DNA Damage and Repair

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 (DDR) system in PDA cells has generated encouraging results. Ubiquitin-specific peptidase 11 (USP11), an enzyme that interacts with the DDR protein BRCA2, was recently discovered to play a key role in DNA double-strand break repair and may be a novel therapeutic target. A systematic high-throughput approach was used to biochemically screen 2,000 U.S. Food and Drug Administration (FDA)-approved compounds for inhibition of USP11 enzymatic activity. Six pharmacologically active small molecules that inhibit USP11 enzymatic activity were identified. An in vitro drug sensitivity assay demonstrated that one of these USP11 inhibitors, mitoxantrone, impacted PDA cell survival with an IC50 of less than 10 nM. Importantly, 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. Interestingly, USP11 silencing in PDA cells also enhanced sensitivity to GEM. These findings establish a preclinical model for the rapid discovery of FDA-approved compounds and identify USP11 as a target of mitoxantrone in PDA.

Implications: This high-throughput approach provides a strong rationale to study mitoxantrone in an early-phase clinical setting for the treatment of PDA. Mol Cancer Res; 11(8); 901–11. ©2013 AACR.

Introduction

Pancreatic ductal adenocarcinoma remains the fourth leading cause of cancer-related death in the United States.

There are approximately 44,000 new cases reported annually in the United States, and the 5-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 is the most commonly used chemotherapeutic agent in both the adjuvant setting following resection and in advanced disease. Gemcitabine is a nucleoside analog that has been a preferred systemic choice for advanced pancreatic ductal adenocarcinoma, as its superiority over 5-fluorouracil (5-FU) was shown in a randomized trial published in 1996; the benefit in the gemcitabine arm was just over 1 month (5.65 vs. 4.41 months; ref. 5). Gemcitabine’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 and hENT1: a transmembrane nucleoside transporter used by certain chemotherapeutics) and new therapeutic strategies [e.g., FOLFIRINOX (leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin) and PARP inhibitors; ref. 8–10] are being more commonly used or are in the pipeline, yet virtually all pancreatic ductal adenocarcinomas develop resistance to the
PARP1 and 25), independent of silencing sensitized cells to death (10). Likely, a synthetic USP11 response pathways, BRCA2, p53

function, or localization of DNA-associated proteins includes for targeted drug discovery. In addition, USP11 proteins therefore comprise a group of understudied candidates for targeted drug discovery. Moreover, a global gene RNAi screen for many deubiquitinating (DUB) enzymes has been shown as a promising therapeutic target in preliminary studies because it regulates stability, function, or localization of DNA-associated proteins including BRCA2, p53, and IkB (32).

Another defined component of this DNA repair complex is ubiquitin-specific peptidase 11 (USP11: also known as ubiquitin-specific protease 11). Dysfunction 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. In addition, USP11 enzymatic activity has been shown as a promising therapeutic target in preliminary studies because it regulates stability, function, or localization of DNA-associated proteins including BRCA2, p53, and IkB (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; ref. 33). Furthermore, a global gene RNAi screen to find a synthetic lethal approach in combination with PARP inhibition (AZD2281) showed 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 seems to play a critical role in DNA damage repair. Thus, we sought to discover inhibitors of USP11 enzyme activity from a large panel of U.S. Food and Drug Administration (FDA)-approved drugs, natural products, and bioactive molecules. Identified compounds were then evaluated for therapeutic efficacy against human pancreatic ductal adenocarcinoma cells and the target, USP11, was evaluated as an available target in human pancreatic ductal adenocarcinoma specimens.

Materials and 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 media (Sigma-Aldrich Co), containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). The cells were grown at 37°C to OD600 of 0.6 and induced with 0.4 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight growth at 15°C, the cells were harvested and lysed by sonication with buffer containing 10 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.25% Triton X-100. The lysate was centrifuged and the supernatant was bound to Glutathione Sepharose 4B resin (GE Healthcare Life Sciences) at 4°C for 2 hours. The supernatant was washed with a buffer containing 0.5 mol/L NaCl, pH 7.3, 10 mmol/L KCl, 10 mmol/L Na2HPO4, and 1.8 mmol/L KH2PO4. USP11 protein was eluted from the resin with buffer (50 mmol/L Tris, pH 8.0, 40 mmol/L reduced glutathione, 1 mmol/L EDTA). The purified USP11 enzyme was detected using SDS-PAGE and Coomassie Blue staining (Invitrogen; Life Technologies Inc).

