog induced hyperglycemia by an opposing modulation of the same glucose uptake mechanism, but by extension also antagonizes rapalog induced activation of the glucose/insulin/PI3K pathway.

When we take into account the overwhelming evidence of anti-cancer synergy between metformin and rapaogs, it follows that their combination would not only account for a better prognosis, but also potentially eliminate side effects beyond hyperglycemia by virtue of a lower combined dose of the two drugs.

Rationale for combining metformin with 2nd or 3rd generation mTOR inhibitors:

Mutations in the mTOR catalytic domain occur frequently in cancer cells that are treated with mTOR-KIs and is one of the most common mechanisms of drug resistance [44]. Metformin could hypothetically overcome this problem because it inhibits mTOR indirectly by blocking complex I of the ETC, thus resulting in AMPK activation [54][55][56][57]. Thus far, 3rd

generation mTOR inhibitors have not reached clinical trials. However, 2nd generation mTOR Kinase inhibitors still suffer the adverse effect of hyperglycemia that plagued 1st generation rapalogs [43][88][89].

A valid concern against using the antidiabetic drug metformin in combination with mTOR inhibitors is that it might induce hypoglycemia in patients who are not experiencing hyperglycemia. However, this should not be a concern since the American Association of Clinical Endocrinologists states that metformin does not cause hypoglycemia in non-diabetics [90]. This statement is supported by a separate study that determined blood glucose does not become significantly lower in healthy individuals treated with metformin [91]. As even further proof of metformin's safety, Bonanni et al. [92] conducted a randomized pre-surgical trial to test levels of the proliferation marker, Ki-67, before and after metformin administration on operable breast cancer patients. In this study, none of the 100 non-diabetic patients experienced hypoglycemia.

Introducing a Hypothetical Mechanism of Synergy Between Metformin and New Generation mTOR inhibitors

Second and third generation mTOR inhibitors function by competitively inhibiting the catalytic domain of mTOR (with third generation inhibitors simultaneously targeting the allosteric site) and this is accomplished by competing with cellular ATP for the active site of the enzyme[41][42][43][44]. Synergism between metformin and new generation mTOR inhibitors has not yet been explored, however, I hypothesize that the two will synergize to an even greater extent than we have seen with first generation rapalogs.

Metformin's mechanism of action is not completely understood; however, it is established that the drug somehow inhibits complex I of the electron transport chain, increases the intracellular AMP:ATP ratio, and activates AMPK [54][55][56][57]. Based on this knowledge, I propose the administration of metformin together with mTOR Kinase Inhibitors will introduce a new mechanism of anti-tumor synergy by virtue of decreasing available intracellular ATP, thus allowing for more efficient competitive inhibition of mTOR's catalytic ATP binding domain. I also predict that greater synergy will occur between metformin and third generation mTOR inhibitors by the same newly proposed mechanism in conjunction with the mechanisms previously discussed that explain the synergy with first generation rapalogs.

The Warburg Effect: why it refutes my hypothetical mechanism

In the 1920s, Otto Warburg and his research group came to the conclusion that cancer cells were primarily using the anaerobic glycolytic pathway even when in an oxygen rich environment. This conclusion came from the observation of extreme glucose intake coupled with unusually high lactate production [93]. He coined the term "aerobic glycolysis" and eventually the utilization of glycolysis in aerobic conditions by cancer cells became known as the Warburg effect. For the

most part, the scientific community as a whole accepted the paradoxical concept that cancer cells predominantly metabolize sugar using the inefficient glycolysis pathway which produces only 2 ATPs per glucose molecule instead of 34-36 ATPs using oxidative phosphorylation in the electron transport chain (ETC) of the inner mitochondrial membrane. A reasonable supplementary idea that cancer cells had mitochondria with defective ETCs was also a long accepted concept [93].

There are several logical theories as to why neoplastic cells would employ such an inefficient metabolic profile in the presence of oxygen. First of all, it assures that the synthesis of ATP can happen even when the cancer cell outgrows the oxygen supply. Additionally, the release of lactic acid lowers the pH of the extracellular microenvironment which in turn helps with tumor cell invasion in addition to suppressing immune system retaliation [94]. Lastly, the metabolic intermediates of glycolysis provide raw material for the synthesis of cellular components during rapid cellular proliferation [94][95]. This last theory is particularly sound because the Warburg effect can also be observed physiologically in rapidly proliferating cells such as embryonic tissue [96] or activated T lymphocytes [97].

