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Minnelide Overcomes Oxaliplatin Resistance by Downregulating DNA Repair Pathway in Pancreatic Cancer

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Abstract

Introduction—Oxaliplatin is part of pancreatic cancer therapy in FOLFIRINOX or GEMOX/XELOX regimen. DNA damage repair is one of the factors responsible for oxaliplatin resistance that eventually develops in this cancer. Triptolide/Minnelide has been shown to be effective against pancreatic cancer in preclinical trials. In this study, we evaluated the efficacy of combination of triptolide and oxaliplatin against pancreatic cancer.

Methods—Highly aggressive pancreatic cancer cells (MIA PaCa-2 and PANC-1) were treated with oxaliplatin (0–10 μ M), low dose triptolide (50nM) or a combination of both for 24–48h. Cell viability, apoptosis and DNA damage was evaluated by appropriate methods. Nucleotide excision repair pathway components were quantitated using qPCR and western blot. Combination of low doses of Minnelide and oxaliplatin was tested in an orthotopic murine model of pancreatic cancer.

Results—Proliferation of pancreatic cancer cells was markedly inhibited by combination treatment. Triptolide potentiated apoptotic cell death induced by oxaliplatin and sensitized cancer cells towards oxaliplatin induced DNA damage by suppressing oxaliplatin induced DNA damage repair pathway. Combination of low doses of Minnelide and oxaliplatin inhibited tumor progression by inducing significant apoptotic cell death in these tumors.

Conclusions—Combination of low doses of Minnelide and oxaliplatin has immense potential to emerge as novel therapeutic strategy against pancreatic cancer.

Keywords

Oxaliplatin; Minnelide; triptolide; NER; DNA damage pancreatic cancer

Introduction

Pancreatic cancer was the 12th most common cancer in the United States in 2014 and yet this malignancy is the fourth leading cause of cancer-related mortality in the United States.

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Conflict of interest

University of Minnesota has filed a patent for Minnelide, which has been licensed to Minneamrita Therapeutics, LLC. AKS is the co-founder and the Chief Scientific Officer of this company.

The 5-year survival rate (2004-2010) is estimated at 6.7%, not a substantial change from 6.2% in 2002 [1]. Although, significant advances have been made in major pancreatic resections, only few patients (10-15%) have a chance to undergo radical surgery as most of the patients present with advanced disease. Additionally, most patients after surgery will develop disease recurrence within the next 2 years [2]. Multiple drugs have been approved as chemotherapy in pancreatic ductal adenocarcinoma (PDAC) recently, like nab-paclitaxel [3]. Platinum compounds are used either as part of a first line regimen (FOLFIRINOX) or a second line (GEMOX, 5-FU/OX) therapy. Despite these advances, overall survival has increased from 6.8 months (gemcitabine alone) to a mere 11.1 months (FOLFIRINOX) [4]. Future research should focus on the development of novel chemotherapy regimens based on the evolving understanding of molecular events in drug resistance.

Platinum compounds including oxaliplatin, display their cytotoxic action by forming DNA adducts (intra and inter-strand crosslinks) contorting the DNA and subsequently causing DNA damage. This DNA kinking is recognized and primarily repaired by the nucleotide excision repair (NER) pathway which contributes to acquired or intrinsic resistance to platinum based therapies [5-7]. NER has a broad spectrum of specificity with no differences observed in the excision of adducts formed by different platinum drugs suggesting that this mechanism of resistance may not be overcome by structurally changing platinum compounds [8]. ERCC1 and, to a lesser extent, other NER pathway proteins (XPF, XPA and XPD) have been associated with poor response and resistance to platinum therapy in various cancers, including PDAC [9-12].

We have previously shown that triptolide (TPL), a diterpenoid triepoxide extracted from the Chinese herb *Tripterygium wilfordii* and its water-soluble prodrug (Minnelide) induces apoptosis in pancreatic cancer cells, both *in vivo* and *in vitro* [13, 14]. Triptolide has also been shown to enhance the cytotoxic effects of other chemotherapeutic agents including platinum agents in various cancers [15-21]. However, the effects of triptolide on the NER pathway and its role in overcoming platinum resistance in pancreatic cancer have not been clearly elucidated until now [15-19].