Fluorescence-based assay for USP11 deubiquitinating activity

Ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) is a fluorogenic substrate for many deubiquitinating (DUB) enzymes 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 (kcat and km) for USP11 at pH 7.8. The reaction was initiated by adding 10 nmol/L USP11 and varying Ub-AMC concentrations from 50 nmol/L to 2.5 μmol/L in a buffer containing 50 mmol/L HEPES (pH 7.8), 0.5 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 0.1 mg/mL bovine serum albumin (BSA) at 25°C. The initial velocities were measured and plotted against the substrate concentration. The plot was fitted to the Michaelis-Menten equation for the linear (V = (Vmax * [S])/(Km + [S])) and the Vmax and Km values were obtained using GraphPad Prism 5 (GraphPad Software). The kcat was equal to Vmax divided by the total enzyme concentration in the assay.
USP11 high-throughput screening assay

High-throughput screening (HTS) assays were conducted at room temperature in black 384-well nonbinding, low-volume plates (No. 3676, Corning Inc. Life Sciences). Each well contained 10 nmol/L USP11 and 200 nmol/L ubiquitin-AMC substrate in assay buffer (50 mmol/L HEPES, 0.5 mmol/L EDTA, 1 mmol/L 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 µmol/L 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 dimethyl sulfoxide (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 µmol/L in each well. Approximately 2,000 compounds were screened for inhibition of USP11 (Spectrum compound library, Microsource Discovery Systems). USP11 activity was determined by measuring the difference in AMC fluorescence between time 0 and 90 minutes on an EnVision microplate reader (Perkin Elmer Inc.).

USP11 hit confirmation assays

HTS hits (≥30% inhibition) were retested in triplicate by monitoring fluorescence over time for 90 minutes to minimize data outliers and artifacts. Confirmed active compounds were also tested in triplicate in the USP11 assay with 10 nmol/L cysteine rather than DTT. Selected compounds were then tested in dose response from 3 nmol/L to 100 µmol/L 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 nmol/L AMC in the absence of enzyme. USP11 assay data were subsequently normalized by calculating the ratio of unquenched to quenched fluorescence using 30 nmol/L AMC substrate alone (34). Normalized data were fit to a sigmoidal dose–response curve, and IC₅₀ values were calculated by nonlinear regression (4-parameter logistic fit) using GraphPad Prism.

Cell culture

Pancreatic ductal adenocarcinoma cell lines (ASPC1, Capan-1, Hs766T, MiaPaCa2, Panc1, and PL5) were purchased from American Type Culture Collection and grown at 37°C and 5% CO₂ in standard 75 cm² flasks. ASPC1 media consisted of RPMI-1640 supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin (all sourced from: Invitrogen). The remaining cell lines were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) with similar supplementation. Hs766T is known to be deficient in the homologous DNA recombination pathway, harboring a nonsense Fanconi anemia complementation group (FANCG) mutation with associated loss of heterozygosity (13). In addition, 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) 3 times. RNA was obtained using the RNaseasy Mini Kit (see manufacturer protocol for details: Qiagen Inc.). RNA was quantified on a Nanodrop 1000 (Thermo-Fisher Scientific) and cDNA template was created using the USB first-strand cDNA Kit (Affymetrix). USP11 mRNA was quantified by quantitative PCR (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, and sennoside) was administered to each well in varying doses along the plate using a serial dilution technique as previously described (36). Cells were grown for 7 days before being washed and lysed with deionized water. Cell viability was quantified by staining for double-stranded DNA with 1:200 concentration of Quanti-IT PicoGreen (Invitrogen) and registering uptake on a 96-well plate reader (Tecan Group Ltd.). Analysis of cell viability at each drug dose was expressed as a percentage of cells remaining compared with the no treatment group. In a manner similar to that used in HTS, normalized data were fit to a sigmoidal dose–response curve and IC₅₀ values were calculated by a nonlinear regression (4-parameter logistic fit) using GraphPad Prism. Statistical significance between drugs was accepted for P < 0.05 by Student t test analysis of IC₅₀ values obtained in 3 or more replicate, independent experiments.

