Mitoxantrone targets human ubiquitin-specific peptidase 11 (USP11) and is a potent inhibitor of pancreatic cancer cell survival.

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related death in the United States with a 95% five-year mortality rate. For over a decade, gemcitabine (GEM) has been the established first-line treatment for this disease despite suboptimal response rates. The development of PARP inhibitors that target the DNA damage repair mechanism in PDA cells has generated encouraging pre-clinical results. Ubiquitin-specific peptidase 11 (USP11), an enzyme that interacts with BRCA2, was recently discovered to play a key role in DNA double-strand break repair and may be a novel therapeutic target. Using a systematic high-throughput approach, we biochemically screened 2000 FDA approved and pharmacologically active compounds for inhibition of USP11 enzymatic activity. We identified six active small molecules that inhibit USP11 enzymatic activity. An in vitro drug sensitivity assay demonstrated that one of these USP11 inhibitors, mitoxantrone, affected PDA cell survival with an IC_{50} of less than 10 nM. Across six different PDA cell lines, two with defects in the Fanconi Anemia/BRCA2 pathway (Hs766T and Capan-1), mitoxantrone is 40 to 20,000-fold more potent than GEM, with increased endogenous USP11 mRNA levels associated with increased sensitivity to mitoxantrone. USP11 silencing in PDA cells also enhanced sensitivity to GEM. These findings establish a model for rapid discovery of FDA approved compounds by complementing in vitro biochemical experiments with cell culture studies. Further, they provide a strong rationale to study mitoxantrone in pre-clinical and early-phase clinical settings for the treatment of PDA.
INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) remains the fourth leading cause of cancer related death in the United States. There are approximately 44,000 new cases reported annually in the U.S., and the five-year mortality remains at 95% (1, 2). Surgical resection remains the only hope for cure, yet only 20% of patients are candidates for resection at the time of diagnosis (3, 4). Single agent gemcitabine (GEM) is the most commonly used chemotherapeutic agent in both the adjuvant setting following resection and in advanced disease. GEM is a nucleoside analog that has been a preferred systemic choice for advanced PDA since its superiority over 5-flourouracil (5-FU) was demonstrated in a randomized trial published in 1996; the benefit in the GEM arm was just over one month (5.65 v 4.41 months) (5). GEM’s superiority in the adjuvant setting is less clear, although toxicity is believed to be less than 5-FU (5-7). Promising biomarkers (e.g., HuR or ELAVL1: an oncogenic RNA binding protein that participates in post-transcriptional gene regulation, hENT1: a transmembrane nucleoside transporter utilized by certain chemotherapeutics) and new therapeutic strategies (e.g., FOLFIRINOX (leucovorin, fluorouracil, irinotecan, and oxaliplatin) and PARP inhibitors (8-10) are being more commonly used or are in the pipeline, yet virtually all PDAs develop resistance to the existing therapies. Thus, the discovery of new and targeted therapies for this disease is urgently needed.

Ideal ‘druggable targets’ are those that are dysregulated in cancer cells compared to normal cells. Genes that are somatically mutated provide an obvious and rational targeted strategy, since only cancer cells harbor mutant copies of the gene. This concept has been in the forefront of cancer biology and drug discovery fields for decades (11-14) with several notable examples of success, such as imatinib (Gleevec) (15, 16). Along these lines, the DNA repair pathway is an attractive target in PDA for several reasons. First, this pathway is well-
characterized and contains many established cancer genes (e.g., BRCA2, Fanconi anemia genes) that are somatically mutated in a proportion of PDA cells (13, 14, 17, 18). Second, chromosomal instability (CIN) is a ubiquitous hallmark of PDA suggesting a central role for DNA repair pathways in tumorigenesis (19-22). Finally, emerging data show that targeting the DNA damage response pathway in PDA is a worthy endeavor in pre-clinical models (23, 24) as well as in patients (25).

While the role of BRCA2-deficiency in pancreatic cancer was recently described, germ-line and somatic mutations of BRCA2 occur in only a minority of pancreatic cancers (17, 18, 26-28). These studies suggest that targeting BRCA2 directly in the majority of patients with PDA (i.e., BRCA +/+ and BRCA +/-) would be unsuccessful. Functionally, BRCA2 participates in the DNA damage response via formation of a complex that participates in double-stranded break repair via homologous recombination (29, 30).

Another defined component of this DNA repair complex is ubiquitin-specific peptidase 11 (USP11: also known as ubiquitin-specific protease 11). Dysregulation of members of the ubiquitin-specific protease family (USP 9X, 9Y, 10, and 25), independent of BRCA2 status, has been associated with increased tumorigenicity in cancer models (31). These proteins therefore comprise a group of understudied candidates for targeted drug discovery. Additionally, USP11 enzymatic activity has shown promise as a therapeutic target in preliminary studies because it regulates stability, function, or localization of DNA-associated proteins including BRCA2, p53, and IkK (32).

