

Therapeutic approaches to *BRCA2*-associated pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a five-year overall survival rate of only 6%. The current treatment strategies are largely ineffective and there is a critical need to identify novel therapeutic approaches. Current research is focused on delineating the various molecular subtypes of PDAC, with the aim of developing more effective personalized therapies targeting specific defects in each PDAC subtype. Since hereditary forms of PDAC are attributed to common genetic driver mutations, each characterized hereditary form of PDAC may represent a subtype of PDAC that could potentially be exploited therapeutically by targeting common molecular defects. One of these hereditary subtypes is *BRCA2*-associated PDAC, which results in tumors that are defective in homology directed DNA repair. Thereby, we have hypothesized that *BRCA2*-associated PDAC may be therapeutically exploited with DNA crosslinking (DCL) agents or poly-ADP ribose polymerase inhibitors (PARPis). To investigate the use of these agents in *BRCA2*-associated PDAC, we tested a panel of DCL agents and PARPis in *BRCA2*-proficient and –deficient cell lines. Consistent with our hypothesis, we found that *BRCA2*-deficient Capan-1 PDAC cells were significantly more sensitive to treatment with DCL agents and PARPis compared to *BRCA2*-proficient MIA PaCa-2 PDAC cells. IC_{50} values in MIA PaCa-2 versus Capan-1 were 38.3 μ M versus 10.2 μ M ($p = 0.015$) for cisplatin, 96.5 μ M versus 24.9 μ M ($p = 0.0287$) for oxaliplatin, 700.3 μ M versus 99.4 μ M ($p = 0.0015$) for carboplatin, 152.7 μ M versus 89.7 μ M ($p = 0.0001$) for veliparib and 58.23 μ M versus 16.0 μ M ($p = 0.0105$) for BMN 673. We provide further support for this observation by showing that shRNA-mediated *BRCA2* knockdown in PANC-1, a *BRCA2*-proficient PDAC cell line, induces sensitivity to

cisplatin and BMN 673 but not to veliparib. These findings were validated in a PDAC murine xenograft model derived from a patient carrying a germline *BRCA2* mutation. End-point tumor volumes of the treatment versus control trial arms were $189.24 \text{ mm}^3 \pm 31.65 \text{ mm}^3$ (SD) versus $520.55 \text{ mm}^3 \pm 62.68 \text{ mm}^3$ (SD) ($p = 0.0004$) for cisplatin and $195.05 \text{ mm}^3 \pm 95.21 \text{ mm}^3$ (SD) versus $520.55 \text{ mm}^3 \pm 62.68 \text{ mm}^3$ (SD) ($p = 0.0005$) for BMN 673. Reduction of tumor proliferation with treatment was assessed by Ki-67 immunohistochemical analysis of end-point tumors and was found to parallel growth inhibition. The percentage of proliferating cells in cisplatin- and BMN 673-treated versus control arm tumor sections was 6.1-fold lower [$1.5\% \pm 0.2\%$ (SD); $p = 0.0008$] and 5.9-fold lower [$1.6\% \pm 1.3\%$ (SD) versus $9.1\% \pm 1.4\%$ (SD); $p = 0.0024$], respectively. Our findings support a personalized treatment approach for *BRCA*-associated PDAC and suggest that BMN 673 be considered for clinical trial in *BRCA*-associated PDAC.

Résumé

Dans la mesure où la plupart des patients atteints du cancer du pancréas succombent à leur maladie en moins de douze mois, un besoin urgent s'impose d'identifier de nouvelles approches thérapeutiques. Les tumeurs associées aux mutations inactivatrices du gène *BRCA2* sont incapables de réparer efficacement les lésions de l'ADN par le biais de la recombinaison homologe, ce qui peut être exploité en termes thérapeutiques, en traitant ces tumeurs à l'aide d'une combinaison d'agents à base de platine ou d'inhibiteurs de la polyADP-ribose polymérase (PARPi). Pour investiguer l'utilité potentielle de ces agents dans le cadre du traitement du cancer du pancréas associé aux mutations dans *BRCA2*, ainsi que sélectionner ceux qui mériteraient une caractérisation *in vivo*, nous avons comparé la susceptibilité de lignées cellulaires de cancer du pancréas déficientes ou non en *BRCA2* à un panel d'agents à base de platine et d'inhibiteurs PARP. Nous avons ainsi observé que les cellules Capan-1, déficientes en *BRCA2*, sont exclusivement sensibles au traitement par des agents qui ciblent sélectivement les défauts de réparation de l'ADN. Les valeurs de CI_{50} des cellules MIA PACA-2 versus Capan-1 sont de 38,3 uM contre 10,2 uM ($p = 0,015$) pour le cisplatine, 96,5 uM contre 24,9 uM ($p = 0,0287$) pour l'oxaliplatine, 700,3 uM contre 99,4 uM ($p = 0,0015$) pour le carboplatine, 152,7 versus 89,7 uM ($p = 0,0001$) et pour veliparib 58,23 versus 16,0 uM ($p = 0,0105$) pour BMN 673. Pour déterminer si cette sensibilité est expliquée par l'absence fonctionnelle de protéine *BRCA2* dans ces cellules, nous avons établi deux lignées cellulaires dans lesquelles *BRCA2* a été inactivé via un traitement de shRNA afin d'effectuer une caractérisation *in vitro* de différents agents thérapeutiques. Malgré une réduction partielle de l'expression de *BRCA2* via les shRNA, nous avons

observé une sensibilisation au cisplatine et au BMN 673 dans les cellules cancéreuses, mais pas au veliparib, indiquant donc que le BMN 673 pourrait être plus sélectif de l'absence fonctionnelle de BRCA2 par rapport au veliparib. De plus, nous avons établi un modèle de xénogreffe *in vivo* en utilisant du tissu tumoral en provenance d'un patient porteur d'une mutation germinale hétérozygote dans *BRCA2*. Nous avons ainsi constaté que le traitement en monothérapie avec le cisplatine et le BMN 673 résulte en une inhibition significative de la croissance de PDX de notre patient. Les volumes finaux des tumeurs une fois le traitement complété, par rapport aux contrôles, sont de $189,24 \pm 31,65 \text{ mm}^3$ contre $520,55 \pm 62,68 \text{ mm}^3$ ($p = 0,0004$) et pour le cisplatine, de $195,05 \text{ mm}^3 \pm 95,21 \text{ mm}^3$ contre $520,55 \pm 62,68 \text{ mm}^3$ ($p = 0,0005$) avec le BMN 673. La réduction de la prolifération des tumeurs due au traitement a été évaluée par un immunomarquage de Ki67 sur les tumeurs ayant atteint leur volume final, et semblerait suivre en parallèle l'inhibition de la croissance. Le pourcentage de prolifération des cellules traitées à base de cisplatine et de BMN 673 versus les tumeurs contrôles était de 6,1 fois plus bas [$1,5\% \pm 0,2\%$ (SD) contre $9,1\% \pm 1,4\%$ (SD); $p = 0,0008$] et 5,9 fois plus bas [$1,6\% \pm 1,3\%$ (SD) contre $9,1\% \pm 1,4\%$ (SD); $p = 0,0024$], respectivement. Nous avons ici pu démontrer l'efficacité *in vitro* et *in vivo* du traitement en monothérapie à base de cisplatine et de BMN 673, apportant ainsi un fondement pour les essais cliniques de chimiothérapie combinant les PARPi et le platine pour les patients atteints du cancer du pancréas porteurs d'une mutation germinale dans le gène *BRCA2*.