In this study, we have evaluated the effects of the combined therapy of oxaliplatin and triptolide, on pancreatic cancer *in vitro* and *in vivo* along with its effects on NER DNA damage repair pathway.

Materials and Methods

Cell culture and reagents

MIA PaCa-2 and PANC-1 cells were obtained from the American Type Culture Collection. Both cell lines were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). All cell lines were grown under standard conditions at 37°C in a humidified atmosphere containing 5% CO₂. DMEM, FBS and PS were obtained from Invitrogen life technologies (MA). Triptolide (TPL) (Calbiochem, EMD Millipore, MA) was dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mg/ml stock and stored at -20°C. For drug treatment, triptolide stock was diluted in growth medium and added as described below. Oxaliplatin (Sigma Aldrich, MO) was

dissolved in DMSO to obtain a 12.5 mM stock and stored at -20°C . For drug treatment, oxaliplatin was diluted in medium and added in indicated concentrations as described below.

Determination of cell viability and analysis of combination effect

MIA PaCa-2 and PANC-1 (4×10^3 cells/well) were seeded into 96-well plates and allowed to adhere for 48 hours at 37°C . Dojindo Cell Counting Kit-8 (Dojindo Molecular Technologies, MD) was used to measure cell viabilities after drug treatments at indicated concentrations for 24 and 48 hours. Briefly, $10\mu\text{L}$ of the tetrazolium substrate was added to each well of the plate and was allowed to incubate at 37°C for 1 hour, after which the absorbance at 450 nm was measured. All experiments were done in triplicates and repeated three independent times. The cell viability rate was calculated by $(\text{OD experiment}/\text{OD Control}) \times 100\%$.

Isobologram analysis was used to determine whether the combination of triptolide and oxaliplatin had a synergistic, additive or antagonistic effect on pancreatic cancer cells. As the combination of oxaliplatin and triptolide was used in a non-constant ratio, a normalized isobologram for the two drugs was constructed automatically using CompuSyn ver. 1.0 (ComboSyn Inc., Paramus, NJ). In this analysis, an additive effect (if points fall on the hypotenuse), synergism (if points fall in the lower left), or antagonism (if points fall in the upper right) is indicated according to the positions of the combination data points in the normalized isobologram. The combination index (CI), also calculated in CompuSyn provided a way to analyze the combined effects using a median-effect plot analysis. CI values <1.0 , $=1.0$, and >1.0 indicate synergistic, additive, and antagonistic effects, respectively.

Flow cytometry detection of cleaved poly ADP-Ribose Polymerase (PARP), active caspase-3 and $\gamma\text{H2A.X}$

MIA PaCa-2 and PANC-1 (7×10^5 cells/plate) were seeded in 10-cm plates, allowed to adhere for 48 hours and treated with triptolide and oxaliplatin at indicated concentrations. Cells were harvested after 24 hours and incubated in fixation/permeabilization solution (BD cytofix/cytopermTM) for 30 minutes on ice. These fixed cells were then stained with PE conjugated monoclonal antibody for cleaved PARP (Asp 214, BD Biosciences, CA), FITC conjugated active caspase-3 (Asp 175, BD Biosciences, CA) and Alexa flour 647 conjugated monoclonal antibody for $\gamma\text{H2A.X}$ (BD Biosciences, CA) for 30 minutes. Cells were then washed and FACS analyses were performed on a BD FACS Canto II (BD Biosciences, CA) using FACS Diva and FlowJo (Tree Star) software. All experiments were repeated three independent times.

Quantitative real-time polymerase chain reaction for ERCC1, XPA, XPB, XPC, XPD, XPF and XPG

MIA PaCa-2 and PANC-1 (7×10^5 cells/plate) were seeded in 10 cm plates and allowed to adhere for 48 hours at 37°C before treatment. Triptolide and oxaliplatin were then added at indicated concentrations and cells were collected after 24 hours of drug treatment. RNA was extracted from cell lines using TRIzol (Life Technologies, MA) reagent according to the manufacturer's instructions. Total RNA ($1\mu\text{g}$) was used to make cDNA using high capacity