Working in concert with BRCA2 in DNA damage response pathways, USP11 protects cancer cells and promotes survival (33). Accordingly, prior work shows decreasing USP11 levels with RNA interference techniques leads to increased sensitivity to DNA damaging agents (such
as mitomycin C) or ionizing radiation in a breast cancer cell line (MCF7) (33). Further, a global gene RNAi screen to find a synthetic lethal approach in combination with PARP inhibition (AZD2281) demonstrated that USP11 silencing sensitized cells to death (10). Likely, a synthetic lethal combination of silencing PARP1 and USP11 is linked to the impaired recruitment of double-strand break repair enzymes RAD51 and 53BP1 to sites of DNA damage. Therefore, inhibition of USP11 in combination with PARP inhibition may represent an unexplored synthetic lethal approach to targeting and killing cancer cells.

Taken together, USP11 appears to play a critical role in DNA damage repair. Thus, we sought to discover inhibitors of USP11 enzyme activity from a large panel of Food and Drug Administration (FDA) approved drugs, natural products, and bioactive molecules. Identified compounds were then evaluated for therapeutic efficacy against human PDA cells and the target, USP11, was evaluated as an available target in human PDA specimens.

METHODS

USP11 Protein Purification

A USP11 pGEX-5x-1 plasmid was a gift from Winston C. Y. Yu (54). USP11 protein was expressed in Rosetta (DE3) cells. Cells were grown in Luria broth (LB) media (Sigma-Aldrich Co, St. Louis, MO, USA), containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The cells were grown at 37°C to OD_{600} of 0.6 and induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight growth at 15°C, cells were harvested and lysed by sonication with buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.25% Triton X100. The lysate was centrifuged and the supernatant was bound to Glutathione Sepharose 4B resin (GE Healthcare Life Sciences, Uppsala, Sweden) at 4°C for 2 hrs. The resin was then washed with a buffer containing 0.5 M NaCl, pH 7.3, 10 mM KCl, 10 mM Na_{2}HPO_{4},
and 1.8 mM KH$_2$PO$_4$. USP11 protein was eluted from the resin with buffer (50 mM Tris, pH 8.0, 40 mM reduced glutathione, 1mM EDTA). The purified USP11 enzyme was detected using SDS-PAGE and Coomassie Blue staining (Invitrogen, Life Technologies Inc, Carlsbad, CA, USA).

**Fluorescence-Based Assay for USP11 deubiquitinating activity**

Ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) is a fluorogenic substrate for many deubiquitinating (DUB) enzyme activity assays, and used in this study to monitor USP11 enzymatic activity. The hydrolysis of the amide bond between ubiquitin and 7-amino-4-methylcoumarin results in increased fluorescence at 440 nm when excited at 355 nm. With this fluorescence-based assay, we determined the steady-state enzyme kinetic rate constants ($k_{cat}$ and $K_m$) for USP11 at pH 7.8. The reaction was initiated by adding 10 nM USP11 and varying Ub-AMC concentrations from 50 nM to 2.5 µM in a buffer containing 50 mM HEPES (pH 7.8), 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg/ml BSA at 25°C. The initial velocities were measured and plotted against the substrate concentration. The plot was fit to equation $V = \left( \frac{V_{max} \cdot [S]}{[S] + K_m} \right)$ and the $V_{max}$ and $K_m$ values were obtained using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). The $k_{cat}$ was equal to $V_{max}$ divided by the total enzyme concentration in the assay.

**USP11 high-throughput screening (HTS) assay**

HTS assays were performed at room temperature in black 384 well non-binding, low-volume plates (No. 3676, Corning Inc. Life Sciences, Tewksbury, MA, USA). Each well contained 10 nM USP11 and 200 nM ubiquitin-AMC substrate in assay buffer (50 mM HEPES, 0.5 mM EDTA, 1mM DTT, 0.1 mg/ml BSA, pH 7.8) for a total volume of 10 µl per well. Wells containing either no enzyme or 10 µM ubiquitin aldehyde (sufficient to completely inhibit
USP11 activity) were run as controls on each plate. A 96-pin manual pintool was validated for compound transfer and found to deliver an average volume of 77 nL (data not shown), resulting in a final DMSO concentration of 0.8%. A DMSO titration confirmed that this concentration had no effect on the assay (data not shown). Test compounds were added to a final concentration of 30 µM in each well. Approximately 2000 compounds were screened for inhibition of USP11 (Spectrum compound library, Microsource Discovery Systems, Gaylordsville, CT, USA). USP11 activity was determined by measuring the difference in AMC fluorescence between time 0 and 90 min on an EnVisionmicroplate reader (Perkin Elmer Inc., Waltham, MA, USA).