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This thesis is dedicated to my parents (RR & CA), with all my heart.

List of Abbreviations

Abbreviation	Definition
5-FU	Fluorouracil
ADP	Adenosine diphosphate
AJ	Ashkenazi Jewish
APTX	Aprataxin
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BD-IPMN	Branch duct IPMN
BER	Base-excision repair
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRCT	BRCA C terminus domain
CA 15-3	Cancer antigen 15-3
CTD	C-terminal domain
DCL	DNA cross-linking
DP	Dual-plate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DSB	Double-strand break
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementation group 1
FAMMM	Familial atypical multiple mole melanoma
FANCF	Fanconi anemia complementation group F
FBS	Fetal bovine serum
FC	French Canadian
GI	Growth inhibition
H2AX	H2A histone family, member X
HBC	Hereditary breast cancer
HBOC	Hereditary breast and ovarian cancer
HDR	Homology-directed repair
H&E	Hematoxylin and eosin
HNPCC	Hereditary non-polyposis colon carcinoma
IC ₅₀	Half-maximal inhibitory dose
IPMN	Intraductal papillary mucinous neoplasm
MMR	Mismatch repair
MPD-IPMN	Main pancreatic duct IPMN
MRE11	MRE11 meiotic recombination 11 homolog A
NAD ⁺	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PALB2	Partner and localizer of BRCA2

PanIN	Pancreatic intraepithelial neoplasia
PAR	Poly-ADP ribose
PARG	Poly(ADP-ribose) glycohydrolase
PARylation	Poly(ADP-ribo)sylation
PARP	Poly(ADP-ribose) polymerase
PARPi	Poly(ADP-ribose) polymerase inhibitor
PBS	Phosphate-buffered saline
PDAC	Pancreatic adenocarcinoma
PDX	Patient-derived xenograft
PJS	Peutz-Jegher's syndrome
PNKP	Polynucleotide kinase 3'-phosphatase
QPCS	Quebec Pancreas Cancer Study
RAD51	DNA repair protein RAD51 homolog 1
RPMI-1640	Roswell Park Memorial Institute
RTCA	Real-time cell analysis
RTV	Relative tumor volume
SCID	Severe combined immunodeficiency
SD	Standard deviation
shRNA	Short hairpin RNA
SSB	Single-strand break
TGI	Tumor growth inhibition
XRCC1	X-ray repair cross-complementing protein 1

Chapter I:

Literature Review

Ia. Overview of pancreatic cancer

The clinical problem

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in Canada and the most fatal gastrointestinal cancer worldwide, with most patients succumbing to their disease within twelve months of diagnosis (Siegel *et al.* 2013). It is estimated that in 2014, 4 700 Canadians will be diagnosed with PDAC and 4 400 will die as a result (Arslan *et al.* 2014). These tragic statistics have not changed in 40 years and 80% of new cases continue to be diagnosed late, with inoperable disease (Hidalgo *et al.* 2010). Unfortunately, therapeutic options currently available for these patients are largely ineffective and even patients who present with operable disease have poor outcomes. Despite surgical advances, survival following curative intent surgery remains low due to early recurrences (Sutton *et al.* 2014). These outcomes suggest that even cases considered resectable at presentation likely have undetectable micrometastatic disease at diagnosis. Together, these observations suggest that surgical advances alone will not conquer this lethal disease without major improvements in systemic therapy. Current research efforts are focused on delineating the molecular subtypes of PDAC with the goal of applying this new knowledge to develop tailored and more effective treatment strategies (Rustgi *et al.* 2014, Biankin *et al.* 2012).

Histological classification of pancreatic neoplasms

The anatomic divisions of the pancreas are the uncinete process, head, neck, body and tail (Figure 1).

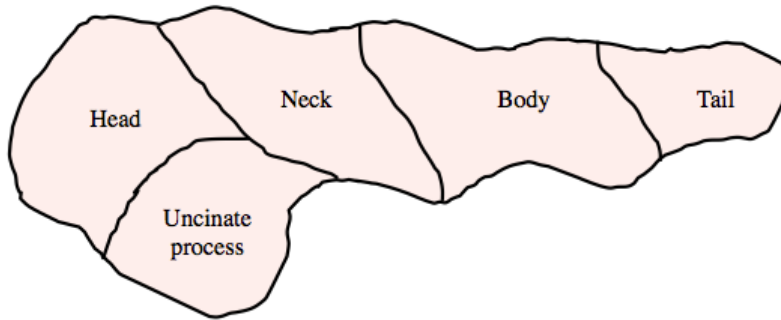


Figure 1. Anatomy of the pancreas.

The pancreas is composed of three predominant cell types, with the exocrine cell types composing the bulk of the gland. These consist of acinar cells and mucinous columnar epithelial cells lining the pancreatic ductal system. Acinar cells produce and secrete digestive enzymes in the pancreatic duct tributaries, which empty into the main pancreatic duct that ultimately empties into the duodenum where the digestive proenzymes are activated (Williams *et al.* 2010). Clusters of endocrine cells, called islets of Langerhans, are present throughout the pancreas and are responsible for the endocrine functions of the gland, including the control of blood glucose levels (Jain *et al.* 2009).

Neoplasms of the pancreas can arise from both its exocrine and endocrine cells, with exocrine neoplasms accounting for the majority of pancreatic neoplasms (Esposito *et al.* 2014). The natural histories and, thus clinical management, of exocrine (pancreatic adenocarcinomas) and endocrine (pancreatic neuroendocrine) neoplasms are distinct. My

thesis focuses on PDAC, the most common histological subtype of pancreatic adenocarcinoma.

Microscopically, PDAC is characterized by infiltrating glands with an intense and pathognomonic desmoplastic reaction (Apte *et al.* 2004). Desmoplasia is the result of marked fibroblast proliferation and increased deposition of extracellular matrix components. While PDAC is the more common histological subtype (90%), acinar cell adenocarcinoma, mucinous cystadenocarcinoma and intraductal papillary-mucinous neoplasm (IPMN) with an invasive component are more rare adenocarcinomas of the pancreas (Distler *et al.* 2014). IPMN is a cystic lesion that grows either from the main pancreatic duct (main-duct IPMN) or branch of the pancreatic duct (side-branch IPMN). These tumors are papillary projections into the duct that may be non-invasive. The malignant potentials of main and side-branch IPMN are different and, consequently the clinical management of main- and side-branch IPMNs is not the same (Marchegiani *et al.* 2014). Finally, histological variants of PDAC have been described, including mucinous noncystic adenocarcinoma, signet-ring cell carcinoma, adenosquamous carcinoma, anaplastic carcinoma, undifferentiated carcinoma with osteoclast-like giant cells, and mixed ductal-endocrine carcinoma (Fitzgerald *et al.* 2008). Regardless of the histological subtype or variant, all forms of PDAC are highly aggressive malignancies, in which vascular, lymphatic and perineural invasion is frequently present.

