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Abilities of berberine and chemically modified berberines to interact with metformin and inhibit proliferation of pancreatic cancer cells

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ABSTRACT

Pancreatic cancer is devastating cancer worldwide with few if any truly effective therapies. Pancreatic cancer has an increasing incidence and may become the second leading cause of death from cancer. Novel, more effective therapeutic approaches are needed as pancreatic cancer patients usually survive for less than a year after being diagnosed. Control of blood sugar levels by the prescription drug metformin in diseases such as diabetes mellitus has been examined in association with pancreatic cancer. While the clinical trials remain inconclusive, there is hope that certain diets and medications may affect positively the outcomes of patients with pancreatic and other cancers. Other natural compounds may share some of the effects of metformin. One "medicinal" fruit consumed by millions worldwide is berberine (BBR). Metformin and BBR both activate AMP-activated protein kinase (AMPK) which is a key mediator of glucose metabolism.

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Glucose metabolism has been shown to be very important in cancer and its significance is increasing. In the following studies, we have examined the effects of metformin, BBR and a panel of modified BBRs (NAX compounds) and chemotherapeutic drugs on the growth of four different human pancreatic adenocarcinoma cell lines (PDAC). Interestingly, the effects of metformin could be enhanced by BBR and certain modified BBRs. Upon restoration of WT-TP53 activity in MIA-PaCa-2 cells, an altered sensitivity to the combination of certain NAX compounds and metformin was observed compared to the parental cells which normally lack WT-TP53. Certain NAX compounds may interact with WT-TP53 and metformin treatment to alter the expression of key molecules involved in cell growth. These results suggest a therapeutic approach by combining certain pharmaceutical drugs and nutraceuticals to suppress the growth of cancer cells.

1. Introduction

Previously, we have summarized many of the key genetic and biological factors implicated in pancreatic cancer (Candido et al., 2018; Abrams et al., 2018, 2019; McCubrey et al., 2018). Although various genetics and environmental factors may be involved in pancreatic cancer, few remedies have been developed to treat this devastating disease beyond surgery and chemotherapy which, unfortunately, only have minimal positive effects on patient survival. Clearly novel, more effective approaches are needed to treat pancreatic ductal adenocarcinoma (PDAC) and the subsequent drug resistance which may arise.

Metformin (N,N-dimethylimidodicarbonimidic diamide) is a type II diabetes drug which also has effects on cancer (Lonardo et al., 2013; Candido et al., 2018). Metformin mainly blocks hepatic gluconeogenesis. Cancer cells normally have high glucose requirements due to their frequent utilization of the inefficient breakdown of glucose by the anaerobic pyruvate pathway (Warburg effect) to generate ATP. Thus, metformin can have anti-cancer effects by modulation of glucose and energy levels in cancer cells. Epidemiological studies have suggested that metformin may have effects on PDAC development.

However, the relationship between metformin use and PDAC development and survival remains controversial (Bosetti et al., 2014). While some studies suggested a benefit for PDAC patients who consumed metformin (Cerullo et al., 2017) other investigations

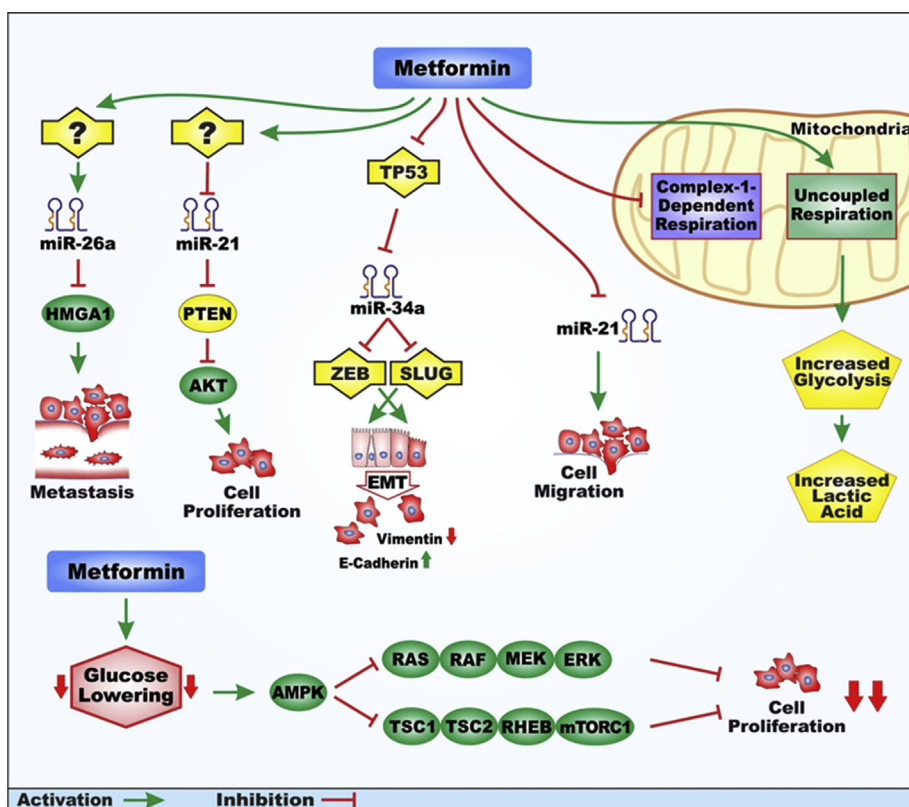


Fig. 1. Effects of Metformin on Metabolism, Gene Expression, Induction of miRs, Cellular Proliferation and Metastasis. Metformin treatment results in the modulation of various metabolic pathways which play key roles in the regulation of cellular proliferation. Various miRs are induced by metformin. This figure depicts briefly, how these miR may affect certain signal transduction and apoptotic pathways which are important in the regulation of cellular growth, malignant transformation, invasion, and drug resistance.

did not demonstrate a statistically significant difference in survival levels (Chaiteerakij et al., 2016). Moreover, in a study comparing metformin consumption to a standard systemic therapy (e.g., cisplatin, epirubicin, capecitabine and gemcitabine), a significant increase in outcome of patients with metastatic pancreatic cancer was not observed (Reni et al., 2016). Data from 907 patients in the Netherlands Comprehensive Cancer Center observational cohort (1998–2011) indicated that there was no association in terms of survival in patients using metformin in PDAC patients (Frouws et al., 2017). However, 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=>]. Thus, the association between metformin use and PDAC is controversial (Chaiteerakij et al., 2016). However, it should be emphasized that metformin has been proposed to have effects on cancer stem cells (CSC) and sensitivity to chemotherapy (Hirsch et al., 2009, 2013; Iliopoulos et al., 2011; Rattan et al., 2011; Bao et al., 2012, 2014; Xu et al., 2018).

1.1. Regulation of key signaling pathways by metformin

Metformin activates 5' AMPK and suppresses mTORC1 activity, a key regulator of cell growth and the target of the immunosuppressive, anti-cancer drug rapamycin (Ming et al., 2014). When AMPK is activated, the pro-proliferative mTORC1 and RAF/MEK/ERK pathways are inhibited. These pathways function to regulate many processes involved in cell growth, the prevention of apoptosis and drug resistance. The PI3K/Pten/Akt/mTORC1 and RAF/MEK/ERK pathways often serve to regulate various growth regulatory mRNAs which have 3' regions structures which make them difficult to translate by phosphorylation of key proteins involved with ribosomes and effect protein translation.

1.2. Regulation of microRNAs (miRs) by metformin

Some of the key molecules involved in drug resistance affected by metformin treatment are miRs. miRs regulate expression of genes. miRs are small non-coding RNAs (ncRNA), 19–25 nucleotides in length, derived from hairpin-shaped precursor molecules encoded by the genomes of animals, plants, and viruses (Esteller, 2011). miRNAs control gene expression and regulate a wide array of biological processes by targeting mRNAs and inducing translational repression or RNA degradation (Adams et al., 2014). Among mammals, miR coding sequences are estimated to account for about 1% of the genome, and yet more than 60% of protein coding genes are regulated by miRs (Friedman et al., 2009). A diagram of the effects of metformin on some miRs and cellular growth and metastasis is presented in Fig. 1. The effects of metformin on miRs is currently under intense study. Some studies have indicated that miRs may be induced by metformin. In a hyperglycemia model consisting of mouse microvascular endothelial cells (MMEC), miR-34a expression altered sirtuin 1 expression as well as post-translation modification of endothelial nitric oxide synthase and angiogenesis. Metformin treatment or inhibition of miR-34a, increased expression of sirtuins and prevented the impairment of angiogenesis. Overexpression of a miR-34a mimic was determined to suppress the effects of metformin (Arunachalam et al., 2016).

Metformin can reduce glucose levels in PDAC cells. This resulted in decreased levels of miR-34a and its downstream targets NOTCH, SLUG and SNAIL. The frequency of PDAC spheroid formations as well as the spheroid sizes were reduced (Cifarelli et al., 2015).

Metformin was also demonstrated to increase the expression of certain miRs in PDAC cells including: let-7a, let-7b, miR-26a, miR-110, miR-200b and miR-200c (Bao et al., 2012). These resulted in the decreased expression of CD44, epithelial cell adhesion molecule (EpCAM), enhancer of zeste homolog 2 (EZH2), NOTCH-1, NANOG and OCT4. Re-expression of miR-26a, mediated by transfection, resulted in decreased expression of EZH2 and EpCAM.

Other studies have shown that metformin can induce miR-34a, which results in the down-regulation of SIRT1/PGC1 α /NRF2 pathway in cells with wild type (WT) TP53 (Do et al., 2014). Metformin resulted in decreased levels of SIRT1 in cells with WT-TP53 but not in cells with mutant TP53. In cells with WT-TP53, metformin induced TP53 and miR-34a and suppressed peroxisome proliferator activated receptor δ (PPAR δ) coactivator 1 α (PGC-1 α). This resulted in the suppression of nuclear factor-like 2 (NRF2), at least in TP53-positive MCF-7 breast cancer cells (Do et al., 2014).

Metformin inhibits certain miRs in rat mesangial cells cultured with high glucose (Wu et al., 2018) High glucose treatment normally results in activation of miRs and the early growth response protein 1 (EGR1) transcription factor. miR-34 can suppress SIRT1 and AMPK which results in EGR1 activation. Metformin suppressed this induction.

Metformin was shown to inhibit the proliferation, migration and invasion in HCT116 and SW-480 colorectal cancer (CRC) cells (Wang et al., 2018). Metformin treatment resulted in the up-regulation of E-cadherin and downregulation of vimentin during the process of epithelial to mesenchymal transition (EMT) of CRC cells. In these studies, metformin induced miR-200a, miR-200c and miR-429 but decreased miR-34a. Metformin treatment also resulted in decreased levels of Snail and ZEB expression.