cDNA reverse transcription kit (Applied Biosystems, NY). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen, CA) according to the manufacturer's instructions. The 2^{-C_t} method was used to calculate the relative expression levels by normalizing to the housekeeping gene 18S. ERCC1, XPA, XPB, XPC, XPD, XPF, XPG and 18S primers were purchased from Life Technologies (MA). Following primer sequences were used:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ERCC1	GAC TGA ATG TCT GAC CAC CG	CTG GGG TCA TCA GGG TAC TT
XPA	GTG TGG CCA TGA ACT GAC A	TGC ACC TAC TCT AGC ACT CA
XPB/ERCC3	ACC GCG ACA AGA AGA AAT CC	CTG ACT CAT CCA CCT GCT TC
XPC	GCC CAT TTA TGG ACA GGG AG	CTC CAC GAC AAT ACC CAA GG
XPD/ERCC2	CCT GGA GCA GCT AGA ATC AG	CTC TGG GTA CCT GGT GGA TA
XPF/ERCC4	CTG GTC CTA GAA AGC AAC CC	CAC CAA GAG CTT CAC TCT CC
XPG/ERCC5	GGA CAT CTT CTG GCT GTT TCC	AGC GCA TTA AAG CGG AGA G

All experiments were done in duplicates and repeated three independent times.

Western blot

MIA PaCa-2 and PANC-1 (7×10^5 cells/plate) were seeded in 10 cm plates and allowed to adhere for 48 hours at 37°C before treatment. Triptolide and oxaliplatin were then added at indicated concentrations, and cells were collected after 24 hours of drug treatment. Cell lysates were prepared by removing the culture media, washing the cells with phosphate buffered saline and then adding Rapid Immuno Precipitation Assay Buffer (Boston BioProducts, MA) with protease (20µL per milliliter of lysis buffer) and phosphatase (10µL per milliliter of lysis buffer) inhibitors at 4°C. The cells in lysis buffer were then scraped from the plates and placed in appropriately labeled microcentrifuge tubes, which were centrifuged at 10,000 rpm for 10 minutes. The total protein content of the supernatant was determined using the Pierce bicinchoninic acid protein estimation kit (ThermoScientific, MA) with bovine serum albumin serving as standards. SDS-PAGE was carried out by loading 40µg protein in 7.5% gels and the gel was transferred to a nitrocellulose membrane using wet transfer method. The membranes were then probed with XPA, XPB, XPC, ERCC1, XPD and XPF antibodies. XPA, XPB and XPC antibodies were obtained from Abcam (MA) and ERCC1, XPD and XPF antibodies were obtained from Cell signaling (MA). Equal protein loading was confirmed by staining with Ponceau S (0.1% Ponceau S [w/v]) (Sigma-Aldrich, MO, USA) in 5% acetic acid [v/v]. Actin expression was used as an internal control. All experiments were repeated two independent times.

Determination of AP1 promoter activity by luciferase reporter assay

AP1 dual luciferase assay was performed using Cignal AP1 reporter kit (Qiagen, CA, USA) according to manufacturer's instruction. Briefly, MIA PaCa-2 and PANC-1 (5×10^4 cells/well) after seeding in a 24 well plate were transfected with 4µL of AP1 reporter plasmid, luciferase negative control and luciferase positive control using attractene transfection

reagent (Qiagen, CA). After 12 hours of transfection, cells were treated with various drugs in DMEM+1% FBS. The luciferase activity was determined 18 hours after drug treatment using a dual-luciferase reporter assay system (Promega, WI), the signal was normalized to Renilla luciferase and values were expressed as relative luciferase units (RLUs).

PDAC orthotopic model

Athymic nude female mice (4–6 weeks old; Charles River Laboratories) were used for the PDAC orthotopic murine model. All procedures were carried out according to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee. Briefly 1×10^5 MIA PaCa-2 cells mixed with Matrigel (BD Biosciences, CA) were injected into the tail of pancreas through a paramedian incision. After 3 weeks, tumor bearing mice were randomized into four treatment groups (vehicle 50 μ L DMSO/week i.p, Minnelide 0.15 mg/kg/day i.p, oxaliplatin 6 mg/kg/week i.p and combination of Minnelide 0.15 mg/kg/day i.p and oxaliplatin 6 mg/kg/week i.p with 7 mice in each group). Treatment was continued for 33 days and animals were euthanized on 34th day.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay for *in situ* apoptosis

Paraffin-embedded PDAC xenograft tissue sections from treated mice were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using In Situ Cell Death Detection Kit, Fluorescein (Roche, IN, USA) according to the manufacturer's instructions. Counterstaining for total cells was done with propidium iodide (PI). Coverslips were applied and fixed with permount. Images were taken on a confocal microscope (Nikon, NY) using a magnification of 20X for overview and 100X for quantifying the images. 3 random high power (100X) field images were taken per tumor section slide. The images were quantified using ImageJ software (NIH, MD) and the mean was calculated for the ratio of total TUNEL positive cells to total PI positive cells.

