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Metformin influences drug sensitivity in pancreatic cancer cells

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive, highly metastatic malignancy and accounts for 85% of pancreatic cancers. PDAC patients have poor prognosis with a five-year survival of only 5–10% after diagnosis and treatment. Pancreatic cancer has been associated with type II diabetes as the frequency of recently diagnosed diabetics that develop pancreatic cancer within a 10-year period of initial diagnosis of diabetes in increased in comparison to non-diabetic patients. Metformin is a very frequently prescribed drug used to treat type II diabetes. Metformin acts in part by stimulating AMP-kinase (AMPK) and results in the suppression of mTORC1 activity and the induction of autophagy. In the following studies, we have examined the effects of metformin in the presence of various chemotherapeutic drugs, signal transduction inhibitors and natural products on the growth of three different PDAC lines. Metformin, by itself, was not effective at suppressing growth of the pancreatic cancer cell lines at concentration less than 1000 nM, however, in certain PDAC lines, a suboptimal dose of metformin (250 nM) potentiated the effects of various chemotherapeutic drugs used to treat pancreatic cancer (*e.g.*, gemcitabine, cisplatin, 5-fluorouracil) and other cancer types (*e.g.*, doxorubicin, docetaxel). Furthermore, metformin could increase anti-proliferative effects of mTORC1 and PI3K/mTOR inhibitors as well as natural products such as berberine and the anti-malarial drug chloroquine in certain PDAC lines. Thus, metformin can enhance the effects of certain drugs and signal transduction inhibitors which are used to treat pancreatic and various other cancers.

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1. Introduction

Pancreatic cancer is a devastating cancer worldwide with no truly effective cures. Surgery and some forms of chemotherapy are clinical options however, the mean 5-year survival is less than 5–10%. By the time that pancreatic ductal adenocarcinoma (PDAC) is diagnosed, PDAC has often spread to distant sites which makes therapy difficult. The increasing incidence and mortality from PDAC makes attempts to cure this disease of paramount importance to people worldwide. Treatment combining surgical resection and chemotherapy is only minimally effective (Siegel et al., 2013). PDAC is strongly associated with environmental factors such as: cigarette smoke, diet, diabetes, obesity, alcohol consumption, gender and low physical activity (Iodice et al., 2008; Arslan et al., 2010; Jiao et al., 2010; Michaud et al., 2010; Li, 2012; Lucenteforte et al., 2012; Maisonnneuve et al., 2012; Bosetti et al., 2014).

The association between type II diabetes chronic inflammation and pancreatic cancer has also been observed in mouse models. In this mouse model, the type-II diabetes drug metformin could suppress cancer cell proliferation in the mice. Chronic inflammation was observed to have only minor effects on the pathophysiology of the established adenocarcinoma in this orthotopic pancreatic transplant model (Zechner et al., 2015). Type II diabetes may be the third highest risk factor for pancreatic cancer, behind cigarette smoking and obesity (Lowenfels and Maisonneuve, 2006; Maisonneuve and Lowenfels, 2015).

PDAC is also associated with mutations at many oncogenes/tumor suppressor genes. Some of the most frequently altered genes are: *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* (Rasheed et al., 2010; Klein, 2012; Fitzgerald et al., 2015; McCubrey et al., 2015). Approximately 90% of pancreatic cancers contain mutations in *KRAS*. These mutations result in an activated KRAS oncoprotein which interacts with downstream signaling pathways leading to continuous cell growth, prevention of apoptosis, senescence, and chemotherapeutic drug resistance (Adamska et al., 2017).

KRAS is often normally activated by various growth factor receptors and is part of the RAS/RAF/ERK, PI3K/PTEN/AKT/mTORC1/GSK-3 and other interacting signaling pathways. The roles of these and other signaling pathways in various disorders, cancer and development have been recently reviewed (McCubrey et al., 2014a, 2014b; Chappell et al., 2016; Ghim et al., 2016; Giudici et al., 2016; Kang et al., 2016; McCubrey et al., 2016; Yang et al., 2016; Cervello et al., 2017; Jhanwar-Uniyal et al., 2017; McCubrey et al., 2017a, 2017b, 2017c, 2017d, 2017e; Pappas et al., 2017; Ruvolo, 2017; Ryuno et al., 2017). As *KRAS* has been frequently observed to be mutated in pancreatic cancer, attempts have been developed to generate effective RAS inhibitors as well as additional signaling molecules in this pathway. Attempts to inhibit the RAS/PI3K/PTEN/Akt/mTORC1/GSK-3 and other signaling pathways have been a central focus in many basic science laboratories and pharmaceutical companies for over three decades due to the involvement oncogenes in this pathway in human cancers (Yang et al., 2013; Fitzgerald et al., 2015; McCubrey et al., 2015; Anderson et al., 2016; Banfic et al., 2016; Cocco et al., 2016; Erneux et al., 2016; Falasca and Ferro, 2016; Fields et al., 2016; Geck and Toker, 2016; Maczis et al., 2016; Perdios et al., 2016; Pyne et al., 2016; Scarlata et al., 2016; Scoumanne et al., 2016; Tanaka et al., 2016; Ando et al., 2017; Carman and Han, 2017; Fujisawa, 2017; Geffken and Spiegel, 2017; Guo et al., 2017; Jang et al., 2017; Leonard and Johnson, 2017; Liu et al., 2017; Matsuzawa, 2017; McCubrey et al., 2017a, 2017b, 2017c, 2017d; Obsil and Obsilova, 2017; Okazaki, 2017; Pyne et al., 2017; Ramos et al., 2017; Ratti et al., 2017; Rebello et al., 2017; Roth and Frohman, 2017; Rusnak and Fu, 2017; Sakane et al., 2017; Scarlata et al., 2017; Vasquez et al., 2017). Multiple signaling pathways may be affected by mutant oncogenes and inflammatory signals as key molecules of one signaling pathway may interact with other important pathways (Coant et al., 2017; Campa and Hirsch, 2017; Ebenezer et al., 2017; Gowda et al., 2017a, 2017b; Hatch et al., 2017; Hermida et al., 2017; Mérida et al., 2017; Nishida et al., 2017; Ricciardi et al., 2017; Ruvolo, 2017; Ruzzene et al., 2017; Schrock et al., 2017; Shears et al., 2017; Steelman et al., 2017; Ramazzotti et al., 2017; Yamauchi et al., 2017).

1.1. Metformin regulates key signaling pathways in diabetes which may have effects on pancreatic cancer

Metformin (N,N-Dimethylimidodicarbonimidic diamide) is a type II diabetes drug which may also have effects on cancer stem cells (CSCs) (Lonardo et al., 2013; Liu et al., 2016). Metformin can activate 5′ AMPK and suppress mTOR activity (Rozengurt et al., 2014) (See Fig. 1). The PI3K/PTEN/Akt/mTORC1 and Raf/MEK/ERK pathways are inhibited when AMPK is activated. These pathways often serve to regulate the translation of mRNAs which have 3'regions which make them difficult to translate (Leibovitch and Topisirovic, 2017).

The effects of metformin on PDAC development and survival remain controversial. Some studies have demonstrated that metformin may improve survival in some PDAC patients which had undergone surgical resection (Cerullo et al., 2017). Another study observed a survival benefit in diabetes patients who used metformin before the development of PDAC (Amin et al., 2016).