Augmentation of drug sensitivity with USP11 silencing

Silencing siRNA oligonucleotides for 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 cm² flasks. The flasks were then washed twice with Opti-Mem and transfection was carried out using Oligofectamine (Invitrogen) as per manufacturer’s instructions with a 25 nmol/L final concentration of USP11 siRNA or control siRNA. Silencing was tested at 36 hours following transfection for each experiment with qPCR analysis as described above.

Testing drug synergy between mitoxantrone and gemcitabine 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 nmol/L. Gemcitabine or ABT-888 (a PARP inhibitor, Abbott Laboratories) was then added to the plate using a serial dilution technique. Cells were allowed to grow for 7 days before PicoGreen cell survival analysis was conducted as described above.
Characterization of USP11 levels in pancreatic ductal adenocarcinoma specimens

Twenty-five human presumptive pancreatic ductal adenocarcinoma tumor samples (all stage I or II disease) and adjacent normal (control) tissues were obtained after surgical resection at Thomas Jefferson University (Institutional review board-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 with 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 11 patients due to a final diagnosis other than pancreatic ductal adenocarcinoma.

Results

A fluorescence-based assay for USP11 activity

The activity of USP11 was monitored by measurement of AMC fluorescence following cleavage of Ub-AMC (Fig. 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 nmol/L to 2.5 µmol/L (Fig. 1A). The data were fit to the Michaelis–Menten equation to obtain the $k_{cat}$ of 0.021 s$^{-1}$ and $K_m$ of 0.12 µmol/L (Fig. 1A). This yielded a $k_{cat}/K_m$ of $1.8 \times 10^5$ M$^{-1}$s$^{-1}$. The kinetic values are comparable with kinetic parameters determined for other DUBs (37, 38).

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 minutes (data not shown). Each well contained 200 nmol/L Ub-AMC substrate. Background wells containing either no enzyme or enzyme with 10 µmol/L ubiquitin aldehyde (sufficient to completely inhibit USP11 activity) yielded no change in fluorescence over 90 minutes. Wells containing USP11 enzyme showed a 5-fold increase in fluorescence over the same 90-minute time period ($Z'$ factor = 0.8), indicating a robust assay. Sensitivity of the assay to graded inhibition was verified by testing increasing concentrations of ubiquitin aldehyde to generate a dose–response curve (IC$_{50}$ of 80 nmol/L, data not shown).

HTS identified 156 of 2,000 compounds that resulted in 30% or more inhibition of USP11 activity (7.8% of compounds tested). Of the 156, there were 73 compounds active on 90-minute retest. Furthermore, 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

Figure 1. Fluorescent-based assay for USP11 activity and identified inhibitor. A, steady state 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.
motifs and other conjugated unsaturated linkages have been shown to react with DTT in assay buffers to generate H$_2$O$_2$, leading to inactivation of cysteine proteases. To exclude DTT-reactive artifacts, we retested hits in an assay buffer containing the less powerful reducing agent cysteine in place of DTT. Retesting the 65 hits in the cysteine-containing buffer revealed only 26 compounds with more 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 6 candidate compounds. These were selected for IC$_{50}$ determination and tested in dose response from 3 to 100 $\mu$mol/L. The IC$_{50}$ of the 6 compounds are shown in Table 1. The structure of one compound, mitoxantrone, is shown in Fig. 1B along with a dose–response reaction between mitoxantrone and Ub-AMC substrate in Fig. 1C.

Evaluating bioavailability of USP11 levels in pancreatic cancer cell lines

USP11 mRNA levels were evaluated in 6 pancreatic cancer cell lines by qPCR analysis. PL5 showed the highest levels of USP11 mRNA with 3-fold higher levels than the second-highest BRCA2-intact cell line, Panc1 (Fig. 2A). MiaPaCa2, Hs766T, and ASPC1 all had relatively low levels of USP11 compared with that seen in PL5. USP11 levels in the BRCA2-mutant cell line, Capan-1, approximated that of the PL5 line (Fig. 2A).