Statistical analysis

Values are expressed as mean $\pm$ SEM. The significance of the difference between the different experimental arms was analyzed by using one way ANOVA with post-hoc test, unpaired student's *t*-test and two sided $P < 0.05$ was considered statistically significant. Data expressed in fold change was analyzed after converting it to the corresponding log₁₀ values.

Results

Treatment with triptolide sensitized pancreatic cancer cells to oxaliplatin

To study the effect of the combination of triptolide and oxaliplatin, we selected two different pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) reported to be resistant to platinum drugs [22]. Effective dose 50 (ED50) for oxaliplatin was determined for both the cell lines following treatment with a wide dose range of this drug. Our results indicated that the ED30 for oxaliplatin for MIA PaCa-2 and PANC-1 was around 7 μ M. We used a lower dose than this dose at 5 μ M. Previous studies from our laboratory indicated that 50nM dose of triptolide resulted in approximately 50% viable MIA PaCa-2 and PANC-1 cells after 48h of treatment. Treatment with this low dose of triptolide (50nM) alone resulted in the expected 50% cell

death, and oxaliplatin (5 μ M) alone did not result in an appreciable decrease in viability of MIA-PaCa-2 and PANC-1 cells in 48h. However, upon combination, a significant decrease in cell viability was observed (**Figures 1a** and **1b**). An isobologram analysis indicated that the combination of triptolide and oxaliplatin was synergistic in nature with a combination index of 0.2-0.7 in both MIA-PaCa-2 (**Figures 1c** and **1e**) and PANC-1 cells. (**Figures 1d** and **1f**).

Combined therapy with triptolide and oxaliplatin increases apoptosis

Apoptosis is the main mechanism by which oxaliplatin induces cell death [5, 7]. We have previously shown that triptolide induces apoptotic cell death in pancreatic cancer cells [13, 23]. Therefore, we examined the effect of combined therapy with triptolide and oxaliplatin on apoptosis. We used cleaved caspase-3 and cleaved PARP levels as markers of apoptosis, which were detected by flow cytometry. Our results show that after 24h of treatment, single agent therapy with 50nM triptolide and 4 μ M oxaliplatin on MIA PaCa-2 (**Figures 2a** and **2b**) and PANC-1 (**Figures 2c** and **2d**) had minimal effect on apoptosis. However, when these pancreatic cancer cell lines were exposed to the combined therapy, the number of cells undergoing apoptosis increased significantly.

Combined therapy with triptolide and oxaliplatin results in increased DNA damage

Since platinum based drugs are known for inducing DNA damage, we next studied the expression of γ H2A.X following triptolide, oxaliplatin and the combination treatment. Treatment with platinum drugs is expected to cause double strand breaks in DNA as a result of formation of platinum adducts. Phosphorylation of H2A.X at S139 is the first response of the cell with the damaged DNA as a result of the drug treatment. This facilitates the recruitment of DNA damage repair proteins to the site of the damage in order to repair the DNA break. However, cells resistant to platinum therapy tend to overexpress the DNA damage repair proteins in order to overcome this deleterious effect. Increase in the expression of phosphorylated γ H2A.X is typically used as a measure of increased DNA damage. Our results showed that when MIA PaCa-2 (**Figure 3a**) and PANC-1 (**Figure 3b**) cell lines were treated with 4 μ M oxaliplatin, had increased expression of γ H2A.X after 24h compared to control. Since triptolide does not cause any DNA damage, treatment with it did not significantly alter the levels of γ H2A.X. When the combination of triptolide and oxaliplatin at the indicated doses was used, an increased expression of γ H2A.X compared to either drug alone was observed. Cells treated with SN-38 (250 nM) served as positive control for this experiment. Based on this observation we concluded that oxaliplatin treatment did cause an increased DNA damage, however, in combination with triptolide this damage was further augmented.