However, other studies have not observed statistically significant better survival in patients who used metformin and had surgery and have suggested that the statistical methods of patient selection were not appropriate in other studies (Chaiteerakij et al., 2016). An additional randomized phase II trial with approximately 60 patients did not demonstrate a benefit with metformin consumption to a standard systemic therapy with cisplatin, epirubicin, capecitabine and gemcitabine patients with metastatic pancreatic cancer. The authors concluded that addition of the metformin dose used to treat type II diabetics did not improve outcome of patients with metastatic pancreatic cancer (Reni et al., 2016). A large study (907 patients) using data from the Netherlands Comprehensive Cancer Center observational cohort (1998–2011) also indicated that there was no association in terms of survival in patients using met-

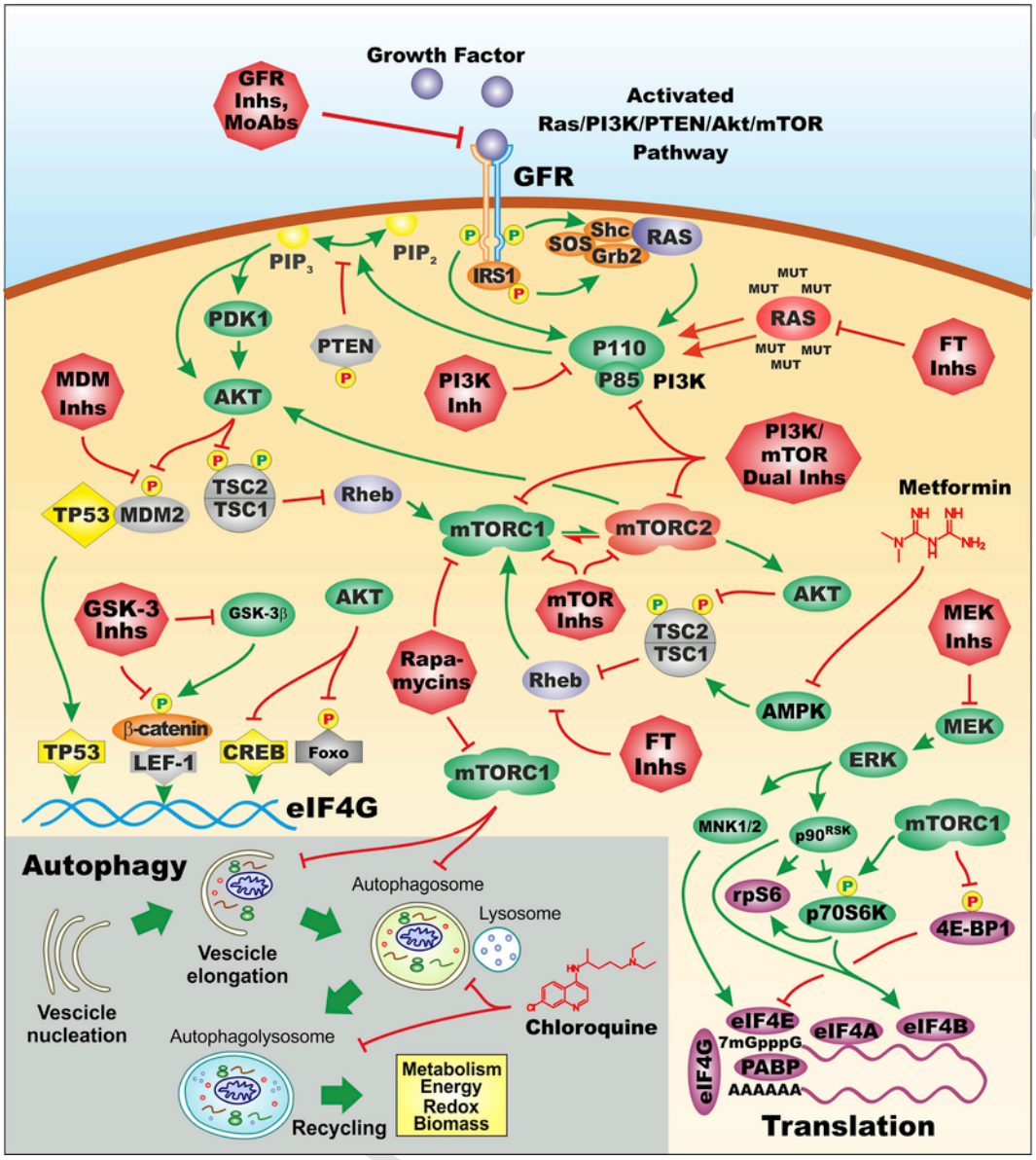

Fig. 1. Overview of Effects of Metformin on Signaling Pathways in Pancreatic Cancer. In this figure, the KRAS/PI3K/PTEN/AKT/mTORC pathway as KRAS is frequently mutated in pancreatic cancer is shown. The effects of metformin on the induction of AMPK and rapamycin on suppression of mTORC1 and induction of autophagy are indicated. In addition, the effects of chloroquine on the suppression of autophagy are indicated. Some sites where effective inhibitors have been developed to either suppress or activate key signaling molecules are indicated.

formin in PDAC patients (Frouws et al., 2017). Finally, some basic research studies with patient derived xenografts (PDX) in mice have indicated that metformin treatment did not inhibit the growth of the PDX in the four different PDX mouse models utilized in that study. The authors proposed that the PDAC cells may have become resistant to metformin by various mechanisms including lack of activation of AMPK or reactivation of mTOR (Lipner et al., 2016). Thus, while some clinicians, epidemiologists and basic scientists have concluded that metformin usage does not improve the survival of PDAC patients, there remains some open questions as to whether metformin can enhance the effects of other drugs which may be used in PDAC and other types of cancers. There are at least 16 clinical trials with the anti-type II diabetes drug metformin and pancreatic cancer https://clinicaltrials.gov/ct2/results?cond = pancreatic + cancer&term = metformin&cntry = &state = &city = &dist =]. Some of these clinical trials are examining the effects of combining metformin and chemotherapeutic drugs, rapalogs and other drugs. These trials differ in their levels of progress and some have been discontinued.

1.2. Signaling pathways regulated by metformin-key roles in many physiological processes and cancer development

The LKB1/AMP activated protein kinase (LMPK) pathway is a key pathway investigated initially in metabolic diseases such as diabetes (Steelman et al., 2016). However, given the interrelationship between metabolism and cancer, the LMPK pathway is now recognized to play important roles in cancer and other diseases (Martelli et al., 2012). This pathway can be activated by the commonly prescribed drugs, such as N,N-dimethylimidodicarbonimidic diamide (metformin) and 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR). AICAR is prescribed to reduce cardiac ischemic injury as well as diabetes care. LKB1/AMPK activation can result in cell cycle arrest, caspase-dependent apoptosis or autophagy. mTORC1-controlled protein translation is suppressed by metformin. Many of the targeted proteins are critical in the growth of malignant cells. Interestingly, the translation of these proteins is not as well inhibited with allosteric mTORC1 inhibitors, such as rapamycin and its derivatives but their translation may be better suppressed by metformin. Thus, the LKB1/AMPK pathway is key in regulating diseases such as diabetes as well as the proliferation and survival of cancer cells. Drugs targeting and activating LKB1/AMPK may be a less toxic and cost-effective treatment option for certain malignancies (Bressanin et al., 2012; McCubrey et al., 2014b).

LKB1 is considered a tumor suppressor gene. Gatekeeper mutations of *LKB1* are responsible for the rare Peutz-Jeghers Syndrome (PJS). PJS is a cancer-prone syndrome (Jansen et al., 2009). LKB1 is a critical regulator of energy/metabolism control as well as polarity. LKB1 exerts it vast effects via diverse effectors (Godlewski et al., 2010; van der Velden and Haramis, 2011). AMPK functions as a metabolic gatekeeper important in many diseases including diabetes, cancer and neurologic disorders. Inhibiting mTORC1 activity by drugs such as metformin and other drugs may not only aid in the treatment of diabetics, but also improve cancer therapies and increase longevity (Liu et al., 2011; Vazquez-Martin et al., 2011; Mashhedi et al., 2011; Oliveras-Ferraros et al., 2011). Rapamycin may also have some similar but not identical effects.