**USP11 hit confirmation assays**

HTS hits (>30% inhibition) were retested in triplicate by monitoring fluorescence over time for 90 min to minimize data outliers and artifacts. Confirmed active compounds were also tested in triplicate in the USP11 assay with 10 mM cysteine rather than DTT. Selected compounds were then tested in dose-response from 3 nM to 100 µM to determine in-vitro IC₅₀ values. In addition to being evaluated in the USP11 assay, compounds were tested for an ability to quench the fluorescence of 30 nM AMC in the absence of enzyme. USP11 assay data was subsequently normalized by calculating the ratio of unquenched to quenched fluorescence using 30 nM AMC substrate alone (34). Normalized data were fit to a sigmoidal dose-response curve and IC₅₀ values were calculated by non-linear regression (4-parameter logistic fit) using GraphPad Prism.

**Cell Culture**

PDA cell lines (ASPC1, Capan-1, HS766T, MiaPaCa2, Panc1, and PL5) were purchased from American Type Culture Collection (Manassas, VA, USA) and grown at 37°C and 5% CO₂ in standard 75 cm² flasks. ASPC1 media consisted of RPMI 1640 supplemented with 10% fetal
bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (all sourced from: Invitrogen). The remaining cell lines were grown in DMEM (Invitrogen) with similar supplementation.

Hs766T is known to be deficient in the homologous DNA recombination pathway, harboring a non-sense FANCG mutation with associated loss of heterozygosity (LOH) (13). Additionally, BRCA2 mutational status in the Capan-1 cell line was confirmed by sequencing analysis of the 6174delT frameshift mutation (35).

**Characterizing cell-line USP11 levels by quantitative PCR**

Cells were trypsinized, collected, and washed by resuspension in PBS (Invitrogen) three times. RNA was obtained using the RNeasy mini kit (see manufacturer protocol for details: Qiagen Inc., Hilden, Germany). RNA was quantified on a Nanodrop 1000 (Thermo-Fisher Scientific, Wilmington, DE, USA) and cDNA template was created using the USB first-strand cDNA kit (Affymetrix, Santa Clara, CA, USA). USP11 mRNA was quantified by qPCR with Taqman gene expression mastermix and FAM-based probes (target: USP11, endogenous control: 18s) on an ABI 7500 Fast analyzer (Life Technologies).

**In-vitro drug sensitivity assays**

One thousand cells per well were cultured in triplicate on a 96-well plate (Thermo Fisher Scientific) and allowed to adhere overnight. Drug (mitoxantrone, gemcitabine, sennoside) was administered to each well in varying doses along the plate utilizing a serial dilution technique as previously described (36). Cells were grown for seven days before being washed and lysed with de-ionized water. Cell viability was quantified by staining for double stranded DNA with 1:200 concentration of Quant-iTPicoGreen (Invitrogen) and registering uptake on a 96 well plate reader (Tecan Group Ltd., Mannedorf, Switzerland). Analysis of cell viability at each drug dose was expressed as a percentage of cells remaining compared to the no treatment group. In a manner
similar to that used in HTS, normalized data were fit to a sigmoidal dose-response curve and IC\textsubscript{50} values were calculated by a non-linear regression (4-parameter logistic fit) using GraphPad Prism. Statistical significance between drugs was accepted for \( p < 0.05 \) by Students t-test analysis of IC\textsubscript{50} values obtained in three or greater replicate, independent experiments.

\textit{Augmentation of drug sensitivity with USP11 silencing}

Silencing siRNA oligonucleotides for \textit{USP11} and a scrambled control siRNA were obtained from Invitrogen (sequences available upon request). PL5 cells were plated at 30\% confluency and allowed to adhere overnight in 75 cm\textsuperscript{2} flasks. The flasks were then washed twice with Opti-mem and transfection was carried out utilizing Oligofectamine (Invitrogen) per manufacturer instructions with a 25 nanomolar final concentration of \textit{USP11} siRNA or control siRNA. Silencing was tested at 36 hours following transfection for each experiment with qPCR analysis as described above.

\textit{Testing drug synergy between mitoxantrone and GEM or PARP inhibition}

PL5 cells and MiaPaCa2 cells were plated in 96 well plates as described above. After adhering to the bottom of the each well, mitoxantrone was added to half the plate to a final concentration of 5 nM. GEM or ABT-888 (a PARP inhibitor, Abbott Laboratories, Princeton, NJ, USA) was then added to the plate using a serial dilution technique. Cells were allowed to grow for seven days before PicoGreen cell survival analysis was completed as described above.

\textit{Characterization of USP11 levels in PDA specimens}

Twenty-five human presumptive PDA tumor samples (all stage I or II disease) and adjacent normal (control) tissues were obtained after surgical resection at Thomas Jefferson University (IRB approved and consented). Core cell blocks were homogenized with a Kontes’ pellet pestle (Thermo Fisher Scientific) and total RNA was extracted using TRIzol reagent per
manufacturer instructions (Invitrogen). RNA was quantified on a Nanodrop 1000 (Thermo Fisher Scientific). Bioavailability of the candidate biomarker USP11 was compared to a well-characterized gene (DCK) with qPCR (targets: USP11, dCK; endogenous control: 18S; all sourced from Life Technologies). Data were normalized to expression levels of USP11 or DCK in each matched adjacent normal tissue sample. Data were excluded in eleven patients due to a final diagnosis other than PDA.

