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Effects of the MDM-2 inhibitor Nutlin-3a on PDAC cells containing and lacking WT-TP53 on sensitivity to chemotherapy, signal transduction inhibitors and nutraceuticals

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ABSTRACT

Mutations at the *TP53* gene are readily detected (approximately 50–75%) in pancreatic ductal adenocarcinoma (PDAC) patients. TP53 was previously thought to be a difficult target as it is often mutated, deleted or inactivated on both chromosomes in certain cancers. In the following study, the effects of restoration of wild-type (WT) TP53 activity on the sensitivities of MIA-PaCa-2 pancreatic cancer cells to the MDM2 inhibitor nutlin-3a in combination with chemotherapy, targeted therapy, as well as, nutraceuticals were examined. Upon introduction of the WT-*TP53* gene into MIA-PaCa-2 cells, which contain a *TP53* gain of function (GOF) mutation, the sensitivity to the MDM2 inhibitor increased. However, effects of nutlin-3a were also observed in MIA-PaCa-2 cells lacking WT-TP53, as upon co-treatment with nutlin-3a, the sensitivity to certain inhibitors, chemotherapeutic drugs and nutraceuticals increased. Interestingly, co-treatment with nutlin-3a and certain chemotherapeutic drug such as irinotecan and oxaliplatin resulted in antagonistic effects in cells both lacking and containing WT-TP53 activity. These studies indicate the sensitizing abilities that WT-*TP53* activity can have in PDAC cells which normally lack WT-

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TP53, as well as, the effects that the MDM2 inhibitor nutlin-3a can have in both cells containing and lacking WT-TP53 to various therapeutic agents.

1. Introduction

1.1. Pancreatic cancer: a devastating cancer with few effective therapeutic options

The most common treatment for pancreatic cancer is surgery. Chemotherapy is also a treatment option which has limited success. Pancreatic cancer is among the top ten most common cancers in the western world. Pancreatic cancer has the highest mortality of any cancer. It is predicted that pancreatic cancer will soon become the second most frequent cause of cancer deaths. The survival rate is less than a year with 5-year survival of only 5–10% (Muniraj et al., 2013; Siegel et al., 2013). Unfortunately, the survival rate has not improved significantly over the past 40 years. Most pancreatic cancers consist of pancreatic ductal adenocarcinoma (PDAC). Clearly more effective approaches to treat PDAC patients are important to improve cancer care.

The development of PDAC is complex and includes both environmental, as well as, genetic risk factors (Klein, 2012). We have recently summarized the risk and genetic factors associated with development of pancreatic cancer (Abrams et al., 2018, 2019; Candido et al., 2018). Some of the risk factors for PDAC development in the USA are race and gender based, as well as, diabetes and obesity. In addition, there are various environmental factors which may contribute to PDAC including: cigarette smoking, alcohol consumption, and low physical activity (Iodice et al., 2008; Arslan et al., 2010; Jiao et al., 2010; Michaud et al., 2010; Lucenteforte et al., 2012; Bosetti et al., 2014).

1.2. Existing therapies for PDAC

Removal of the diseased pancreas is a common therapy for PDAC patients (Kommalapati et al., 2018). Combining surgical resection and chemotherapy is also performed but is only minimally effective. A key problem in PDAC therapy is that by the time PDAC has been diagnosed, it has already metastasized, often making treatment very difficult if not impossible (Kommalapati et al., 2018; Ruarus et al., 2018; Skelton et al., 2018).

FOLFIRINOX is a chemotherapeutic regimen which was conceived in the early 2010's. It yields somewhat better results (4 months longer survival) than standard gemcitabine therapy (Conroy et al., 2011, 2013; Faris et al., 2013; Thota et al., 2014). The FOLFIRINOX regime consist of four drugs namely: FOL = folinic acid (leucovorin), a vitamin B derivative that reduces the side effects of, F = fluorouracil, a pyrimidine analog that becomes introduced into DNA and blocks replication, IRIN = irinotecan (Camptosar), a topoisomerase inhibitor, that inhibits DNA uncoiling and duplication and OX = oxaliplatin (Eloxatin), a platinum-based antineoplastic agent, which inhibits DNA repair and replication.

Combining gemcitabine (Gemzar) and protein-bound paclitaxel (nab-paclitaxel, Abraxane) has been evaluated in the treatment of certain patients with advanced pancreatic cancer (Von Hoff et al., 2011). This therapy was approved by the US Food and Drug Administration since 2013. The combination of gemcitabine and nab-paclitaxel may have less side effects than FOLFIRINOX therapy.

1.3. Pancreatic cancer genetics: some common oncogenes/tumor suppressor genes and some novel culprits

It turns out that PDACs have mutations at certain oncogene/tumor suppressor genes which are frequently mutated in human cancer including: *KRAS*, *TP53*, *CDKN2A*, and *SMAD4* (Rasheed et al., 2015; Klein, 2012, 2013). At least 90% of PDACs contain activating mutations in *KRAS*. The constitutively-activated KRAS oncoprotein interacts and often mobilizes many downstream signaling pathways (*e.g.,* PI3K/Akt/mTOR and Raf/MEK/ERK) leading to: proliferation in absence of regulatory growth factors, cell cycle progression, prevention of apoptosis, senescence and chemotherapeutic drug resistance.

The genetic status of: *KRAS*, *TP53*, isocitrate dehydrogenase 1 (*IDH1*), *NRAS* and *BRAF,* as well as, the expression of SMAD4 and CDKN2A/p16 were examined in fifteen long-term PDAC survivors. Long-term PDAC survivors are defined as patients who have survived for longer than 55 months as most PDAC cancer patients succumb less than 20 months after tumor resection. Lack of mutation at *KRAS* and *TP53* and expression of SMAD4 were associated with a 2-fold longer survival than patients who had mutations at or lost expression of these genes (Masetti et al., 2018).

Many of the genes associated with familiar PDAC are also implicated in sporadic PDAC. These include: *ATM, BRCA2, DPC4, PALB2, PIK3CA, SMAD* and others (Blackford et al., 2009; Roberts et al., 2012, 2016; Klein, 2013; Wolpin et al., 2014; Norris et al., 2015). Network analysis (Zhang et al., 2017) is also being used to identify genes which may be involved in PDAC. Novel genes implicated in tumor diversity and evolution are being identified by restriction-site associated DNA sequencing (RADseq) (Perry et al., 2017). Patient-derived xenografts made from human PDAC tumor specimens are also being created to identify additional genes that are aberrantly expressed in PDAC. These animal models may also allow the screening of novel therapeutics useful in treating PDAC (Jung et al., 2016).

1.4. Roles of TP53 in PDAC

The transcription factor TP53 controls the expression of many genes and microRNAs (miRs). These genes and miRs (*e.g.*, $p21^{Cip-1}$, NOXA, PUMA, miR-34a) and many others are involved in the regulation of cell cycle progression, apoptosis, cellular senescence and other critical biological processes important in normal growth, as well as, cancer progression and drug resistance (Chen et al., 1996; Ko and Prives, 1996; Olivier et al., 2002). *TP53* is one of the most frequently mutated oncogenes in human cancers.

Recently, the NOP14 nucleolar protein (NOP14) was detected at higher levels in PDAC tumors and metastatic tissue samples than in normal controls. This increased expression of NOP14 resulted in enhanced cell motility. In contrast, suppression of NOP14 decreased invasiveness. Intriguingly, mutant TP53 was demonstrated to be a target of NOP14. NOP14 increased mutant TP53 mRNA stability. NOP14 and mutant TP53 had effects on cell cycle progression as they suppressed $p21^{Cip-1}$ expression at both the transcriptional and post-transcriptional levels. This may have resulted from the induction of the miR-17–5p. Thus, certain genes and proteins may interact with mutant TP53 to alter cell cycle progression, as well as, metastasis (Du et al., 2017).

1.5. Gain of function (GOF) mutant TP53 proteins-devilish mutations in disguise

Many TP53 mutant proteins have a single amino acid change. They are often missense mutations. These mutations can result in changing the levels of the TP53 proteins, by increasing the protein levels due to altered degradation of the normally labile TP53 protein. Certain mutant TP53 proteins have GOF activity. That is, they have additional "functions or characteristics" which WT-TP53 lacks. These mutations can occur at the *TP53* gene in critical residues that result in altered transcriptional programs for TP53 and some may also inhibit the normal functions of TP53.

GOF mutations may have more effects than deletions of the *TP53* gene in pancreatic cancer. GOF mutations may affect genome stability and responses to chemotherapeutic drugs such as altering induction of cellular senescence. The R248W, R273H and R175H GOF *TP53* mutations are predicted to be present in approximately 15% of *TP53* mutations, thus they may occur in 8% of all human cancers as *TP53* is predicted to be mutated in 50% of human cancers (Liu et al., 2010). The MIA-PaCa-2 cell line contains the R248W *TP53* mutation (Morton et al., 2010). *TP53* GOF mutations such as R248W may alter the ability of TP53 to bind DNA and transcribe certain genes. Also, these mutations affect the ability of TP53 to interact with other transcription factors (TP63, TP73) and anti-apoptotic proteins such as BCLXL and may lead to the deregulation of apoptosis. The R248W and R282W *TP53* mutations are associated with shorter patient survival. These *TP53* mutations have been referred to as "mortality mutations" (Xu et al., 2014). Some other studies have indicated that *TP53* GOF knock-in mice do not show altered survival in comparison to mice lacking *TP53* (p53−/−) but the mice containing the *TP53* GOF knock-in genes exhibit a broader tumor spectrum (Hanel et al., 2013).

Multiple effects have been demonstrated for the R248W *TP53* mutant protein. In knock-in mice, it was demonstrated that the R248W TP53 mutant protein could interact with the MRE11 nuclease and prevent the binding of the MRE11-Rad50-NBS1 (MRN) complex to double strand DNA breaks (DSB). This prevented ataxia-telangiesctasia mutated (ATM) activation, an important molecule involved in DNA repair (Song et al., 2007). ATM is also mutated in certain PDAC patients (Roberts et al., 2012).