Molecular pathogenesis of PDAC

A progression model for PDAC development has been proposed based on histological and genetic alterations observed in PDAC and its pre-invasive predecessor lesions. Similar to the progression in the colon from normal colonic epithelium to invasive carcinoma, the normal ductal epithelium progresses to dysplastic lesions to invasive adenocarcinoma (Klöppel *et al.* 2014). Dysplastic ductal cells acquire atypical features and evolve to lesions called pancreatic intraepithelial neoplasia (PanIN). PanINs are small (<5mm) ductal lesions that develop within the small caliber pancreatic ducts, typically in the head of the pancreas (Iacobuzio-Donahue *et al.* 2012). They are classified into 3 grades: PanIN-1 (subdivided into PanIN-1A and PanIN-1B), PanIN-2 and PanIN-3. PanIN-1 lesions are characterized by mucinous differentiation of ductal cells with minimal cellular atypia, whereas PanIN-3 lesions correspond to *in situ* carcinoma. Progression from PanIN-1 to PanIN-3 is accompanied by an accumulation of genetic alterations in *KRAS2*, *CDKN2A*, *TP53* and *SMAD4*. *KRAS2* mutations are typically acquired early in the progression to invasive carcinoma and are present in most lesions (90%). They can be used as markers for the detection of PanINs, but offer no indication of histological grade. *TP53* or *SMAD4* mutations, however, which occur in later stages, may suggest the presence of a high-grade precursor lesion or invasive carcinoma (Wolfgang *et al.* 2013). Despite these advances in understanding the molecular progression of normal epithelium to invasive cancer, the genetic subtypes of PDAC remain largely unknown.

The molecular pathogeneses of acinar pancreatic cells and IPMNs to invasive adenocarcinoma likely follow similar progression models. IPMNs are macroscopically

visible cystic neoplasms that are classified as either main-duct IPMN or side-branch IPMN, as previously described. Approximately 70% of main-duct IPMNs may progress to invasive PDAC (Iacobuzio-Donahue *et al.* 2014). If detected early, surgery may offer the chance of a cure, with 5-year survival rates of 88% for benign and non-invasive IPMN lesions and 30-60% for more invasive lesions. As with PanINs, IPMNs are associated with genetic mutations in genes including *KRAS*, *TP53*, *RNF43* and *GNAS*. This accumulation of genetic alterations is thought to drive the progression of pancreatic cancer, and may begin with inherited germline mutations in *BRCA2*, *p16/CDKN2A*, *STK11*, *PALB2*, *ATM* and *PRSS1*, with additional mutations acquired as precursor lesions develop into invasive ductal carcinoma (Iacobuzio-Donahue *et al.* 2012, Jones *et al.* 2009).

Treatment of PDAC

As with most other cancer types, treatment and prognosis of PDAC is highly reliant on accurate staging of the disease and categorizing each case as either localized (stage I/II), locally advanced (stage III) or metastatic (stage IV) disease. The only curative modality for PDAC is surgical resection. However, only 20% of new diagnoses are operable since the majority of patients present with metastatic or unresectable locally advanced disease (Wolfgang *et al.* 2013).

In the case of localized operable disease, patients undergo surgical resection of the primary pancreatic lesion, sometimes preceded by neoadjuvant therapy and adjuvant therapy after surgery (Wolfgang *et al.* 2013). The benefit of neoadjuvant therapy remains controversial, as it delays potentially curative surgery, and is often reserved for borderline resectable cases with vascular abutment or involvement. With the shifting of pancreatic

resections to high volume centres over the past three decades, the peri-operative mortality of pancreatic surgery has decreased to 1-3%. However, despite these surgical advances the five-year survival rate remains only 15-27% (Kumar *et al.* 2013, Katz *et al.* 2009). These statistics may be explained by the systemic nature of PDAC, and the likely presence of undetectable micrometastases at the time of diagnosis (Nakao *et al.* 2006).

Unfortunately, approximately 80% of new PDAC diagnoses present with either metastatic or a non-operable locally advanced disease. These patients are treated with systemic cytotoxic therapy to control their disease and manage their symptoms. Gemcitabine was adopted as the standard of care for metastatic PDAC as the result of a pivotal trial in the 1990s in which it was found to improve median one-year survival from 2% with treatment with fluorouracil (5-FU) to 18% (Shi *et al.* 2012). Gemcitabine treatment was also associated with decreased pain intensity, reduced daily analgesic consumption and an improvement in Karnofsky performance status (Crooks *et al.* 1991). Throughout the 2000s, numerous trials have evaluated the efficacy of gemcitabine combinations including marimastat, tipifarnib, exatecan, irinotecan, pemetrexed, 5-FU, capecitabine, cisplatin, oxaliplatin, erlotinib, bevacizumab and cetuximab (Valsecchi *et al.* 2014). Unfortunately, none of these gemcitabine combinations have demonstrated improved efficacy, except for a modest improvement in median overall survival with gemcitabine and erlotinib (Moore *et al.* 2007). More recently, phase 3 clinical trials have identified two new regimens for the management of metastatic PDAC. In 2011, Conroy *et al.* showed an increased overall survival in patients with metastatic PDAC with FOLFIRINOX treatment, which is a combination regimen of four drugs (leucovorin, 5-FU, irinotecan and oxaliplatin, Conroy *et al.* 2011). The median overall

survival was 11.1 months in the FOLFIRINOX arm compared to 6.8 months in the gemcitabine arm. However, more adverse events were observed in the FOLFIRINOX group, with patients exhibiting increased cytopenias, neutropenic fever, diarrhea, vomiting and peripheral neuropathy. Despite increased cytotoxicity, 31% of patients in the FOLFIRINOX group displayed a significantly decreased quality of life, compared to 66% in the gemcitabine group (95% CI, 0.30 to 0.70; $p < 0.001$). As such, FOLFIRINOX was found to confer a survival advantage and represents a suitable option for the treatment of metastatic PDAC in patients with a good performance status. In an even more recent phase 3 trial, Von Hoff and colleagues showed that patients with metastatic PDAC have improved overall survival, progression-free survival, and response rate with nab-paclitaxel plus gemcitabine compared to gemcitabine alone treatment (Van Hoff *et al.* 2013). The median overall survival was 8.5 months in the nab-paclitaxel-gemcitabine group compared to 6.7 months in the gemcitabine alone group. However, rates of peripheral neuropathy and myelosuppression were also increased. Despite increased toxicities with these two new regimens, FOLFIRINOX and nab-paclitaxel-gemcitabine provide patients with metastatic disease and good performance therapeutic options with improved efficacy.

Personalized medicine strategies with tailored approaches aimed at targeting specific genetic and molecular defects in subtypes of PDAC may offer patients even greater treatment efficacies with potentially less secondary effects. Advances in our understanding of the genetic subtypes of PDAC will help with the design of such tailored therapeutic strategies. PDAC cases arising from *BRCA1*, *BRCA2* and *PALB2* germline mutations are examples of genetic PDAC subtypes in which there may be an avenue for

tailored systemic therapies. These tumors have defects in DNA repair and may be uniquely sensitive to treatments that target their inherent DNA repair deficiencies (Tischkowitz *et al.* 2009, Lowery *et al.* 2011, Golan *et al.* 2014, Smith *et al.* 2015).

Ib. Hereditary contributions to the development of PDAC

Approximately 10% of PDAC cases are attributable to genetic predisposition (Hruban *et al.* 2010). The evidence for hereditary forms of PDAC is based on several case-control studies demonstrating that patients with PDAC are more likely to have a relative affected with PDAC than are healthy controls (Shirts *et al.* 2010, Brune *et al.* 2010, Jacobs *et al.* 2010, Klein *et al.* 2004, Ghadirian *et al.* 2002, Tersmette *et al.* 2001, Ghadirian *et al.* 1991). Modeling analyses using these families favor autosomal dominant models of inheritance (Klein *et al.* 2002). In addition, twin studies suggest that heritable factors may account for up to 36% of PDAC risk (Lichtenstein *et al.* 2000). A third line of evidence that genetic predisposition is an important risk factor for PDAC comes from hereditary syndromes where the tumor spectrum includes PDAC.