RESULTS

A fluorescence-based assay for USP11 activity

The activity of USP11 was monitored by measurement of AMC fluorescence following cleavage of Ub-AMC (Figure 1A). With this assay, we obtained a steady-state enzyme kinetic rate constant for USP11 at pH 7.8. The initial velocities were determined at different substrate concentrations varying from 50 nM to 2.5 µM (Figure 1A). The data were fit to the Michaelis–Menten equation to obtain the $k_{\text{cat}}$ of 0.021 s$^{-1}$ and $K_m$ of 0.12 µM (Figure 1A). This yielded a $k_{\text{cat}}/K_m$ of $1.8 \times 10^5$ M$^{-1}$·s$^{-1}$. The kinetic values are comparable to kinetic parameters determined for other DUBs (37, 38).

High-throughput screening (HTS) for inhibition of USP11 enzymatic activity

The deubiquitinating activity of USP11 was tested for each compound at a concentration close to its $K_m$. In the absence of USP11 inhibition, initial modeling found that fluorescence increased with cleavage of the Ub-AMC substrate in a linear fashion for at least 150 min (data not shown). Each well contained 200 nM Ub-AMC substrate. Background wells containing either no enzyme or enzyme with 10 µM ubiquitin aldehyde (sufficient to completely inhibit USP11 activity) yielded no change in fluorescence over 90 min. Wells containing USP11
enzyme demonstrated a five-fold increase in fluorescence over the same 90 minute time period (Z’ factor = 0.8), indicating a robust assay [60]. Sensitivity of the assay to graded inhibition was verified by testing increasing concentrations of ubiquitin aldehyde to generate a dose-response curve (IC₅₀ of 80 nM, data not shown).

HTS identified 156 of 2000 compounds which resulted in 30% or greater inhibition of USP11 activity (7.8% of compounds tested). Of the 156, there were 73 compounds active on 90 minute retest. Further, 8 known highly fluorescent compounds and fluorescence quenchers were eliminated, leaving 65 compounds for evaluation. Inspection of the structures of the remaining hits revealed that many were likely to be artifacts; for example, redox-active compounds containing quinonoid motifs and other conjugated unsaturated linkages have been shown to react with DTT in assay buffers to generate H₂O₂, leading to inactivation of cysteine proteases [61]. To exclude DTT-reactive artifacts, we retested hits in an assay buffer containing the less powerful reducing agent cysteine in place of DTT [62]. Retesting the 65 hits in the cysteine-containing buffer revealed only 26 compounds with greater than 30% inhibition of USP11 activity. Compounds active in the presence of cysteine were selected for further study.

**Identifying USP11 inhibitors**

Many of the 26 hits active in the presence of cysteine were eliminated from further study due to structural features that rendered them undesirable as candidates for a novel class of selective protease inhibitor (including electrophilic thiol-reactive metals, Michael acceptors, and very complex macrocyclic structures). Several common dyes and antiseptics were also excluded, leaving six candidate compounds. These were selected for IC₅₀ determination and tested in dose-response from 3 to 100 µM. The IC₅₀ of the six compounds are shown in Table 1. The structure
of one compound, mitoxantrone, is shown in Figure 1B along with a dose-response reaction between mitoxantrone and Ub-AMC substrate in Figure 1C.

**Evaluating bioavailability of USP11 levels in pancreatic cancer cell lines**

USP11 mRNA levels were evaluated in six pancreatic cancer cell lines by qPCR analysis. PL5 demonstrated the highest levels of USP11 mRNA with three-fold higher levels than the second-highest BRCA2-intact cell line, Panc1 (Figure 2A). MiaPaCa2, Hs766T, and ASPC1 all had relatively low levels of USP11 compared to that seen in PL5. USP11 levels in the BRCA2 mutant cell line, Capan-1, approximated that of the PL5 line (Figure 2A).