1.3. Pancreatic cancer stem cells (CSCs) and potential targeting approaches

The concept of CSCs has emerged in different types of cancers including PDAC (Fitzgerald and McCubrey, 2014; Fitzgerald et al., 2015; Xu et al., 2015; Steelman et al., 2016; McCubrey et al., 2017c). The PDAC CSCs contain certain stem-like properties. These properties include: the expression of certain cell surface antigens, the increased expression of proteins that are involved in chemo-therapeutic drug resistance (drug pumps) and expression of certain transcription factors.

More recently, the expression of various micro RNAs (miRs) have been associated with CSCs (Steelman et al., 2016; McCubrey et al., 2017c). Attempts to target CSCs with various drugs and other methods has emerged as the CSCs are thought to be responsible for cancer initiation, drug resistance and reemergence after various therapeutic approaches.

The CSCs often may differ from the bulk of the tumor cells (BC) present in the cancer patient as the CSCs have properties of stem cells. CSCs may be obtained from certain established PDAC lines based on the expression of certain cell surface markers and their ability to forms spheroids in culture. CSCs have increased expression of CD44, CD24, CD133, aldehyde dehydrogenase-1 (ALDH1), epithelial-specific antigen (ESA), activin receptor type-1B (ALK4), CXCR4, CXCL12 and Notch compared to the BCs (Fitzgerald and McCubrey, 2014; Fitzgerald et al., 2015).

Often cells are characterized by their growth properties. The adherent cells (Adh.) appear to differ from spheroid forming cells. Spheroid forming cells may have some characteristic of CSC. The spheroid forming cells is more of a morphological criterion. In the following studies, we have focused only on Adh. cells as we have compared three different PDAC lines which may have different growth properties and differential abilities to generate spheroid cells.

The KRAS-JNK signaling axis is also important in the self-renewal and tumor initiating properties of pancreatic CSCs. Suppression of KRAS activity resulted in inhibition of the JNK pathway and negatively affected the pancreatic CSC population in pancreatic cancer pre-established xenograft models (Okada et al., 2014).

Upregulation of the hedgehog [HH] pathway was observed in drug-resistant BxPC-3 and Panc3.27 pancreatic cells. The slower cycling pancreatic cells were more invasive and had a higher tumorigenic potential compared to the faster cycling cells. The slower cycling cells had morphological properties consistent with cells which had undergone epithelial mesenchymal transition (EMT) (Dembinski and Krauss, 2009). HH inhibitors may be effective in targeting pancreatic CSCs if they are combined with other drugs/inhibitors (Sandhiya et al., 2013).

The natural product cyclopamine targets smoothened (SMO) which is an important component in the HH pathway. Vismodegib is a HH inhibitor developed by Genetech (LoRusso et al., 2011). It also targets smoothly SMO and results in the inactivation of GLI1 and GLI2 transcription factors. Vismodegib is FDA approved to treat basal cell carcinoma patients and is also being evaluated with pancreatic, colorectal and other cancer patients (Sekulic et al., 2012, 2017; Sandhiya et al., 2013). However, a clinical trial with combined gemcitabine and vismodegib treatment in PDAC did not show an enhancement of survival in an unselected cohort (Catenacci et al., 2015).

Suppression of either mTOR by rapamycin or HH by cyclopamine was not by themselves sufficient to effectively eliminate the pancreatic CSC pool. In contrast, combining mTORC1 and HH inhibitors suppressed the pancreatic CSCs (Mueller et al., 2009). Interestingly, rapamycin reduced the stemness properties (viability and sphere formation ability) of CD133 pancreatic cancer cells while

suppression of mTORC1 and inhibition of HH activity had different effects on pancreatic cancer stem-like cells (Matsubara et al., 2013).

Metformin treatment was observed to have negative effects on cell survival, clonogenicity, sphere forming capacity in both gemcitabine-sensitive and resistant PDAC. Metformin inhibited the expression of CSC markers, CD44, EpCAM, enhancer of zeste homolog 2 (EZH2), Notch-1, Nanog and Oct4. In contrast, metformin induced the expression of various miRs including: let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c. The expression of these miRs are often detected at different levels in pancreatic cancer Adr. cells and pancreatic sphere colonies (Bao et al., 2012, 2014).

In spheroid forming PDAC models, a different pattern of miR expression was observed consisting of miR-99a, miR-100, miR-125b, miR-192, and miR-429 than in Adh. cells (Jung et al., 2011). Holoclone forming cells were isolated from pancreatic cancer BxPC-3 cells that were enriched in PDAC CSCs. Proteins such as CXCR4, Polycomb complex protein (BMI1), GLI1, and GLI were expressed in the holoclones. In addition, miR-214, miR-21, miR221, miR-222 and miR-155 were observed (Tan et al., 2011). These results indicate that treatment of PDAC cells with metformin may alter the expression of various miRs which have different targets.

Certain drugs may target adherent and spheroid cells differently. Drugs which affect metabolism such as rapamycin and metformin may have more effects on spheroid than adherent cells. The anti-malarial drug chloroquine has effects on pancreatic spheroid CSCs (Balic et al., 2014a, 2014b). Chloroquine has effects on the C-X-C chemokine receptor type 4 (CXCR4) and internalizes it. This makes cells less responsive to chemokine (C-X-C motif) ligand 12 (CXCL12). HH signaling is decreased and there are lower levels of PTCH and SMO on the cell surface. In addition, chloroquine effects the levels of GLI and Cyclin D1 proteins in the cytoplasm and suppresses ERK and STAT5 activation (Balic et al., 2014a).

1.4. Catabolic sources of energy and metabolic reprogramming in CSCs-effects of metformin

Breast and other types of CSCs have been observed to have enhanced ability to utilize catabolic sources of energy under starvation circumstances. Metabolic reprogramming may be an adaptive strategy by which CSCs survive during nutrient deprivation. Metabolic reprogramming during cancer progression is important (Cuyas et al., 2014). Other models documenting the importance of glycolysis and tumor metabolism have been presented (Alfarouk et al., 2014).

Elevated glucose uptake is a manifestation present in many metastatic tumors. The expression of mutant oncogenes can contribute to this phenomenon. RAS pathway activation increases glycolytic flux into lactate. The hepatocyte growth factor (HGF) binds the MET tyrosine kinase receptor. This can result in activation of RAS and downstream ERK. This leads to increased motility and glucose uptake. This may regulate the metabolism of certain breast and other cancer types as well as influence blood-flow in the tumors (Natan et al., 2014).

The effects of metformin and the related biguanide phenformin were examined in an SRC-inducible model of transformation as well as in mammosphere-derived breast CSCs by metabolomics to evaluate how these drugs might preferentially effect CSCs. During the process of cellular transformation, treatment with the biguanides suppressed the increase of glycolytic intermediates and decreased tricarboxylic acid (TCA) intermediates at certain stages of transformation. In contrast, in the breast CSCs, the effects on glycolytic and TCA intermediates were not as pronounced but reduction in nucleotide triphosphates levels were documented which could suppress nucleotide synthesis. Thus, the biguanides inhibited mitochondrial complex 1, while their metabolic effects differed at various stages of the transformation process (Janzer et al., 2014).

Metformin was proposed to induce metabolic reprogramming of chemoresistant breast cells. Metformin stimulated metabolism in ALDH-bright but not ALDH-low cells. Metformin treatment altered glutathione metabolism in the ALDH-bright cells in comparison to the ALDH-low cells. As in other studies, metformin altered miR expression in ALDH-bright cells. Interestingly, many of the affected miRs were believed to target metabolic pathways. Thus metformin may reduce the differences between the chemoresistant ALDH-bright and chemosensitive ALDH-low cells (Cioce et al., 2014).