Triptolide in combination with oxaliplatin augmented DNA damage by suppressing Nucleotide Exchange Repair (NER) pathway

To understand how triptolide augmented the DNA damage induced by oxaliplatin, we next studied the Nucleotide Exchange Repair (NER) pathway. NER pathway is composed of a number of proteins that form the repair complex. To assess the effect of triptolide and/or oxaliplatin on the rate-limiting proteins of NER pathway (XPA, XPB, XPC, XPD, XPG,

XPF and ERCC1), we evaluated the mRNA and protein expression levels of these components after treating MIA-PaCa2 and PANC-1 cells with triptolide and oxaliplatin. Our results showed that mRNA expression of NER pathway components in MIA PaCa-2 (**Figures 4a** and **4b**) and PANC-1 (**Figures 4c** and **4d**) cells increased after treatment with oxaliplatin (4 μ M) while they decreased after treatment with 50nM triptolide. Interestingly, when treated with a combination of triptolide and oxaliplatin, the expression of the NER pathway components showed an overall decrease. Thus, triptolide (50 nM) treatment abrogated oxaliplatin induced increase in the mRNA levels of the above mentioned component proteins of NER.

This was further confirmed when we studied the protein expression of XPA, XPB, XPC, ERCC1, XPD and XPF that are key components of the NER pathway. Oxaliplatin treatment led to an increase in expression of these proteins (**Figure 5a**) while triptolide therapy led to a decreased expression (**Figure 5b**).

Triptolide sensitizes pancreatic cancer cells to oxaliplatin by inhibiting AP1 activity

One of the resistance mechanisms to oxaliplatin is mediated via overexpression of the DNA repair protein ERCC1, which in turn is induced by the transcription factor AP1 [24]. Since ERCC1 was overexpressed following treatment with oxaliplatin, and it decreased following treatment with triptolide, we next examined the effect of these drugs on the AP1 transcription activity. To examine this, we measured AP1 transcriptional activity in MIA PaCa-2 (**Figure 6a**) and PANC-1 (**Figure 6b**) cell lines using a dual luciferase based reporter assay. Oxaliplatin therapy increased AP1 transcriptional activity in both cell lines after 18 hours of treatment (as expected), while triptolide inhibited AP1 transcriptional activity. When added in combination, AP1 activity was significantly decreased. Cells treated with PMA (Phorbol 12-myristate 13-acetate) (10 ng/mL) served as positive control.

Combination of Minnelide and oxaliplatin was superior in inhibiting PDAC tumor xenograft progression than either drug alone

Combination of Minnelide (water soluble prodrug of triptolide) and oxaliplatin was tested in a murine orthotopic tumor model of pancreatic cancer. PDAC tumors treated with the combination of Minnelide and oxaliplatin were significantly smaller in volume and weight as compared to tumors in other treatment groups (**Figures 7a** and **7b**). Animals treated with vehicle and oxaliplatin developed ascites and were morbid looking while animals treated with combination therapy did not have any cancer associated morbidity. Body weight of animals was used as a marker for possible serious adverse effects of therapy. Animals were weighed before and after the end of treatment (**Figure 7c**). TUNEL assay was used as a DNA damage and apoptosis marker and tumors treated with the combination therapy had significantly higher TUNEL positive cells as compared to tumors in other treatment groups (Vehicle: 7.3 ± 1 ; Minnelide 0.15 mg/kg/day: 12.1 ± 1.9 ; oxaliplatin 6 mg/kg/week: 10.1 ± 1.2 ; Minnelide 0.15 mg/kg/day + oxaliplatin 6 mg/kg/week: 68.84 ± 7.2 , $p < 0.0001$) (**Figures 7d** and **7e**).

Discussion

Platinum compounds like cisplatin and oxaliplatin are alkylating-like agents that induce cancer cell death by forming intra-strand and inter-strand platinum adducts with DNA leading to DNA damage [5, 7]. One of the early events immediately following DNA damage is phosphorylation of H2A (histone 2A) variant protein, H2A.X at serine 139, resulting in formation of γ H2A.X. “ γ H2A.X” foci represent DNA damage in a 1:1 manner in response to various cytotoxic and ionizing radiations including platinum compounds leading to their use as a biomarker to measure DNA damage [25-28]. In our study, treatment with oxaliplatin alone resulted in increased γ H2A.X compared to that seen in triptolide treatment alone. This was an expected observation, as triptolide has almost no deleterious effect on DNA. When added in combination with oxaliplatin, γ H2A.X levels increased significantly in pancreatic cancer cell lines MIA-PaCa-2 and PANC-1 (**Figures 3a** and **3b**). This suggests that triptolide either sensitizes the DNA to oxaliplatin induced damage or prevents repair of the existent damage.