If we are to believe that all neoplastic cells are functioning by utilizing aerobic glycolysis metabolism, then my hypothesis that metformin's ATP lowering effects (via ETC inhibition) increases mTOR kinase inhibitor efficiency would not hold true.

The Reverse-Warburg Effect: why it resurrects my hypothesis

Advances in research techniques over the past decade or so have uncovered that the glucose metabolism of cancer cells is not as black and white as we once thought it was. It has become increasingly evident that the Warburg effect is not a consistency, and that there are in fact many types of cancer cells with highly functional and active mitochondria [93][98][99][100][101][102][103].

A model called the Reverse Warburg Effect describes that it is actually the surrounding stroma and fibroblasts that are engaged in aerobic glycolysis, not the neoplastic cells themselves. This cancer associated stroma then transports lactate and factors needed for rapid proliferation to the neoplastic cells, which then utilize the fuel to proliferate and produce ATP by oxidative phosphorylation. In this model the neoplastic cell and neighboring stromal cell engage in an intimate metabolic coupling which allows for maximum ATP production and proliferation at the same time [104][105][106][107].

Just like the Warburg effect, the Reverse-Warburg effect can also be observed physiologically. This can be seen in the brain, as the astrocytes (which function as supporting/stromal cells in the brain) utilize aerobic glycolysis, and then feed lactate to surrounding neurons, which then use the lactate to produce ATP via oxidative phosphorylation [108].

It has been demonstrated that various cancer cells will adapt their metabolic profile depending on the amount of glucose available in their microenvironment. Studies have shown that cells cultured in an environment with high levels of glucose will significantly switch to aerobic glycolysis [109][110][111]. Previous studies may have in fact skewed the results of cancer

metabolism studies because of unrealistically large amounts of glucose in the cell culture media, leading to reduced oxidative phosphorylation. Many cancer metabolism studies have cultured cells in 10-25 mM of glucose even though cells are typically exposed to 4-6 mM of glucose in physiological conditions, with even lower glucose concentrations seen in a tumor microenvironment [93][107]. The anti-cancer properties of metformin have actually been demonstrated alongside epithelial cancer cells with hyper activated oxidative phosphorylation in earlier studies [112], and metformin has actually contributed to the concept of cancer cells metabolizing glucose by oxidative phosphorylation in low glucose settings.

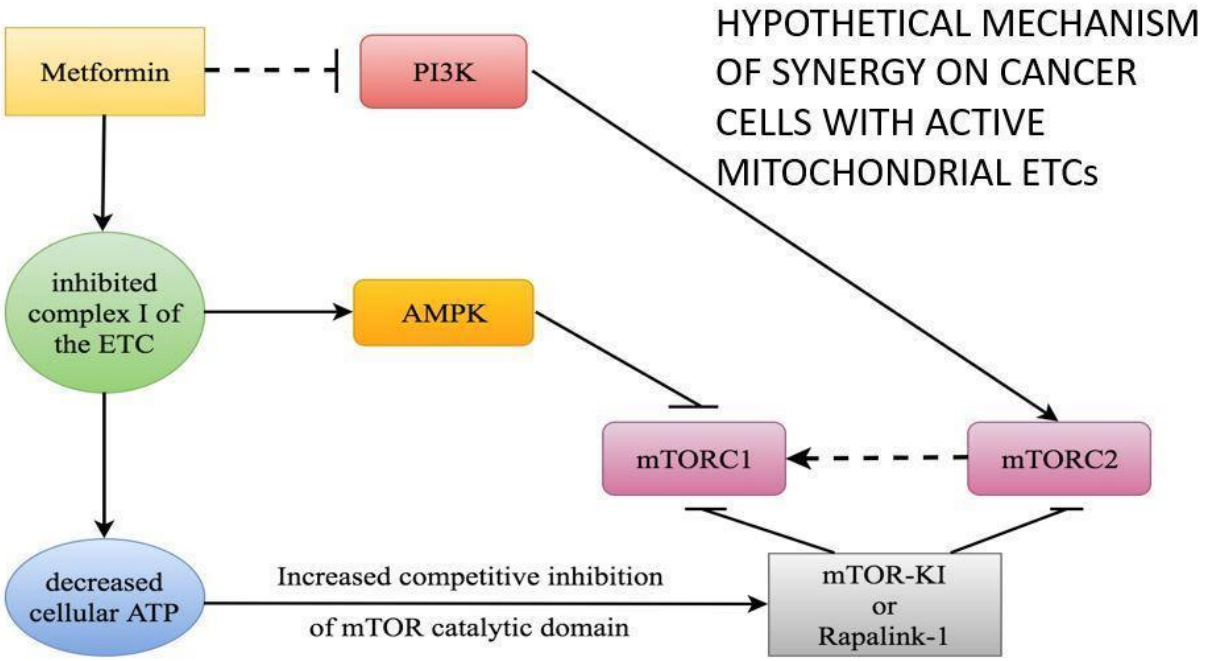
A 2016 study demonstrated that metformin and cisplatin (a common chemotherapeutic drug) displayed synergistic anti-tumor effects on esophageal squamous cancer cells in a low glucose microenvironment [113]. The concept of cancer cell lines operating on mitochondrial oxidative phosphorylation under low glucose conditions was yet again demonstrated in 2017 [114], this time through the combination of metformin and the rapalog everolimus. In addition to the demonstrated synergism of the two drugs on the breast cancer cell lines MCF-7 and MDA-MB-231, researchers demonstrated that everolimus was much more effective in a high glucose microenvironment, while the opposite was true when cancer cell lines were treated with metformin alone. Metformin's cytotoxic effects on the cancer cell lines increased when available glucose was reduced, thus supporting the idea that metformin's effects on the cancer cells were downstream of mitochondrial ETC inhibition [114].