The R248W-TP53 mutant protein increases the expression of the growth–regulated oncogene 1 (GRO1 a.k.a. CXCL1, a small cytokine, growth promoting protein) and decreases the expression of the inhibitor of DNA Binding 2 (ID2, a transcriptional regulator) which are important in growth regulation of cells containing the mutant *TP53* gene (Brosh and Rotter, 2009). CXCL1 may play key roles in the pancreatic tumor microenvironment and tumor development. CXCL1 is a potent chemoattractant for myeloid cells. The necrosome and CXCL1 have been shown recently to be important in PDAC (Seifert et al., 2016), however, the roles of mutant (s) and WT TP53 are not known. The necrosome induces CXCL1. Necroptosis (programmed necrosis) is another form of programmed cell death and is important in PDAC.

Various missense TP53 proteins regulate (induce or repress) the expression of other genes including: *FAS* (a.k.a., CD95), *ID2*, multi-drug resistance-1 (*MDR1, ABCB1*), c-Myc (*MYC*) and NF-kappaB2 (*NFKB2*) and many others (Brosh and Rotter, 2009). The spectrum of genes regulated by mutant TP53 missense proteins is often different than the proteins regulated by WT-TP53. Certain TP53 mutant proteins can form heterotetramers with WT-TP53. The C-terminal basic domain of the WT-TP53 protein has been shown to inhibit the activity of mutant R248W and other TP53 mutant proteins (Yan and Chen, 2010; Zhang et al., 2016a).

Previously, we and others have examined the roles of *TP53* in the sensitivity of hematopoietic, prostate and pancreatic cells and other cancer types to chemotherapeutic drugs and, in some cases, radiation (Lehmann et al., 2007' McCubrey et al., 2008; Chappell et al., 2012; Abrams et al., 2017; Steelman et al., 2017; Abrams et al., 2018; Abrams et al., 2019). We observed that introduction of dominant negative (DN)-TP53 into FL5.12 hematopoietic cells increased their chemoresistance (McCubrey et al., 2008; Abrams et al., 2017; Steelman et al., 2017). The biological effects of addition of WT-*TP53* or DN-*TP53* to cells which either lack or have WT-*TP53* have been examined in prostate cancer cells (Lehmann et al., 2007; Chappell et al., 2012). Introduction of WT-*TP53* into prostate cancer, DU145 cells which expressed mutant TP53, resulted in increased chemosensitivity and radiosensitivity (Lehmann et al., 2007; Chappell et al., 2012). Introduction of WT-*TP53* also increased the induction of cellular senescence after radiation treatment and sensitivity to the MDM2 inhibitor nutlin-3A when the cells were treated with the TP53-inducer doxorubicin (Chappell et al., 2012). Introduction of WT-*TP53* increased TP53-mediated luciferase activity in prostate cancer cells which had defective TP53, while introduction of DN-*TP53* suppressed TP53 luciferase activity in LNCaP cells with WT-*TP53* (Lehmann et al., 2007; Chappell et al., 2012). In addition, introduction of WT-*TP53* suppressed the ability of prostate cells with mutant *TP53* (DU145 and PC3) to form colonies in soft agar (Chappell et al., 2012), while introduction of WT-*TP53* increased the ability of prostate cancer cells with WT-

TP53 activity to form colonies in soft agar (Chappell et al., 2012). The ability of cells to form colonies in soft agar has been associated with malignant progression (Shin et al., 1975). WT-TP53 activity was critical in controlling the induction of pre-mature cellular senescence after radiation treatment of prostate cancer cells (Lehmann et al., 2007).

Introduction of WT-TP53 was observed to increase the sensitivity of certain PDAC cells to certain chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals (Abrams et al., 2018, 2019). Reprogramming TP53 is a potent target for pancreatic cancer therapy (Saghatelian et al., 2017). Thus, TP53 is a key regulatory molecule in processes involved in malignant progression, chemosensitivity and radiosensitivity.

1.6. TP53 roles in PDAC metastasis

Dense fibrotic stroma is associated with PDAC and can contribute to tumor growth, metastasis and lead to drug resistance. Quiescent pancreatic stellate cells (PSCs) often become activated during PDAC growth which can lead to increased growth factor signaling. This can result in extracellular matrix deposition and fibrosis. The E3 ubiquitin-protein ligase mouse double minute 2 homolog (MDM2) inhibitor nutlin-3a can activate TP53 and this then regulates PSC activation and modulates PDAC fibrosis. This results in transcriptional changes which lead to PSC quiescence (Saison-Ridinger et al., 2017).

TP53 mutations have been frequently detected during the transition from benign pancreatic intra-epithelial neoplasia to the highly aggressive, invasive and metastatic PDAC. These mutations can play critical roles in PDAC progression and may result in loss of TP53's anti-proliferative effects. However, some missense mutations result in GOF mutations. Platelet-derived growth factor receptor b (PDGFRb) was demonstrated to be involved in the invasiveness of PDAC cells. The p73/NF—Y complex was suppressed in these cells which in turn inhibited invasion *in vivo*. PDGFRb expression in pancreatic, colon and ovarian cancer patients was associated with poor disease-free survival (Weissmueller et al., 2014).

1.7. Increasing TP53 activity by targeting with the MDM2 inhibitor Nutlin-3a

The level of the labile TP53 protein is controlled, in part, by MDM2. Nutlin-3a is an example of a MDM2 inhibitor which has been characterized in multiple cancer types (Vassilev et al., 2004). Various small molecule MDM2 inhibitors are under investigation and some are being evaluated in clinical cancer trials (Vu and Vassilev, 2011; Khoo et al., 2014). Recently, we have summarized MDM2 and related MDM4 proteins and their inhibitors and some of the mechanisms of resistance to these inhibitors (McCubrey et al., 2017). The potential for inhibitors such as nutlin-3a and other MDM2 inhibitors either alone or in combination with chemotherapeutic drugs to suppress the growth of tumor cells containing WT-TP53 or mutant TP53 has been discussed (Ambrosini et al., 2007; Zhang et al., 2011; Conradt et al., 2013).

The fruits *Garcinia mangostana* L and *Garcinia hanburyi* contain prenylated xanthones alpha-mangostin and gambogic acid respectively. These compounds have some natural MDM2 inhibitory activity (Leão et al., 2013). Thus, MDM2 represents a target which has been selected by evolution of nutraceuticals.

1.8. Reactivating mutant TP53

An alternative approach of TP53-directed therapeutics is to reactivate TP53 activity in *TP53*-mutant cancers. APR-246, [(a.k.a., PRIMA-1, PRIMA-1 (MET)] is a small molecule drug that binds critical cysteine residues in the core binding domain of mutant TP53 and results in reactivation of TP53 activity (Bykov et al., 2002; Zache et al., 2008; Zandi et al., 2011). These mutant TP53 "reactivators" may function as chaperones and bind mutant TP53 and related TP63 and TP73 proteins. These TP53 "reactivators" stabilize the proteins and maintain the correctly folded protein conformation.

PRIMA-1 (MET) (APR-246) has been evaluated in some clinical trials with hematopoietic prostate and ovarian cancers (Lehmann et al., 2012). PRIMA-1 (MET) and other MDM2 inhibitors have been examined on cancer stem cells (Zhang et al., 2016b). PRIMA-1 (MET) enhanced the effects of the poly ADP ribose polymerase (PARP) inhibitor AZD2281 (olaparib) in non-small cell lung cancer cells (Deben et al., 2016). PRIMA-1 (MET) also increased the sensitivity of V600E/K *BRAF* mutant melanomas to the BRAF inhibitor vermurafenib (Krayem et al., 2016). PRIMA-1 (MET) (APR-246) will suppress cisplatin- and doxorubicin-resistance in ovarian cancer cells (Mohell et al., 2015).

1.9. Targeting signal transduction pathways induced by chemotherapy

Chemotherapy can induce TP53 and NF-κB and other molecules such as Raf/MEK/ERK and PI3K/PTEN/AKT/mTOR and other pathways which are involved in the resistance to multiple therapeutic approaches (Abrams et al., 2017; Steelman et al., 2017). Many signaling pathways, nutraceuticals and natural products interact with NF-κB and TP53 and other pathways which are involved with drug resistance (McCubrey et al., 2017, 2018). In addition, the TP53 and NF-κB pathways may be induced by nutraceuticals.

As stated previously, *TP53* is frequently mutated in PDAC. Multiple signaling pathways may be affected by mutant TP53 (Solomon et al., 2012; Abrams et al., 2017; Steelman et al., 2017; McCubrey et al., 2018). Mutations in *TP53* have pleiotropic effects, which can contribute to cancer progression.

In the following studies, we have investigated the effects of the MDM2 inhibitor nutlin-3a on a pancreatic cell line, MIA-PaCa-2 which has a GOF TP53 mutation and in a derivative cell line which has an introduced WT-TP53 gene to determine the effects of var-

ious chemotherapeutic drugs, signal transduction inhibitors, natural products on the sensitivity to nutlin-3a. We observed that in some cases the cells lacking WT-TP53 were sensitive to the nutlin-3a when combined with chemotherapeutic drugs suggesting that the MDM2 inhibitor may have effects on certain PDAC cell with TP53 GOF mutations. An overview of the effects of nutlin-3a, chemotherapeutic drugs, signal transduction inhibitors on TP53, miR-34a and downstream signal pathways important in PDAC as well as the progression of other cancers is presented in Fig. 1.