**Sensitivity to mitoxantrone correlates with cell line USP11 levels.**

Proposed compounds (sennoside A, sennoside B, tetrochlorophthalonitrile, epirubicin hydrochloride, rutoside, mitoxantrone) were tested in the high-USP11 PL5 cell line (data not shown). A range of doses were used between zero and 10 mM for each compound. Mitoxantrone alone appeared to have potent effects on cell survival in this cell line. The drug was subsequently tested at various doses (between 0 and 300 nM) for an effect on survival in all five cell lines (Figure 2B). Results were correlated with USP11 levels (Figure 2C). Two BRCA2-intact cell lines containing high amounts of USP11 mRNA, PL5 and Panc1, were the most sensitive to treatment with mitoxantrone with IC$_{50}$ concentrations less than one nanomolar (Figure 2C). MiaPaCa2 cells and ASPC1 cells (moderate expressing USP11 cell lines) were both sensitive to mitoxantrone but required a higher dose (approximately 10 nM) to reach an IC$_{50}$ (Figure 2B). The lowest USP11 level was found in the Hs766T cell line and an IC$_{50}$ was not reached in this cell line until approximately 1000 nM concentration (Figures 2B and 2C). A regression analysis of BRCA2 wild-type lines, with corresponding r-value, is displayed with Figure 2C.
Since USP11 has been implicated in the homologous repair pathway (31,32,33), we explored PDA lines previously described to harbor defects in this pathway, to assess whether mitoxantrone would induce synthetic lethality in these cells. We evaluated the Hs766T cell line, defective in the Fanconi anemia pathway (13) and the BRCA2 mutant cell line, Capan-1. The Hs766T line (low USP11 levels) and the Capan-1 line (high USP11 levels) were relatively resistant to mitoxantrone-induced cell death (Figure 2B, and 2C) compared to the other PDA cell lines. Taken together, these results suggest PDA lines defective in the Fanconi anemia/BRCA2 (FA/BRCA) pathway would not be uniformly sensitive to a USP11 inhibitor (mitoxantrone).

**PDA cells are hypersensitive to mitoxantrone as compared to gemcitabine**

Relative potency of mitoxantrone compared to GEM was tested in the PL5 cell line (Figure 2D). The IC$_{50}$ for mitoxantrone remained in the low nM range (1-20 nM in all replicates) while sensitivity to GEM (as defined by IC$_{50}$) was over one-thousand fold less (p < 0.001). Specifically, the IC$_{50}$ for GEM was in the 1-100 µM range (Figure 2D). The relative activity of another compound identified by high-throughput screening, sennoside B, is shown for comparison (Figure 2D).

An observed IC$_{50}$ concentration for mitoxantrone near 1 nM (Figures 2C and 2D) is well below the concentration achieved in the plasma with intravenous administration in human patients (39). Alberts and colleagues showed that a typical dose of mitoxantrone (12 mg/m$^2$ administered over a 30 minute period) resulted in a plasma concentration ranging between 1 and 10 ng/mL (or approximately 20-200 nM). Of note, an autopsy evaluation in one patient 35 days after administration of mitoxantrone demonstrated that pancreatic-tissue specific drug penetration was amongst the highest in the body at approximately 1000 ng mitoxantrone per
gram of pancreatic tissue (second only to liver drug levels of approximately 1100 ng mitoxantrone per gram of hepatic tissue).

**Silencing of USP11 in PDA cell lines with high baseline USP11 decreases cellular survival with mitoxantrone treatment**

The cell line with the highest endogenous USP11 expression, PL5, was used to test whether further inhibition of USP11 by siRNA silencing would augment the effect of pharmacologic USP11 inhibition with mitoxantrone. Using USP11-targeted siRNA oligos, the USP11 mRNA levels were reduced by approximately 60% compared to a control transfected cell line (Figure 3A). Reduced amounts of USP11 mRNA caused a small decrease in growth rates in a high USP11 expression cell line (PL5) (Supplemental Figure 1). Though not reaching statistical significance, this decrease may suggest vulnerability of these cells to cytotoxic therapies that could account for our below findings when testing these isogenic cell lines.

Compared to cells transfected with scrambled control siRNA, cells with reduced USP11 expression demonstrated increased sensitivity to mitoxantrone with a statistically significant IC\textsubscript{50} decrease by 3-fold (p < 0.001), confirming that this cell line is particularly susceptible to USP11 inhibition (Figure 3B). Additionally, when GEM sensitivity is tested in the setting of USP11 silencing a similar effect is detected. Compared to control siRNA, USP11 silencing increases PL5 sensitivity to treatment with GEM, reducing the IC\textsubscript{50} by 67%, from 0.75 µM to approximately 0.25 µM (p=0.012, Figure 3C). When a PARP inhibitor (ABT-888) is tested in the setting of USP11 silencing there is little-to-no decrease in cellular survival (p=0.34, Figure 3D). These data demonstrate that specifically targeting USP11 (via siRNA) can sensitize PDA cells to both mitoxantrone and GEM. These experiments cannot rule out synergistic off-target effects (unrelated to USP11 targeting) of mitoxantrone (48-50).
Treatment with mitoxantrone does not sensitize cells to GEM or PARP inhibitor therapies

The PL5 (Figures 4A and 4B) and MiaPaCa2 (Figures 4C and 4D) cell lines were used to study whether a synergistic effect occurred when cells were co-treated with mitoxantrone (as an inhibitor of USP11) plus either GEM or PARP inhibition (ABT-888) (40). Cellular survival was measured over a range of gemcitabine doses (Figures 4A and 4C) and a range of PARP inhibitor doses (Figures 4B and 4D) both with and without co-treatment with mitoxantrone at 5 nM. This mitoxantrone dose was chosen based on IC_{50} values obtained in preceding experiments. In contrast to the findings with USP11 silencing, no differences in sensitivity to GEM were found when PL5 and MiaPaCa2 cells were co-treated with mitoxantrone (p=0.88, 0.65). Similarly, in both cell lines there were no differences in sensitivity to PARP inhibition when cells were co-treated with mitoxantrone (p=0.41, 0.78).