Metformin-resistance in breast and other cancer types may result in cells with a metastatic stem-like phenotype. Resistance to metformin lead to expression of many genes encoding: migration and invasion factors, stem cell markers, pro-metastatic lipases and components of the degradome. Downregulation of genes involved in the G_2/M DNA damage checkpoint regulation that maintain genome stability as well as alteration of gene expression with pro-autophagic features were also observed (Oliveras-Ferraros et al., 2014; Menendez et al., 2014).

The effects of metformin and hyperthermia were examined on MIA-PaCa-2 pancreatic and MCF-7 and MDA-MB-231 breast cancer cells. Metformin was determined to be preferentially cytotoxic to CD44high/CD24low MCF-7 cells and CD44high/CD24high Mia-PaCa-2 cells. The CD44high/CD24low and CD44high/CD24high phenotypes are associated with breast and pancreatic CSCs respectively. Exposure of the cells to 42 °C for 1 h enhanced the ability of metformin to kill BC cancer cells as well as CSCs. Hyperthermia was determined to activate AMPK and inactivate mTORC1 and downstream p70S6K. Thus, hyperthermia could enhance the cytotoxic effects of metformin on both breast and pancreatic CSC in this experimental model system (Lee et al., 2014).

Metformin has effects on the expression of genes involved in metabolism in breast and other cancer cell types. Metformin suppressed fatty acid synthetase (FASN) in triple negative breast cancer (TNBC) by inducing the expression miR-193b. miR-193b bound the 3′UTR of FASN. Importantly, the effects of miR-193b were observed in TNBC but not in non-transformed breast epithelial cells. Metformin was shown to induce miR-193 which reduced mammosphere formation in TNBC lines (Wahdan-Alaswad et al., 2014).

Metformin may be synthetically lethal with glucose withdrawal in breast and other types of CSCs. CSCs have increased reliance on Warburg-like aerobic glycolysis to maintain CSC stemness and immortality. These stemness properties are lost upon metformin

treatment. Metformin inhibited the ability of oncogenes such as HER2 to prevent apoptosis under conditions of glucose-deprivation of HER2-positive breast CSCs (Menendez et al., 2012).

2. Results

Given the various documented effects of metformin on various types of cancer cells and the different potential mechanisms by which it could induce these different activities, we examined the abilities of metformin to suppress growth in three different pancreatic cancer cell lines ASPC-1, BxPC-3 and MIA-PaCa-2. As observed in Fig. 2, treatment with metformin by itself with concentrations up to 5000 nM did not elicit growth inhibitory effects on BxPC-3 or MIA-PaCa-2 cells as IC_{50} values were not obtained. Treatment of ASPC-1 cells with 5000 nM metformin did result in an IC_{50}. It is not surprising that metformin is not strongly toxic on cells as it is used for daily treatment of certain type II diabetes patients. The metformin levels in patient's serum taking this drug is approximately 1–20 μM. The levels of metformin in the serum depends on the kidney glomerular filtration rate (GFR) activity levels (Frid et al., 2010).

2.1. Effects of combining a suboptimal dose of metformin with drugs used to treat pancreatic cancer patients

The effects of combining a suboptimal dose of metformin (250 nM) with chemotherapeutic drugs used to treat pancreatic cancer patients and the HH inhibitor visomedigib were examined in Figs. 3 and 4. The experiments in the individual panels were both performed on the same day and most of these experiments in the two figures were performed the same day. Addition of a suboptimal dose of metformin lowered the gemcitabine IC_{50} from 700 nM to 25 nM (28-fold) in ASPC-1 cells (Fig. 3, Panel A). Addition of a suboptimal dose of metformin lowered the gemcitabine IC_{50} from 130 nM to 9 nM (14.4-fold) in BxPC-3 cells (Fig. 3, Panel B). Addition of a suboptimal dose of metformin lowered the gemcitabine IC_{50} from 9 nM to 3 nM (3-fold) in MIA-PaCa-2 cells (Fig. 3, Panel C). These experiments demonstrated that suboptimal doses of metformin could lower the concentration of gemcitabine required to reach the IC_{50} for three different PDAC lines. Furthermore, these experiments document the differential sensitivity of these three PDAC lines to gemcitabine, almost a 100-fold difference in sensitivity between MIA-PaCa-2 cells and ASPC-1 cells.

Addition of a suboptimal dose of metformin lowered the 5-fluorouracil IC_{50} from 9 μM to 0.25 μM (36-fold) in ASPC-1 cells (Fig. 3, Panel D). Addition of a suboptimal dose of metformin lowered the 5-fluorouracil IC_{50} from 3.5 nM to 0.5 nM (7-fold) in BxPC-3 cells (Fig. 3, Panel E). Addition of a suboptimal dose of metformin lowered the 5-fluorouracil IC_{50} from 5 μM to 0.6 μM (3-fold) in MIA-PaCa-2 cells (Fig. 3, Panel F).

Addition of a suboptimal dose of metformin lowered the cisplatin IC_{50} from 15 μM to 0.7 μM (21.4-fold) in ASPC-1 cells (Fig. 4, Panel A). Addition of a suboptimal dose of metformin lowered the cisplatin IC_{50} from approximately 200 μM–1 μM (200-fold) in BxPC-3 cells (Fig. 4, Panel B). Addition of a suboptimal dose of metformin did not lower the cisplatin IC_{50} in MIA-PaCa-2 cells (Fig. 4, Panel C). These experiments demonstrated that suboptimal doses of metformin could lower the concentration of cisplatin required to reach the IC_{50} for two different, ASPC-1 and BxPC-3 PDAC lines but not in MIA-PaCa-2 cells.

Vismodegib is a small molecule inhibitor which targets HH signaling and is being used to treat basal cell carcinoma (Sekulic et al., 2012, 2017). It is also being evaluated in other cancers such as PDAC (Catenacci et al., 2015). Addition of a suboptimal dose of metformin did not lower the vismodegib IC_{50} in either ASPC-1 or BxPC-3 cells (Fig. 4, Panels D and E), however, it did decrease growth observed in BxPC-3 cells at high concentrations of vismodegib (Fig. 4, Panel E). It also decreased growth and reduced the IC_{50} in MIA-PaCa-2 cells (Panel F).

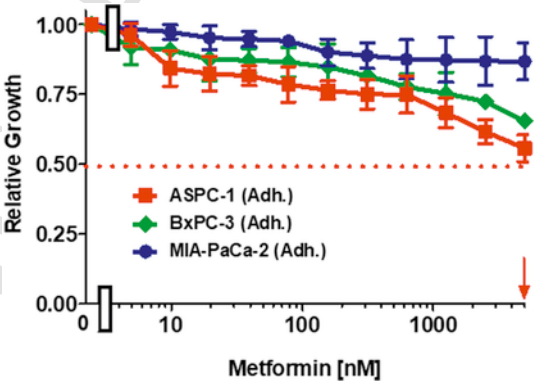

Fig. 2. Effects of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (red squares), BxPC-3 (green diamonds) and MIA-PaCa-2 (blue circles) and MCF-7 (red circles) cells were titrated with different concentrations of metformin for 4 days before MTT analysis was performed. Arrows on the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