2. Materials and methods

2.1. Cell lines and tissue culture

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 to all TP53 target sequences in TP53-responsive genes and results in loss of its tumor suppressor properties (Brosh and Rotter, 2009; Solomon et al., 2012). In addition, the *TP53* GOF mutation may result in the transcription of genes which are not normally targets of WT-TP53. MIA-PaCa-2 cells also have an activating mutation at *KRAS* (G12C) and they have elevated PI3K/AKT pathway activity. MIA-PaCa-2 cells were obtained from the ATCC (Rockville, MD, USA). MIA-PaCa-2 cells were infected with the pLXSN empty viral vector (Miller and Rosman, 1989) or a retroviral vector encoding WT-TP53 (Eliyahu et al., 1989) as described (Abrams et al., 2018) and stable pools isolated in the presence of medium containing 2 mg/ml geneticin (Sigma-Aldrich, Saint Louis, MO, USA).

Cells were cultured in medium containing 5% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA) as described in (Abrams et al., 2017, 2018, 2019; Steelman et al., 2017; Candido et al., 2018). Tissue culture medium (Dulbecco's modified Eagles medium, DMEM), antibiotics containing L-glutamine and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). Nutraceuticals, chemotherapeutic drugs, and signal transduction inhibitors were purchased from either Selleckchem (Houston, TX USA) or Sigma-Aldrich.

2.2. Methylthiazol tetrazolium assays

Methylthiazol tetrazolium (MTT) assays were performed to ascertain the sensitivity of the pancreatic cancer cells containing and lacking an introduced WT-*TP53* gene, or a control empty retroviral pLXSN control vector to chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals as described (Abrams et al., 2018, 2019). To test the hypotheses that the various IC_{50}s in the pancreatic cancer cells containing or lacking WT-TP53 that were treated with chemotherapeutic drugs, signal transduction inhibitors, nutraceuticals and nutlin-3a were statistically different, student's T tests were performed by Graph Pad Prism (QuickCals) statistical analysis.

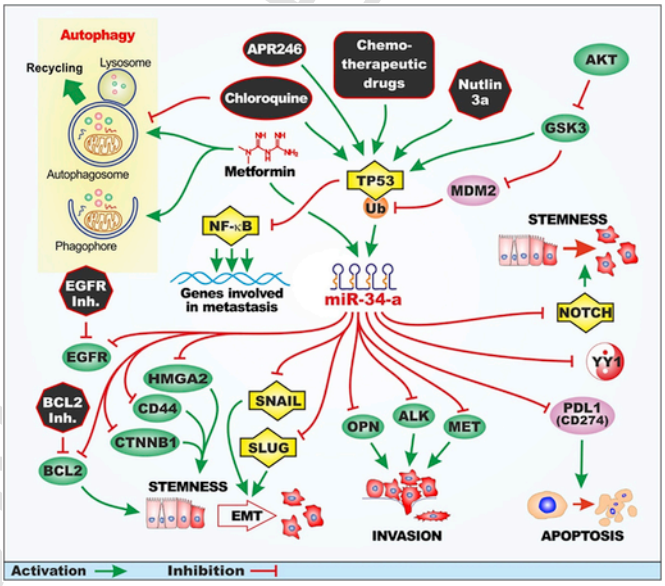

Fig. 1. Overview of Effects of Nutlin-3 on TP53 and miR-34a Expression. The effects of various drugs on the TP53 and miR-34a are indicated. The sites of interaction of various signal transduction pathway inhibitors are indicated. The effects of miR-34a activation on important pathways involved in cancer progression and other physiological processes are indicated.

3. Results

3.1. Introduction of WT-TP53 alters the sensitivity of MIA-PaCa-2 cells to various chemotherapeutic drugs and signal transduction inhibitors

MIA-PaCa-2 cells were infected with the empty retroviral vector pLXSN as a control for infection with the retrovirus encoding WT-TP53. The experiments in this manuscript were performed with adherent (Adh.) cells. It is important to state whether the MIA-PaCa-2 cells are adherent or spheroid as they will also grow non-adherently as spheroids. MTT analyses were used to determine the effects of TP53 on the chemosensitivity of MIA-PaCa-2 + WT-TP53 (Adh.) cells to the MDM2 inhibitor. The IC_{50} for nutlin-3a in MIA-PaCa-2 + WT-TP53 (Adh.) cells was approximately 10,000 nM (Fig. 2, Panel A). In contrast, an IC_{50} for nutlin-3a was not obtained in MIA-PaCa-2 + pLXSN (Adh.) cells with up to 10,000 nM documenting the resistance of cells lacking WT-TP53 to this MDM2 inhibitor. The IC_{50}s for gemcitabine were also examined. In MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) the IC_{50}s for gemcitabine were 6 nM and 0.4 nM respectively, approximately a 15-fold increase in sensitivity in cells containing WT-TP53. (Fig. 2, Panel B). These results demonstrate the effects that WT-TP53 can have on the sensitivity of the cells to various drugs. The effects of introduction of WT-TP53 on the sensitivity to various chemotherapeutic and nutraceuticals are summarized in Table 1.

3.2. Effects of the MDM2 inhibitor Nutlin-3a on sensitivity to chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals

The effects of combining gemcitabine with a constant dose of 500 nM nutlin-3a were examined in MIA-PaCa-2 + pLXSN and MIA-PaCa-2 + WT-TP53 (Fig. 2, Panels C & D). The IC_{50} for nutlin-3a in MIA-PaCa-2 + pLXSN (Adh.) was approximately 6 nM (Fig. 2, Panel C). Inclusion of a constant sub-IC_{50} dose of 500 nM nutlin-3a reduced the gemcitabine IC_{50} 1.5-fold to 4 nM. The IC_{50} for gemcitabine in MIA-PaCa-2 + WT-TP53 (Adh.) cells was approximately 0.6 nM (Fig. 1, Panel D), Addition of 500 nM nutlin-3a reduced the IC_{50} 2-fold to 0.3 nM. The effects of a sub-IC_{50} dose of nutlin-3a in the presence and absence of WT-TP3 on the IC_{50}s of various chemotherapeutic drugs and nutraceuticals are summarized in Table 2.

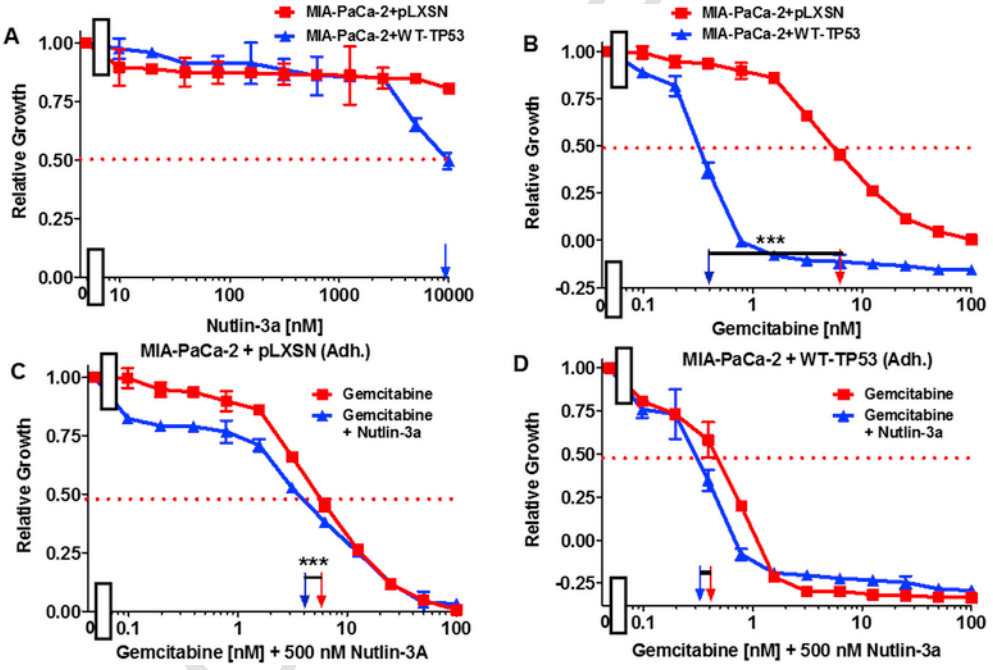

Fig. 2. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Nutlin-3a and Gemcitabine. MIA-PaCa-2 + pLXSN (red squares), and MIA-PaCa-2 + WT-TP53 (blue triangles) cells were titrated with different concentrations of Panel: A) nutlin-3a and B) gemcitabine for 4 days before MTT analysis was performed. 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 MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with gemcitabine and is extremely statistically significant. The experiments in panels A and B were performed on the same days. These experiments were repeated 4 times and similar results were obtained. Adh. = adherent cells. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of gemcitabine (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel C, the two-tailed *P* value equals 0.0058 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with gemcitabine and nutlin-3a and is extremely statistically significant. In Panel D, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with gemcitabine and nutlin-3a and is considered to be extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Effects of WT-TP53 on sensitivity to chemotherapeutic drugs and nutraceuticals (IC50 analysis)[a].

Presence of WT-TP53→	- WT-TP53	+ WT-TP53	Fold Change with introduction of WT-TP53
Drug/Nutraceutical↓			
Gemcitabine	6 nM	0.4 nM	15 X↓
Cisplatin	23,000 nM	2,000 nM	11.5 X↓
Irinotecan	4,000 nM	2,000 nM	2 X↓
Oxaliplatin	400 nM	400 nM	–
Mitoxantrone	8 nM	5 nM	1.6 X↓
BCL2 Inh. (ABT737)	2,000 nM	2,000 nM	–
Berberine	1800 nM	700 nM	2.6 X↓
Resveratrol	30,000 nM	100,000 nM	3.3 X↑
Curcumin	30,000 nM	20,000 nM	1.5 X↓
Garcinia Cambogia	100,000 ng/ml	100,000 ng/ml	–

[a] IC_{50} and statistical analyses were determined as described (Abrams et al., 2018, 2019; Candido et al., 2018). Statistical analysis is described in materials and methods and results section. All IC_{50} values are presented in nM.