Metformin also suppressed miR-27a expression in PDAC cells which resulted in increased expression of zinc finger and BR-C, ttk and bab (BTB) domain containing 10 (ZBTB10). ZBTB10 is a suppressor of the SP1, SP3 and SP4 transcription factors. Metformin treatment resulted in a decreased growth of the PDAC cells both *in vitro* and in orthotopic tumor models (Nair et al., 2013).

1.3. Regulation of signal transduction pathways, cellular growth, and apoptosis by berberine

Berberine (BBR) is a commonly-consumed nutraceutical. We have recently summarized the effects of BBR on many important processes involved in cell growth (Abrams et al., 2018a; Abrams et al., 2018b; McCubrey et al., 2017, 2018). The pleiotropic effects on various properties on the regulation of cell growth and apoptosis have also been critically evaluated recently (Farooqi et al., 2019).

Below, we summarize some of the more novel aspects about BBRs which may explain some of their diverse effects on cellular proliferation and drug resistance. BBR can also activate AMPK (Pan et al., 2017). BBR has been shown to modulate the activity of reactive oxygen species (ROS) levels (Chatterjee et al., 2015). ROS can alter the activity of many signaling and apoptotic pathways including RAS/RAF/MEK/ERK and mTORC1 pathways (Burgeiro et al., 2011; Hsu et al., 2012; McCubrey et al., 2017, 2018). BBRs can also have effects on NF- κ B, TP53 and other transcription factors which are key controllers of gene regulation, DNA repair and metastasis. A diagram summarizing the effects of BBR on gene expression, cellular migration, DNA/chromatin structure and cell cycle progression is presented in Fig. 2.

Some of the potential anti-diabetic and anti-cancer effects of BBRs are their abilities to localize to the mitochondria and inhibit the electron transport chain and activate AMPK and suppress mTOR activity (Wang et al., 2010). The PI3K/PTEN/AKT/mTORC1 and RAF/MEK/ERK pathways are inhibited when AMPK is activated.

1.4. BBR can alter gene expression through DNA binding

Some of the more intriguing effects of BBR and modified BBRs may be due to their interactions with nucleic acids. BBR may bind DNA and RNA via the nitrogen atom at the 7-position in the alkaloid BBR skeleton. Different modified BBRs may have distinct interactions with nucleic acids which confer diverse effects on gene expression. Interactions between BBRs and nucleic acids may inhibit telomerases and topoisomerases as well as gene transcript levels by interacting with the TATA-binding protein and TATA-box present in certain gene promoters (Gatto et al., 1996; Qin et al., 2007; Bhowmik et al., 2012a Xiao et al., 2012). Thus, different modified BBRs might have diverse effects on gene expression and cellular growth which could be exploited commercially.

In addition, BBRs may induce double strand DNA breaks and cell cycle arrest (Wang et al., 2012). BBRs can also influence gene expression by inhibiting histone deacetylase (HDAC) activity. BBR repressed total HDAC and class I, II and IV HDAC activity by hyperacetylation of histones in A549 lung cancer cells. This resulted in decreases in prostaglandin-endoperoxide synthase 2 (COX2),

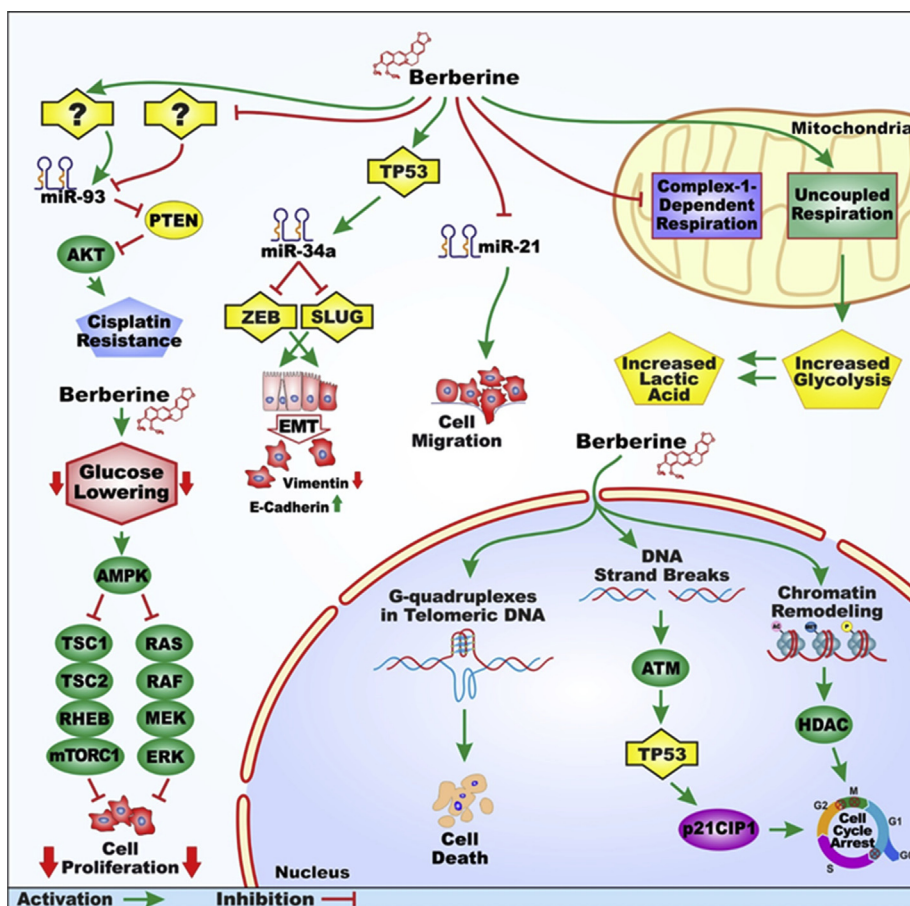


Fig. 2. **Effects of Berberine on Metabolism, Gene Expression, Induction of miRs, Cellular Proliferation and Metastasis.** Berberine has effects on many cellular processes including: metabolism, regulation of gene expression by interaction with DNA and chromatin in the nucleus and signaling pathways. Various miRs are induced by BBR. The mechanisms by which they may affect certain signal transduction and apoptotic pathways which are important in the regulation of cellular growth, malignant transformation, invasion, and drug resistance are depicted briefly in this figure.

matrix metalloproteinase (MMP2), MMP9 and tumor necrosis factor- α (TNF- α) expression and increases in TP53 and p21^{Cip-1} levels. BBR was determined to regulate apoptotic activity (Kalaiarasi et al., 2016). In this system, BBR could have both direct and indirect effects on DNA by altering chromatin structure and TP53 expression. Thus, there are important interactions between BBR and TP53.

1.5. BBR effects on cisplatin-resistance

Cisplatin normally functions by the induction of DNA strand breaks. BBR can sensitize breast cancer cells to cisplatin and inhibit growth, however, in some cases cisplatin treatment is ineffective due to the development of drug-resistance. BBR may increase the effectiveness of cisplatin in inducing DNA strand breaks by augmenting the activation of caspase-3, caspase-9 and apoptosis (Zhao et al., 2016).

In the cisplatin-sensitive OV2008 cell line and in the resistant C13* subline, BBR differently affected cell growth of sensitive and resistant cells. BBR suppressed the growth of cisplatin-resistant cells more than the sensitive counterparts. This was demonstrated to occur by altered expression of genes encoding folate cycle enzymes, such as dihydrofolate reductase (DHFR) and thymidylate synthase (TS). BBR also affected polyamine metabolism. This occurred by upregulation of the key catabolic enzyme, spermidine/spermine N1-acetyltransferase (SSAT). BBR stimulated SSAT induction by the spermine analogue N1, N12 bisethyl-spermine (BESpm). BESpm downregulated DHFR mRNA more than TS mRNA. BBR treatment reversed the resistance of the cells to cisplatin and BESpm (Marverti et al., 2013).

1.6. BBR effects on signaling pathways implicated in drug resistance

Combining BBR with signal transduction pathway inhibitors can inhibit the drug resistance of certain cancers. BBR can interact with the epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor (HER2) inhibitor lapatinib and suppress the resistance of HER2+ breast cancer (Zhang et al., 2016). In lapatinib-resistant cells, lapatinib induces c-MYC/pro-NRF2 and GSK-3 β signaling pathways. This results in stabilization of NRF2. In contrast, BBR treatment prevents some aspects of the growth promoting effects of lapatinib such as the induction of c-MYC expression. The combination of BBR and lapatinib decreased c-MYC expression, lapatinib-resistance and cell growth.

BBR inhibited the NF- κ B and the STAT3 pathways in cholangiocarcinoma. This induced cell cycle arrest at the G₁ phase which suppressed cancer cell growth (Puthdee et al., 2017). Reduced CYCLIN D1 and CYCLIN E levels and increased levels of activated STAT3 and ERK1/2 were observed.

BBR also inhibits EGFR signaling and increased the effects of the EGFR inhibitors erlotinib and cetuximab in gastric cancer cells, both in *in vitro* and *in vivo* xenograft studies. These results are important as cetuximab plus chemotherapy has been examined in phase 2 clinical trials, however, the combination alone did not provide enough benefit for justification of performing phase 3 clinical trials. BBR was determined to inhibit activation of EGFR in tumors and the combination of BBR and cetuximab was more effective than treatment with either agent by itself. Part of the biochemical mechanism for growth inhibition resulted from decreased phosphorylation of STAT3 as well as decreased expression of BCLXL and cyclin D1 (Wang et al., 2016).

The PI3K/PTEN/AKT/mTORC1/GSK-3 signaling pathway is often regulated by nutraceuticals such as BBR. This pathway plays critical roles in: diabetes, cardiovascular diseases, inflammation, neuropathology, obesity, as well as cancer (McCubrey et al., 2017; Candido et al., 2018; Duda et al., 2018; McCubrey et al., 2018).

BBR affected AKT expression and its effects on EMT (Kou et al., 2016) by suppressing the migratory and invasive abilities of B16 melanoma cells. BBR altered the pluripotency of EMT-associated factors. BBR treatment suppressed phosphorylation of PI3K and AKT and downregulated retinoic acid receptor alpha (RARalpha) while increasing RARbeta and RARgamma expression. The PI3K inhibitor, LY294002, which will suppress AKT activity, had similar effects as BBR on most of the examined biochemical properties, except for RARgamma regulation. Importantly, BBR could reverse EMT in B16 melanoma cells.

BBRs also has effects on the RAF/MEK/ERK signaling pathway. BBR inhibited the proliferation of MGC 803 gastric cancer cells *in vitro* and *in vivo* xenograft studies in mice (Li et al., 2016). BBR was determined to inhibit interleukin-8 (IL-8) expression. BBR affected p38^{MAPK}, ERK and c-Jun N-terminal kinase (JNK) expression.