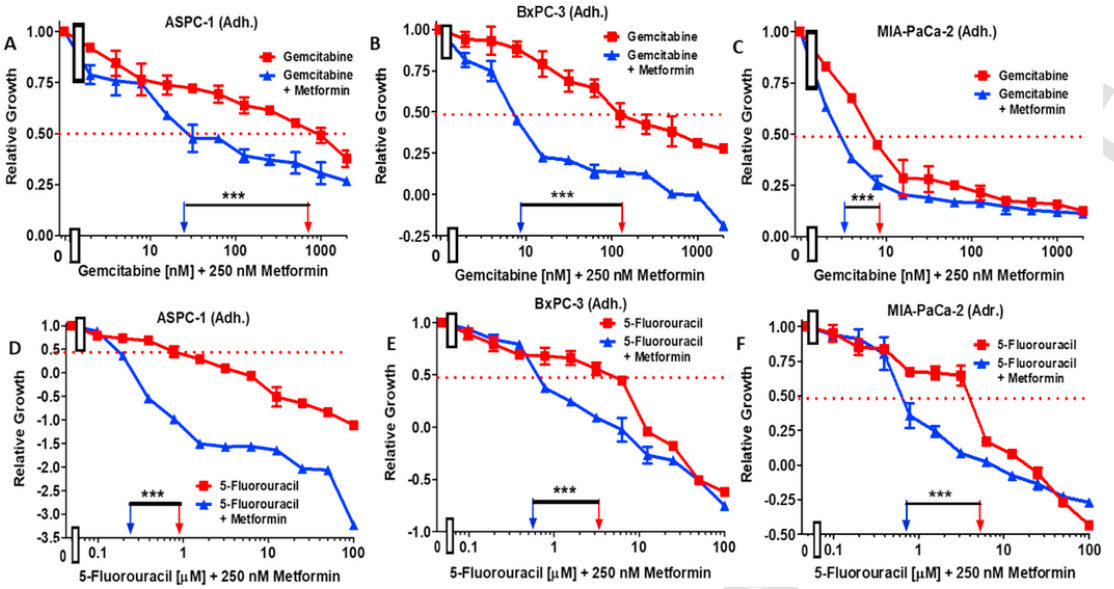

Fig. 3. Effects of Gemcitabine and 5-Fluorouracil in the Absence and Presence of Suboptimal Doses of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (Panel A), BxPC-3 (Panel B) and MIA-PaCa-2 (Panel C) were titrated with different concentrations of gemcitabine in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel A, the two-tailed *P* value is less than 0.0001 between gemcitabine and gemcitabine and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between the gemcitabine and the gemcitabine and metformin-treated BxPC-1 cells and considered to be extremely statistically significant. In Panel C, the two-tailed *P* value is less than 0.0001 between the gemcitabine and the gemcitabine and metformin-treated MIA-PaCa-2 cells are less than 0.0001 and considered to be extremely statistically significant. ASPC-1 (Panel D), BxPC-3 (Panel E) and MIA-PaCa-2 (Panel F) were titrated with different concentrations of 5-fluorouracil in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel D, the two-tailed *P* value equals 0.0002 between the 5-fluorouracil and the 5-fluorouracil and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel E, the two-tailed *P* value is less than 0.0001 between the 5-fluorouracil and the 5-fluorouracil and metformin-treated BxPC-3 cells is less than 0.0001 and considered to be extremely statistically significant. In Panel F, the two-tailed *P* value is less than 0.0001 between the 5-fluorouracil and the 5-fluorouracil and metformin-treated MIA-PaCa-2 cells and considered to be extremely statistically significant. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Effects of combining a suboptimal dose of metformin with commonly used chemotherapeutic drugs

The effects of metformin on two commonly used chemotherapeutic drugs (doxorubicin and docetaxel) were examined (Fig. 5). Addition of a suboptimal dose of metformin lowered the doxorubicin IC_{50} from 130 nM to 6 μM (21.7-fold) in ASPC-1 cells (Fig. 5, Panel A). Addition of a suboptimal dose of metformin lowered the growth of BxPC-3 cells when cultured in doxorubicin up to approximately 400 nM, but did not lower the doxorubicin IC_{50} in BxPC-3 cells which was approximately 600 nM (Fig. 5, Panel B). Addition of a suboptimal dose of metformin lowered the doxorubicin IC_{50} in MIA-PaCa-2 cells from approximately 90 nM–45 nM (about 2-fold) (Fig. 5, Panel C). These experiments demonstrated that suboptimal doses of metformin could lower the concentration of doxorubicin required to reach the IC_{50} for two different PDAC lines, ASPC-1 and MIA-PaCa-2 lines but not in BxPC3 cells. These experiments also demonstrated that some PDAC lines such as BxPC3 are more resistant to doxorubicin than ASPC-1 and MIA-PaCa-2 cells.

Addition of a suboptimal dose of metformin lowered the docetaxel IC_{50} from 6.5 nM to 0.2 nM (3.3-fold) in ASPC-1 cells (Fig. 5, Panel D). Addition of a suboptimal dose of metformin did not lower the docetaxel IC_{50} in BxPC-3 cells (Fig. 5, Panel E). If anything, the suboptimal dose of docetaxel inhibited the suppressive effects of docetaxel in BxPC-3 cells Addition of a suboptimal dose of metformin did not lower the docetaxel IC_{50} in MIA-PaCa-2 cells (Fig. 5, Panel F).

2.3. Effects of combining a suboptimal dose of metformin with drugs used with PI3K/PTEN/Akt/mTORC pathway inhibitors

Most PDACs have mutations in *KRAS* which result in increased activation (Rasheed et al., 2010; Klein, 2012; Fitzgerald et al., 2015; McCubrey et al., 2015). KRAS can be an upstream activator of PI3K. Thus, the targeting of KRAS, PI3K and mTORC1 has been an active area in basic pancreatic cancer research. One of the effects of metformin is the suppression of mTORC1 activity which leads to the induction of autophagy. Likewise, the mTORC1 blocker and the dual PI3K/mTOR inhibitor NVP-BE235 will both block mTORC1 which will lead to the induction of autophagy.

Addition of a suboptimal dose of metformin did not lower the rapamycin IC_{50} in ASPC-1 cells (Fig. 6, Panel A). In contrast, addition of a suboptimal dose of metformin lowered the growth and rapamycin IC_{50} in BxPC-3 cells from approximately 45 nM–0.85 nM