Table 2
Effects of Nutlin-3a on sensitivity to chemotherapeutic drugs in the absence and presence of WT-TP53 (IC_{50} analysis)[a].

Presence of WT-TP53→	–	–	–	+	+	+
Drug/Nutraceutical↓	Presence of 500 nM Nutlin-3a		Fold Change	Presence of 500 nM Nutlin-3a		Fold Change
	–	+		–	+	
Gemcitabine	6 nM	4 nM	1.5 X↓	0.6 nM	0.3 nM	2 X↓
Cisplatin	23,000 nM	500 nM	46 X↓	2,000 nM	500 nM	4 X↓
Irinotecan	4,000 nM	12,000 nM	3 X↑	2,000 nM	10,000 nM	5 X↑
Oxaliplatin	400 nM	1,300 nM	3.3 X↑	400 nM	1,300 nM	3.3 X↑
Mitoxantrone	8 nM	2 nM	4 X ↓	5 nM	2 nM	2.5 X↓
BCL2 Inh. (ABT-737)	2,000 nM	60 nM	35 X↓	2,000 nM	100 nM	10 X↓
Berberine	1,800 nM	700 nM	2.6 X↓	700 nM	400 nM	1.8 X↓
Resveratrol	30,000 nM	1,500 nM	20 X↓	100,000 nM	13,000 nM	7.7 X↓
Curcumin	30,000 nM	20,000 nM	1.5 X↓	20,000 nM	5,000 nM	4 X↓
Garcinia Cambogia	100,000 ng/ml	12,000 ng/ml	8.3 X↓	100,000 ng/ml	6,000 ng/ml	16.7 X↓

[a] IC_{50} and statistical analyses were determined as described (Abrams et al., 2018, 2019; Candido et al., 2018). Statistical analysis is described in materials and methods and results section. All IC_{50} values are presented in nM.

The IC_{50} for cisplatin in MIA-PaCa-2 + pLXSN (Adh.) cells was approximately 23 nM (Fig. 3, Panel A), while the IC_{50} for MIA-PaCa-2 + WT-TP53 (Adh.) incubated in the presence of cisplatin was approximately 11.5-fold lower (Fig. 3, Panel B). Addition of 500 nM nutlin-3A reduced the IC_{50} for MIA-PaCa-2 + pLXSN (Adh.) from 23 to 0.5 μM, approximately 46-fold (Panel A). Addition of 500 nM nutlin-3a, reduced the IC_{50} for cisplatin 4-fold in MIA-PaCa-2 + WT-TP53 cells from approximately 2 to 0.5 μM (Panel B).

The effects of introduction of WT-TP53 into MIA-PaCa-2 cells on the sensitivity to other drugs frequently used in chemotherapy were also examined. MIA-PaCa-2 + pLXSN (Adh.) were more resistant to docetaxel (Fig. 3, Panel C) than MIA-PaCa-2 + WT-TP53 cells (Fig. 3, Panel D). Addition of 500 nM nutlin-3a decreased the IC_{50} for docetaxel approximately 50-fold in MIA-PaCa-2-TP53 cells from 50 to 1 nM (Panel D).

The effects of introduction of WT-TP53 into MIA-PaCa-2 cells on the sensitivity to irinotecan and oxaliplatin in the presence and absence of 500 nM Nutlin-3A were determined (Fig. 4). MIA-PaCa-2 + WT-TP53 (Adh.) cells were approximately 2-fold more sensitive to irinotecan than MIA-PaCa-2 + pLXSN (Adh.) cells as IC_{50}s of 2 and 4 μM were observed respectively (Fig. 4, Panels A & B). However, when the MIA-PaCa-2 + pLXSN (Adh.) an MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with 500 nM Nutlin-3a and irinotecan (Panels A & B), interesting results were observed as they became less sensitive to irinotecan and the IC_{50}s increased to approximately 12 and 10 μM, respectively.

When the MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with oxaliplatin, IC_{50}s of approximately 0.4 μM were observed (Fig. 4, Panels C & D). Thus, the presence of WT-TP53 did not appear to alter the chemosensitivity to oxaliplatin. Addition of 500 nM nutlin-3a decreased the sensitivity to oxaliplatin approximately 3.3-fold to 1.3 μM, in MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells in oxaliplatin indicating that nutlin-3A inhibited the effects of oxaliplatin.

The effects of introduction of WT-TP53 into MIA-PaCa-2 cells on the sensitivity to mitoxantrone and the EGFR/HER2 inhibitor ARRY 543 were examined in the presence and absence of 500 nM nutlin-3a (Fig. 5). MIA-PaCa-2 + pLXSN (Adh.) were more resistant to both mitoxantrone (Panel A) and the ARRY-543 EGFR/HER2 inhibitor (Panel C) than MIA-PaCa-2 + WT-TP53 cells (Panels B & D). The mitoxantrone IC_{50} in MIA-PaCa-2 + pLXSN (Adh.) decreased approximately 4-fold, from 8 to 2 nM, when the cells were treated with 500 nM nutlin-5a. The IC_{50} for mitoxantrone in MIA-PaCa-2 + WT-TP53 (Adh.) cells was approximately 5 nM (Fig. 5, Panel B) and decreased approximately 2.5-fold, to 2 nM, when the cells were also treated with 500 nM nutlin-3a.

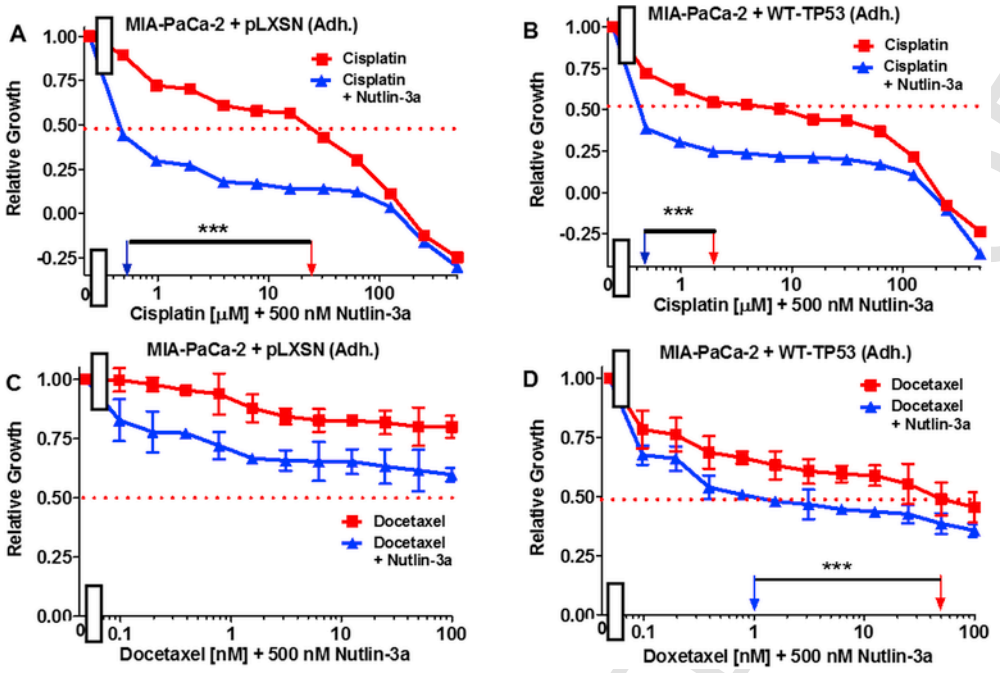

Fig. 3. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Cisplatin and Docetaxel in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of cisplatin (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangle) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with cisplatin and nutlin-3a and is extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with cisplatin and nutlin-3a and is considered to be extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 4 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of docetaxel (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel D, the two-tailed P value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with docetaxel and nutlin-3a and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The IC_{50} for the EGFR/HER2 inhibitor ARRY-543 was not reached when MIA-PaCa-2 + pLXSN (Adh.) cells were treated with up to 2,000 nM ARRY-543 (Fig. 5, Panel C). In contrast, when the cells were treated with 500 nM nutlin-3a, an IC_{50} of 800 nM was observed. Thus, the addition of nutlin-3a lowered the amount of ARRY-543 required to reach an IC_{50} dose >2.5 fold.

The IC_{50} for ARRY-543 in MIA-PaCa-2 + WT-TP53 (Adh.) cells was approximately 2,000 nM (Fig. 5, Panel D). Addition of nutlin-3a did not lower the IC_{50} in MIA-PaCa-2 + WT-TP53 (Adh.) cells significantly but did lower the level of relative growth of the cells (Fig. 5, Panel D).

The effects of introduction of WT-TP53 on the sensitivity of MIA-PaCa-2 cells to the BCL2/BCLXL inhibitor ABT-737 and the Hedgehog pathway inhibitor vismodegib were examined (Fig. 6). The IC_{50} for the ABT-737 BCL2/BCLXL inhibitor in MIA-PaCa-2 + pLXSN (Adh.) cells was approximately 2,000 nM (Fig. 6, Panel A). Addition of 500 nM nutlin-3A sensitized both cell lines to ABT-737. Addition of a constant dose of 500 nM nutlin-3a decreased the IC_{50} for ABT-737 in MIA-PaCa-2 + pLXSN + pLXSN (Adh) approximately 33-fold, to 60 nM (Fig. 6, Panel A). The IC_{50} for the ABT-737 in MIA-PaCa-2 + WT-TP53 (Adh.) cells was approximately 2,000 nM (Fig. 6, Panel B). Addition of 500 nM nutlin-3a decreased the IC_{50} 20-fold to approximately 100 nM (Fig. 6, Panel B).

The IC_{50} for the hedgehog pathway inhibitor vismodegib in MIA-PaCa-2 + pLXSN cells was not reached when the cells were treated with up to 10,000 nM vismodegib (Fig. 6, Panel C). In contrast, when the cells were treated with 500 nM nutlin-3a, an IC_{50} of 5,000 nM was observed which represents at least a 2-fold increase in sensitivity. When MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with vismodegib, an IC_{50} of approximately 10,000 nM was observed (Fig. 6, Panel D). Addition of 500 nM nutlin-3a increased the sensitivity of the cells approximately 66.7-fold as an IC_{50} of 150 nM was observed.