BBR altered the activity of the hedgehog (Hh) signaling pathway in medulloblastoma cells. Some medulloblastoma cells may exhibit growth addiction to the Hh signaling as their proliferation is dependent on activation of this pathway. BBR inhibited the Hh pathway in these cells by suppressing SMO activity. BBR was determined to inhibit Hh-dependent growth both *in vitro* and *in vivo* (Wang et al., 2015).

1.7. BBR effects on miRs

BBR affected the cisplatin-sensitivity of A2780 ovarian cancer cells. The miR-93/PTEN/AKT signaling pathway was determined to be important in the induction of apoptosis and G₀/G₁ arrest. BBR treatment resulted in decreased levels of miR-93 cisplatin-resistant cells which resulted in their conversion to cisplatin-sensitive cells. In the absence of miR-93, PTEN tumor suppressor activity reappeared and inhibited AKT activity and apoptosis occurred. These studies established a regulatory link between miR-93, PTEN and AKT which could be modified by BBR treatment. BBR treatment may also eliminate the effects that miR-93 has on PTEN and relieve cisplatin-resistance in ovarian cancer cells (Chen et al., 2015).

BBR treatment an also relieve the cisplatin-resistance of gastric cancer cells by upregulation of miR-203 and suppression of the

anti-apoptotic protein BCLW. miR-203 can bind mRNAs encoding BCLW and inhibit their expression and induce apoptosis (You et al., 2016). BBR can inhibit drug transporter expression in MCF-7 breast cancer cells. The expression of drug transporters is associated with both drug resistance and cancer stem cells (Kim et al., 2008).

BBR can also relieve the cisplatin-resistance of ovarian cancer cells by inhibiting the expression of miR-21. This occurred in part by loss of inhibition of the programmed cell death 4 (PDCD4) tumor suppressor gene (Liu et al., 2013). Previously it had been shown that in glioblastoma cells, miR-21 effected negatively the expression of matrix metalloproteinase (MMP) inhibitors, namely reversion inducing cysteine rich protein with kazal motifs (RECK) and tissue inhibitor of metalloproteinases (TIMP) (Gabriely et al., 2008). This can affect MMP9 expression and invasion.

BBR also suppressed miR-21 expression in HCT116 CRC cells (Lü et al., 2018). This resulted in decreased cancer cell viability and increased apoptosis. BBR also increased integrin subunit beta 4 (ITG β 4) and PDCD4 expression in these cells. BBR can also suppress miR-21 expression in glioma cells (Luo et al., 2017). miR-21 can promote invasion and migration in glioma cells by activation of SOX2 and β -catenin signaling.

Thus, metformin and BBR can relieve the drug resistance of cancer cells by various mechanisms. This is a rapidly emerging field and we have described pathways regulated by metformin and BBR. The regulation of miR expression by metformin and BBR may play critical roles in relieving chemotherapeutic drug resistance.

1.8. Chemically-modified BBRs—Enhancing effects of BBR by medicinal chemistry

We have recently reviewed various modified BBR compounds that have been developed (McCubrey et al., 2017; Abrams et al., 2018; Abrams et al., 2019). In this current manuscript, we will summarize some novel advances in the field. Various medicinal chemical approaches have been made to design more active BBRs and other nutraceuticals (Bhowmik et al., 2012b; Guamán Ortiz et al., 2014; Bhowmik and Kumar, 2016). NO-donating proto-BBRs have been developed which display more potent activity against certain tumor cells such as HepG2 hepatocellular carcinoma cells (Chen et al., 2017). These compounds release NO and the anti-tumor effects of certain compounds could be inhibited by a NO scavenger. Compound 15a developed by this group exhibited a broad anti-tumor efficacy and was selective against hepatocellular tumor cells as opposed to normal liver LO-2 cells. Compound 15a was determined to block HepG2 cells in the G2 phase of the cell cycle and induce apoptosis.

A panel of position-13 substituted BBRs was developed by Naxospharma in Milan Italy (Bhowmik et al., 2012b; Tillhon et al., 2012; Pierpaoli et al., 2013; Guaman Ortiz et al., 2014a; Guaman Ortiz et al., 2014b; Guaman Ortiz et al., 2015; Pierpaoli et al., 2015; Pierpaoli et al., 2018). These compounds exhibit enhanced activity in comparison to BBR against various cancer cells including colon

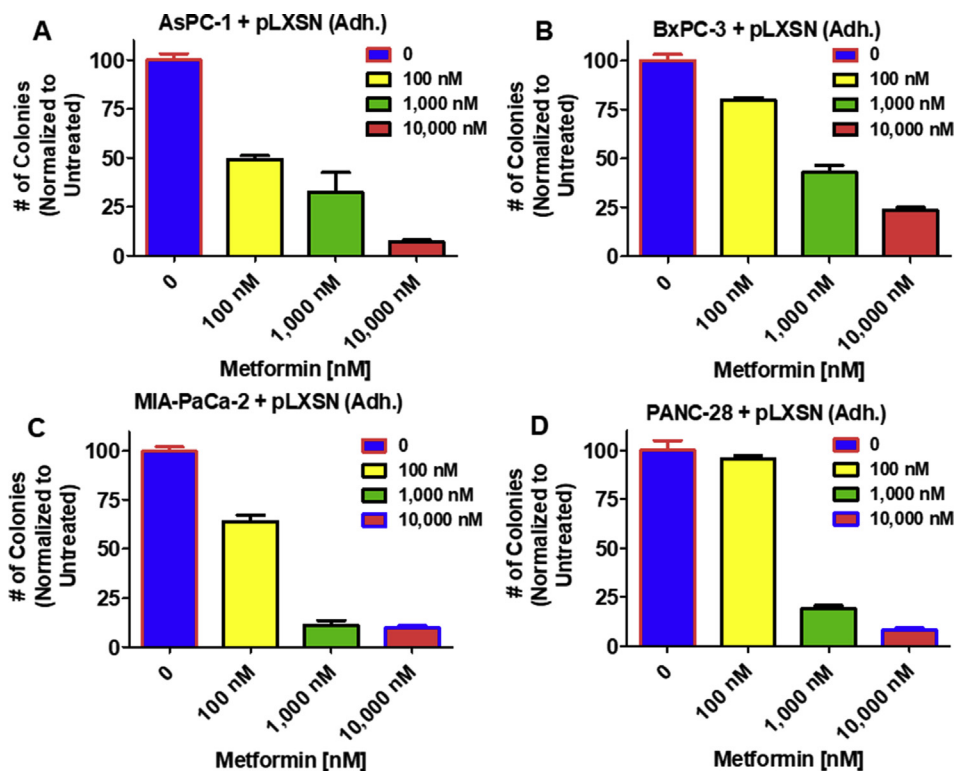


Fig. 3. Effects of Metformin on Colony Formation in Four PDAC Cell Lines. The effects of different concentrations of metformin on the colony forming ability of: AsPC-1 + pLXSN (Adh.) (Panel A), BxPC-3 + pLXSN (Adh.) (Panel B), MIA-PaCa-2 + pLXSN (Adh.) (Panel C) and PANC-28 + pLXSN (Adh.) (Panel D) cells are presented. Colonies were normalized with each drug-treated cell line to the control no drug treated plates.

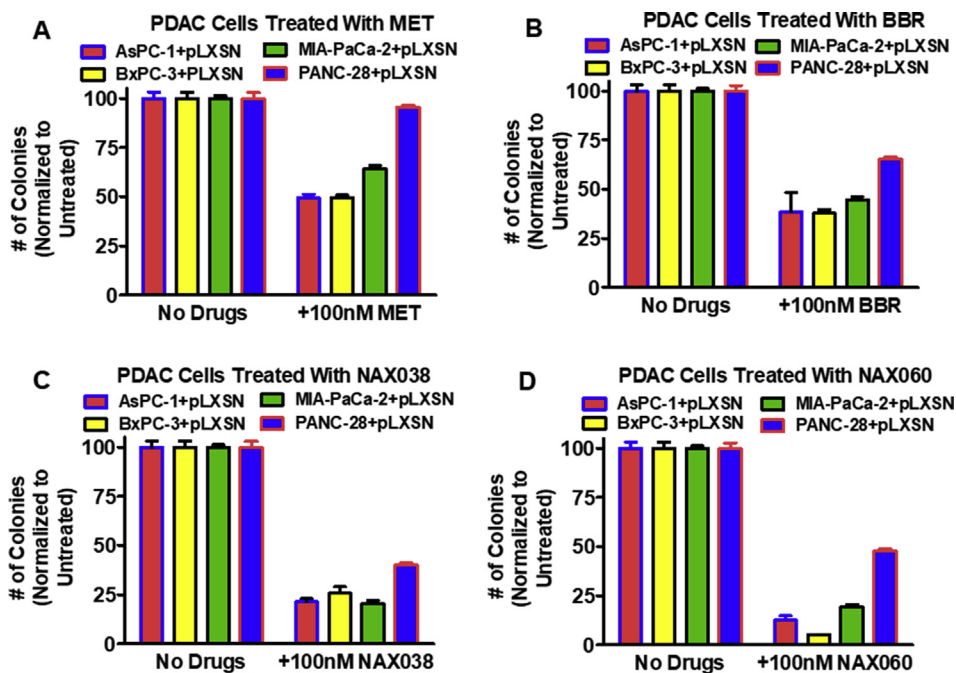


Fig. 4. Effects of 100 nM Dose of Metformin, BBR, NAX038 and NAX060 on Colony Formation in Four PDAC Cell Lines. The effects of no drugs (all panels) or 100 nM metformin (MET) (Panel A), 100 nM BBR (Panel B), 100 nM NAX038 (Panel C) and 100 nM NAX060 (Panel D) on the colony forming ability of: AsPC-1 + pLXSN (Adh.) (red bars), BxPC-3 + pLXSN (Adh.) (yellow bars), MIA-PaCa-2 + pLXSN (Adh.) (green bars) and PANC-28 + pLXSN (Adh.) (blue bars) cells are presented. Colonies were normalized with each drug-treated cell line respectively, to the control no drug-treated plates.

and breast cancer (Guaman Ortiz et al., 2015; Pierpaoli et al., 2015; Pierpaoli et al., 2018).