USP11 mRNA in human PDA tumor specimens

As a reference, we tested mRNA levels of deoxycytidine kinase (dCK), a previously reported biomarker (41), and stratified the tumors as USP11 mRNA expression as low or high relative to the levels in adjacent normal tissue. Consistent with the previous study (38), 25% of tumor samples had low dCK mRNA levels (less than adjacent normal tissue), 50% had moderate dCK mRNA levels (between 1- and 5-fold relative to adjacent normal tissue), and 25% had high dCK mRNA levels (greater than 5-fold compared to adjacent normal tissue; Figure 5). USP11 levels were similarly quantified. Out of 14 informative cases, 5 had low USP11 (36%), 3 had moderate expression (21%), and 6 had high expression (43%). These data support the notion that USP11 is available and detectable in PDA specimens (Figure 5).

DISCUSSION
In recent years, there has been growing interest in finding new indications for FDA approved compounds against novel therapeutic targets and diseases (42, 43). Unlike novel agents with unknown pharmacokinetics and safety profiles, drugs that already have been FDA-approved may be rapidly evaluated in phase II settings for new indications. This is a common strategy used by drug companies to expand indications and potentially realize a larger return on an investment. Miltefosine is a notable example, in which the drug was developed as an anti-neoplastic drug, and is now used as an anti-protozoal agent to treat visceral leishmaniasis (44, 45). In cancer, drugs that are successful for one tumor type are often tested in clinical trials against other tumor types. For a lethal disease like pancreatic cancer, this strategy is certainly attractive because it can avoid the costly and timely drug development pipeline. Therefore, the model presented here represents an innovative high-throughput strategy to screen literally thousands of previously FDA-approved compounds for novel indications.

Clinically, mitoxantrone has been used for the last decade in the treatment of acute myeloid leukemia, hormone-refractory prostate cancer, and multiple sclerosis (46, 47). While the mechanism of action remains incompletely described, it is thought that mitoxantrone intercalates with DNA resulting in DNA strand breaks. Additionally, it has been characterized as a DNA type II topoisomerase inhibitor in a bacterial model (48-50). Topoisomerase II is a key enzyme in the DNA replication fork and is important in rapidly dividing cells such as cancer cells. This study does not rule out other mitoxantrone mechanisms of action in PDA cells, and perhaps mitoxantrone’s superior activity may be correlated to its ability to target multiple targets at once in PDA cells.

Human USP11 is a deubiquitinating enzyme that works in concert with BRCA2 to facilitate DNA homologous recombination by recruiting components of the DNA repair complex
As such, USP11 is thought to be particularly important in tumors with functional BRCA2, including PDA where greater than 90% of tumor specimens from patients with sporadic (non-familial) tumors have preserved BRCA2 function (22, 24). Further highlighting the role of USP11 in DNA damage and cancer is recent evidence that augmentation of the ubiquitination status of certain FA/BRCA pathway genes sensitizes cells to DNA damaging agents (10, 33, 38, 51). Lastly, prior reports have documented the effects USP11 function may have in broad-reaching pathways in response to DNA damage through effects on p53 and IkK pathway modulation (32).

With these mechanisms in mind, we found mitoxantrone to inhibit USP11 enzymatic activity robustly in vitro (Figure 1) and characterized the effects of this FDA approved compound in four PDA cell lines with wild-type (functional) BRCA2, one cell line with FANCG mutation but wild-type BRCA2, and one cell line with mutated BRCA2 (Figure 2, Supplemental Figure 1). We were able to detect USP11 by quantitative PCR analysis in all PDA cell lines screened (Figure 2) and found a direct correlation between higher levels of baseline USP11 mRNA and increased sensitivity to mitoxantrone-induced cell death in FA/BRCA2 intact PDA cells (Figure 2). The failure of mitoxantrone to induce cell death despite high USP11 levels in a BRCA2 mutant cell line suggests that intact BRCA2 function may be an important mediator of USP11 targeted therapy, a finding that requires further exploration.

Furthermore, previously it was shown that the Hs766T line, due to harboring a FANCG mutation, was hypersensitive to DNA crosslinking agents (12). The fact that Hs766T cells were relatively resistant to mitoxantrone compared to the other FA/BRCA2-proficient lines suggests that mitoxantrone will not work in a synthetic lethal manner (similar to crosslinking agents) in FA/BRCA-deficient cells. Taken together, tumors expressing higher levels of USP11 (perhaps
in tumors with an intact FA/BRCA2 pathway) would appear to be good candidates for treatment with mitoxantrone.