It is well known that the efficacy of platinum therapy in PDAC is limited, due to inherent or acquired resistance to these drugs [29-31]. Among the molecular events, DNA damage repair pathways are one of the major factors responsible for the platinum resistance. Nucleotide Excision Repair (NER) pathway repairs the bulky DNA lesions such as those formed by UV radiation, platinum compounds and polycyclic aromatic hydrocarbons [32].

The interaction between various component proteins of NER pathway (comprised of approximately 30 proteins *in vivo* and 7 core proteins *in vitro*): XPD (ERCC2), XPB (ERCC3), XPA, RPA, TFIIH, XPG (ERCC5) and complex of XPF (ERCC4) and ERCC1 have been well worked out [33]. Following DNA damage, these proteins recognize the damage site and repair it, thereby making the cell resistant to the platinum therapy [9, 34]. ERCC1-XPF complex is the best studied component of NER pathway in relation to the role it plays in resistance to platinum therapy. *In vitro*, it has been shown that mRNA and protein levels of ERCC1 positively correlate with resistance to platinum drugs in various cancer cell lines [35-38]. Similar results have been obtained in multiple clinical studies spread across wide variety of solid organ cancers [10, 11, 39-42], including pancreatic cancer [12, 43]. These and many other similar findings have led to the recent consideration of ERCC1-XPF complex as a novel therapeutic target to overcome platinum chemoresistance [9]. As expected, we found that oxaliplatin treatment increased the mRNA and protein levels of core components of NER pathway while triptolide had a reverse effect (**Figure 5**). In combination therapy as well, triptolide was able to successfully suppress oxaliplatin induced increase in the mRNA and protein levels of these NER components (**Figures 4** and **5**), without affecting the cellular transcriptional machinery (Supplementary Figure 1). This explained the increased γ H2A.X levels in the combination therapy compared to either drug alone. We conjectured that in oxaliplatin treatment alone, the cells were undergoing DNA damage, and since ERCC1 and other NER components were overexpressed in these cells, the damaged DNA was repaired resulting in minimal effect on cell viability. Triptolide by itself did not induce any DNA damage and downregulated the expression of the NER pathway proteins. As a result, when treated in combination with oxaliplatin, there was a profound effect on both γ H2A.X expression as well as cell viability. This indicated that

MIA-PaCa-2 and PANC-1 cells though inherently capable of repairing DNA damage induced by oxaliplatin, were unable to do so in the presence of triptolide as the NER pathway was downregulated. The upregulation of NER pathway in response to the DNA damage is specific to oxaliplatin treatment as other chemotherapeutic drugs (paclitaxel and rapamycin) at similar effective concentrations did not affect this pathway (Supplementary Figure 2).

Transcriptional regulation of ERCC1 has been shown to be mediated by AP1 (activator protein-1) transcription factor, which binds to the promoter elements upstream of ERCC1 5' coding region [44]. Abolition of AP1 activity has been shown to resensitize the platinum resistant cancer cells to cisplatin [45, 46]. It has also been demonstrated that for the DNA repair system to work best, all component proteins should be up or down regulated together [6, 47, 48]. We found that oxaliplatin therapy induces the AP1 transcription activity in our system. Low concentration of triptolide (50nM) suppressed the AP1 transcriptional activity to negligible levels. A dominant triptolide effect was seen in combination therapy as well (**Figure 6**) which ultimately led to the suppression of NER proteins. This effect was specific

The synergistic effect of low doses of Minnelide and oxaliplatin was seen in orthotopic tumors derived from MIA PaCa-2 pancreatic cancer cells. *In vivo*, tumors treated with combined therapy were much smaller (**Figure 7a** and **7b**) and there was a significant increase in TUNEL positive cells in these tumors as compared to tumors treated with either drug alone (**Figure 7e** and **7f**). Additionally, there was no significant change in the body weight of mice in any treatment group (**Figure 7c**). This is important as it shows that combination of low doses of Minnelide and oxaliplatin do not have potential serious adverse effects.