BBR has a condensed polyaromatic buckled structure due to partial saturation in one of the rings and provides an attractive natural lead for the introduction of various chemical modifications. In this respect, the BBR derivatives (NAX compounds) developed by Naxospharma (Milan, Italy) bear (un)substituted(hetero)aromatic moieties bonded to position 13 of the parent alkaloid skeleton through a hydrocarbon linker of variable length, in a fashion to possibly create a geometric propensity for additional stacking-type, non-covalent aromatic interactions with cellular targets. Aromatic interactions are ubiquitous in nature and their geometry plays a central role in the molecular interaction with biological macromolecules. The 13-phenylalkyl and 13-diphenylalkyl BBR derivative NAX compounds were determined to result in telomeric DNA G-quadruplexes. The NAX042 and NAX053 compounds were examined in terms of telomeric G-quadruplexes in this system (Ferraroni et al., 2016). NAX042 and NAX053 were the best compounds in terms of simulating the interaction with human telomeric DNA G4 structures.

In the following studies, we have investigated the abilities of NAX compounds to interact with metformin and inhibit the growth of PDAC cell lines. We have investigated the effects of the introduction of one phenyl moiety (NAX012, NAX042), two phenyl moieties (benzhydryl) (NAX035, NAX053) and one heterocycle (pyridine) (NAX075, NAX077). In the monophenyl series, we investigated also the effects of single or multiple electron-releasing (NAX038, NAX054) or electron-withdrawing (NAX014, NAX060, NAX111) substituents. The effects of two different lengths of the hydrocarbon linker were also examined. Some of the NAX compounds could interact with metformin and inhibit proliferation better than others. The presence of WT-TP53 was determined to influence the electiveness of certain NAX compounds upon co-treatment with metformin.

2. Materials and methods

2.1. Cell lines and tissue culture

The MIA-PaCa-2 PDAC (ATCC[®] CRM-CRL-1420[™]) was recovered from a 65-year old Caucasian male PDAC patient (Deer et al., 2010). These cells were obtained from the ATCC (Rockville, MD, USA). The MDA-PANC-28 cell line (CVCL_3917) was isolated from a female patient (Frazier et al., 1996) and is often abbreviated PANC-28. PANC-28 cells were generously provided by Dr. Shrikanth A. G. Reddy, (MD Anderson Cancer Center, Houston, TX USA), where the PANC-28 cell line was isolated originally. The AsPC-1 PDAC cell line (ATCC[®] CRL-1682[™]) was recovered from a 62-year old female PDAC patient (Deer et al., 2010). BxPC-3 cells were derived from a 61-year old PDAC patient (ATCC[®] CRL-1687[™]). BxPC-3 cells have mutant *TP53* but WT *KRAS* (Deer et al., 2010).

Cells were cultured in Dulbecco's modified Eagles medium, (DMEM), (Invitrogen, Carlsbad, CA), antibiotics containing L-glutamine medium supplemented with 5% fetal bovine serum (FBS) purchased from Atlanta Biologicals, Atlanta, GA, USA) as described in

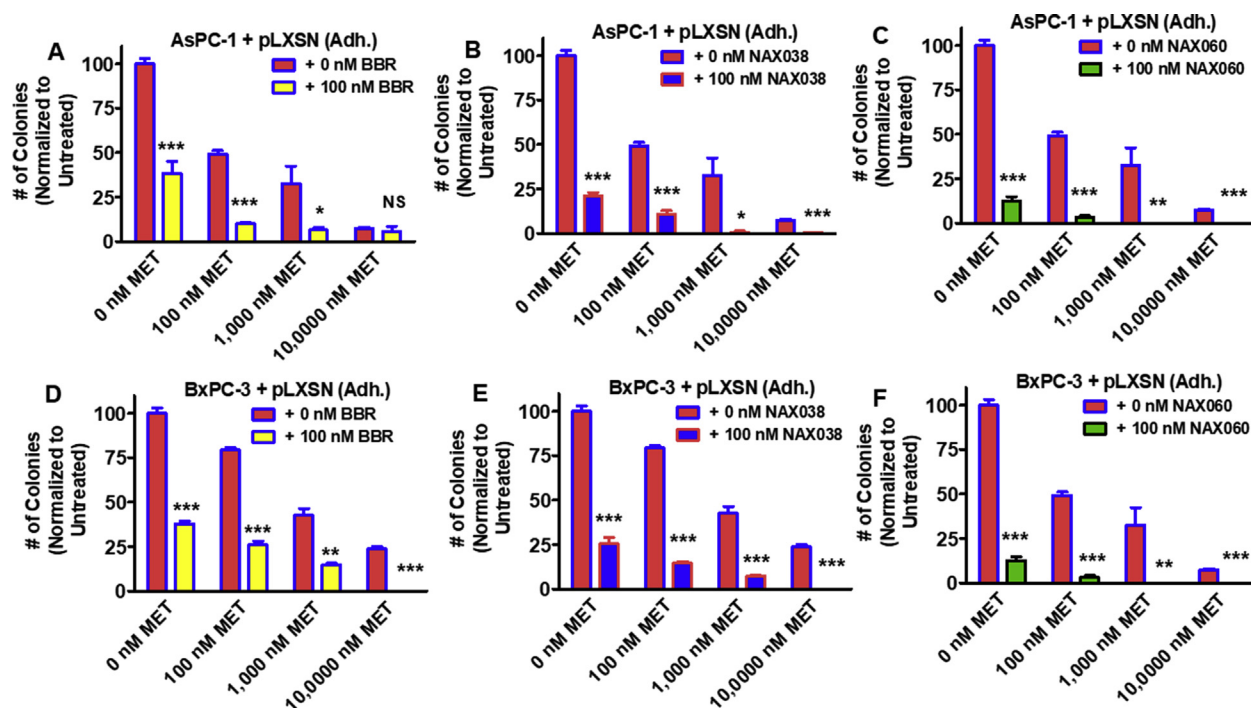


Fig. 5. Effects of Different Doses of Metformin in the Presence of No Drugs, 100 nM Berberine, 100 nM NAX038 or 100 nM NAX060 on Colony Formation in AsPC-1 and BxPC-3 PDAC Cells. The effects of either no BBR or 100 nM BBR on the colony formation in the presence of different doses of metformin (MET) in AsPC-1 + pLXSN (Adh.) (Panel A) and BxPC-3 + pLXSN (Adh.) (Panel D) cells were determined. The effects of either no NAX038 or 100 nM NAX038 on the colony formation in the presence of different doses of metformin (MET) in AsPC-1 + pLXSN (Adh.) (Panel B) and BxPC-3 + pLXSN (Adh.) (Panel E) were determined. The effects of either no NAX060 or 100 nM NAX060 on the colony formation in the presence of different doses of metformin (MET) in AsPC-1 + pLXSN (Adh.) (Panel C) and BxPC-3 + pLXSN (Adh.) (Panel F) cells were determined. Different treated cell lines were normalized to untreated respective cell line. Statistical analyses were performed in comparison with BBR, NAX038 or NAX060 treated cells vs. metformin-alone treated cells where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, ** $P < 0.005$, * $P < 0.05$, NS = not statistically significant.

(Abrams et al., 2018a; Abrams et al., 2018b). NAX compounds were synthesized, purified and provided by our collaborator and co-author, Dr. Paolo Lombardi (Naxospharma, Milan, Italy) (Guamán Ortiz et al., 2014, Guamán Ortiz et al., 2015; Pierpaoli et al., 2013, 2015, 2018). BBR was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Infection of cells with a retroviral vector encoding WT-TP53

PDAC cell lines were infected with either a retroviral vector encoding WT-TP53 or the empty pLXSN vector as a control as described (Abrams et al., 2018, 2019). Stably infected cell lines were isolated in the presence of 2 mg/ml G418 (geneticin) Sigma-Aldrich. Pools were established after approximately four weeks in culture (Abrams et al., 2018, 2019).

2.3. Methylthiazole tetrazolium assays

Methylthiazole tetrazolium (MTT) assays were performed to determine the sensitivity of the pancreatic cancer cells to the modified NAX compounds and berberine as described (Abrams et al., 2018, 2019).

To test the hypothesis that the various IC_{50} s in the PDAC cells treated with the NAX compounds were statistically different than in the same cells treated the same day with the parental BBR, student's T tests were performed using Graph Pad Prism (QuickCals) statistical analysis.

2.4. Colony formation assays

Cells were seeded at 500 cells per well into three wells of six well Falcon plates (Falcon/Corning, Durham, NC, USA). Cells were treated with the indicated concentrations of chemotherapeutic drugs, metformin, BBR or NAX compounds. Colonies were propagated for approximately two weeks. When the colony size was approximately forty to 100 cells, the number of colonies were determined after giemsa staining of the colonies in the wells. The number of colonies were determined in three wells per condition and the average and standard deviation determined. The number of colonies in each condition were normalized to the untreated samples.