Estrogen and 4-hydroxytamoxifen (4HT) can have effects on PDAC cells such as MIA-PaCa-2 cells as they express the estrogen receptor (Guo et al., 2004). With the concentrations of estrogen and 4HT employed in these experiments, neither estrogen nor 4HT had pronounced effects on MIA-PaCa-2 cells which either lacked (Panels A & C) or contained WT-TP53 (Panels B or D) (Fig. 7). However, the effects of estrogen could be enhanced upon treatment with 500 nM nutlin-3a in MIA-PaCa-2 cells + WT-TP53 (Panel B). Likewise, the effects of 4HT could be enhanced upon treatment of both cell lines with 500 nM nutlin3a (Panels C & D) as an IC_{50} of approximately 100 nM was observed.

The effects of the anti-diabetes drug metformin, which induces autophagy, and the anti-malarial drug chloroquine which inhibits autophagy were examined on cells containing and lacking WT-TP53 in the presence and absence of 500 nM nutlin-3a (Fig. 8). Cells

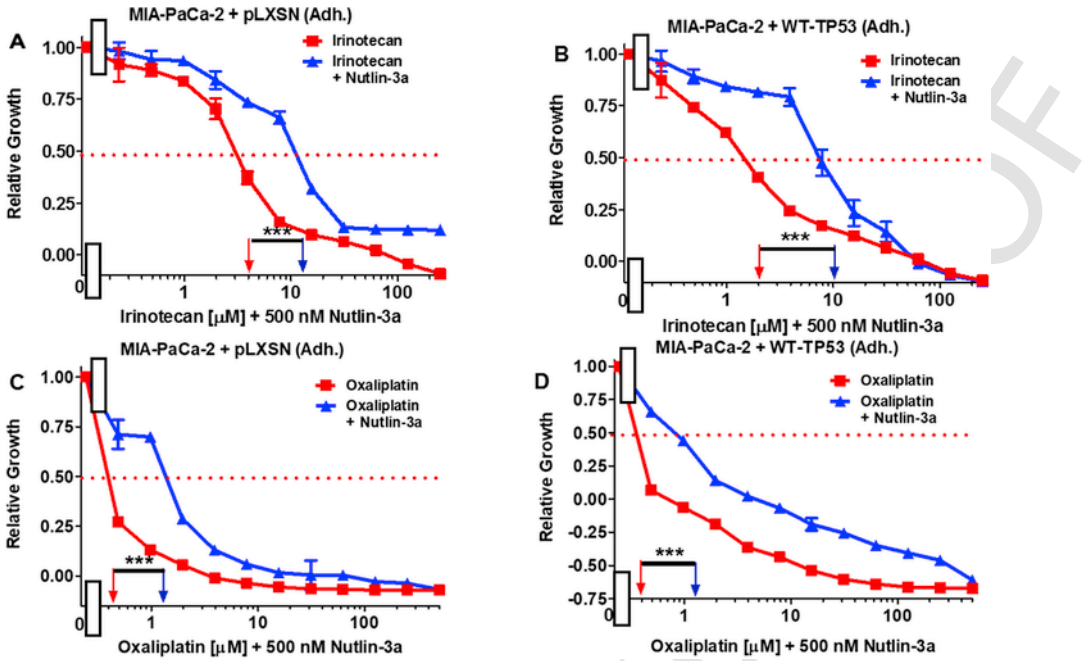

Fig. 4. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Irinotecan and Oxaliplatin in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of irinotecan (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with irinotecan and nutlin-3a and is extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with irinotecan and nutlin-3a is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of oxaliplatin (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel C, the two-tailed *P* value is 0.0027 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with oxaliplatin and nutlin-3a and is very statistically significant. In Panel D, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with oxaliplatin and nutlin-3a is less than 0.0001 and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

containing WT-TP53 were more sensitive to metformin (Panel B) and chloroquine (Panel D) than cells lacking WT-TP53 (Panels A & C). Inclusion of 500 nM nutlin-3a sensitized cells containing and lacking WT-TP53 to metformin (Panels A & B) and chloroquine (Panels C & D). Addition of 500 nM nutlin-3a to MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with metformin resulted in a 3.3-fold drop in the IC_{50} from 10,000 nM to 3,000 nM (Panel B). Upon addition of 500 nM nutlin-3a to the chloroquine-treated MIA-PaCa-2 + WT-TP53 cells resulted in a 7.5-fold decrease in the IC_{50} from 15 to 2.5 μM (Panel D).

The effects of the nutraceuticals berberine and resveratrol were examined on cells containing and lacking WT-TP53 in the presence and absence of 500 nM nutlin-3a (Fig. 9). Cells containing WT-TP53 were more sensitive to berberine (Panel B) than cells lacking WT-TP53 (Panel A) an IC_{50}s of 700 and1,800 nM were observed. The IC_{50} for berberine in MIA-PaCa-2 + pLXSN cells was approximately 1,800 nM (Fig. 9, Panel A). Upon addition of 500 nM nutlin-3a, the IC_{50} for berberine dropped approximately 2.6-fold, to 700 nM. The IC_{50} for berberine in MIA-PaCa-2 + WT-TP53 cells was approximately 700 nM (Fig. 9, Panel B). Upon addition of 500 nM nutlin-3a, the IC_{50} dropped approximately 1.8-fold, to 400 nM.

When MIA-PaCa-2 + pLXSN (Adh.) cells were treated with resveratrol, an IC_{50} of approximately 30 μM was observed (Fig. 9, Panel C). When the cells were treated with 500 nM nutlin-3a and different doses of resveratrol, the IC_{50} dropped approximately 20-fold, to 1.5 μM. When MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with resveratrol, an IC_{50} of approximately 100 μM was observed (Fig. 9, Panel D). When the same cells were treated with 500 nM nutlin-3a, the resveratrol IC_{50} dropped approximately 7.7-fold, to 13 μM. Thus, cells with WT-TP53 were less sensitive to resveratrol than cells containing WT-TP53.

The effects of the nutraceuticals curcumin and garcinia gambogia were examined on cells containing and lacking WT-TP53 in the presence and absence of 500 nM nutlin-3a (Fig. 10). The IC_{50}s for curcumin in MIA-PaCa-2 + pLXSN (Adh.) cells (Fig. 10, Panel A) and MIA-PaCa-2 + WT-TP53 (Adh.) cells (Fig. 10, Panel B) were approximately 30 and 20 μM, respectively. Treatment of MIA-PaCa-2 + pLXSN (Adh.) cells (Fig. 10, Panel A) and MIA-PaCa-2 + WT-TP53 (Adh.) cells (Fig. 10, Panel B) with 500 nM nutlin-3a reduced the IC_{50}s for curcumin approximately 1.5 and 4-fold, to 20 and 5 μM, respectively.

When MIA-PaCa-2 + pLXSN (Adh.) cells were treated with garcinia cambogia, an IC_{50} of approximately 100 μg/ml was observed (Fig. 10, Panel C). When the cells were also treated with 500 nM nutlin-3a, the IC_{50} dropped approximately 8.3-fold–12 μg/ml. When MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with garcinia cambogia, an IC_{50} of approximately 100 μg/ml was observed (Fig. 10, Panel D). When the same cells were treated with 500 nM nutlin-3a, the IC_{50} dropped approximately 16.7-fold, to 6 μg/ml.

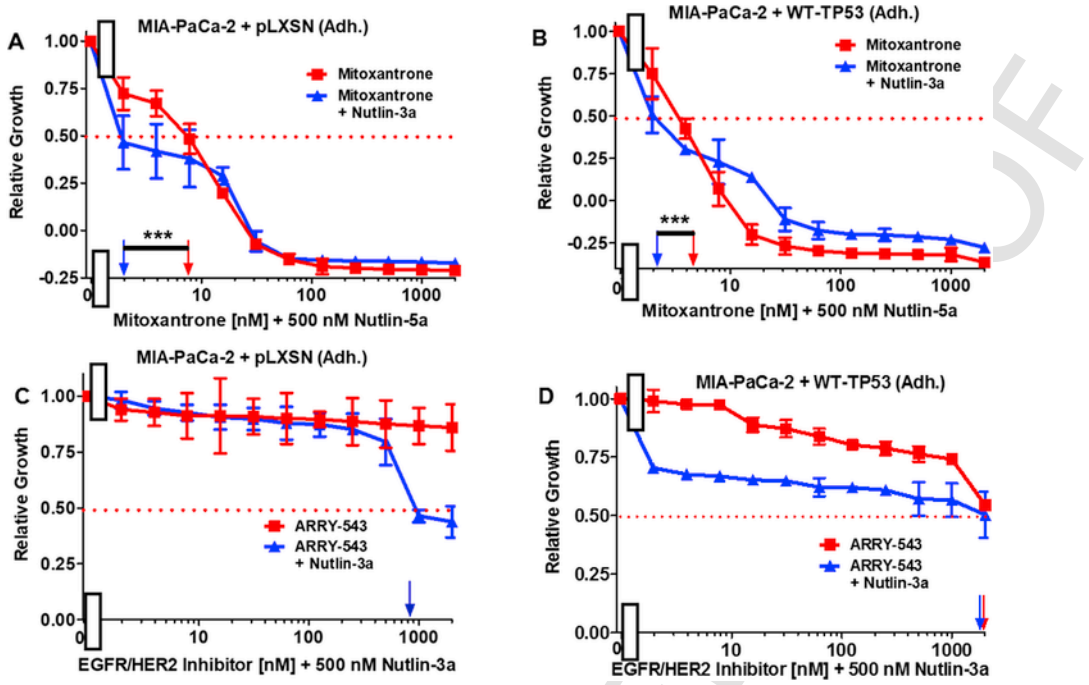

Fig. 5. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Mitoxantrone and the EGFR/HER2 Inhibitor ARRY543 in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of mitoxantrone (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value equals 0.0002 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with mitoxantrone and nutlin-3a and is extremely statistically significant. In Panel B, the two-tailed *P* value equals 0.0009 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with mitoxantrone and nutlin-3a and is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of ARRY543 (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Enhancement of effects of chemotherapeutic drugs upon inclusion of the MDM-2 inhibitor Nutlin-3a in MIA-PaCa-2 cells in the presence and absence of WT-TP53