These hereditary syndromes include Peutz-Jegher's syndrome (PJS), familial atypical multiple mole melanoma (FAMMM), hereditary non-polyposis colorectal cancer (HNPCC), hereditary pancreatitis, and hereditary breast and ovarian cancer syndrome (HBOC). Of these inherited conditions, germline mutations in *BRCA2* are the most common inherited contributors to increased risk of PDAC (Klein *et al.* 2012). These recognized syndromes, however, account for only a small fraction of hereditary PDAC and one of the most important questions in the field remains the identification of the genetic causes of familial PDAC where known genes are not implicated.

Peutz-Jeghers syndrome

PJS is an autosomal dominant cancer syndrome, typically associated with germline mutations in the *STK11* gene (also known as *LKB1*) and characterized by benign hamartomatous polyps in the gastrointestinal tract and stomach, as well as hyperpigmented macules on the lips and oral mucosa (Giardiello *et al.* 2010). When mutated, the *STK11* tumor suppressor gene leads to uncontrolled cell growth, and the formation of noncancerous polyps and cancerous tumors. Along with an increased risk of developing GI tract cancers, cervical cancer, ovarian cancer and breast cancer, individuals with PJS have a 132-fold increased risk of PDAC compared to the general population, and an 11-32% lifetime risk of developing PDAC (Klein *et al.* 2012).

Familial atypical multiple mole melanoma

FAMMM is an autosomal dominant genodermatosis characterized by the presence of multiple, usually over 50, melanocytic nevi, a family history of melanoma, and germline mutations in *p16/CDKN2A* (Rustgi *et al.* 2014, de Snoo *et al.* 2008, Goldstein *et al.* 1995). *CDKN2A* encodes two main transcripts, p16(INK4a) and p14(ARF). p14(ARF) is implicated in cell cycle control, by acting as a stabilizer of the tumor suppressor protein P53. In the normal cell cycle, p16(INK4a) and p14(ARF) are critical mediators of G1/S phase transition, by inhibiting the cyclin-dependent kinases CDK4 and CDK6, consequently preventing the phosphorylation of the retinoblastoma protein RB1. This inhibits the release of transcription factors that induce progression to the S phase, under normal circumstances. Germline *CDKN2A* mutations disrupt the function of p16(INK4a) and p14(ARF), leading to improper G1 to S phase progression and uncontrolled cell growth and proliferation (McWilliams *et al.* 2010). Along with an

associated 13- to 22-fold increased risk of melanoma, individuals with FAMMM have a 38-fold increased risk of developing PDAC, compared to the general population, and a 17% lifetime risk (Klein *et al.* 2012).

Hereditary non-polyposis colorectal cancer

HNPCC is a genetically heterogeneous cancer syndrome typically associated with early onset colon cancer as a result of germline mutations in the *MSH2*, *MLH1*, *PMS1*, *PMS2* and *MSH6* mismatch repair (MMR) genes, which are critical for proper DNA mismatch repair (Wolfgang *et al.* 2013). Of the MMR genes implicated in HNPCC, mutations in *MSH2* are responsible for 60% of HNPCC cases, while mutations in *MLH1* account for 30%, classifying these genes as tumor suppressor genes (Peltomaki *et al.* 2003). Defects in DNA repair by MMR leads to microsatellite instability, a hallmark in many human cancers. HNPCC can be subdivided into 1) Lynch syndrome I (site-specific familial colon cancer) and 2) Lynch syndrome II (extra-colonic carcinoma), which is associated with increased risk of stomach cancer, endometrial cancer, biliary tract cancer, urinary tract cancer and a 8.6-fold increased risk of PDAC compared to the general population, and a 3.98% lifetime risk of developing PDAC (Klein *et al.* 2012).

Hereditary pancreatitis

Unlike the hereditary cancer syndromes with clearly defined clinical features as described in previous sections, hereditary pancreatitis is a chronic inflammatory, hereditary pancreatitis is a chronic inflammatory syndrome, which is associated with a 53-fold increased risk and a lifetime risk of 30-40% of developing PDAC (Lowenfels *et al.* 1997). This genetic syndrome is characterized by inherited germline mutations in the *PRSS1* and *SPINK1* genes, which cause an autosomal dominant or autosomal recessive

form of hereditary pancreatitis, respectively (Klein *et al.* 2012). Individuals with hereditary pancreatitis suffer from episodes of acute inflammation of the pancreas during childhood, leading to chronic inflammation by early adulthood. *PRSSI* encodes the enzyme trypsinogen, which is secreted by the pancreas, transported to the small intestine where it is converted to trypsin to aid in food digestion. Individuals harboring germline mutations in *PRSSI* express a form of trypsinogen that is prematurely converted to trypsin while still in the pancreas, causing tissue damage in the pancreas and triggering an inflammatory immune response. This chronic tissue damage leads to fibrosis of functional pancreatic tissue, loss of pancreatic function, decreased production of digestive enzymes and insulin, which collectively results in the disruption of normal digestion, weight loss, and in some cases, the onset of diabetes mellitus. The chronic inflammatory state is also thought to underlie the increased lifetime risk of developing PDAC, which is estimated at 30-40% in these patients (Rustgi *et al.* 2014, Vitone *et al.* 2005).

Hereditary breast and ovarian cancer syndrome

HBOC is most often caused by inherited germline mutations in *BRCA1* and *BRCA2*, and is characterized by an increased risk of breast cancer, ovarian cancer, male breast cancer, prostate cancer and PDAC (Klein *et al.* 2012, van Asperen *et al.* 2005). HBOC is suspected in families with multiple blood relatives diagnosed with breast cancer, ovarian cancer, or both, the presence of both primary breast and ovarian cancers in the same individual, or early age of onset of breast cancer (<50 years of age). Confirmation HBOC is obtained through genetic mutation testing of affected individuals. PDAC may also occur in families with Hereditary Breast Cancer (HBC) syndrome. HBC has been used to characterize kindred with breast cancer but without ovarian cancer

clustering, and is, at least, partially accounted by *BRCA1* and *BRCA2* germline mutations (Tonin *et al.* 2000). The approximate lifetime risk of developing breast cancer is 40-80%, 11-40% for ovarian cancer, 1-10% for male breast cancer, 40% for prostate cancer, and 1-7% for PDAC in *BRCA1* and *BRCA2* mutation carriers (Klein *et al.* 2012, Risch *et al.* 2006).

Of the genes that are currently associated with increased PDAC predisposition, germline mutations in *BRCA2* account for the majority of hereditary PDAC. *BRCA2* germline mutations are estimated to underlie 5-19% of the hereditary fraction of PDAC (Iqbal *et al.* 2012). Moreover, germline mutations in *BRCA2* contribute more readily to PDAC risk compared to *BRCA1*. A recent study suggested that the relative risk for PDAC in mutation carriers is 2.26 (95% CI=1.26-4.06, P=0.004) for *BRCA1* and 3.51 (95% CI =1.87-6.58, P=0.0012) for *BRCA2* (Iqbal *et al.* 2012). This correlates with a second study that showed the lifetime risk of PDAC to be 2-7% in *BRCA2* mutation carriers, 1-3% in *BRCA1* mutation carriers, and, 0.50% in the general population (Petrucelli *et al.* 2013). In certain populations where specific founder mutations in *BRCA1* and *BRCA2* are more common, such as the Ashkenazi Jewish (AJ) and French Canadian (FC) population, the contribution of these mutations to PDAC predisposition may be more significant (Axilbund *et al.* 2012). It is estimated that 1/40 individuals of AJ decent harbor a *BRCA1* or *BRCA2* mutation, compared to 1/345-1/1000 individuals in the general population (Petrucelli *et al.* 2013). The contribution of FC *BRCA1* and *BRCA2* founder mutations to PDAC is currently under investigation (Smith *et al.* 2015).