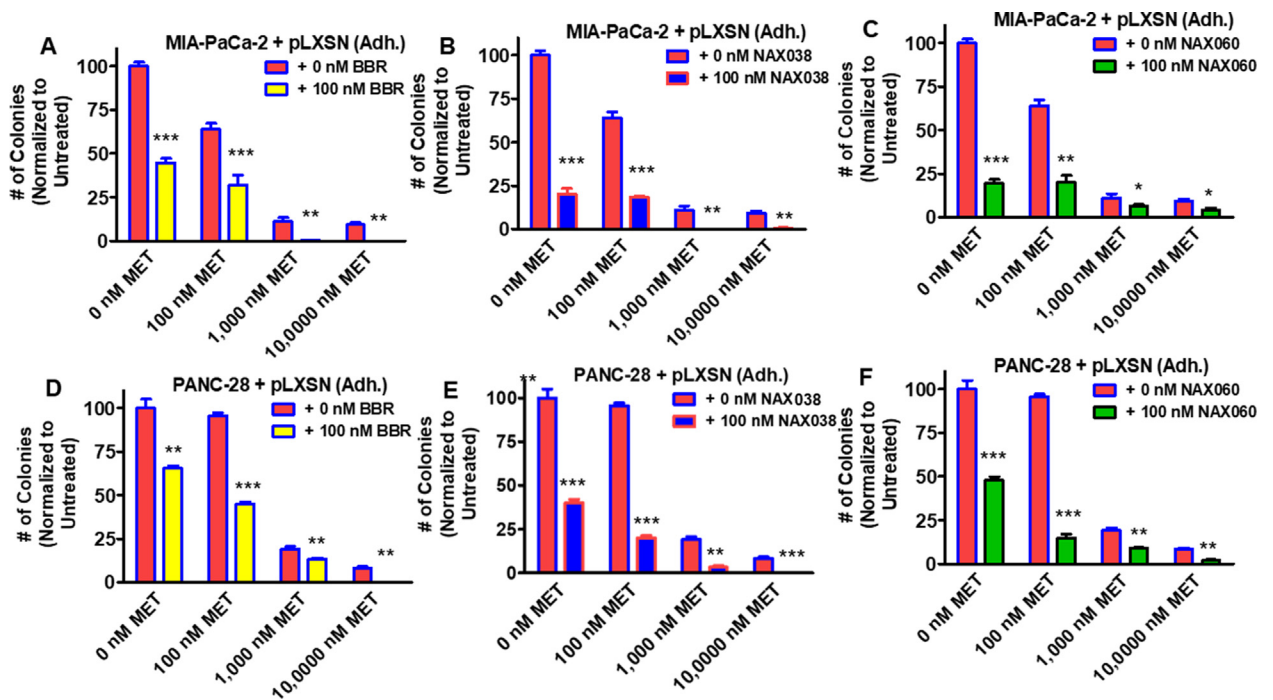


Fig. 6. Effects of Different Doses of Metformin in the Presence of No Drug, 100 nM Berberine, 100 nM NAX038 or 100 nM NAX060 on Colony Formation in MIA-PaCa-2 and PANC-28 PDAC Cells. The effects of either no BBR or 100 nM BBR on the colony formation in the presence of different doses of metformin (MET) in MIA-PaCa-2 + pLXSN (Adh.) (Panel A) and PANC-28 + pLXSN (Adh.) (Panel D) were determined. The effects of either no NAX038 or 100 nM NAX038 on the colony formation in the presence of different doses of metformin (MET) in MIA-PaCa-2 + pLXSN (Adh.) (Panel B) and PANC-28 + pLXSN (Adh.) (Panel E) were determined. The effects of either no NAX060 or 100 nM NAX060 on the colony formation in the presence of different doses of metformin (MET) in MIA-PaCa-2 + pLXSN (Adh.) (Panel C) and PANC-28 + pLXSN (Adh.) (Panel F) were determined. Different treated cell lines were normalized to untreated respective cell line. Statistical analyses were performed in comparison with BBR, NAX038 or NAX060 treated cells vs. metformin-alone treated cells where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, ** $P < 0.005$, * $P < 0.05$, NS = not statistically significant.

Statistics were performed as described above.

3. Results

3.1. Effects of metformin on PDAC colony formation

The effects of different doses of metformin on the colony formation of AsPC-1 + pLXSN (Panel A), BxPC-3 + pLXSN (Panel B), MIA-PaCa-2 + pLXSN (Panel C) and PANC-28 + pLXSN (Panel D) are presented in Fig. 3. These are four different PDAC cell lines. In these experiments, adherent (Adh.) cells were used. These cells had been infected with the pLXSN retrovirus as a control for retroviral infection as experiments presented in Figs. 9–15 and other studies were performed with cells infected with a WT-TP53 virus (Abrams et al., 2018, 2019). Dose-dependent effects of metformin were observed. In general, AsPC-1 and MIA-PaCa-2 cells were more sensitive to metformin than either BxPC-3 or PANC-28 cells, however, the growth of all four PDAC lines was suppressed by over 50% when 1000 nM and greater concentrations of metformin were used.

3.2. Effects of low doses (100 nM) of metformin, BBR and NAX compounds on colony formation in PDAC cells

For comparison purposes, a diagram of the effects of: 100 nM metformin, 100 nM BBR, 100 nM NAX038 and 100 nM NAX060 on the four different PDAC cell lines is present in Fig. 4. The PANC-28 cell line was the most resistant to 100 nM metformin (Panel A). All four cell lines were more sensitive to either 100 nM NAX038 or 100 nM NAX060 (Panels C and D) than either 100 nM metformin or 100 nM BBR (Panels A and B).

3.3. Effects of BBR, NAX038 and NAX060 on different doses of metformin on colony formation in four PDAC cell lines

Figs. 5 and 6 document the effects of addition of different concentrations of metformin on the colony formation of AsPC1 + pLXSN (Adh.) (Fig. 5, Panels A–C), BxPC-3 + pLXSN (Adh.) (Fig. 5, Panels D–F), MIA-PaCa-2 + pLXSN (Adh.) (Fig. 6, Panel A–C) and PANC-28 + pLXSN (Adh.) (Fig. 6, Panels D–F). As presented previously, metformin inhibited colony formation in a dose-

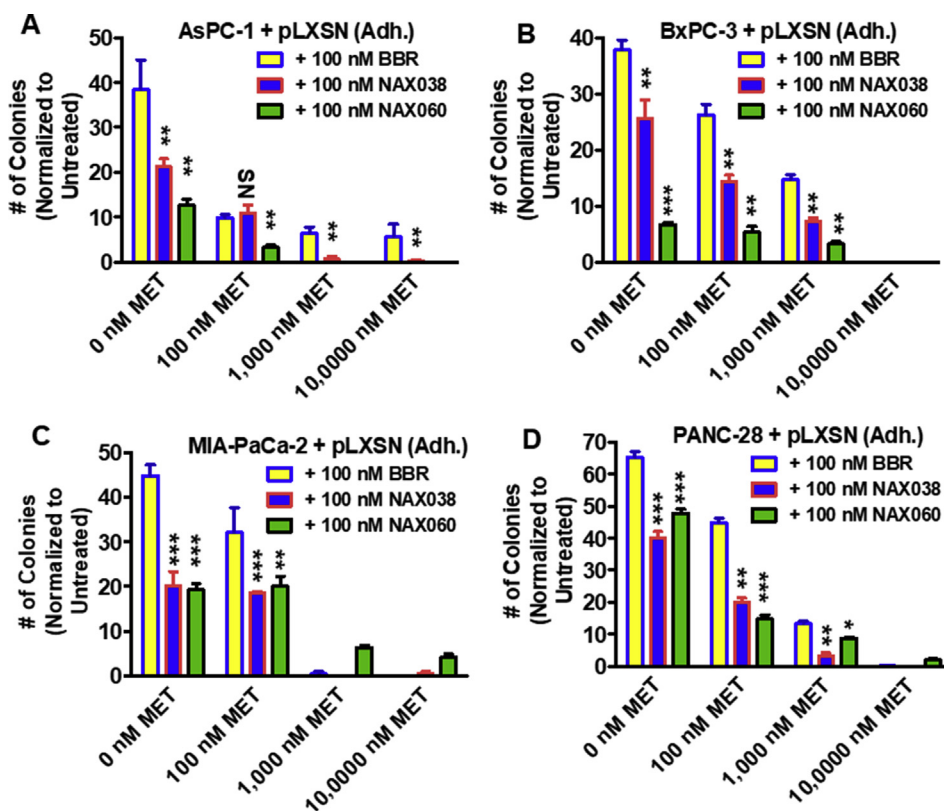


Fig. 7. Effects of 100 nM Dose of BBR, NAX038 and NAX060 on Colony Formation in the Presence of Different Concentrations of Metformin in Four PDAC Cell Lines. The effects of 100 nM BBR (yellow bars) 100 nM NAX038 (blue bars) and 100 nM NAX060 (green bars) on the colony forming ability of: AsPC-1 + pLXSN (Adh.) (Panel A), BxPC-3 + pLXSN (Adh.) (Panel B), MIA-PaCa-2 + pLXSN (Adh.) (Panel C) and PANC-28 + pLXSN (Adh.) (Panel D) are presented. Colonies were normalized with each drug treated cell line to the control no drug treated plates. Statistical analyses were performed in relationship to metformin and NAX038 or NAX060 vs BBR treated where appropriate by the Student T test on the means and standard deviations of various treatment groups. *** = $P < 0.0001$, ** $P < 0.005$, * $P < 0.05$, NS = not statistically significant.

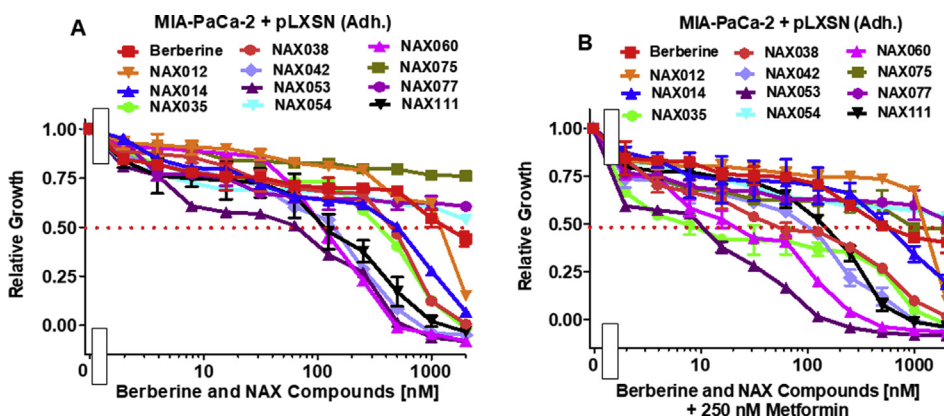


Fig. 8. Effects of the Nutraceuticals Berberine And NAX Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) Cells Cultured in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells cultured with BBR or the different NAX compounds. Panel B) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells exposed to the different NAX compounds in the presence of 250 nM metformin. All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

dependent fashion. Addition of 100 nM BBR augmented the effects of metformin-mediated inhibition of colony formation in AsPC-1 + pLXSN (Adh.) (Fig. 5, Panel A), BxPC-3 + pLXSN (Adh.) (Fig. 5, Panel D), MIA-PaCa-2 + pLXSN (Adh.) (Fig. 6, Panel A) and PANC-28 + pLXSN (Adh.) (Fig. 6, Panel D).