A goal of our studies was to examine the effects on the MDM-2 inhibitor nutlin-3a on the sensitivity of MIA-PaCa-2 cells to chemotherapeutic drugs, signal transduction inhibitors and nutraceuticals in the presence and absence of WT-TP53. Previously, we observed that the sensitivity of MIA-PaCa-2 cells containing WT-TP53 to 5-fluorouracil and doxorubicin could be enhanced by inclusion of 250 nM nutlin-3a (Abrams et al., 2018). In this same study, we also observed that the sensitivities of MIA-PaCa-2 cells containing or lacking WT-TP53 to the proteasomal inhibitor MG132 or the PI3K/mTOR inhibitor NVP-BEZ235 were enhanced by the addition of 250 nM nutlin-3a (Abrams et al., 2018). Thus, there are also mechanisms by which nutlin-3a can sensitize cells to certain signal transduction inhibitors in the absence of WT-TP53. It should be pointed out that the MIA-PaCa-2 cells have the R248W *TP53* GOF mutation. The effects of nutlin-3a in cells containing *TP53* GOF mutation have not been well investigated. In the current study, we increased the sub-IC_{50} dose of nutlin-3a to 500 nM to determine if the higher concentration of nutlin-3a might induce more effects on the IC_{50} of various drugs in cells which either had or lacked WT-TP53. A dose of 500 nM nutlin-3a is a sub-IC_{50} dose for these cells (Fig. 2, Panel A).

Nutlin-3a treatment will increase TP53 activity in cells with WT-TP53. This can result in the enhancement of miRs including miR-34a, miR-192, miR-194, and miR-215 (Pichiorri et al., 2010; Rihani et al., 2015). These miRs may regulate key genes involved in proliferation, apoptosis, drug resistance, epithelial mesenchymal transition (EMT), invasion and metastasis.

In this study, we demonstrated that infection of MIA-PaCa-2 cells with a retrovirus encoding WT-*TP53* increased their sensitivity to: gemcitabine, cisplatin, docetaxel, irinotecan, mitoxantrone, vismodegib, chloroquine, and berberine but not resveratrol. In addition, the addition of 500 nM nutlin-3a sensitized the MIA-PaCa-2 cells to many chemotherapeutic drugs, signal transduction inhibitor and natural products in the presence and absence of WT-TP53.

Our studies indicate that introduction of WT-TP53 into PDAC cells which had GOF *TP53* mutations resulted in increased sensitivity to gemcitabine. Mutant *TP53* has been shown previously to increase the resistance of PDAC cells to gemcitabine (Fiorini et al.,

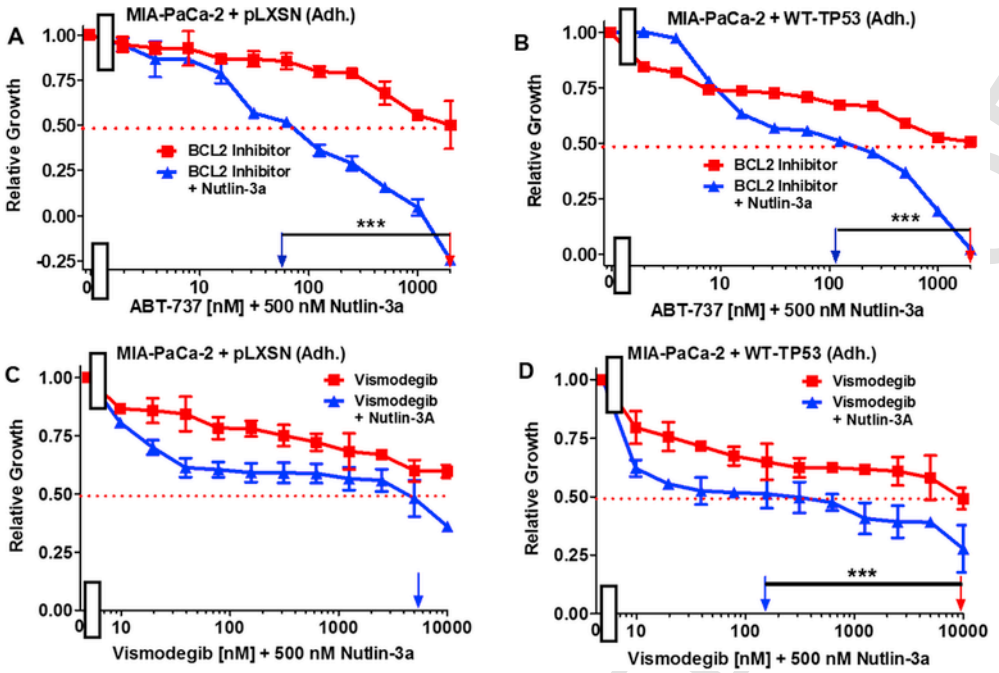

Fig. 6. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to ABT-737 and Vismodegib in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of ABT-737 (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value equals 0.0002 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with irinotecan and nutlin-3a and is extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with cisplatin and nutlin-3a and is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of vismodegib (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel D, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with vismodegib and nutlin-3a and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2015). In these studies, gemcitabine was observed to stabilize mutant TP53 proteins in the nuclei. This resulted in expression of Cdk1 and cyclin B1. Treatment with p53-reactivating molecules (CP-31398 and RITA) led to suppression of growth and induction of apoptosis in PDAC cells which had either WT or GOF mutant *TP53* but not in *TP53*-null cells. Co-treatment with gemcitabine and the TP53-reactivating compounds (See Section 4) had synergistic effects which were observed in both WT and mutant *TP53*-containing cell lines, but these synergistic effects were not observed in *TP53*-null cells. Gemcitabine and TP53-reactivator treatment resulted in phosphorylation of TP53 at Ser15, apoptosis and autophagosome formation. Suppression of autophagy was determined to enhance the effects of gemcitabine and the CP-31398 treatment. These studies demonstrate the potential for combined therapy of gemcitabine, TP53-reactivators and autophagy inhibitors in PDAC tumors which are either WT-*TP53* or GOF-*TP53* mutant, but not in *TP53*-null PDAC cells.

Loss of WT-TP53 activity induces the JAK/STAT pathway and gemcitabine-resistance in PDAC mouse models (Wörmann et al., 2016). This has been associated with PDAC growth and stromal modification. Moreover, loss of TP53 activity and elevation of JAK2/STAT signaling was associated with poor patient survival.

The effects of a novel MDM2 inhibitor PXN822 and nutlin-3a were examined on murine pancreatic cancer cells that differed in their presence and absence of WT-TP53 (Conradt et al., 2013). The authors observed that PXN822 treatment could synergistically enhance the sensitivity to etoposide in a TP53-indepenent fashion. Also, the authors demonstrated that PXN822 could enhance the sensitivity to doxorubicin. Both etoposide and doxorubicin inhibit topoisomerase II. The authors also demonstrated that MDM2 inhibitors delayed the repair of double strand DNA breaks. MDM2 was determines to be involved in the repair of etoposide-induce DNA double breaks. MDM2 was determined to bind the MRE11–RAD50–Nijmegen breakage syndrome (NBS) 1 DNA repair complex at NBS1 (Nibrin).

We observed that introduction of WT-*TP53* increased the sensitivity of MIA-PaCa-2 cells to cisplatin. Cisplatin is a chemotherapeutic drug which is being evaluated as an additional drug in combination with other drugs in PDAC therapy (Borazanci et al., 2018). The role of WT-*TP53* in sensitivity to cisplatin in PDAC cells has not been described well in the scientific literature.

Cisplatin, carboplatin and doxorubicin were demonstrated to increase the cytotoxic effects of nutlin-3a in a panel of mutant TP53 cell lines (Ambrosini et al., 2007). In TP53-mutant peripheral nerve sheath (MPNST) and TP53-null HC116 cells, nutlin-3a was determined to enhance the effects of cisplatin. In the human dedifferentiated liposarcoma LS141 and WT-TP53 HCT116, nutlin-3 induced the down regulation of the E2F transcription factor 1 (E2F1). This downregulation was determined to be proteasome-depen-