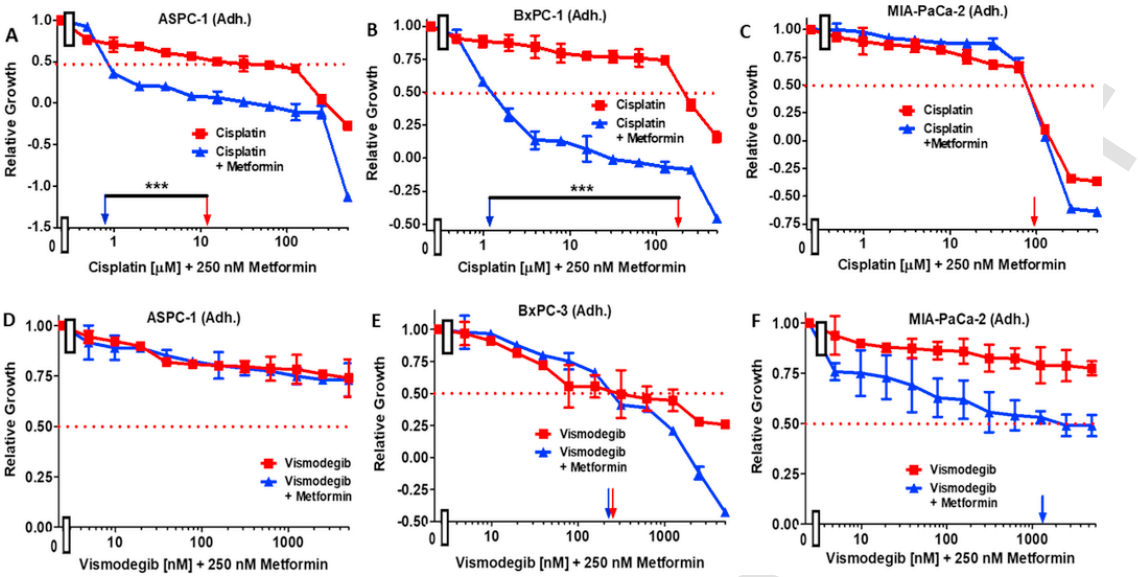

Fig. 4. Effects of Cisplatin and Vismodegib in the Absence and Presence of Suboptimal Doses of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (Panel A), BxPC-3 (Panel B) and MIA-PaCa-2 (Panel C) were titrated with different concentrations of cisplatin in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel A, the two-tailed *P* value is less than 0.0001 between cisplatin and cisplatin and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between the cisplatin and the cisplatin and metformin-treated BxPC-1 cells and considered to be extremely statistically significant. ASPC-1 (Panel D), BxPC-3 (Panel E) and MIA-PaCa-2 (Panel F) were titrated with different concentrations of vismodegib in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

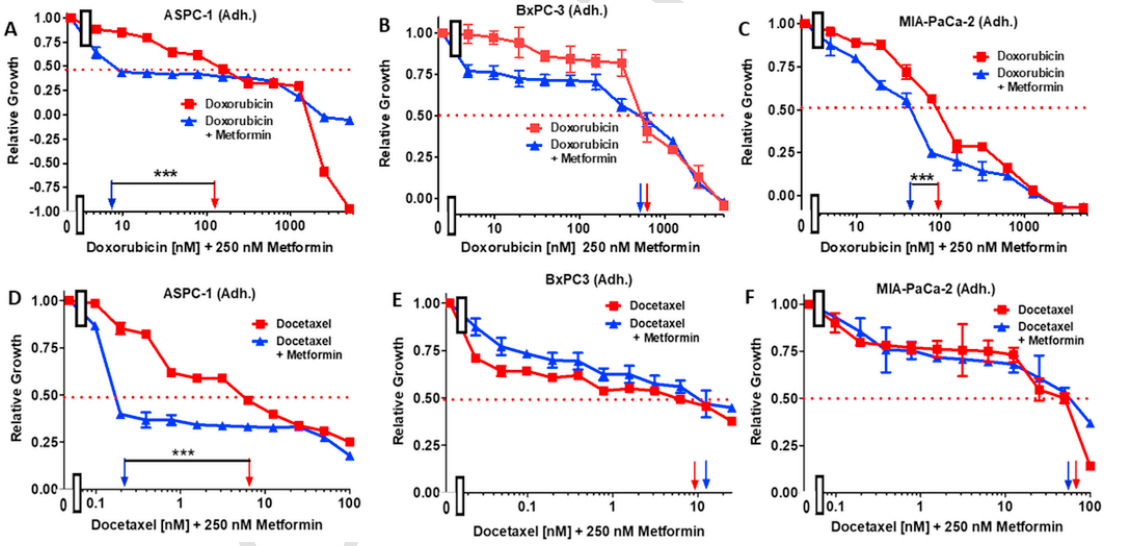

Fig. 5. Effects of Doxorubicin and Docetaxel in the Absence and Presence of Suboptimal Doses of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (Panel A), BxPC-3 (Panel B) and MIA-PaCa-2 (Panel C) were titrated with different concentrations of doxorubicin in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel A, the two-tailed *P* value is less than 0.0001 between doxorubicin and doxorubicin and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel C, the two-tailed *P* value is less than 0.0001 between the doxorubicin and the doxorubicin and metformin-treated MIA-PaCa-2 cells are less than 0.0001 and considered to be extremely statistically significant. ASPC-1 (Panel D), BxPC-3 (Panel E) and MIA-PaCa-2 (Panel F) were titrated with different concentrations of docetaxel in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel D, the two-tailed *P* value is less than 0.0001 between the docetaxel and the docetaxel and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

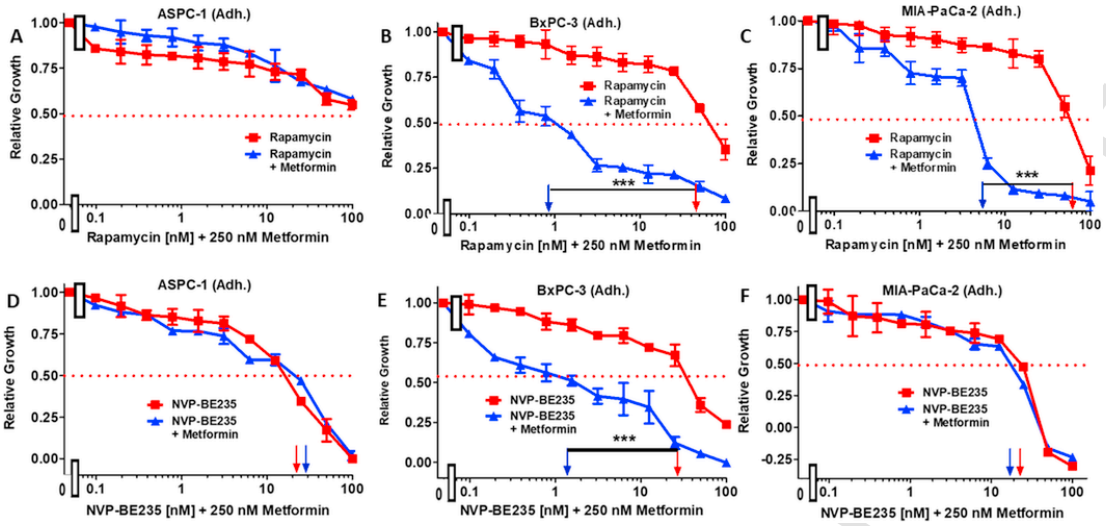

Fig. 6. Effects of Rapamycin and NVP-BE235 in the Absence and Presence of Suboptimal Doses of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (Panel A), BxPC-3 (Panel B) and MIA-PaCa-2 (Panel C) were titrated with different concentrations of rapamycin in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel B, the two-tailed *P* value is less than 0.0001 between the rapamycin and the rapamycin and metformin-treated BxPC-1 cells and considered to be extremely statistically significant. In Panel C, the two-tailed *P* value is less than 0.0001 between the rapamycin and the rapamycin and metformin-treated MIA-PaCa-2 cells are less than 0.0001 and considered to be extremely statistically significant. ASPC-1 (Panel D), BxPC-3 (Panel E) and MIA-PaCa-2 (Panel F) were titrated with different concentrations of NVP-BE235 in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel E, the two-tailed *P* value is less than 0.0001 between the NVP-BE-235 and the NVP-BE235 and metformin-treated BxPC-3 cells is less than 0.0001 and considered to be extremely statistically significant. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(approximately 53-fold) (Fig. 6, Panel B). Addition of a suboptimal dose of metformin lowered the rapamycin IC_{50} in MIA-PaCa-2 cells from approximately 60 nM–5 nM (about 12-fold) (Fig. 6, Panel C). These experiments demonstrated that suboptimal doses of metformin could lower the concentration of rapamycin required to reach the IC_{50} for two different PDAC lines, BxPC-3 and MIA-PaCa-2 lines but not in AsPC-1 cells. Interestingly, BxPC-3 is reported to have wild-type (WT) KRAS, while ASPC-1 and MIA-PaCa-2 cells have mutant KRAS genes (Deer et al., 2010; Barretina et al., 2012; Hamidi et al., 2014).