In acknowledgment that cancer cell cultures are commonly incubated in 25 mM of glucose, Yongxian Zhuang et al. [115] showed that metformin was cytotoxic to the breast cancer cell lines MCF7, MDAMB231, SKBR3 and the ovarian cancer cell lines OVCAR3 and PA-1 in cell cultures containing 0-5 mM of glucose, yet the same cancer cell lines began losing their sensitivity to metformin when incubated in cultures containing 10 mM or higher of glucose. Next, the study demonstrated that metformin significantly lowered intracellular ATP in cells incubated in 2.5 mM, whereas it did not effect ATP production when cells were incubated in 25 mM of glucose. In addition to the above mentioned effects, researchers were also able to reverse aerobic glycolysis (Warburg effect) when culture media was switched from 25 mM of glucose to 2.5 mM of glucose. Western Blotting demonstrated that metformin treatment in 2.5 mM of glucose significantly reduced concentrations of phosphorylated (activated) Akt, reduced concentrations of the phosphorylated (deactivated) translation suppressor 4EBP1, and reduced the concentrations of phosphorylated (activated) translation factor S6K.

Finally, within the same study, Yongxian Zhuang et al. [115] demonstrated that metformin was ineffective in a low blood glucose environment using mice that were injected with mouse mammary cancer 4T1 cells. Mice subjected to a low carbohydrate ketogenic diet lowered their blood glucose serum levels from 6 mM to 3 mM and responded to metformin with drastically reduced tumor growth compared to the conventional diet control group which showed no signs of tumor growth inhibition. Interestingly, mice that were solely on the low carbohydrate ketogenic diet also showed signs of inhibited tumor growth without the administration of any drugs.

In conclusion, these studies validate the idea that a great assortment of cancers do in fact utilize oxidative phosphorylation, and they do so in a manner that is positively correlated with diminished glucose availability. When we embrace these newly exposed metabolic shifts that occur in neoplastic cells, together with the discovery that many cancer cell lines can and do have

highly functioning and active mitochondrial electron transport chains (ETCs), a new mechanism becomes plausible in describing the likely synergism of metformin and new generation mTOR inhibitors. The idea of metformin's ETC inhibition and the resulting decrease in cellular ATP contributing to the efficiency of competitive mTOR Kinase Inhibitors begins to sound like a plausible synergistic mechanism.