Clinical screening of individuals at increased risk for PDAC

One strategy to improve detection rates of early stage PDAC is to implement early detection screening programs for individuals at high lifetime risk for PDAC. A role for screening programs is supported by recent estimates that PDAC develops over a 10-15 year period following the initiating cancer cell mutation, providing significant lead-time for screening (Yachida *et al.* 2010). Unfortunately, screening strategies for individuals at risk on the basis of family history have been largely ineffective, creating patient anxiety around screening without clinical benefit (Al-Sukhni *et al.* 2012). These disappointing results call for screening strategies with increased sensitivities to detect early pre-malignant lesions rather than early invasive cancers. In addition, successful screening programs also require precise patient selection criteria. Thereby, a full understanding of the genetic risks associated PDAC will allow for clinical resources to be directed to these more specific and rigorous screening programs.

Ic. Therapeutic opportunities for *BRCA1*- and *BRCA2*-associated tumors

Tumors arising in the context of a germline *BRCA1* or *BRCA2* mutation may be particularly sensitive to agents that exploit defects in DNA repair. Breast and ovarian cancer patients harboring germline *BRCA1* and *BRCA2* mutations have been successfully treated with targeted therapy regimens including DNA crosslinking (DCL) agents and poly(ADP)-ribose polymerase inhibitors (PARPi) (O’Sullivan *et al.* 2014). However, aside from a handful of clinical case reports suggesting favorable responses and outcomes in *BRCA1*- and *BRCA2*-associated PDAC, the efficacy of these agents in this PDAC subtype remains widely uncharacterized (Lowery *et al.* 2011, Golan *et al.* 2014).

In addition to tumors that arise in the context of *BRCA1* and *BRCA2* germline mutations, there are sporadic tumors with “BRCA-like” features (Helleday *et al.* 2008). The term “BRCA-like” refers to the characteristics or traits that seemingly sporadic cases share with those occurring in the context of a defined germline *BRCA1* or *BRCA2* mutation (Turner *et al.* 2004). The major identified clinical “BRCA-like” behavior is the susceptibility of these tumors to DCL agents and other DNA damaging agents (Rigakos *et al.* 2012). Although the molecular characteristics accompanying these clinical observations have not yet been clearly defined, evidence from “BRCA-like” breast and ovarian tumor data suggests an underlying genetic defect in DNA repair, caused by mutations in genes implicated in the homology-directed repair (HDR) pathway (De Summa *et al.* 2013, Muggia *et al.* 2014). Genetic events such as promoter methylation and silencing of *BRCA1*, loss or disruption of *RAD51*, *ATM*, *ATR*, *CHK1*, *CHK2*, *FANCD2* and *FANCA* have been reported in “BRCA-like” breast and ovarian tumors harboring HDR defects, and similar changes may also be present in “BRCA-like” PDAC (Gudmundsdottir *et al.* 2006).

With the advent of personalized medicine, a better understanding of the molecular characteristics underlying this inherent HDR defect will enable clinicians to extend the use of DCL agents, PARPis and other DNA damaging agents to cancers arising in *BRCA1* and *BRCA2* germline mutation carriers and to “BRCA-like” tumors. One major limitation is the lack of predictive biomarkers available for the identification of “BRCA-like” tumors (Tutt *et al.* 2005). Based on observations in breast and ovarian cancers, proposed predictive biomarkers of PARPi sensitivity include *BRCA1* and *BRCA2* somatic mutation or methylation, *ATM* mutation, *MRE11*-dominant negative mutations in MMR-

deficient tumors, *FANCF* promoter methylation and PTEN deficiency (Burgess *et al.* 2014). These observations may assist in the development of assays to evaluate HDR defects, in order to rapidly and efficiently identify patients who may benefit from therapy targeting this inherent defect in DNA repair. As a result, the utility of PARPis and DCL agents may exceed previously defined clinical expectations.

HDR

Several mechanisms of DNA damage repair exist, including two well-defined mechanisms for the repair of double stranded breaks (DSBs) [HDR and non-homologous end-joining (NHEJ)] and four mechanisms for the repair of single-stranded breaks (SSBs) [base excision repair (BER), nucleotide excision repair (NER), MMR and trans-lesional synthesis] (Aparicio *et al.* 2014). DNA errors are generated due to normally occurring replication errors, exposure to ultraviolet light, therapeutic and ambient radiation and chemicals, which are closely monitored and quickly repaired by these mechanisms, keeping DNA damage in check (Aparicio *et al.* 2014). Normally occurring SSBs are repaired by one of the four mechanisms of SSB repair. If left unrepaired, these single-strand gaps in DNA may generate the more detrimental form of DNA damage, DSBs, when encountered by a replication fork. DSBs must be repaired by either the high-fidelity HDR pathway, mediated by BRCA1 and BRCA2, or through the low-fidelity NHEJ system (Dietlein *et al.* 2014). The HDR pathway is initiated when DSBs are detected by sensor proteins ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) kinases. The sensor proteins detect DNA damage and direct the recruitment of additional mediator proteins, which direct the formation of macro-complexes and further recruit effector proteins necessary for DNA damage repair.

Specifically, H2A histone family, member X (H2AX) is recruited to the site of the DSB, which is phosphorylated in an ATR-dependent manner and subsequently recruits BRCA1. Phosphorylated BRCA1 recruits additional proteins responsible for mediating HR, including the partner and localizer of BRCA2 (PALB2) and BRCA2. BRCA2 recruits the main HDR effector protein RAD51 homolog 1 (RAD51) through physical interaction with RAD51 via BRC repeats in exon 11 of BRCA2 as well as the C-terminal domain (CTD) in exon 27 of BRCA2. RAD51 is directed to SSB sites, where it catalyzes the critical step of HDR: homologous pairing and strand invasion. Accordingly, tumor cells arising in the context of germline mutations in *BRCA1*, *BRCA2* or *RAD51*, harbor an inherent defect in HDR-mediated repair of DNA damage, rendering these cells uniquely sensitive to agents targeting the DNA repair pathway (Figure 2, Peng *et al.* 2011, Kaelin *et al.* 2005, Alvarez-Gonzalez *et al.* 1995).

Poly (ADP-ribose) polymerase (PARP)

Synthetic lethality

The synthetic lethal interaction between *BRCA1* or *BRCA2* loss and PARP inhibition may be therapeutically exploited using PARPis, and has been explored in *BRCA1*- and *BRCA2*-associated breast and ovarian cancer, among other cancer subtypes (Dedes *et al.* 2013). The basis for this strategy relies on the DNA repair roles of BRCA1, BRCA2, and PARP proteins (Dedes *et al.* 2013). Under PARP inhibition, naturally occurring SSBs accumulate within the cell, as PARP proteins are a critical mediator of SSB repair (McCabe *et al.* 2006). Accumulated SSBs are consequently converted to DSBs during DNA replication, which cannot be repaired in BRCA1 and BRCA2-

deficient cells, as DSB repair is highly reliant on proper BRCA1 and BRCA2 function. As a result, PARPi use in BRCA2-deficient tumor cells leads to an accumulation of DSBs, stalled replication forks, and an insurmountable level of DNA damage, consequently leading to cell death (Ame *et al.* 2014, Kaelin *et al.* 2005, Lord *et al.* 2008). Although the sensitivity of *BRCA1*- and *BRCA2*-associated PDAC to PARPis is not well characterized, the efficacy of PARPis in treating breast and ovarian cancers with *BRCA1* or *BRCA2* mutations suggests a role for PARPis in *BRCA1*- and *BRCA2*-associated PDAC subtype (Ang *et al.* 2013, Audeh *et al.* 2010, Metzger *et al.* 2013).