Of note, using qPCR, we found that nearly half of human PDA tumor specimens had a high level of USP11 mRNA (figure 5). Recognizing that mRNA expression measurements were contaminated by normal cells within the tumor stroma (52), immunohistochemical analyses of USP11 expression would be more informative, and are currently being developed.

We present *in vitro* data indicating that mitoxantrone potency exceeds that of gemcitabine (approximately 1000-fold in the PL5 cell line, Figure 2D). Mitoxantrone targeting specificity was tested by evaluating drug sensitivity after USP11 silencing (Figure 3). With the proposed novel target (i.e. *USP11*) silenced by approximately 60%, we altered sensitivity to mitoxantrone-induced cell death. These findings suggest that at least part of mitoxantrone’s mechanism of action in PDA cells is in indeed via disruption of USP11 enzymatic activity, rather than solely through mechanisms identified in prior work (i.e. DNA intercalation or topoisomerase II inhibition) (48-50).

Interestingly, sensitivity to GEM with USP11 knockdown was also increased, suggesting a powerful synergy between inhibition of the USP11 and GEM (Figure 3). To our knowledge, this is the first report suggesting a synergistic link between an anti-*USP11* treatment strategy and a nucleoside analog like GEM. We were unable to reproduce this synergy with GEM when using mitoxantrone instead of a siRNA strategy to inhibit USP11 (Figure 4). Given the rapid resistance to GEM that develops clinically, this study raises the possibility that an siRNA silencing approach may be superior to co-treatment with a pharmacologic inhibitor when targeting *USP11* as a strategy to break GEM resistance. Unlike prior work, we were unable to confirm synergism
between USP11 inhibition and PARP inhibition (10) (albeit the testing of just one PARP inhibitor, ABT-888) in our PDA model.

Mitoxantrone has been used previously for the treatment of pancreatic cancer. A pair of dated European trials from Beger and colleagues compared celiac artery infusion of mitoxantrone, 5-fluorouracil, folinic acid, and cisplatinum in patients with resected PDA (53, 54). Both studies were small (<30 patients) and single-arm studies, with comparisons to historical institutional control patients who did not receive any adjuvant treatment. Nevertheless, survival was favorable in the treatment group. In addition, there is a dated randomized study performed by the Southwest Oncology Group that compared single-agent mitoxantrone to a multi-drug regimen (fluoruracil, adriamycin, mitomycin, and streptozotocin) in patients with advanced pancreatic cancer (55). In 24 patients, no response was observed with mitoxantrone and the median survival was only 4 months. It should be emphasized that the study patients had relatively poor performance status (Karnofsky score; median=2), and that patients with a comparable performance status who have received GEM (standard-of-care) have little-to-no benefit (56). Modern first-line palliative chemotherapy trials for PDA (e.g., FOLFIRINOX) rarely include patients who have such poor performance status. Therefore, it is reasonable to conclude that the efficacy of mitoxantrone in appropriately selected patients with PDA remains unknown. More recently, mitoxantrone in combination with doxorubicin and irinotecan was used in drug-eluting bead therapy in a murine model of PDA and shown to decrease tumor proliferation and increase apoptosis (57). Lastly, in a high-throughput screen for compounds demonstrating synthetic lethality with Tumor Necrosis Factor-α Related Apoptosis Inducing Ligand (TRAIL) therapy, mitoxantrone was shown to reduce tumor cell viability at concentrations below 1 µM in pancreatic cancer cell lines (58).
In summary, we determined that *USP11* is a therapeutic target in pre-clinical models of PDA, and identified mitoxantrone as a potent inhibitor of the DNA repair enzyme, USP11. Additionally, drug response was associated with baseline *USP11* expression and potency far exceeded results observed with gemcitabine. This study lays the framework to: 1) explore *USP11* as a promising ‘druggable’ target and biomarker in additional pre-clinical PDA models; and 2) utilize mitoxantrone as a bioavailable lead compound for treating this lethal disease.

ACKNOWLEDGMENTS

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References

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Figure Legends

Figure 1: Fluorescent-based assay for USP11 activity and identified inhibitor.
(A) State-steady study of USP11 activity. (B) Structure of mitoxantrone. (C) Dose-dependent inhibition of USP11, as measured by cleavage of Ub-AMC substrate, in the presence of different concentrations of mitoxantrone. Axis depicting drug concentration is displayed in logarithmic scale for clarity.