Our group has previously described that triptolide and its water soluble prodrug, Minnelide (currently in Phase I trial), have been very effective in different preclinical models of pancreatic and other cancers as single agent as well as in combination with other anti-cancer agents [14, 20, 21, 49]. Our study shows that triptolide cooperates with oxaliplatin to induce apoptosis of pancreatic cancer cells *in vitro* and this synergy is mediated via suppression of the NER pathway by triptolide. We have also successfully tested the combination of low doses of both Minnelide and oxaliplatin in an orthotopic murine model of pancreatic cancer. Other groups have also shown the efficacy of combination of triptolide and platinum compounds in various cancers but ours is the first study that sheds light on the effects of triptolide on NER pathway [15-19]. A schematic representation of effects of triptolide and oxaliplatin on AP1 and NER pathway is shown in **Figure 8**. In addition to the downregulation of NER pathway, we cannot exclude that triptolide and oxaliplatin might have effects on other molecular targets in cancer cells like heat shock proteins, cell cycle regulatory proteins and other transcription factors.

Conclusions

Combination of low doses of Minnelide and oxaliplatin has immense potential to emerge as a novel therapeutic strategy against pancreatic cancer. Our findings form the foundation of future clinical trials to combat this devastating disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Primary Discussant

Jeffrey B. Matthews, M.D. (Chicago, IL)

Despite advances in surgical therapy for pancreatic cancer, we have made precious little impact on overall survival in this disease due in part to slow progress in identifying effective chemotherapeutic agents, or combinations of agents, against this difficult tumor. You have shown that Minnelide, a synthetic prodrug of triptolide, increased cell death and reduced drug resistance to oxaliplatin. These results are interesting and suggest a novel approach to combat resistance to platinum based chemotherapy. Nevertheless, our enthusiasm is always tempered by past experience. The road to successful cancer drug development is unfortunately littered with compounds that were effective in cell lines and murine models only to fail in the human disease. We eagerly await the results of your Phase 1 clinical trial.

I have two questions for you. First, because triptolide is derived from a Chinese herb I am wondering

- 1.) Whether the concentrations used in your experiments and trials are similar to what would be achieved in its use as a traditional Chinese medicine? Are there any specific toxicities and side effects?
- 2.) Regarding mechanism of action. In previous publications from your lab, you suggested that heat shock protein 70 was involved in the pro-apoptotic action of Minnelide, and other work suggests involvement of reactive oxidant species and NfKB signaling. In today's work, you focus on the nucleotide excision repair pathway. Can you connect the dots for us? Is there a single molecular target for Minnelide that might explain both its own apoptotic action and its effect on platinum resistance? ”

Closing Discussant

Dr. Modi

1. The extracts of *Tripterygium wilfordii* hook F (TWHF) contain around 80 active components with triptolide being one of them. Therefore, the direct comparison between the concentrations of triptolide achieved by administration of various extracts of Chinese herbal medicine and Minnelide is not feasible.

In the Phase I clinical trial, 27 patients have been enrolled so far, with 24 evaluable for toxicity. The therapy has been generally well tolerated with the only common toxicity being hematologic, but one patient experienced reversible cerebellar dysfunction at the highest dose. Hematologic toxicity has been notable for rapid onset and rapid recovery from neutropenia—often within 2-3 days of dose interruption. We have used these reference doses obtained from the trial in our experiments.

2. The exact molecular target of triptolide in a cancer cell is still not completely known. The cellular pathways are an intricate network of interconnected signaling. We have previously shown that triptolide inhibits Sp1 activity leading to decreased NF- κ B signaling and thereby decreased HSP 70 levels. AP1 has also been shown to regulate pro-survival pathways like NF- κ B and vice-versa. Promoter regions of AP1 are predicted to have Sp1 and NF- κ B binding sites as well (www.genecards.org). So it is possible that triptolide affects these upstream events that culminate into AP1 inhibition leading to triggering of apoptotic pathways and overcoming platinum resistance.

The proposed mechanism of action of how triptolide inhibits AP1

Figure 1.