Sensitivity to mitoxantrone correlates with cell line USP11 levels

Proposed compounds (sennoside A, sennoside B, tetrochlorophthalonitrile, epirubicin hydrochloride, rutoside, and mitoxantrone) were tested in the high-USP11 PL5 cell line (data not shown). A range of doses were used between 0 and 10 nmol/L for each compound. Mitoxantrone alone seemed to have potent effects on cell survival in this cell line. The drug was subsequently tested at various doses (between 0 and 300 nmol/L) for an effect on survival in all 5 cell lines (Fig. 2B). Results were correlated with USP11 levels (Fig. 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 1 nmol/L (Fig. 2C). MiaPaCa2 cells and ASPC1 cells (moderate expressing USP11 cell lines) were both sensitive to mitoxantrone but required a higher dose (approximately 10 nmol/L) to reach an IC$_{50}$ (Fig. 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 1,000 nmol/L concentration (Fig. 2B and C). A regression analysis of BRCA2 wild-type lines, with corresponding R-value, is displayed in Fig. 2C.

As USP11 has been implicated in the homologous repair pathway (31, 32,33), we explored pancreatic ductal adenocarcinoma 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 cell line (low USP11 levels) and the Capan-1 line (high USP11 levels) were relatively resistant to mitoxantrone-induced cell death (Fig. 2B and C) compared with the other pancreatic ductal adenocarcinoma cell lines. Taken together, these results suggest pancreatic ductal adenocarcinoma lines defective in the Fanconi anemia/BRCA2 (FA/BRCA) pathway would not be uniformly sensitive to a USP11 inhibitor (mitoxantrone).

Pancreatic ductal adenocarcinoma cells are hypersensitive to mitoxantrone as compared with gemcitabine

Relative potency of mitoxantrone compared with gemcitabine was tested in the PL5 cell line (Fig. 2D). The IC$_{50}$ for mitoxantrone remained in the low nmol/L range (1–20 nmol/L in all replicates), whereas sensitivity to gemcitabine (as defined by IC$_{50}$) was over 1,000-fold less ($P < 0.001$). Specifically, the IC$_{50}$ for gemcitabine was in the 1 to 100 $\mu$mol/L range (Fig. 2D). The relative activity of another compound identified by HTS, sennoside B, is shown for comparison (Fig. 2D).

An observed IC$_{50}$ concentration for mitoxantrone near 1 nmol/L (Fig. 2C and D) 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 nmol/L; 39). Of note, an autopsy evaluation in 1 patient 35 days after administration of mitoxantrone showed that

<table>
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<tr>
<th>Compound</th>
<th>Sennoside A</th>
<th>Mitoxantrone</th>
<th>Sennoside B</th>
<th>Tetrachlorophthalonitrile</th>
<th>Epirubicin</th>
<th>Rutoside</th>
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<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>
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<tr>
<td>IC$_{50}$ (µmol/L)</td>
<td>2.77</td>
<td>3.15</td>
<td>9.62</td>
<td>28.6</td>
<td>34.0</td>
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pancreatic-tissue–specific drug penetration was amongst the highest in the body at approximately 1,000 ng mitoxantrone per gram of pancreatic tissue (second only to liver drug levels of approximately 1,100 ng mitoxantrone per gram of hepatic tissue).

Silencing of USP11 in pancreatic ductal adenocarcinoma 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 with a control transfected cell line (Fig. 3A). Reduced amounts of USP11 mRNA caused a small decrease in growth rates in a high USP11 expression cell line (PL5; Supplementary Fig. S1). 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 with cells transfected with scrambled control siRNA, cells with reduced USP11 expression showed increased sensitivity to mitoxantrone with a statistically significant IC₅₀ decrease by 3-fold ($P < 0.001$), confirming that this cell line is particularly susceptible to USP11 inhibition (Fig. 3B). In addition, when gemcitabine sensitivity is tested in the setting of USP11 silencing, a similar effect is detected. Compared with control siRNA, USP11 silencing increases PL5 sensitivity to treatment with gemcitabine, reducing the IC₅₀ by 67%, from 0.75 μmol/L to 0.25 μmol/L.