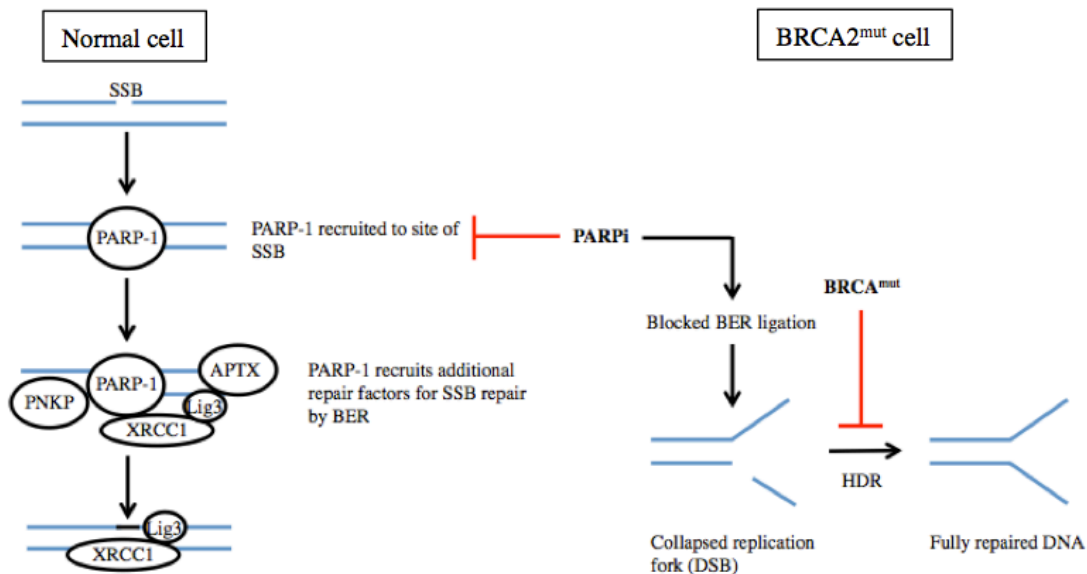


Figure 2. Synthetic lethality between *BRCA* loss and PARP-1 inhibition. Left (normal cell): SSBs, which arise naturally during normal cell processes, are repaired by PARP-1 and associated effector proteins: XRCC1-Ligase3, APTX, PNKP. When this DNA repair process is inhibited, as in the case of PARP-1 inhibition by PARPi treatment, SSBs remain unrepaired and form DSBs upon reaching a replication fork. Right (*BRCA2*^{mut} cell): In *BRCA*-deficient cells collapsed replication forks are not repaired, leading to irreparable DNA damage and cell death (McCabe *et al.* 2005).

PARP protein family

The PARP family of proteins is heavily implicated in a number of cellular processes, including DNA repair and cell death (Gagne *et al.* 2006). Of the 17 members of the PARP family, PARP-1 is the best understood and is involved mostly in DNA damage response, specifically the repair of SSBs by BER (Ame *et al.* 2014). The PARP-1 protein is composed of three N-terminal DNA binding zinc finger motifs, a BRCA1 C-terminus-like motif (BRCT), an auto-modification domain, and the C-terminal catalytic domain that transfers ADP-ribose from nicotinamide adenine dinucleotides (NAD⁺) substrates to target proteins, resulting in the attachment of linear or branched poly(ADP-ribose) (PAR) polymers. These polymers are composed of repeating ADP-ribose units, linked via glycosydic ribose-ribose 1''→2' bonds (Alvarez-Gonzalez *et al.* 1995, Otto *et al.* 2005). Of all ADP-ribosyltransferases, PARP-1 is the most abundant and is responsible for the poly(ADP-ribosyl)ation or PARylation of the majority of proteins involved in DNA repair, along with the less abundant PARP-2. The enzymatic activity of PARP-1 and other PARP family member proteins relies heavily on the presence of NAD⁺, which is hydrolyzed to generate ADP-ribose units for PARylation of protein targets and the attachment of PAR (Kim *et al.* 2005, Otto *et al.* 2005). Levels and availability of NAD⁺ may be exploited as a means of PARP enzyme control as it has been described that the availability of NAD⁺ correlates with the length of PAR synthesis by PARP-1 *in vitro* and also regulates the effect of PARP-1 on chromatin structure and transcription (Kim *et al.* 2005). PARP-1 proteins are implicated in multiple DNA repair pathways, including the SSB, DSB and BER pathways. SSBs are detected by PARP-1, which binds to the damaged DNA through the PARP-1 double zinc finger DNA binding

domains, inducing autoPARylation and recruitment of DNA repair proteins, XRCC1 and Mre11, generating a functional protein complex capable of repairing DNA damage. AutoPARylation of PARP-1 leads to an accumulation of negatively charged ADP-ribose molecules, inducing its dissociation from the DNA, a necessary step for the completion of DNA repair. Upon dissociation, PAR chains are rapidly hydrolyzed by poly(ADP-ribose) glycohydrolase (PARG), generating free mono and oligo(ADP-ribose) and returning PARP-1 to its preactivated state (Ame *et al.* 2004, Alvarez-Gonzalez *et al.* 1995, Gagne *et al.* 2006, Gagne *et al.* 2012).

PARPis for the treatment of BRCA2-associated PDAC

Mechanism of action

Recent studies demonstrate that in BRCA1- and BRCA2-deficient cells treated with a PARP-1 inhibitor, there is a substantial increase in chromosomal instability as well as decreased formation of RAD51 foci, which indicates reduced DSB repair by HDR (Metzger *et al.* 2013). This suggests that PARPi-mediated repression of BER, the main mechanism of SSB repair, leads to the accumulation of SSBs, which are converted to DSBs during S phase. These DSBs then cause replication fork collapse, which cannot be repaired in cells lacking functional BRCA1 and BRCA2 proteins, and consequently, functional HDR machinery (McCabe *et al.* 2006).

PARPis have two defined mechanisms of action: the most well characterized being its role as a catalytic inhibitor of PARP-1/2 (Ashworth *et al.* 2008). The structure of PARPis includes a nicotinamide moiety that competes with NAD⁺, which is critical for PARP-mediated PARylation of target proteins (Otto *et al.* 2005). A novel PARPi

mechanism of action has been described, proposing that PARP-1 is trapped on DNA by PARPis, generating cytotoxic PARP-1-DNA complexes, which reduces PARP-1 availability for DNA damage repair, causing replication and transcription fork blockage, leading to DNA breakage (Murai *et al.* 2014). This mechanism is independent from the ability of PARPis to inhibit PARP catalytic activity and may explain the difference in cytotoxic potential observed among clinically tested PARPis. For example, although olaparib, veliparib and niraparib all exhibit a similar ability to inhibit PARP catalytic activity, they differ greatly in terms of cytotoxicity (Rouleau *et al.* 2010). This may be explained by the difference in their ability to “poison PARP” by trapping PARP-1-DNA complexes. Bulkier drugs such as olaparib and niraparib, but not veliparib, bind readily to the NAD⁺ site, allosterically enhancing DNA binding of PARP (Murai *et al.* 2014). In these studies, olaparib and niraparib were found to stabilize toxic PARP-1-DNA complexes more tightly than veliparib, which was 10-fold less cytotoxic than olaparib and niraparib (Murai *et al.* 2014).