The effects of 100 nM NAX038 were also examined in combination with metformin. Addition of 100 nM NAX038 augmented the

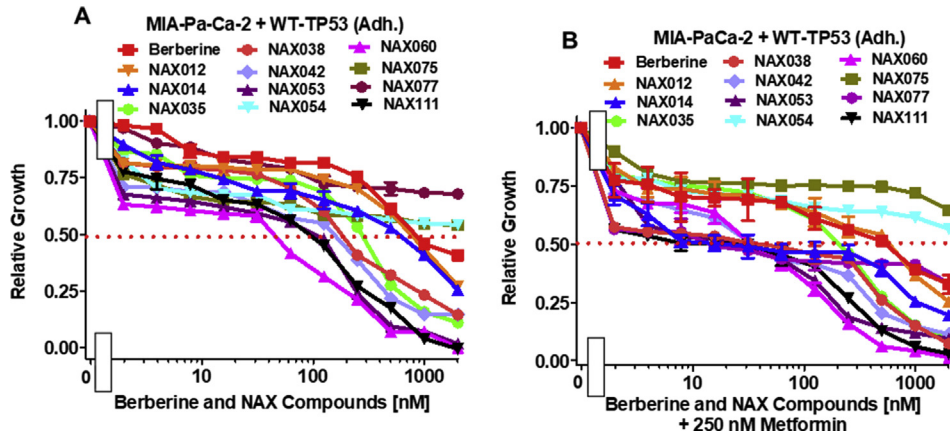


Fig. 9. Effects of the Nutraceuticals Berberine And NAX Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + WT-TP53 (Adh.) Cells Cultured in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells cultured with BBR or the different NAX compounds. Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells exposed to the different NAX compounds in the presence of 250 nM metformin. All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

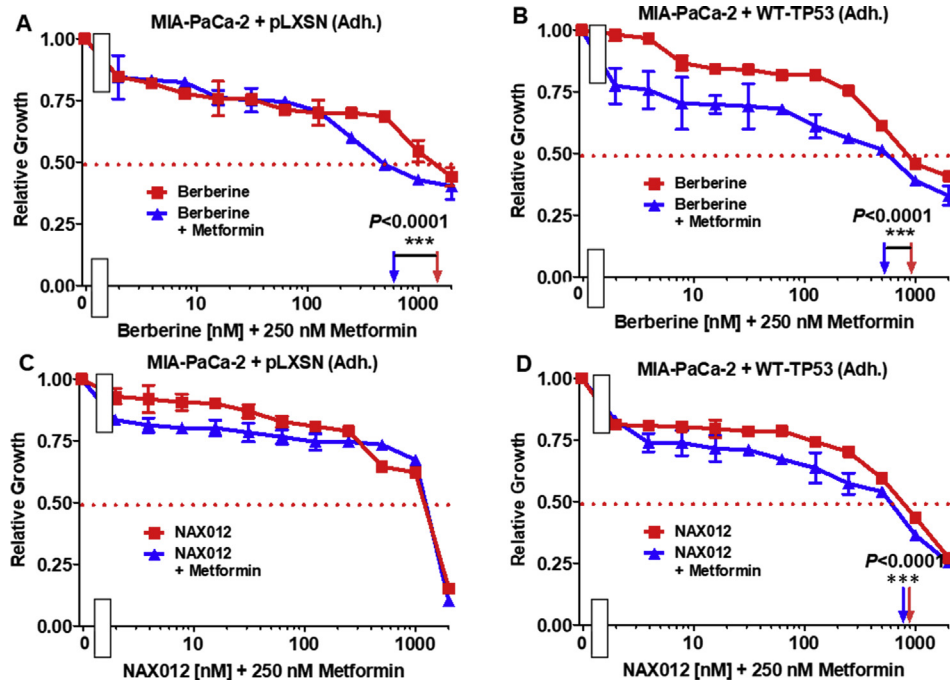


Fig. 10. Effects of the Nutraceutical Berberine and the NAX012 Compound on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of berberine (solid red squares) or different concentrations of berberine and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of berberine (solid red squares) or different concentrations of berberine and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX012 (solid red squares) or different concentrations of NAX012 and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX012 (solid red squares) or different concentrations of NAX012 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

effects of metformin-mediated inhibition of colony formation in AsPC-1 + pLXSN (Adh.) (Fig. 5, Panel B), BxPC-3 + pLXSN (Adh.) (Fig. 5, Panel E), MIA-PaCa-2 + pLXSN (Adh.) (Fig. 6, Panel B) and PANC-28 + pLXSN (Adh.) (Fig. 6, Panel E).

The effects of 100 nM NAX060 were also examined in combination with metformin. Addition of 100 nM NAX060 augmented the effects of metformin-mediated inhibition of colony formation in AsPC-1 + pLXSN (Adh.) (Fig. 5, Panel C), BxPC-3 + pLXSN (Adh.) (Fig. 5, Panel F), MIA-PaCa-2 + pLXSN (Adh.) (Fig. 6, Panel C) and PANC-28 + pLXSN (Adh.) (Fig. 6, Panel F). Addition of 100 nM

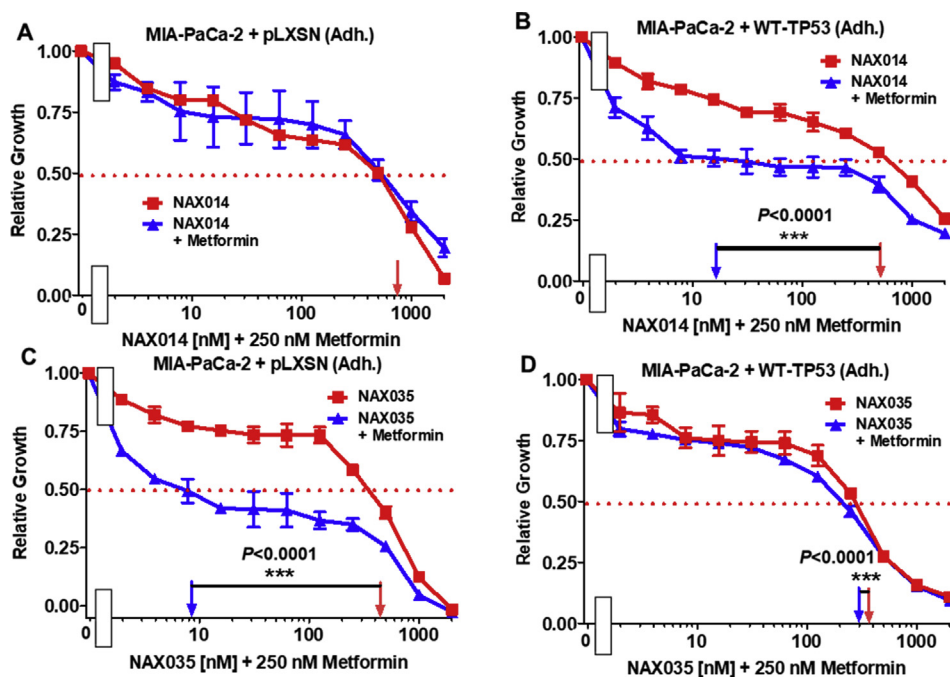


Fig. 11. Effects of the NAX014 and NAX035 Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX014 (solid red squares) or different concentrations of NAX014 and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX014 (solid red squares) or different concentrations of NAX014 and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX035 (solid red squares) or different concentrations of NAX012 and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX035 (solid red squares) or different concentrations of NAX035 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

BBR, NAX038 and NAX060 augmented the ability of metformin to inhibit colony formation in AsPC-1 + pLXSN (Adh.) (Fig. 5, Panel C), BxPC-3 + pLXSN (Adh.) (Fig. 5, Panel F), MIA-PaCa-2 + pLXSN (Adh.) (Fig. 6, Panel C) and PANC-28 + pLXSN (Adh.) cells (Panel 6, Panel F).

3.4. Effects of combination of metformin and 100 nM BBR, NAX038 or NAX060

A more condensed depiction of the effects of BBR, NAX038 and NAX060 on different concentrations of metformin is presented in Fig. 7. Addition of 100 nM BBR, NAX038 or NAX060 reduced the concentration of metformin required for growth inhibition in the four different cell lines. In general, NAX038 and NAX060 suppressed proliferation in AsPC-1 + pLXSN (Adh.) and BxPC-3 + pLXSN (Adh.) in combination with metformin more than BBR (Fig. 7, Panels A and B). 100 nM NAX038 and NAX060 suppressed colony formation more than BBR in MIA-PaCa-2 + pLXSN (Adh.) when the cells were treated 0 and 100 nM metformin (Fig. 7, Panel C). 100 nM NAX038 and NAX060 suppressed colony formation more than BBR when the PANC-28 + pLXSN (Adh.) were treated with 0, 100 and 1000 nM metformin (Fig. 7, Panel D).

3.5. Titrations of MIA-PaCa-2 + pLXSN and MIA-PaCa-2 + WT-TP53 cells with berberine, NAX compounds and a Sub-IC₅₀ dose of 250 nM metformin

The effects of titrating MIA-PaCa-2 + pLXSN and MIA-PaCa-2 + WT-TP53 cells with different concentration of BBR, NAX compounds either by themselves (Figs. 8 and 9, A Panels) or also with 250 nM metformin (Figs. 8 and 9, B Panels) were determined by MTT analysis. In addition, the effects of the BBR and NAX compounds were plotted separately (Figs. 10–15). The differences in the effects of the various NAX compounds in the presence and absence of 250 nM metformin are summarized in Table 1 and Table 2.

As observed in Fig. 10, Panel A, the IC₅₀ for BBR in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 1600 nM. Addition of 250 nM metformin resulted in a 2.7-fold decrease in the IC₅₀ to 600 nM. The IC₅₀ for BBR in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 900 nM. Addition of 250 nM metformin resulted in a 1.8-fold decrease in the IC₅₀ to 500 nM (Fig. 10, Panel B).

The IC₅₀ for NAX012 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 1300 nM Addition of 250 nM metformin did not result in a change in the IC₅₀ (Fig. 10, Panel C). The IC₅₀ for NAX012 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 900 nM. Addition of 250 nM metformin resulted in a 1.6-fold decrease in the IC₅₀ to 550 nM (Fig. 10, Panel D).

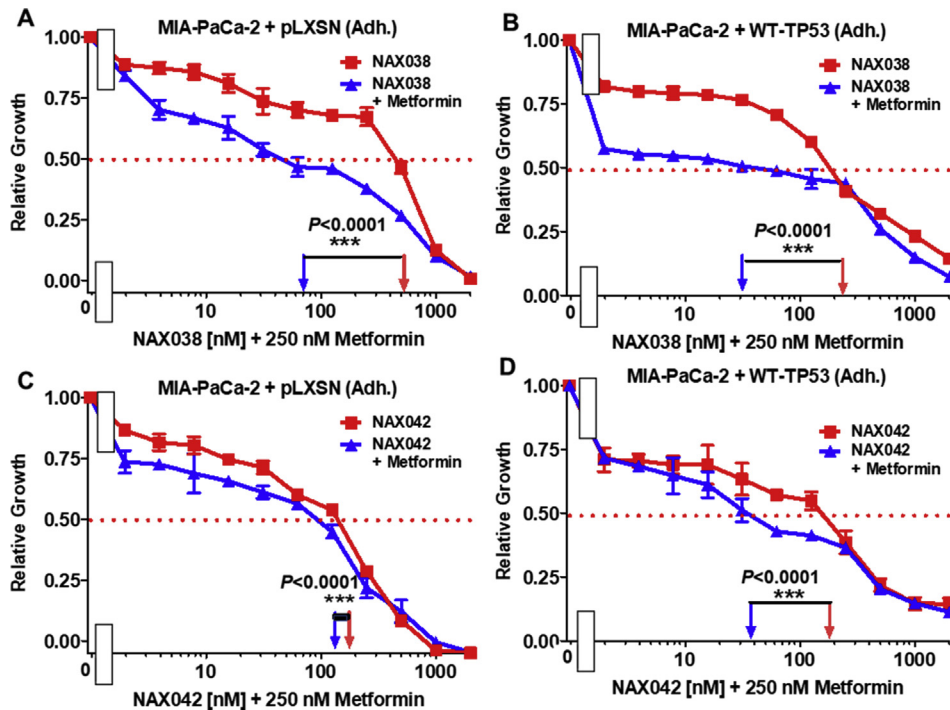


Fig. 12. Effects of the NAX038 and NAX042 Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX038 (solid red squares) or different concentrations of NAX038 and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX038 (solid red squares) or different concentrations of NAX038 and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX042 (solid red squares) or different concentrations of NAX042 and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX042 (solid red squares) or different concentrations of NAX042 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

The IC₅₀ for NAX014 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 750 nM. Addition of 250 nM metformin did not result in a change in the IC₅₀ (Fig. 11, Panel A). The IC₅₀ for NAX014 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 500 nM. Addition of 250 nM metformin resulted in a 33.3-fold decrease in the IC₅₀ to 15 nM (Fig. 11, Panel B).