Addition of a suboptimal dose of metformin did not lower the dual PI3K/mTORC NVP-BE235 inhibitor IC_{50} in ASPC-1 cells (Fig. 6, Panel D). Addition of a suboptimal dose of metformin did lower the NVP-BE235 IC_{50} in BxPC-3 cells from 28 to 1.3 nM, approximately 21.5-fold (Fig. 6, Panel E). Addition of a suboptimal dose of metformin did not lower the NVP-BE235 IC_{50} in MIA-PaCa-2 cells (Fig. 6, Panel F).

2.4. Effects of combining a suboptimal dose of metformin with the nutraceutical berberine and the anti-malarial drug chloroquine

Berberine is a natural product obtained from various berries and fruits (McCubrey et al., 2017a, 2017b, 2017e). It can have multiple effects on cells, one is on the induction of NF-kappa B, another may be the induction of AMPK. The plant natural product berberine has recently been shown to inhibit the growth of breast and pancreatic cancer cells (McCubrey et al., 2017e). Berberine may also target and activate LKB1/AMPK (McCubrey et al., 2017a; Pan et al., 2017). Berberine may also target various types of CSCs (Lin et al., 2017).

Chloroquine is a drug which is commonly used to eliminate malarial disease. The effects of treatment of PDAC cells with suboptimal doses of metformin and either berberine or chloroquine are presented in Fig. 7.

Addition of a suboptimal dose of metformin lowered the berberine IC_{50} from in ASPC-1 cells from approximately 1800 nM–30 nM, approximately 60-fold (Fig. 7, Panel A). Addition of a suboptimal dose of metformin lowered the growth and berberine IC_{50} in BxPC-3 cells from approximately 600 nM–20 nM (approximately 30-fold) (Fig. 6, Panel B). In contrast, addition of a suboptimal dose of metformin did not lower the berberine IC_{50} in MIA-PaCa-2 cells (Fig. 7, Panel C). These experiments demonstrated that suboptimal doses of metformin could lower the concentration of berberine required to reach the IC_{50} for two different PDAC lines, ASPC-1 and BxPC-3 lines but not in MIA-PaCa-2 cells.

Addition of a suboptimal dose of metformin lowered the chloroquine IC_{50} in ASPC-1 cells from 2 μM to 0.35 μM (approximately 5.7-fold) (Fig. 7, Panel D). Addition of a suboptimal dose of metformin lowered the chloroquine IC_{50} in BxPC-3 cells from 10 to 0.15 μM approximately 67-fold (Fig. 7, Panel E). Addition of a suboptimal dose of metformin did lower the chloroquine IC_{50} in MIA-PaCa-2 cells from greater than 20 μM to approximately 0.2 μM (approximately 100-fold) (Fig. 7, Panel F). Thus, the effects of chloroquine in all three PDAC lines can be enhanced by a suboptimal dose of metformin.

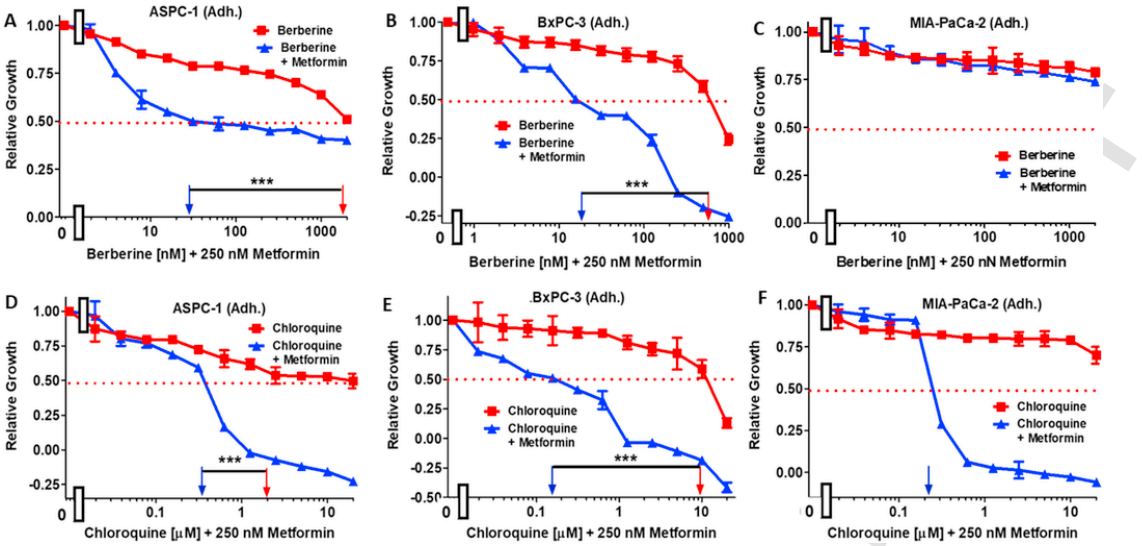

Fig. 7. Effects of Berberine and Chloroquine in the Absence and Presence of Suboptimal Doses of Metformin on the Proliferation of Three Pancreatic Cancer Cell Lines. ASPC-1 (Panel A), BxPC-3 (Panel B) and MIA-PaCa-2 (Panel C) were titrated with different concentrations of berberine in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel A, the two-tailed *P* value is less than 0.0001 between berberine and berberine and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between the berberine and the berberine and metformin-treated BxPC-1 cells and considered to be extremely statistically significant. ASPC-1 (Panel D), BxPC-3 (Panel E) and MIA-PaCa-2 (Panel F) were titrated with different concentrations of chloroquine in the absence (red squares) and presence (blue triangles) of suboptimal doses of 250 nM metformin. Arrows on the X-axis indicate where the IC_{50}s can be estimated. In Panel D, the two-tailed *P* value is less than 0.0001 between the chloroquine and the chloroquine and metformin-treated ASPC-1 cells and considered to be extremely statistically significant. In Panel E, the two-tailed *P* value is less than 0.0001 between the chloroquine and the chloroquine and metformin-treated BxPC-3 cells is less than 0.0001 and considered to be extremely statistically significant. These experiments were repeated 3 times and similar results were obtained. Adh. = adherent cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Conclusions

Metformin is one of the most frequently prescribed drugs to treat type-II diabetes. It acts by inducing AMPK which in turn suppresses mTORC1 that results in the induction of autophagy. Metformin treatment by itself did not inhibit growth of PDAC cell lines as much as 5-fluorouracil or gemcitabine which are used to treat PDAC patients. However, a suboptimal dose of metformin could lower the IC_{50}s for various chemotherapeutic drugs. The most substantial effects were observed with 5-fluorouracil and gemcitabine in all three PDACs examined. Metformin also enhanced the effects of cisplatin in the BxPC-3 cell line. Gemcitabine, 5-fluorouracil and cisplatin are all nucleoside analogues. The significance of these observations is being examined with other nucleoside analogues used to treat PDAC. Metformin showed some effects on the effects on doxorubicin (a topoisomerase inhibitor) in AsPC-1, BxPC3 and MIA-PaCa-2 cells. In contrast, metformin did not appear to have effects at this suboptimal concentration when combined with docetaxel (microtubule depolymerization blocker) in the PDAC lines examined. These two other chemotherapeutic drugs act by different mechanisms than the nucleoside analogues.