TPL and oxaliplatin synergize in decreasing viability of pancreatic cancer cell lines MIA PaCa-2 and PANC-1. MIA PaCa-2 (a) and PANC-1 (b) cells were incubated with TPL (50 nM) and/or oxaliplatin (5μM) for 48 hours. Normalized isobologram for triptolide and oxaliplatin in the MIA PaCa-2 (c) and PANC-1 (d) cell lines. Data points located at lower left inside the triangle indicate synergism. Fractional affected-combination index (Fa-CI) plot in combination index analysis in the MIA PaCa-2 (e) and PANC-1 (f) cell lines.

Combination index values <1 , $=1$ or >1 indicate synergism, additivity and antagonism respectively. Data are mean $\pm$ SEM, $n=3$, $*p<0.05$.

Figure 2.

TPL enhances oxaliplatin induced apoptosis in pancreatic cancer cell lines MIA PaCa-2 and PANC-1. MIA PaCa-2 and PANC-1 cells were incubated with TPL (50 nM) and/or oxaliplatin (4 μM) for 24 hours. TPL (200 nM) was used a positive control. Cleaved Caspase-3 levels in MIA PaCa-2 cells (a) and PANC-1 cells (b). Cleaved PARP levels in MIA PaCa-2 cells (c) and PANC-1 cells (d). Data are mean ± SEM, n=3, *p<0.05 compared to control and each drug alone.

Figure 3.

Combination therapy with TPL and oxaliplatin increases DNA damage. MIA PaCa-2 and PANC-1 were incubated with TPL (50 nM) and oxaliplatin (4 μM) for 24 hours. SN-38 was used as a positive control. γH2A.X levels in MIA PaCa-2 (a) and PANC-1 (b) cancer cells. Data are mean ± SEM, n=3, *p<0.05 compared to control and each drug alone.

Figure 4.

TPL abrogates oxaliplatin induced mRNA expression of various components of Nucleotide Excision Repair (NER) pathway. MIA PaCa-2 (a, b) and PANC-1 (c, d) pancreatic cancer cells were incubated with TPL (50 nM) and/or oxaliplatin (4 μM) for 24 hours. mRNA levels of ERCC1, XPA, XPB, XPC, XPD, XPF and XPG were tested in MIA PaCa-2 and PANC-1 cancer cells. Data are mean ± SEM, n=3. *p<0.05 compared to control and oxaliplatin.

a.

MIA PaCa-2

b.

PANC-1

Figure 5.

Oxaliplatin increases the protein expression of key components of NER pathway while triptolide decreases it. Effect of TPL (50 nM) and/or oxaliplatin (4 μM) on the protein levels of XPA, XPB, XPC, ERCC1, XPD and XPF in MIA PaCa-2 (a) and PANC-1 (b) cancer cells.

Figure 6.

TPL abrogates oxaliplatin induced upregulation of AP1 promoter activity. Effect of TPL (50 nM) and/or oxaliplatin (4 μM) on AP1 promoter activity in MIA PaCa-2 (a) and PANC-1 (b) pancreatic cancer cells. Phorbol 12-Myristate 13-Acetate (PMA: 10ng/ml) was used as a positive control. Data are mean ± SEM, n=4. *p<0.05 compared to control and oxaliplatin.

Figure 7.

Combination treatment of Minnelide and oxaliplatin suppressed tumor growth *in vivo* by inducing apoptotic cell death in tumor cells. MIA PaCa-2 cells (1×10^5) were injected into the tail of pancreas of 4-6 weeks old athymic nude mice (7 mice /group). Animals were randomized into 4 groups and treatment was started 3 weeks after surgery: saline with 50 μ l DMSO, Minnelide 0.15 mg/kg/day, oxaliplatin 6 mg/kg/week and Minnelide combined with oxaliplatin. To assess the toxicity profile, body weight was plotted at start and end of treatment (c). At day 33, the animals were euthanized and tumors weight (a) and volume (b)

were documented. Fixed tissue in 10 % neutral buffered formalin was used for TUNEL staining later (d and e). Data are mean $\pm$ SEM, n=7. *p<0.05 compared to all other groups.

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Figure 8. Schematic diagram showing the inhibitory effects of triptolide on oxaliplatin induced upregulation of nucleotide excision repair pathway. Triptolide inhibits AP1 driven transcription of various genes of the NER pathway.