To date, therapeutic exploitation of this synthetic lethality using targeted agents has been met with success in *BRCA1*- and *BRCA2*-associated breast and ovarian cancers. Through phase 1 and 2 studies, it has been established that the use of olaparib, a third-generation PARPi, was associated with only mild side effects and elicited significant responses in germline *BRCA2* mutation carriers with breast or ovarian cancer (Lee *et al.* 2014). Additionally, phase 1 and 2 trials evaluating administration of olaparib or veliparib, third-generation PARPis, in combination with platinum-based DCL agents such as carboplatin or cisplatin also elicited favorable responses in *BRCA1*- and *BRCA2*-associated breast and ovarian cancers (Ratner *et al.* 2012, Sandhu *et al.* 2011). However,

limited data exists to support the use of these agents in *BRCA1*- and *BRCA2*-associated PDAC. Collectively, these observations indicate that although the possibility to markedly improve outcomes in *BRCA1*- and *BRCA2*-associated PDAC is tangible, novel therapeutic agents or new insights into the use of combination therapy are critical.

The most promising PARPi currently available is the most recently developed BMN 673, (8*S*,9*R*)-5-fluoro-8-(4-fluorophenyl)-9-(1-methyl-1*H*-1,2,4-triazol-5-yl)-8,9-dihydro-2*H*-pyrido[4,3,2-*de*]phthalazin-3(7*H*)-one, a potent stereo-selective inhibitor of PARP-1 and -2 (Smith *et al.* 2015). Similar to earlier-generation PARPis, BMN 673 inhibits the catalytic activity of PARP-1/2, impairing the BER-mediated pathway of SSB repair. However, BMN 673 is ~100-fold more potent at trapping PARP-1 to DNA at sites of SSBs compared to earlier-generation PARPis, such as olaparib and rucaparib, which exhibit equivalent PARP trapping capacities. This advantage in PARP trapping may explain the 10-fold greater cytotoxic potency of BMN 673 compared to veliparib, rucaparib and olaparib (Murai *et al.* 2014). Furthermore, the selectivity of BMN 673 and consequent absence of off-target effects was demonstrated by the resistance of DT40 cells lacking PARP-2 to BMN 673, whereas PARP-proficient DT40 cells were sensitive to nanomolar concentrations of BMN 673 (Murai *et al.* 2014). Phase 1 trials for BMN 673 are complete, with the primary dose-limiting toxicity being thrombocytopenia and phase 3 clinical trials for patients harboring germline *BRCA* mutations and locally advanced/metastatic breast cancer are currently underway (NCT01945775). Although preliminary, these results suggest the potential of BMN 673 in the treatment of *BRCA*-associated malignancies, including *BRCA2*-associated PDAC (Smith *et al.* 2015, Murai *et al.* 2014).

Challenges to PARPi development

The development of PARPi-based therapy in the context of HDR-deficient tumors is one of the best-known examples of a synthetic lethal approach to targeted cancer therapy. However, there are many challenges to be addressed before PARPis may be considered a success.

Firstly, the development of chemo-resistance seen with almost all chemotherapeutic approaches also occurs with PARPi treatment. Many mechanisms of PARPi resistance exist, including 1) the development of secondary *BRCA2* mutations that restore functional HR, 2) increased drug export, as well as 3) circumventing the loss of HDR through upregulation of other pathways (Ashworth *et al.* 2008). There have been several reports of tumors arising in a *BRCA2*-mutation carriers acquiring secondary *BRCA2* mutations that restore the open reading frame, thus reestablishing normal *BRCA2* function and DNA repair by HDR (Galluzzi *et al.* 2012). For example, through exome sequencing of DNA derived from tissue samples from the primary breast tumor and metastasis of a male patient harboring a germline *BRCA2* mutation, novel secondary *BRCA2* mutation were reported in the metastasis that appeared to restore *BRCA2* protein function. The patient had been treated with olaparib and initially demonstrated a marked response, with CA15-3 levels decreasing notably. However, signs of resistance to therapy were observed after 10 months of olaparib treatment (Barber *et al.* 2013). These reports are the first to demonstrate the correlation between secondary *BRCA2* mutations and clinical resistance to PARPi (Barber *et al.* 2013, Lord *et al.* 2008). As is the case with numerous chemotherapeutic agents, resistance to PARPi may be caused by increased drug export. It has been demonstrated that PARPis such as olaparib are extruded from

tumor cells by the P-glycoprotein drug efflux transporter *in vivo* (Bouwman *et al.* 2013). Finally, cells may develop resistance to PARPi by circumventing *BRCA1* and *BRCA2* mutation-mediated HDR loss (Ashworth *et al.* 2008). There have been reports of 53BP1 loss in *BRCA1*-deficient mammary tumors correlating with HDR restoration and resistance to HDR-deficiency targeted therapy. These tumor cells seem to have a partially restored HDR pathway, indicated by the presence of RAD51 foci and increased resistance to DNA damaging agents (Jaspers *et al.* 2013)

Secondly, accurate means of identifying patients who will respond to tailored therapy and benefit from PARPi treatment must be developed (Zaremba *et al.* 2007). Currently, *BRCA1* and *BRCA2* mutation status is seemingly the only known and validated predictive biomarker for the use of PARPi therapy (Barber *et al.* 2013). However, as previously mentioned, secondary somatic *BRCA2* mutations may restore functional HDR and render the tumor resistant to PARPis, suggesting that screening for these secondary *BRCA2* mutations may also be necessary (Barber *et al.* 2013). In other cases, *BRCA1* and *BRCA2* loss may be attributed to methylation and may only be detected by testing for *BRCA1* and *BRCA2* protein expression. Additionally, the HDR pathway may be defective in certain cases with functional *BRCA1* and *BRCA2* proteins, wherein the HDR deficiency is due to an unknown molecular defect, termed “BRCA-like” cases (Tutt *et al.* 2005). Therefore, in these tumors, germline *BRCA1* and *BRCA2* mutation status does not serve as an informative predictive biomarker of PARPi efficacy. A genetic and molecular understanding of these “BRCA-like” tumors is needed to identify additional subsets of patients who may also benefit from tailored therapy with PARPis (McCabe *et al.* 2006).

One method of identifying the “BRCA-like” tumors subset involves the quantification of RAD51 focus formation following induction of DNA damage. RAD51, as previously described, is a critical mediating protein of HDR and serves as a marker for DNA damage repair by HDR. In this assay, tumors are irradiated to induce DNA damage, and subsequently analyzed by immunohistochemistry to assess radiation-induced RAD51 focus formation, and geminin as a marker of proliferation. RAD51 score is then determined by the proportion of proliferative cells with RAD51 foci, with a low RAD51 score indicating defective HDR. This is a valuable tool that may be used to evaluate HDR capacity in the context of seemingly sporadic wild-type *BRCA1* and *BRCA2* PDAC tumors, and may identify additional patients harboring “BRCA-like” tumors who may benefit from a tailored therapy approach (Graeser *et al.* 2010).