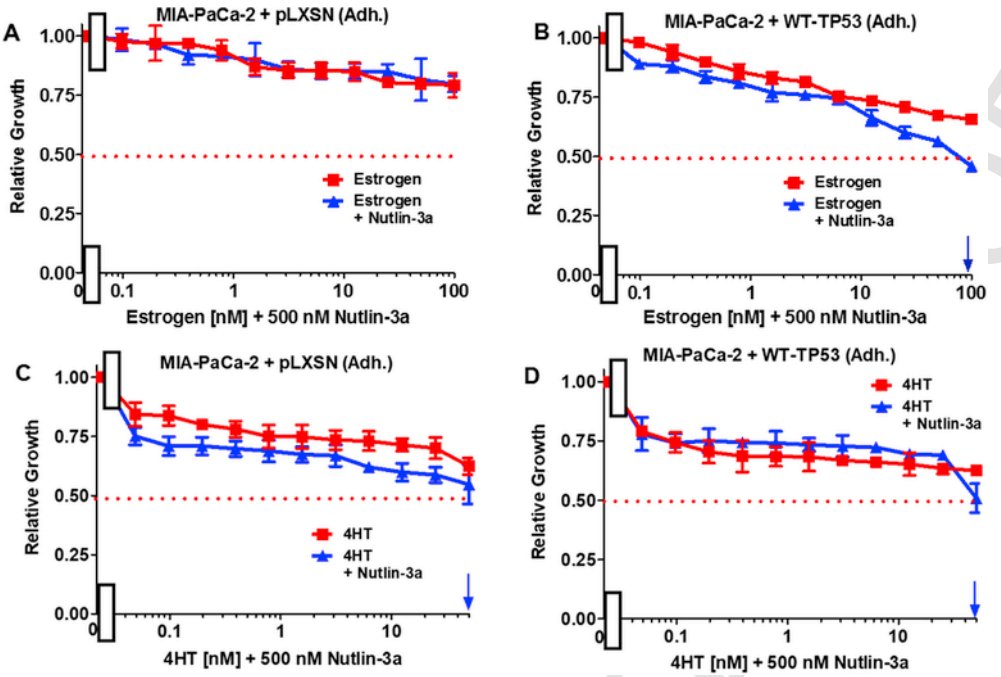

Fig. 7. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Estrogen and 4HT in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of estrogen (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of 4HT (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dent. In contrast, in MPNST and HCT116p53$^{-/-}$ cells, nutlin-3a was determined to inhibit the binding of E2F1 to MDM2. This resulted in E2F1 activation in the presence of cisplatin-induced DNA damage. E2F1 normally interacts with the retinoblastoma (RB) protein to regulate G_1/S phase transition. Nutlin-3a was postulated to induce E2F1 in cells lacking WT-TP53 in response to treatment with certain chemotherapeutic drugs. These studies point to a role for E2F1 in the sensitivity of cells with mutant TP53 to treatment with nutlin-3a and cisplatin. Interestingly, we determined that nutlin-3a could synergize with the proteasome inhibitor MG132 in a TP53-independent fashion previously in both MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) [Abrams et al., 2018].

Interestingly, combined treatment with nutlin-3a and the topoisomerase-1 inhibitor irinotecan or the platinum-based drug oxalplatin did not result in increased cell death, in fact, the combination appeared antagonistic. These results are important as irinotecan and oxaliplatin are components of the FOLFIRINOX regime.

In our study, the topoisomerase I inhibitor irinotecan did not synergize with nutlin-3a either in the presence and absence of functional TP53 in MIA-PaCa-2 or MIA-PaCa-2 + WT-TP53. In fact, treatment with nutlin-3a appeared to decrease the effects of irinotecan as an antagonistic response was observed. This lack of increased response to irinotecan could be due to many different factors. Firstly, the differences between topoisomerase I and topoisomerase II.

Topoisomerase I cleaves single strand DNA and does not need ATP. In contrast, topoisomerase II cleaves double stranded DNA and requires ATP. The topoisomerase II inhibitors, etoposide, doxorubicin and mitoxantrone may cause more DNA double strand breaks which synergize with the MDM2 inhibitor-induced delays in repair DSB. Synergy may not be present with the single strand DNA breaks induced by irinotecan and the nutlin-3a co-treatment. The reasons for antagonism are not clear. It could be a difference in drug resistance transporters induced by irinotecan in comparison to etoposide, doxorubicin and mitoxantrone. Nutlin-3a could induce some drug transporters which inhibit the uptake of irinotecan.

Also in our study, nutlin-3a did not appear to increase the response of cells to oxaliplatin. Some studies have observed that in cells with WT-TP53, synergy was observed between the MDM2 inhibitor MI-219 and oxaliplatin (Azmi et al., 2011). In these studies, only cells reported to have WT-TP53 were used, moreover quite high concentrations of the MI-219 and oxaliplatin were utilized in the co-treatment studies, 15 μM each. In our studies, only 500 nM nutlin-3a was used in the combination experiments. The IC_{50}s for treatment with oxaliplatin the absence of nutlin-3a in the absence and presence of WT-TP53 were approximately 400 nM. These lower drug doses might explain why we did not observe synergy between oxaliplatin and nutlin-3a treatment. The reasons for antagonism are not so clear. Some of the drug transporters responsible for oxaliplatin are regulated by TP53. However, it is not so clear how they are regulated in cells with TP53 GOF mutations.

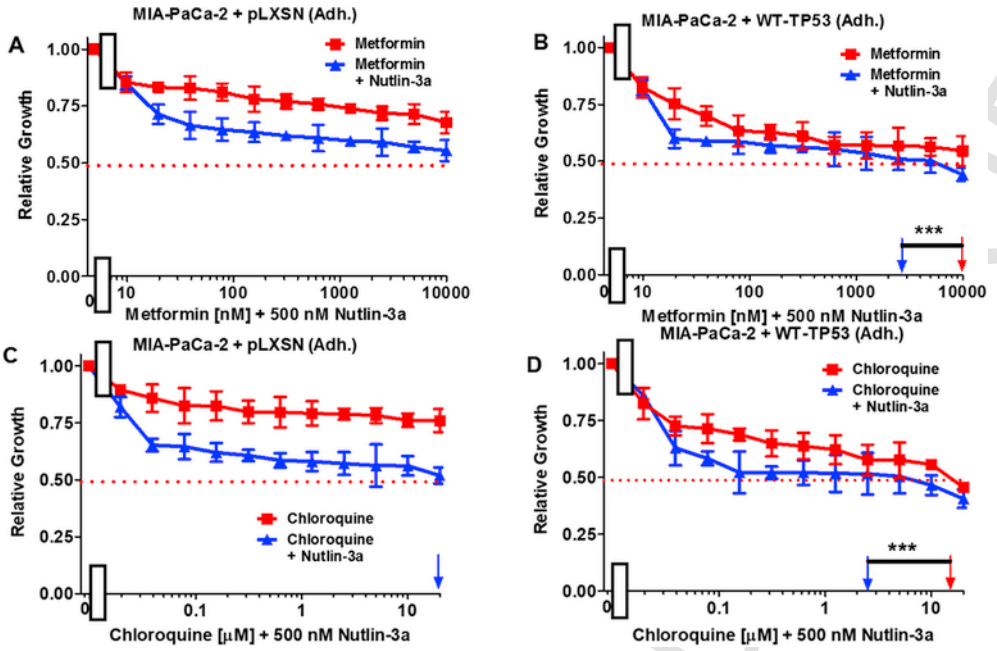

Fig. 8. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Metformin and Chloroquine in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of metformin (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with metformin and nutlin-3a and is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of chloroquine (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel D, the two-tailed *P* value equals 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with chloroquine and nutlin-3a and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Mitoxantrone is a drug that is used to treat multiple sclerosis patients, as well as, certain individuals with hematopoietic and prostate cancers. Mitoxantrone is a topoisomerase II inhibitor. We observed that introduction of WT-TP53 into MIA-PaCa-2 cells increased their sensitivity to mitoxantrone (Fig. 5, Panel B). Addition of 500 nM nutlin-3a increased the sensitivity of MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells to mitoxantrone.

Other studies have observed that nutlin-3 can reverse mitoxantrone-resistance in Saos-2 human osteosarcoma cells by inhibiting breast cancer resistance protein (ABCG2) (Zhang et al., 2011). Likewise, we observed that the topoisomerase II inhibitor mitoxantrone could also synergize with the MDM2 inhibitor nutlin-3a in suppressing the growth o MIA-PaCa-2 and MIA-PaCa-2 + WT-TP53 cells in a TP53-independent fashion.

Nutlin-3a had some effects on both MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells when they were treated with the EGFR/HER2 inhibitor ARRY-543 (Fig. 5, Panels C & D). While MIA-PaCa-2 + pLXSN (Adh.) cells were not very sensitive to ARRY-543 when they were treated with up to 2000 nM, when they were treated with both ARRY-543 and nutlin-3a, they became more sensitive and an IC_{50} of approximately 800 nM was observed. An IC_{50} of approximately 2,000 nM was observed when MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with ARRY-543. While the IC_{50} did not change when these cells were treated with ARRY-543 and nutlin-3a, the relative level of growth decreased indicating that the nutlin-3a inhibitor was having more effects in the cells which had WT-TP53. Various miRs regulate EGFR expression (Yin et al., 2013; McCubrey et al., 2018). The effects of combining EGFR inhibitors and various TP53 activating agents could be an important clinical approach as EGFR is frequently abnormally expressed in various cancers.

Nutlin-3 treatment sensitized both MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells to the BCL2/BCLXL inhibitor ABT-737. BCL2 is a target of various miRs including miR-34a (Ji et al., 2008, 2009). It is conceivable that nutlin-3a treatment increased TP53 activity which sensitized the cells to the ABT-737 BCL2/BCLXL inhibitor.

Vismodegib is a hedgehog (Hh) pathway inhibitor. Vismodegib inhibits sonic hedgehog (SHH) which is a critical ligand responsible for activation of the pathway. Vismodegib is used in the treatment of certain basal cell carcinomas and has been investigated in pancreatic cancer patients (Kumar et al., 2015). Treatment with nutlin-3a increased the sensitivity of both MIA-PaCa-2 + pLXSN and MIA-PaCa-2 + TP53 (WT) cells to vismodegib. miR-602 and miR-608 have been shown to regulate SHH expression (Akhtar et al., 2015). Combining vismodegib with WT-TP53 reactivating agents could become an effective therapeutic approach for certain cancers.