The IC₅₀ for NAX035 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 450 nM. Addition of 250 nM metformin resulted in a 52.9-fold decrease in the IC₅₀ to 8.5 nM (Fig. 11, Panel C). The IC₅₀ for NAX035 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 400 nM. Addition of 250 nM metformin resulted in a 1.3-fold decrease in the IC₅₀ to 300 nM (Fig. 11, Panel D).

The IC₅₀ for NAX038 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 500 nM. Addition of 250 nM metformin resulted in a 6.4-fold decrease in the IC₅₀ to 70 nM (Fig. 12, Panel A). The IC₅₀ for NAX038 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 220 nM. Addition of 250 nM metformin resulted in a 7.3-fold decrease in the IC₅₀ to 300 nM (Fig. 12, Panel B).

The IC₅₀ for NAX042 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 180 nM. Addition of 250 nM metformin resulted in a 1.2-fold decrease in the IC₅₀ to 150 nM (Fig. 12, Panel C). The IC₅₀ for NAX042 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 200 nM. Addition of 250 nM metformin resulted in a 5-fold decrease in the IC₅₀ to 40 nM (Fig. 12, Panel D).

The IC₅₀ for NAX053 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 100 nM. Addition of 250 nM metformin resulted in a 6.7-fold decrease in the IC₅₀ to 15 nM (Fig. 13, Panel A). The IC₅₀ for NAX053 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 130 nM. Addition of 250 nM metformin resulted in a 3.3-fold decrease in the IC₅₀ to 40 nM (Fig. 13, Panel B).

The IC₅₀ for NAX054 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 2000 nM. Addition of 250 nM metformin did not result in a decrease in the IC₅₀ (Fig. 13, Panel C). The IC₅₀ for NAX054 in MIA-PaCa-2 + WT-TP53 (Adh) cells was > 2000 nM. Addition of 250 nM metformin resulted in a slight decrease in the IC₅₀ to 2000 nM (Fig. 13, Panel D).

The IC₅₀ for NAX060 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 180 nM. Addition of 250 nM metformin resulted in a 6.4-fold decrease in the IC₅₀ to 28 nM (Fig. 14, Panel A). The IC₅₀ for NAX060 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 60 nM. Addition of 250 nM metformin resulted in a 1.5-fold decrease in the IC₅₀ to 40 nM (Fig. 14, Panel B).

The IC₅₀ for NAX075 in MIA-PaCa-2 + pLXSN (Adh) cells was > 2000 nM. Addition of 250 nM metformin resulted in a > 2-fold decrease in the IC₅₀ to 1000 nM (Fig. 14, Panel C). The IC₅₀ for NAX075 in MIA-PaCa-2 + WT-TP53 (Adh) cells was not observed in the absence and presence of 250 nM (Fig. 14, Panel D).

The IC₅₀ for NAX077 in MIA-PaCa-2 + pLXSN (Adh) cells was > 2000 nM. Addition of 250 nM metformin resulted in a > 2-fold

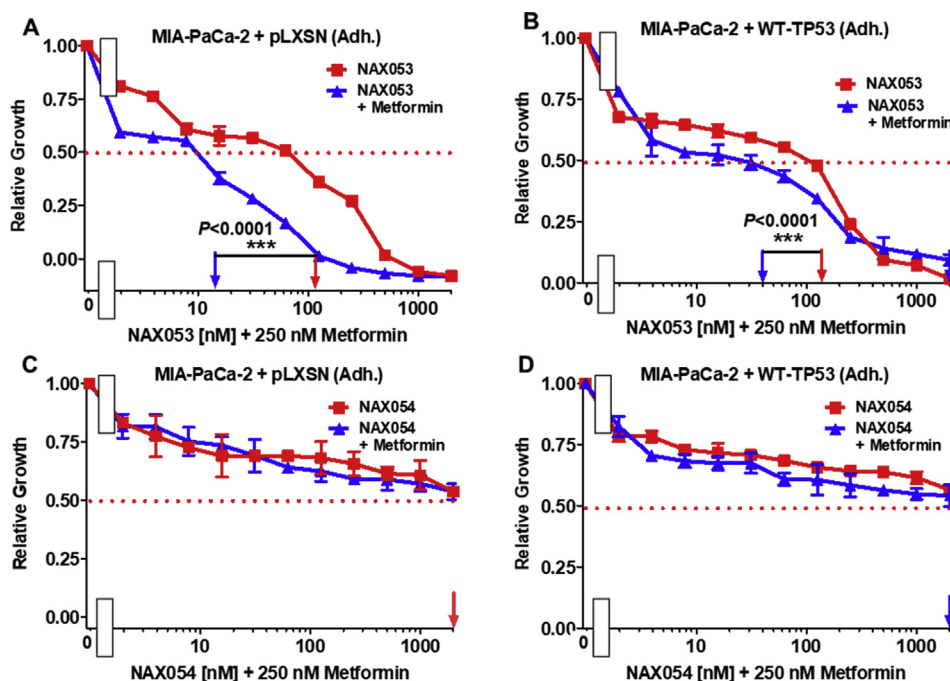


Fig. 13. Effects of the NAX053 and NAX054 Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX053 (solid red squares) or different concentrations of NAX053 and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX054 (solid red squares) or different concentrations of NAX053 and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX053 (solid red squares) or different concentrations of NAX054 and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX054 (solid red squares) or different concentrations of NAX054 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

decrease in the IC₅₀ to 1000 nM (Fig. 15, Panel A). The IC₅₀ for NAX077 in MIA-PaCa-2 + WT-TP53 (Adh) cells was not observed upon addition with up to 2000. However, upon addition of 250 nM metformin, an IC₅₀ of approximately 20 nM was observed, a decrease of > 100-fold. (Fig. 15, Panel B).

The IC₅₀ for NAX111 in MIA-PaCa-2 + pLXSN (Adh) cells was approximately 200 nM. Addition of 250 nM metformin did not result in a change in the IC₅₀ (Fig. 15, Panel C). The IC₅₀ for NAX11 in MIA-PaCa-2 + WT-TP53 (Adh) cells was approximately 150 nM. Addition of 250 nM metformin resulted in a 16.8-fold decrease in the IC₅₀ to 9 nM (Fig. 15, Panel D).

4. Discussion

Metformin is a commonly prescribed drug for the treatment of type-II diabetes. It is a glucose lowering drug which is given to diabetic patients in the absence of insulin treatment. Metformin effects the AMPK pathway which regulates TSC1/TSC2/RHEB/mTORC1 signaling and metabolism, energy, redox and biomass (Candido et al., 2018). When AMPK is activated the pro-proliferative RAS/MEK/ERK and PI3K/PTEN/AKT/mTORC1 pathways are suppressed.

miRs have important effects on mitochondria (Li et al., 2012). One of the key targets of metformin is the mitochondria where it can alter bioenergetics (Andrzejewski et al., 2014). Metformin can have divergent effects on miR expression. Some studies have indicated that metformin may induce the expression of certain miRs. Other investigations have indicated that metformin may suppress the expression of the same miR under different experimental conditions and divergent tissue types.

Metformin can have profound effects on mitochondrial dysfunction in breast cancer cells (Sharma & Kumar., 2018). In these studies, metformin influenced the migration and induction of apoptosis in breast cancer cell lines. Metformin also altered the expression of many genes including: BCL2, BAX, MMP2, MMP9, miR-21 and miR-155.

Metformin can also have effects on drug-resistant prostate cancer cells by targeting mitochondrial complex-1. This was demonstrated to impair the proliferation and invasiveness of the drug-resistant PC3-DR cells but not drug-sensitive PC3 cells. Decreased expression of miR-205 is associated with drug resistance and EMT in PC3-DR cells. Induced re-expression of miR-205 was demonstrated to shift mitochondrial oxidative phosphorylation to Warburg metabolism and resulted in increased sensitivity to docetaxel (Ippolito et al., 2016).

Metformin can induce mitochondrial-regulated apoptosis in oral cancer cells. Metformin treatment of the oral cancer cell line KB1

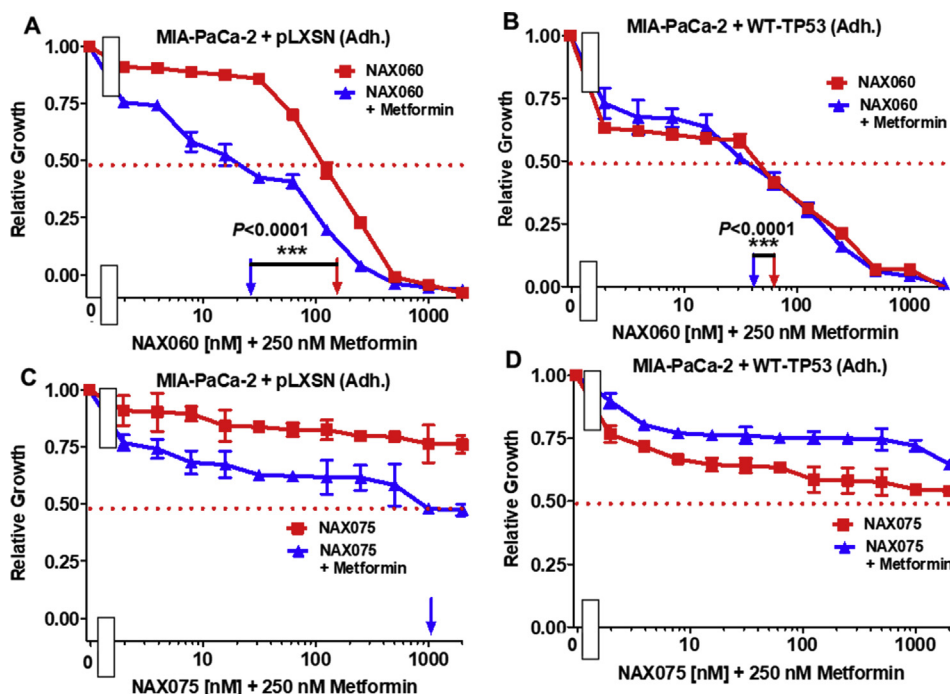


Fig. 14. Effects of the NAX060 and NAX075 Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX060 (solid red squares) or different concentrations of NAX060 and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX060 (solid red squares) or different concentrations of NAX060 and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX075 (solid red squares) and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX075 (solid red squares) or different concentrations of NAX075 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

resulted in decreased anti-apoptotic MCL1 expression. In contrast, the expression of pro-apoptotic BAX and BIM were increased upon metformin treatment. Part of the effects of metformin may be due to miR-26a expression as metformin increased its expression. miR-26a mimics also decreased MCL1 expression and increased the induction of apoptosis in these cells (Wang et al., 2016).