Discussion, and Further Consideration For Combining Rapalogs with Metformin in Treating Breast Cancer Patients

Synergism between metformin and new generation mTOR inhibitors have yet to be demonstrated, however, metformin's inhibitory effects on the PI3K pathway, and known synergism with first generation rapalogs also warrant further clinical research in specific cancers. In addition to directly inhibiting mTORC1 downstream of AMPK activation, metformin's inhibition of the PI3K pathway may be exploited to treat cancers that commonly feature aberrant insulin receptor and/or insulin like growth factor-1 (IGF-1) receptor signaling. Many cancers feature over expression of both insulin and IGF receptors, and breast cancers are one of the most prominent examples [116]. Upregulation of both insulin and IGF receptors is seen in virtually every subtype of breast cancer, and patient prognosis is negatively correlated with the degree of upregulation [117]. As discussed previously, the insulin receptor is a mitogenic PI3K activating pathway, and the IGF receptor is no different, utilizing the same downstream adaptor protein Insulin Receptor Substrate-1 (IRS-1) before activating the PI3K pathway [118].

Metformin's AMPK dependent action of inhibiting gluconeogenesis in the liver and upregulating GLUT4 receptors in muscles have more than just a glucose lowering effect in the blood serum. The insulin levels that drop in response to lowered blood sugar may contribute to a better prognosis, as breast cancer morbidities have been shown to be directly correlated with high

serum insulin levels [119]. In this context, it is worth mentioning that insulin also binds and activates IGF receptors, although with much less affinity than insulin receptors [118]. Moreover, several studies have demonstrated that metformin downregulates the mRNA expression of PI3K in itself, and this likely also contributes to metformin's efficacy against breast cancers [51][62][63]. The fact that IGF-1 receptors activate the PI3K pathway utilizing the same IRS-1 adaptor protein as insulin receptors is not to be understated, as the mutant BRCA-1 variety of breast cancer has been shown to upregulate IGF-1 receptors in several studies [120][121]. Moreover Abramovitch et al. [122] demonstrated that the BRCA-1 gene is in itself is a suppressor of the IGF-1R transcriptional promoter.

As discussed previously, metformin's ability to inhibit the PI3K pathway goes beyond its modulation of serum insulin levels; its ability to downregulate mRNA transcription of PI3K may be instrumental for inhibiting downstream IGF-1 receptor signaling. Research has already shown that inhibiting the IGF-1R/PI3K/AKT pathway with small molecule inhibitors reduces the proliferation of BRCA-1 mutant cells [123]. Metformin's potential efficacy towards BRCA-1 mutant breast cancer could have a great impact, especially with the recent advent of genetic testing, in which screening for the mutated gene is becoming commonplace. With metformin's documented inhibition of breast cancer proliferation and low risk of hypoglycemia in non-diabetics, high risk BRCA-1 mutant carriers may one day be prescribed a daily low dose prophylactic of the drug.

As further exploitation of metformin's inhibitory actions on PI3K, its potential use may be indicated even more so against breast cancers that upregulate human epidermal growth factor receptor 2 (HER2), which activates PI3K downstream of its stimulation. HER2 positive breast cancers account for approximately 15-30 percent of breast cancers [124][125] and are generally characterized as being more aggressive than HER2 negative variants [126].

Just like metformin, rapamycin and its analogs have been shown to exert antiproliferative effects on a wide array of cancers, with particularly good efficacy at combating breast cancer. Synergy between the two drugs has in fact already been demonstrated with breast cancer cells [65][114], and given their safe track record, warrant further consideration to enter clinical trials. The synergism observed between these two drugs could be effectively utilized against BRCA-1 mutant and/or HER2 positive breast cancers.

It is additionally worthy to remain open minded about combining either of these drugs with other novel cancer therapies that face the challenges of acquired resistance. For example, metformin has been shown to drastically reduce treatment resistance to the HER2 monoclonal antibody trastuzumab when applied to breast cancer xenografts [127]. Trastuzumab is one of the first line treatments used to treat HER2 positive breast cancers, but has the unfortunate association of acquired resistance and cardiac toxicity in susceptible patients [128].

Finally, it is imperative to iterate that both metformin and rapalogs are limited in vivo as antiproliferative drugs, both on their own and together, and as such, other than possibly being utilized as prophylactics, must be used in unison with other drugs that exert cytotoxic effects. Synergisms of two drugs that have the same effect are a clinically important attribute, as side effects often accompany individual drugs, and these can be potentially diminished when the combined therapeutic dosage is less than either of the two individual dosages.

In this exciting new era of precision medicine it has become evident that we are just scratching the surface when it comes to personalized treatments for individual patients. With every two steps forward in treatment, cancer cells retaliate and send us one step in reverse. Nonetheless, we continue to stride ahead with new discoveries and improvements on old concepts, and I am excited for the future that lies ahead.

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