Figure 2: Sensitivity to mitoxantrone-induced cell death correlates with baseline USP11 levels in PDA.
(A) Baseline USP11 mRNA levels in six separate pancreatic cancer cell lines as analyzed by RT-qPCR and normalized to 18S reveals that PL5 cells have the highest expression followed by Capan-1 (the BRCA2 mutant line) and Panc1 cells. (B) Pico Green survival assay (7 days): Panc1 and PL5 cell lines have the highest susceptibility to mitoxantrone, followed by ASPC1 and MiaPaCa2 while Capan-1 and Hs766T cells appear relatively resistant at nM concentrations of the drug. (C) Integrating panels A and B, increased baseline USP11 levels correlate with increased sensitivity to mitoxantrone in BRCA2 wild-type PDA cells. The BRCA2 mutated cell line, Capan-1, is shown as an empty diamond for comparison. A power-based trend line is included with accuracy of fit modeled with r-value for BRCA2 intact lines. (D) Mitoxantrone was more potent than GEM at inducing cell death in the PL5 cell line (high baseline USP11 levels) with an IC$_{50}$ of roughly 1000 fold less than GEM (<1 nM mitoxantrone versus > 0.75 uM gemcitabine). Sennoside B is included as an example of relative potency of agents identified in our high-throughput screen. Axes representing drug concentration are depicted in logarithmic scale for clarity.
Figure 3: Silencing USP11 results in increased sensitivity to gemcitabine and mitoxantrone.

(A) Silencing of USP11 via siRNA reduces the level of USP11 RNA by approximately 60% compared to scrambled siRNA transfection as analyzed by RT-qPCR and normalized to 18S.

(B-D) Solid lines (—) represent cells treated with control siRNA while dotted lines (---) represent cells treated with USP11 siRNA. Knockdown of USP11 enhances mitoxantrone (B) and GEM (C) potency but fails to alter sensitivity to PARP inhibition using ABT-888 (D). Note the x-axes (drug concentration) (B-D) are in log scale.

Figure 4: Mitoxantrone treatment does not act synergistically with GEM or ABT-888.

Solid lines (—) represent co-treatment with PBS-vector control and dotted lines (---) represent co-treatment with mitoxantrone (5 nM). In the PL5 cell line, mitoxantrone administration failed to demonstrate GEM synergy (A) or ABT-888 synergy (B) as determined by Pico Green assay analysis. Similarly in the MiaPaCa2 cell line, co-administration of mitoxantrone with GEM (C) or ABT-888 (D) also failed to demonstrate synergistic potency. Note the x-axes (drug concentration) are in log scale.

Figure 5: Detection of dCK and USP11 mRNAs in human PDA specimens.

Box and whisker plots demonstrating the range of DCK mRNA and the proposed biomarker, USP11 mRNA seen in human PDA tumor samples normalized to adjacent normal pancreatic tissue. 18s expression was used in each sample as an endogenous control and biomarker expression is stratified into low, moderate (mod), and high expression tumors (n=14 samples).
Table 1: The IC_{50} (µM) value and drug use of the six confirmed USP11 inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sennoside A</th>
<th>Mitoxantrone</th>
<th>Sennoside B</th>
<th>Tetrachloro-isophthalonitrile</th>
<th>Epirubicin</th>
<th>Rutoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug use</td>
<td>Cathartic</td>
<td>Antineoplastic</td>
<td>Cathartic</td>
<td>Antifungal</td>
<td>Antineoplastic</td>
<td>Vascular protectant</td>
</tr>
<tr>
<td>IC_{50} (µM)</td>
<td>2.77</td>
<td>3.15</td>
<td>9.62</td>
<td>28.6</td>
<td>34.0</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Figure 1

K\textsubscript{cat} = 0.021 s\textsuperscript{-1}

K\textsubscript{m} = 0.12 µM

A

B

Mitoxantrone

C
Figure 2

A

USP11 mRNA normalized to 18s

ASPC1 Capan-1 Hs766T MiaPaCa2 Panc1 PL5

B

Cell survival (%)

Mitoxantrone concentration (nM)

ASPC1 Capan-1 Hs766T MiaPaCa2 Panc1 PL5

C

Mitoxantrone IC50 (nM) note: logarithmic scale

Hs766T Capan-1

MiaPaCa2

ASPC1

Panc1 PL5

y = 16.1^x -1.25
r = 0.77

D

Cell survival (%)

Drug concentration (nM)

GEM Mitoxantrone Sennoside B

Note: logaritmic scale
Figure 3

A

USP11 mRNA normalized to 18s

Control siRNA

USP11 siRNA

B

Mitoxantrone concentration (nM)

Cell survival (%)

C

Gemcitabine concentration (µM)

Cell survival (%)

D

ABT-888 concentration (µM)

Cell survival (%)
Figure 5

Human PDA tumor sample biomarker level relative to adjacent normal tissue

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Low</th>
<th>Mod</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative levels: low, mod, high.
Molecular Cancer Research

Mitoxantrone targets human ubiquitin-specific peptidase 11 (USP11) and is a potent inhibitor of pancreatic cancer cell survival


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