Figure 2. Sensitivity to mitoxantrone-induced cell death correlates with baseline USP11 levels in pancreatic ductal adenocarcinoma. A, baseline USP11 mRNA levels in 6 separate pancreatic cancer cell lines as analyzed by quantitative real-time PCR (qRT-PCR) and normalized to 18S reveal 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, whereas Capan-1 and Hs766T cells appear relatively resistant at nmol/L concentrations of the drug. C, integrating (A) and (B), increased baseline USP11 levels correlate with increased sensitivity to mitoxantrone in BRCA2 wild-type pancreatic ductal adenocarcinoma 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 gemcitabine (GEM) at inducing cell death in the PL5 cell line (high baseline USP11 levels) with an IC₅₀ of roughly 1,000 fold less than GEM ($<1$ nmol/L mitoxantrone vs. $>0.75$ μmol/L gemcitabine). Sennoside B is included as an example of relative potency of agents identified in our HTS. Axes representing drug concentration are depicted in logarithmic scale for clarity.
approximately 0.25 µmol/L ($P = 0.012$, Fig. 3C). When a PARP inhibitor (ABT-888) is tested in the setting of USP11 silencing, there is little-to-no decrease in cellular survival ($P = 0.34$, Fig. 3D). These data show that specifically targeting USP11 (via siRNA) can sensitize pancreatic ductal adenocarcinoma cells to both mitoxantrone and gemcitabine. 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 gemcitabine or PARP inhibitor therapies**

The PL5 (Fig. 4A and B) and MiaPaCa2 (Fig. 4C and D) cell lines were used to study whether a synergistic effect

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**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 with scrambled siRNA transfection as analyzed by qRT-PCR and normalized to 18S. B to D, solid lines (—) represent cells treated with control siRNA, whereas 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 cotreatment with PBS-vector control and dotted lines (---) represent cotreatment with mitoxantrone (5 nmol/L). In the PL5 cell line, mitoxantrone administration failed to show GEM synergy (A) or ABT-888 synergy (B) as determined by the Pico Green assay analysis. Similarly, in the MiaPaCa2 cell line, coadministration of mitoxantrone with GEM (C) or ABT-888 (D) also failed to show synergistic potency. Note the $x$-axes (drug concentration) are in log scale.
occurred when cells were cotreated with mitoxantrone (as an inhibitor of USP11) plus either gemcitabine or PARP inhibition (ABT-888; ref. 40). Cellular survival was measured over a range of gemcitabine doses (Fig. 4A and C) and a range of PARP inhibitor doses (Fig. 4B and D) both with and without cotreatment with mitoxantrone at 5 nmol/L. 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 gemcitabine were found when PL5 and MiaPaCa2 cells were cotreated with mitoxantrone (P = 0.88, 0.65). Similarly, in both cell lines, there were no differences in sensitivity to PARP inhibition when cells were cotreated with mitoxantrone (P = 0.41, 0.78).

**USP11 mRNA in human pancreatic ductal adenocarcinoma tumor specimens**

As a reference, we tested mRNA levels of deoxycytidine kinase (dCK), a previously reported biomarker (41), and stratified the tumors’ USP11 mRNA expression as low or high relative to the levels in adjacent normal tissue. Consistent with previous studies (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 (more than 5-fold compared with adjacent normal tissue; Fig. 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 pancreatic ductal adenocarcinoma specimens (Fig. 5).

![Figure 5](image-url). Detection of dCK and USP11 mRNAs in human pancreatic ductal adenocarcinoma specimens. Box and whisker plots showing the range of dCK mRNA and the proposed biomarker, USP11 mRNA seen in human pancreatic ductal adenocarcinoma 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).

**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.

Mitoferrine is a notable example, in which the drug was developed as an antineoplastic drug, and is now used as an antiprotozoal agent to treat visceral leishmaniasis (44, 45). In cancer, drugs that are successful for 1 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. In addition, 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 pancreatic ductal adenocarcinoma cells, and perhaps mitoxantrone’s superior activity may be correlated to its ability to target multiple targets at once in pancreatic ductal adenocarcinoma 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 (31, 33). As such, USP11 is thought to be particularly important in tumors with functional BRCA2, including pancreatic ductal adenocarcinoma where more than 90% of tumor specimens from patients with sporadic (nonfamilial) 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). Finally, prior reports have documented the effects USP11 function may have in broad-reaching pathways in response to DNA damage through effects on p53 and IκK pathway modulation (32).