Metformin administration may have effects on miR-155/miR-144 which is important in the regulation of mitochondrially-bound hexokinase II (mtHKII). mtHKII has important roles in the resistance of cancer cells in avoiding cell death as well as protecting against ischemia-reperfusion (IR) injury. Some of the effects of the cardioprotection against reperfusion were shown to be due to the mitochondria, such as preventing outer mitochondrial membrane breakage and subsequent cytochrome C release (Nederlof et al., 2014).

BBR is consumed by millions of people as it is contained in fruits. It is also prepared as a dietary supplement and present in capsules. As a nutraceutical, BBR is consumed for the prevention and suppression of various diseases. Various attempts by multiple investigators have been performed to increase BBR's effectiveness. In our studies, we examined the effects of modified BBRs (NAX compounds) on colony formation in four different PDAC cell lines. Some of the NAX compounds inhibited proliferation more than other NAX compounds and the parental BBR both in terms of MTT assays and IC₅₀ determination and in colony formation abilities (Abrams et al., 2018b). Interestingly, the effects of NAX038 and NAX060 compounds were similar on all four PDAC cell lines in terms of colony formation assays. Metformin increased the ability of some, but not all, NAX compounds to inhibit proliferation.

MIA-PaCa-2 cells have a gain of function mutation at *TP53* (Deer et al., 2010) and an activating mutation at *KRAS*. We have examined previously the effects of metformin and BBR on these cells (Abrams et al., 2018a; Abrams et al., 2018b; Candido et al., 2108; McCubrey et al., 2018). AsPC-1 and BxPC3 cells also have *TP53* mutations. AsPC-1 has mutations at *KRAS* while BxPC3 is reported to have WT-*KRAS*. miR pathways would be predicted to be dysregulated in these PDAC cells as *TP53* is mutated and miRs can also influence the *KRAS* pathway. Since *TP53* and *KRAS* are frequently mutated in PDAC, elucidating the effectiveness of drugs and nutraceuticals which can function in the presence of these mutations is important.

The increased effectiveness of certain NAX compounds in the presence of metformin in inhibiting proliferation could be due to multiple mechanisms. Some of the more effective NAX compounds could induce more DNA damage which could synergize with the effects of metformin and result in cell death. Some of the NAX compounds could induce more ROS or WT-TP53 than others which would stimulate the induction of miRs and apoptosis. The induction of autophagy is also a possible mechanism which could explain the differences of the NAX compounds effectiveness. Elucidation of the mechanisms by which the different NAX compounds in

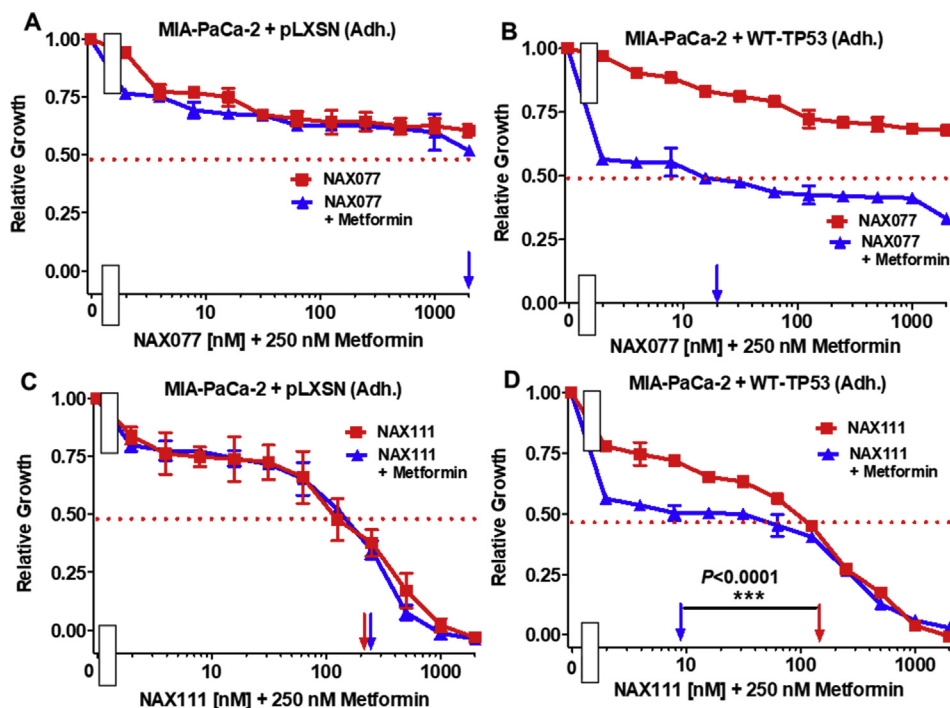


Fig. 15. Effects of the NAX077 and NAX111 Compounds on the Proliferation and IC₅₀s of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) Cells in the Absence and Presence of 250 nM Metformin. Panel A) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX077 (solid red squares) or different concentrations of NAX077 and 250 nM metformin (solid blue triangles). Panel B) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX077 (solid red squares) or different concentrations of NAX077 and 250 nM metformin (solid blue triangles). Panel C) MTT analysis of MIA-PaCa-2 + pLXSN (Adh.) cells plated with different concentrations of NAX111 (solid red squares) or different concentrations of NAX111 and 250 nM metformin (solid blue triangles). Panel D) MTT analysis of MIA-PaCa-2 + WT-TP53 (Adh.) cells plated with different concentrations of NAX111 (solid red squares) or different concentrations of NAX111 and 250 nM metformin (solid blue triangles). All the experiments indicated in this figure were performed on the same day. These experiments were repeated 3 times and similar results were obtained.

Table 1

Effects of Berberine and NAX Compounds on MIA-PaCa-2 + pLXSN (Adh.) Cells.^a

Compound	IC ₅₀	IC ₅₀ with 250 nM Metformin	Fold Difference of IC ₅₀ with 250 nM Metformin	Statistical Significance
Berberine (BBR)	1600 ± 17 nM	600 nM ± 5.4 nM	2.7 X ↓	P < 0.0001
NAX012	1300 ± 2.9 nM	1300 nM ± 2.9 nM	-	-
NAX014	750 ± 6.3 nM	750 nM ± 16 nM	-	-
NAX035	450 ± 11.5 nM	8.5 nM ± 1 nM	52.9 X ↓	P < 0.0001
NAX038	500 ± 12 nM	70 nM ± 2.8 nM	6.4 X ↓	P < 0.0001
NAX042	180 ± 1.3 nM	150 nM ± 1 nM	1.2 X ↓	P < 0.0001
NAX053	100 ± 1 nM	15 nM ± 0.4 nM	6.7 X ↓	P < 0.0001
NAX054	2000 nM	2000 nM	-	-
NAX060	180 ± 12.6 nM	28 nM ± 2.5 nM	6.4 X ↓	P < 0.0001
NAX075	> 2000 nM	1000 nM ± 4.9 nM	> 2 X ↓	-
NAX077	> 2000 nM	2000 nM	> 2 X ↓	-
NAX111	200 ± 11.3 nM	200 nM ± 7.8 nM	-	-

^a Determined by MTT Analysis as presented in Abrams et al. (2018), Candido et al. (2018); Abrams et al. (2019).

combination with metformin inhibit proliferation could further the usage of modified BBRs (NAX compounds) in cancer therapy as well as other diseases. It is conceivable that type-II diabetes patients who are treated with metformin to alleviate their diabetes also consume BBR either as a fruit or a dietary supplement. This combination could have enhanced anti-cancer effects.

In our studies, we have for the first time shown the effects of introduction of WT-TP53 on the effects of modified BBRs in response to a suboptimal dose of 250 nM metformin. Certain NAX compounds (e.g., NAX014, NAX038, NAX042, NAX077 and NAX111) elicited elevated sensitivity in the presence of WT-TP53 and metformin while other NAX compounds (e.g. NAX053, NAX060, NAX075) showed greater effect on sensitivity to the addition of 250 nM metformin in the absence of WT-TP53. The relationships between BBR and DNA binding and TP53 are not well established. It is possible that various NAX compounds can interact with DNA and WT-TP53 in different fashions which could be useful in certain cancer treatments. Further elucidation of the effects of NAX

Table 2
Effects of berberine and NAX compounds on MIA-PaCa-2 + WT-TP53 (Adh.) cells.^a

Compound	IC ₅₀	IC ₅₀ with 250 nM Metformin	Fold Difference of IC ₅₀ with 250 nM Metformin	Statistical Significance
Berberine (BBR)	900 ± 1.9 nM	500 nM ± 5 nM	1.8 X ↓	P < 0.0001
NAX012	900 ± 9 nM	550 nM ± 0.8 nM	1.6X ↓	P < 0.0001
NAX014	500 ± 2.9 nM	15 nM ± 0.5 nM	33.3 X ↓	P < 0.0001
NAX035	400 ± 8 nM	300 nM ± 6 nM	1.3 X ↓	P < 0.0001
NAX038	220 ± 3.2 nM	30 nM ± 0.4 nM	7.3 X ↓	P < 0.0001
NAX042	200 ± 2.6 nM	40 nM ± 0.8 nM	5 X ↓	P < 0.0001
NAX053	130 ± 2 nM	40 nM ± 0.8 nM	3.3 X ↓	P < 0.0001
NAX054	> 2000 nM	2000 nM	–	–
NAX060	60 ± 0.8 nM	40 nM ± 1.2 nM	1.5 X ↓	P < 0.0001
NAX075	> 2000 nM	> 2000 nM	–	–
NAX077	> 2000 nM	20 nM	> 100 X↓	–
NAX111	150 ± 2.3 nM	9 nM ± 0.3 nM	16.6 X↓	P < 0.0001

^a Determined by MTT Analysis as presented in Abrams et al. (2018), Candido et al. (2018); Abrams et al. (2019).

compounds and metformin in the presence and absence of WT-TP53 could further our understanding of how BBR and modified derivatives function in suppressing growth of pancreatic and other cancer cell types.

Conflicts of interest

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

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