Although, initially one might think that metformin, rapalogs and PI3K/Akt inhibitors might have similar effects, they have some different properties which may lead to their combined usage might prove more effective. For example, while metformin suppresses the proliferation of T-acute lymphoblastic leukemias (T-ALL) cell lines and primary T-ALL patient samples, metformin induced autophagy and apoptosis and suppressed proliferation. The effects of metformin on control $CD4^+$ T-lymphocytes were also examined. Metformin was determined to be less toxic against $CD4^+$ T-lymphocytes from healthy donors than against either the T-ALL cell lines or primary patient cells. These studies documented the dephosphorylation of downstream targets of mTORC1. In addition, inhibition of mRNA translation in metformin-treated T-ALL cells were seen. Importantly, a similar inhibition of translation was not observed after rapamycin treatment. Intriguingly, in primary patient samples, metformin targeted the side population of T-ALL cell lines as well as a putative leukemia initiating cell (LIC) subpopulation ($CD34^+$, CD7-, CD4-). Metformin exhibited anti-leukemic activity, which supports further development of LKB1/AMPK pathway activators as potential clinical candidates for T-ALL and other cancer therapies (Grimaldi et al., 2012).

AMPK is also important in BCR-ABL-induced chronic myeloid leukemia (CML) and AMPK pathway activators may prove useful as combination drug therapy (with BCR-ABL inhibitors) for this disease (Martelli et al., 2012). AMPK can also be activated by rapamycin (Habib, 2011). These studies also demonstrated that the effects of rapamycin and metformin are not always identical. In our studies, we observed that suboptimal doses of metformin could enhance the effects of rapamycin or the PI3K/mTOR dual inhibitor NVP-BEZ-235 in certain PDAC lines which indicate that while these inhibitors and metformin may have some similar downstream targets, there may be others which upon suppression can eliminate cellular proliferation more effectively.

Metformin could also enhance the effects of the mTORC1 blocker rapamycin and the PI3K/mTOR inhibitor NVP-BE235 in both the BxPC-3 and MIA-PaCa-2 cells but not in the ASPC-1 cells. BxPC-3 cells are not reported to have mutant KRAS, but both ASPC-1 and MIA-PaCa-2 both have mutant KRAS. All three cell lines have mutant *KRAS* but they differ in the presence of other key genetic mutations. These studies document that different approaches to suppress the PI3K pathway and mTORC1 can be effective. That is, even though rapamycin and NVP-BE-235 do act in part to inhibit mTORC, metformin can enhance their abilities to suppress cell growth in at least two PDAC models.

Berberine is used for the treatment and prevention of various diseases world-wide now (McCubrey et al., 2017a, 2017b, 2017c, 2017d, 2017e; McCubrey and Cocco, 2017). Berberine are naturally present in many fruits and plants including: *Berberis aetnensis C. Presl.*, *Berberis aristata*, *Berberis vulgaris*, *Coptis chinensis*, *Coptis japonica*, *Coptis rhizome*, *Hydrastis canadensis*, *Phellondendron amurense* and *Tinosora cordifolia*. Berberine is an isoquinoline quaternary alkaloid (a 5,6-dihydrodibenzo[a,g]quinolizinium derivative). The potential beneficial effects of BBR are being investigated in at least 35 clinical trials (McCubrey et al., 2017a).

Berberine can modulate the expression of various genes that function in the regulation of cell growth including: BCL2, BCLXL, PARP1, Beclin-1, TP53, $p21^{Cip1}$, MMP9 (Tillhon et al., 2012). Among the many effects of berberine which have been described it may induce double strand DNA breaks and cell cycle arrest (Wang et al., 2012).

Berberines can form complexes with nucleic acids, such as DNA and RNA by binding the nitrogen atom at the 7-positon in the alkaloid BBR skeleton. These complexes may interfere telomerases and topoisomerases (Gatto et al., 1996; Kim et al., 1998; Qin et al., 2007; Bhowmik et al., 2012). Berberines may also affect mRNA transcription by interacting with the TATA-binding proteins and the TATA-boxes contained in certain promoter regions of anti-apoptotic genes such as BCL2 and subsequently influence gene transcription (Wang et al., 2011; Xiao et al., 2012).

Berberine can affect many different physiological processes as well as cancer and non-alcoholic fatty liver disease (Zhu et al., 2016; McCubrey et al., 2017a, 2017b, 2017c, 2017d, 2017e). Treatment of ASPC-1 and BxPC-1 cells with suboptimal dose of metformin lowered the concentrations of berberine required to reach the IC_{50}. In contrast, the combined effects of berberine and metformin on suppression of proliferation were not observed on MIA-PaCa-2 cells. Berberine and other natural products that we consume in our diet may have effects on the induction of reactive oxygen species which can have anti-cancer effects via stimulation of apoptosis and other growth regulatory processes (Park et al., 2015; Cusimano et al., 2017).

The anti-malarial drug chloroquine is known to inhibit the induction of autophagy. Thus, in theory the addition of chloroquine and metformin could have opposing effects. However, we observed that the combined treatment of a suboptimal dose of metformin enhanced the effects of chloroquine on the suppression of growth in all three pancreatic cell lines examined. Likewise blockade of MEK inhibitor induced autophagy has been shown to sensitize certain cancer cells to the PI3K/AKT inhibitor NVP-BKM120 (Ren et al., 2016).

These three pancreatic cell lines differ in the frequency and presence of sphere forming cells in them upon growth under normal culture conditions. There are often many sphere forming cells present in MIA-PaCa-2 cells. Sphere forming cells have some characteristics of CSCs. It will be interesting to determine whether the sphere forming cells from the various PDAC lines have different sensitivities to metformin and various other drugs and nutraceuticals.

Our studies document that while some drug combinations with metformin will result in a lowering of the drug concentration required to meet the IC_{50} in all three cell lines examined, some metformin and drug combinations do not lead to a lowering of the drug concentration required to reach the IC_{50}s. These results are important as in cancer patients, there is heterogeneity in pancreatic cancer cell lines in terms to their responses to certain drugs and drug combinations. Thus, we should not expect that some drug combinations will work with all cancer patients.

4. Materials and methods

4.1. Cell lines

The MIA-PaCa-2 PDAC (ATCC® CRM-CRL-1420™) carcinoma cell line was derived from a 65-year old Caucasian male (Deer et al., 2010). MIA-PaCa-2 cells have the R248W TP53 GOF mutation. The R248W TP53 mutation present in MIA-PaCa-2 cells is a missense point mutation in the central DNA binding domain which abrogates its DNA contact (Liu et al., 2010). This TP53 mutation results in a TP53 protein that is unable to bind specifically TP53 target sequences in TP53-responsive genes and results in loss of its tumor suppressor properties (Song et al., 2007; Solomon et al., 2012). MIA-PaCa-2 cells also have an activating mutation at KRAS (G12C) and they have elevated PI3K/AKT pathway activity.

The ASPC-1 cell line (ATCC® CRL-1682™), an adenocarcinoma, was derived from a 62-year old female (Deer et al., 2010). It has mutant *KRAS* and *TP53* genes.

The BxPC-3 cell line (ATCC® CRL-1687™), an adenocarcinoma, was derived from a 61-year-old woman (Deer et al., 2010). Interestingly, the BxPC-3 cells express WT-*KRAS* (Barretina et al., 2012) but it has mutant *TP53* genes.

4.2. Tissue culture and treatment of cells with signal transduction inhibitors and doxorubicin

Tissue culture medium was obtained from Invitrogen (Carlsbad, CA, USA). Nutraceuticals, chemotherapeutic drugs, signal transduction inhibitors were purchased from either Selleckchem (Houston, TX USA) or Sigma-Aldrich (Saint Louis, MO, USA). MIA-PaCa-

2 and MCF-7 cells were titrated with the different nutraceuticals, signal transduction inhibitors, chemotherapeutic and other drugs as described (Chappell et al., 2012; Abrams et al., 2017; Steelman et al., 2018). Statistical analysis was performed using GraphPad Prism.

Conflicts of interest

The authors declare that they have no conflicts of interest with publication of this manuscript.

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