With these mechanisms in mind, we found mitoxantrone to inhibit USP11 enzymatic activity robustly in vitro (Fig. 1) and characterized the effects of this FDA-approved compound in 4 pancreatic ductal adenocarcinoma cell lines with wild-type (functional) BRCA2, one cell line with FANCG mutation but wild-type BRCA2, and one cell line with mutated BRCA2 (Fig. 2 and Supplementary Fig. 1). We...
Mitoxantrone Inhibits USP11 and Pancreatic Cancer Cell Growth

were able to detect USP11 by quantitative PCR analysis in all pancreatic ductal adenocarcinoma cell lines screened (Fig. 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 pancreatic ductal adenocarcinoma cells (Fig. 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 with the other FA/BRCa2-proficient lines suggests that mitoxantrone will not work in a synthetic lethal manner (similar to crosslinking agents) in FA/BRCa2-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 pancreatic ductal adenocarcinoma tumor specimens had a high level of USP11 mRNA (Fig. 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 1,000-fold in the PL5 cell line, Fig. 2D). Mitoxantrone targeting specificity was tested by evaluating drug sensitivity after USP11 silencing (Fig. 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 pancreatic ductal adenocarcinoma 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; refs. 48–50).

Interestingly, sensitivity to gemcitabine with USP11 knockdown was also increased, suggesting a powerful synergy between inhibition of the USP11 and gemcitabine (Fig. 3). To our knowledge, this is the first report suggesting a synergistic link between an anti-USP11 treatment strategy and a nucleoside analog like gemcitabine. We were unable to reproduce this synergy with gemcitabine when using mitoxantrone instead of a siRNA strategy to inhibit USP11 (Fig. 4). Given the rapid resistance to gemcitabine that develops clinically, this study raises the possibility that a siRNA silencing approach may be superior to cotreatment with a pharmacologic inhibitor when targeting USP11 as a strategy to break gemcitabine resistance. Unlike prior work, we were unable to confirm synergism between USP11 inhibition and PARP inhibition (ref. 10; albeit the testing of just one PARP inhibitor, ABT-888) in our pancreatic ductal adenocarcinoma 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-FU, folinic acid, and cisplatinum in patients with resected pancreatic ductal adenocarcinoma (53, 54). Both studies were small (<30 patients) and single-arm studies, in comparison with 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 conducted by the Southwest Oncology Group (SWOG) that compared single-agent mitoxantrone to a multidrug regimen (fluorouracil, 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 gemcitabine (standard-of-care) have little-to-no benefit (56). Modern first-line palliative chemotherapy trials for pancreatic ductal adenocarcinoma (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 pancreatic ductal adenocarcinoma remains unknown. More recently, mitoxantrone in combination with doxorubicin and irinotecan was used in drug-eluting bead therapy in a murine model of pancreatic ductal adenocarcinoma and was shown to decrease tumor proliferation and increase apoptosis (57). Finally, in a HTS for compounds showing synthetic lethality with TNF-α–related apoptosis inducing ligand (TRAIL) therapy, mitoxantrone was shown to reduce tumor cell viability at concentrations below 1 μmol/L in pancreatic cancer cell lines (58).

In summary, we determined that USP11 is a therapeutic target in preclinical models of pancreatic ductal adenocarcinoma, and identified mitoxantrone as a potent inhibitor of the DNA repair enzyme, USP11. In addition, drug response was associated with baseline USP11 expression and potency far exceeded results observed with gemcitabine. This study lays the framework to: (i) explore USP11 as a promising “druggable” target and biomarker in additional preclinical pancreatic ductal adenocarcinoma models; and (ii) use mitoxantrone as a bioavailable lead compound for treating this lethal disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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www.aacjournals.org Mol Cancer Res; 11(8) August 2013 909

Published OnlineFirst May 21, 2013; DOI: 10.1158/1541-7786.MCR-12-0699

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Acknowledgments

The authors thank Winston C. Y. Yu at the National Health Research Institutes (NHRI) for the USP11 pGEX-5x-1 plasmid.

References


Grant Support

This work was supported in part by a grant from Delaware Health Sciences Alliance (to Z. Zhuang, A. Napper, and J.R. Brody). AACR-PanCAN grant # 10-20-25-BROD, ACS grant # RSG-10-19-01-G2D, and grants from the WW Smith Foundation (C1004 and C1104, to J.R. Brody), and from the Lisa Walter Hayes Foundation (to R.A. Burkhart). This work was also, in part, supported from resources provided by the “Fund a Cure for Pancreatic Cancer” project. We are also grateful for Abbott Laboratories (Abbott Park, Illinois).

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Received December 18, 2012; revised March 28, 2013; accepted May 8, 2013; published OnlineFirst June 4, 2013.

Published OnlineFirst May 21, 2013; DOI: 10.1158/1541-7786.MCR-12-0699
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