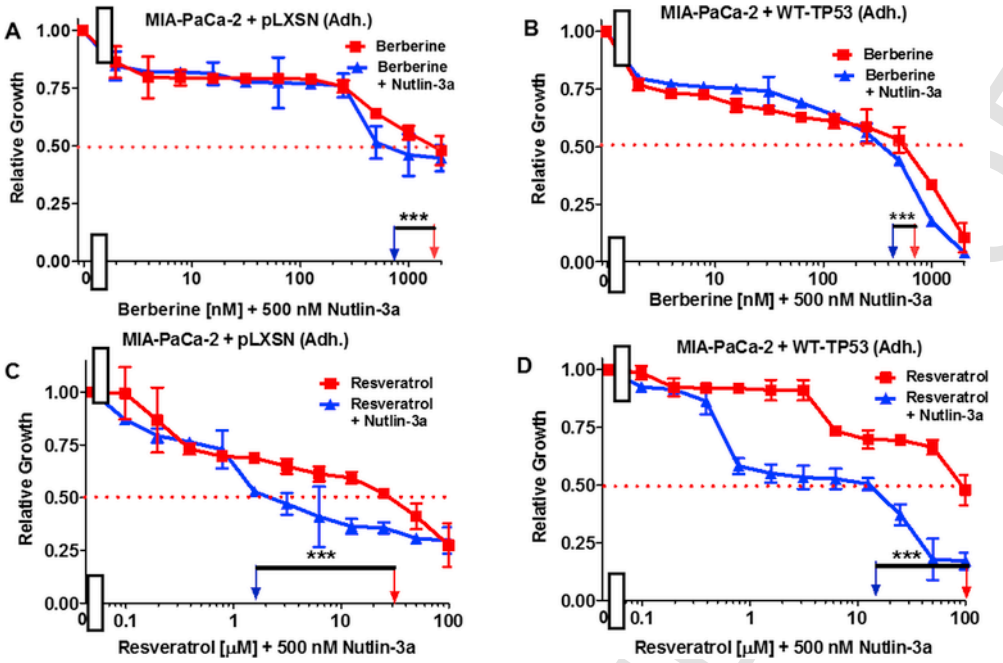

Fig. 9. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Berberine and Resveratrol in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of berberine (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + pLXSN (Adh.) cells treated with irinotecan and nutlin-3a and is considered extremely statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with berberine and nutlin-3a and is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 3 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of resveratrol (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel C, the two-tailed P value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with resveratrol and nutlin-3a and is extremely statistically significant. In Panel D, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with resveratrol and nutlin-3a and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In contrast, addition of nutlin-3a to either estrogen or 4HT treated cells had a relatively minor effect on their IC_{50}s (Fig. 7). Although the experiments with estrogen and 4HT did not yield impressive results with MIA-PaCa-2 + pLXSN cells, they do serve as a control in the sense that they confirm that 500 nM nutlin-3a treatment was not toxic to the cells. Addition of 500 nM nutlin-3a did increase the sensitivity of MIA-PaCa-2 + WT-TP53 (Adh.) cells to both estrogen and 4HT.

Nutlin-3a had some modest effects on cells treated with metformin and chloroquine. Metformin can have effects on miR-34 and other miRs (Arunachalam et al., 2016; Bao et al., 2012; Do et al., 2014; Cifarelli et al., 2015; Noren Hanel et al., 2016; Wang et al., 2018; Wu et al., 2018). Chloroquine can activate TP53 and induce apoptosis in glioblastoma cells (Kim et al., 2010). Addition of nutlin-3a could enhance the effects of metformin and chloroquine.

The effects of the nutraceuticals such as berberine and curcumin on various human diseases and conditions have been summarized recently (McCubrey et al., 2017, 2018; Candido et al., 2018). The roles that WT-*TP53* or mutant *TP53* play in the sensitivities to nutraceuticals such as berberine, resveratrol and curcumin remain controversial. Some studies have proposed important functions for WT-TP53 activity in sensitivity to the nutraceuticals (Choi et al., 2009; Katiyar et al., 2009; Chuang et al., 2017). To our knowledge the effects of nutlin-3a on the sensitivities of PDAC cells to various nutraceuticals in the presence and absence of WT-TP53 have not been examined.

Other studies have not demonstrated requirements of WT-TP53 activity for the effects of berberine (Wang et al., 2012; Liu et al., 2013). Also, the various cells and cell lines examined may have different types of *TP53* mutations. The differences in the requirement for WT-TP53 activity may reside in the choice of cells or cell lines which were compared, as well as, the concentrations of berberine used in the studies. Interestingly, some studies have indicated roles for the TP53-regulator MDM2 in the sensitivity of cells to berberine (Zhang et al., 2010). Berberine may induce TP53 in some cells which leads to induction of miRs important in regulation of cell growth (Wang et al., 2014). We did observe that introduction of WT-TP53 did increase the sensitivity of the MIA-PaCa-2 cells to berberine. In contrast, introduction of WT-TP53 made the MIA-PaCa-2 + WT-TP53 cells more resistant to resveratrol. Addition of nutlin-3a enhanced the effects of berberine in the presence and absence of WT-TP53. Likewise, treatment with 500 nM nutlin-3a increased the effects of resveratrol.

Some studies have observed the effects of curcumin are TP53-dependent (Jee et al., 1998; Choudhuri et al., 2002, 2005). Other studies have observed the effects of curcumin are independent of functional WT-TP53 (Watson et al., 2010). We observed that intro-

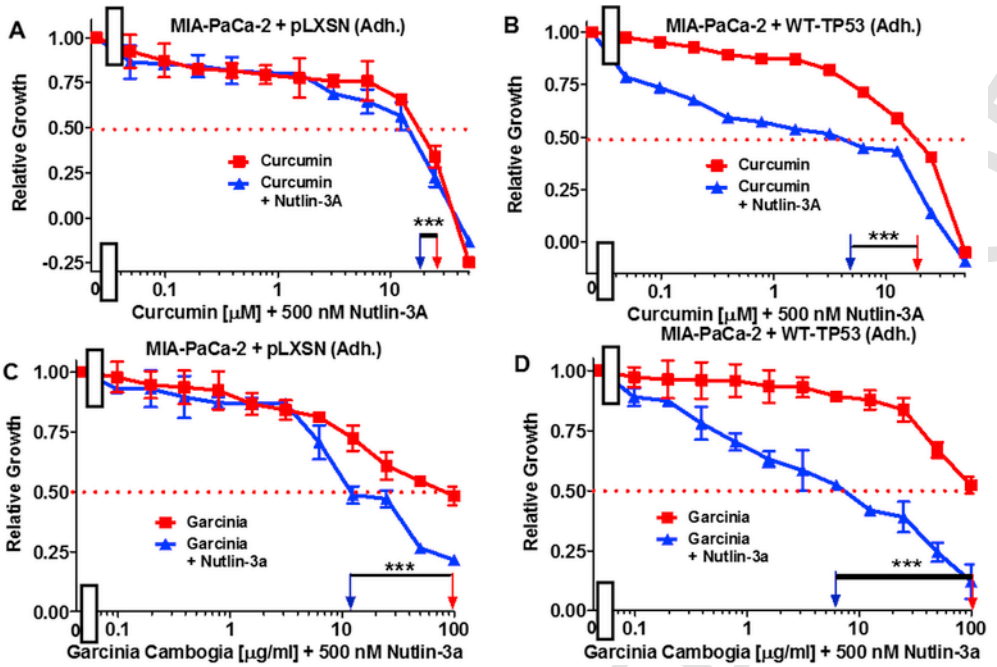

Fig. 10. Effects of Introduction of WT-*TP53* Gene on the Sensitivity of MIA-PaCa-2 Cells to Curcumin and Garcinia Cambogia in the Presence and Absence of Nutlin-3a. In Panel A) MIA-PaCa-2 + pLXSN (Adh.) and in Panel B) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of curcumin (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel A, the two-tailed *P* value between MIA-PaCa-2 + pLXSN (Adh.) cells treated with curcumin and nutlin-3a equals 0.0012 which is considered to be very statistically significant. In Panel B, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with curcumin and nutlin-3a and is extremely statistically significant. The experiments in Panels A and B were performed on the same days. These experiments were repeated 4 times and similar results were observed. In Panel C) MIA-PaCa-2 + pLXSN (Adh.) and in Panel D) MIA-PaCa-2 + WT-TP53 (Adh.) cells were treated with different concentrations of garcinia cambogia (solid red squares) and a constant dose of 500 nM nutlin-3a (solid blue triangles) and then MTT analysis was performed after 4 days. In Panel C, the two-tailed P value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with garcinia cambogia and nutlin-3a and is extremely statistically significant. In Panel D, the two-tailed *P* value is less than 0.0001 between MIA-PaCa-2 + WT-TP53 (Adh.) cells treated with garcinia cambogia and nutlin-3a and is extremely statistically significant. The experiments in Panels C and D were performed on the same days. These experiments were repeated 4 times and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

duction of WT-TP53 resulted in a 1.5-fold increase in sensitivity to curcumin. Upon addition of 500 nM nutlin-3a, the IC_{50}s dropped approximately 1.5–4-fold in MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells, respectively.

MIA-PaCa-2 + pLXSN (Adh.) and MIA-PaCa-2 + WT-TP53 (Adh.) cells had equal sensitivities to garcinia cambogia. Nutlin-3a increased the sensitivities of both cells lines to garcinia cambogia, 10 and 16.7-fold, respectively.

Treatment with nutraceuticals such as berberine, curcumin and resveratrol will result in the induction of various miRs. The induction of some of the miRs may have beneficial effects on human health (Tili et al., 2010; Bai et al., 2014; Li et al., 2014; Dhar et al., 2015; Yang et al., 2015; McCubrey et al., 2017; Mirzaei et al., 2018; Otsuka et al., 2018a; Otsuka et al., 2018b). Some of the effects of co-treatment of cells with nutlin-3a, berberine, curcumin and resveratrol may be enhanced induction of TP53 and the induction of miR by the nutraceuticals.

Our studies indicate that some PDAC cells will be sensitive to the MDM2 inhibitor nutlin-3a even in the absence of WT-TP53. Our studies are important as they may expand the types of cancers that could be treated with MDM2 inhibitors. Additional studies to confirm the "roles" of the TP53 GOF mutation in these cells in the sensitivity to MDM2 inhibitors are important.

Conflicts of interest

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

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