DCL agents for the treatment of BRCA2-associated PDAC

DCL agents have traditionally been used in the treatment of numerous cancer types, including bladder, head and neck, lung, ovarian, testicular and PDAC, as well as sarcomas (Kobayashi *et al.* 2014). DCL agents such as cisplatin, carboplatin and oxaliplatin induce their anti-cancer effect mainly through the generation of DNA crosslinks, which interfere with DNA replication and transcription, activating the DNA damage response and inducing cell death by apoptosis if left unrepaired (Albers *et al.* 2014). Treatment with platinum-based DCL agents often leads to an initial favorable therapeutic response, characterized in some cases by partial response or disease stabilization. However, patients are frequently intrinsically resistant to DCL agents or may develop resistance to these agents over the course of treatment (Colluci *et al.* 2010).

In addition to the development of resistance, the cytotoxicity associated with DCL agents, including renal toxicity, allergic reactions, decreased immunity to infections, gastrointestinal disorders, as well as neurotoxic and ototoxic side effects, is often dose-limiting (Kobayashi *et al.* 2014).

Development of DCL agents

Of the three most commonly used DCL agents (cisplatin, oxaliplatin and carboplatin), *cis*-diamminedichloroplatinum(II), commonly known as cisplatin, was the first to be approved for the treatment of a wide variety of solid neoplasms (Arslan *et al.* 2014). However, the high cytotoxicity of cisplatin led to the development of second-generation platinum, namely *cis*-diammine (cyclobutane-1,1-dicarboxylate-*O,O'*) platinum(II) (carboplatin), which forms DNA adducts identical to cisplatin, but is not associated with the same undesired side effects accompanying cisplatin treatment (Arslan *et al.* 2014). Carboplatin does, however, have a myelo-suppressive effect, which significantly decreases the blood cell and platelet output of bone marrow. Additionally, due to the similarities in mechanism of action, most cisplatin-resistant tumors are also resistant to carboplatin treatment. This led to the development of a novel platinum agent, [(1*R*,2*R*)-cyclohexane-1,2-diamine](ethanedioato-*O,O'*) platinum(II) (oxaliplatin), which exhibits distinct pharmacological and immunological characteristics to both carboplatin and cisplatin. However, although cisplatin-resistant tumors are generally found to be sensitive to treatment with oxaliplatin, the cross-resistance across these DCL agents remains a challenge (Colluci *et al.* 2010, Albers *et al.* 2014).

Mechanism of action and development of chemoresistance

Cisplatin, carboplatin and oxaliplatin are neutral platinum (II) complexes containing two amine ligands as well as two additional ligands for further binding to DNA strands. Upon entry into the cell cytoplasm, cisplatin is activated, during which one or both cis-chloro groups are replaced by water molecules to generate the highly reactive aquated form of cisplatin (Bajrami *et al.* 2014). Their mechanism of action relies on the ability of these reactive cisplatin molecules to bind DNA, favoring nucleophilic N7-sites on purine bases, generating DNA-DNA inter- and intra-strand adducts, as well as protein-DNA complexes. Once a replication fork encounters a DNA crosslink during DNA replication, replication is stalled and the replication fork collapses, forming a DSB at this position. One strand of the collapsed replication fork can be resected, generating a substrate for subsequent strand invasion. DNA crosslinks interfere with numerous cellular processes, including DNA replication, transcription and DNA repair. The end effect is DNA damage, replication arrest and induction of apoptosis if the DNA damage is left unrepaired (Galluzzi *et al.* 2012, Bajrami *et al.* 2014).

DNA damage generated by DCL agents distorts the helical structure of DNA, which is detected by the NER pathway as well as the MMR pathway. DSBs generated by stalled replication forks are detected and repaired by the HDR machinery of the cell. When the extent of DNA damage induced by DCL agents is limited, an arrest in the S and G2 phases is induced, to allow for DNA repair and re-establishment of DNA integrity. However, if DNA damage is irreparable, cells become committed to apoptotic cell death (Rabik *et al.* 2007).

NER is the principal mechanism of DCL agent-induced DNA damage repair. During this process, the DNA lesion is recognized and the excision repair cross-complementation group 1 (ERCC1) protein is recruited to the site of DNA damage to excise nucleotides on either side of the DNA lesion (Bohanes *et al.* 2011). Following excision of the lesion, DNA is resynthesized and ligated. *In vitro* data indicates that intrinsically low levels of ERCC1, as is the case in metastatic testicular cancer cells, correlate with an increased sensitivity to cisplatin. Additionally, reduction of ERCC1 expression by antisense RNA was also found to improve cisplatin sensitivity in ovarian cancer cell lines (Metzger *et al.* 2013).

The MMR system, conversely, may detect DNA lesions induced by DCL agents, but does not participate in the resolution and repair of DNA damage. Mut proteins, of the MMR system, are mainly responsible for the recognition of mismatched or unmatched DNA base pairs or insertion loops and initiate excision of these DNA lesions (Yang *et al.* 2000). In the case of DCL-induced DNA damage, Mut proteins recognize the DNA damage, are unable to repair these adducts and consequently generate a pro-apoptotic signal. In tumors cells lacking a functional MMR system, the signaling pathways leading to the induction of apoptosis are not activated, allowing DNA damage to accumulate in proliferating cells (Martin *et al.* 2008).

Id. Rationale

Despite the promising opportunities for personalized therapies in *BRCA*-associated PDAC, there is a lack of preclinical data comparing the various existing DCL agents and PARPis to rationalize the selection of agents for clinical trial. My dissertation aims to address this research need. Since *BRCA2* germline mutations account for the majority of *BRCA*-associated PDAC, I focused my dissertation on *BRCA2*-associated PDAC. In addition, since BMN 673 has been postulated to have advantageous therapeutic features compared to other agents in its class with lower toxicity than DCL agents, I was particularly interested in evaluating BMN 673.

Ie. Hypothesis

I hypothesize that *BRCA2*-associated PDAC is sensitive to DCL agents and PARPis, and that the novel PARPi BMN 673 is the most selective agent for *BRCA2*-deficiency in its drug class and has equivalent cytotoxicity to the most efficacious DCL.

If. Specific Aims

1. To compare the *in vitro* sensitivities of MIA PaCa-2 (*BRCA2*-proficient) versus Capan-1 (a PDAC cell line harboring the AJ founder germline *BRCA2*:6174delT mutation) against a panel of clinically relevant DCL agents (cisplatin, oxaliplatin and carboplatin) and PARPis (veliparib and BMN 673).
2. To determine if *BRCA2* knockdown in a *BRCA2*-proficient PDAC cell line is sufficient to induce sensitivity to the agents that are found in Aim 1 to be most efficacious.

3. To validate the *in vitro* findings of Aims 1 and 2 in a preclinical trial using a *BRCA2*-associated PDAC patient-derived xenograft (PDX) model.

Chapter II:

Increased *in vitro* and *in vivo* sensitivity of *BRCA2*-associated pancreatic adenocarcinoma to the poly(ADP-ribose) polymerase-1/2 inhibitor BMN 673

Preface and Contribution of Authors

Increased *in vitro* and *in vivo* sensitivity of *BRCA2*-associated pancreatic adenocarcinoma to the poly(ADP-ribose) polymerase-1/2 inhibitor BMN 673

The methodology and results of my dissertation are presented in manuscript format. The manuscript has been submitted to *Cancer Letters*. The following details the contributions of each co-author.

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Alexandra-Zoe Andrei

I performed all experiments, apart from establishing the PDX model (and subsequent passaging) and *BRCA2* mutation testing. I also performed the data analyses with the assistance of the co-authors as described below. In addition, I prepared the initial draft of this manuscript and performed all revisions following review of the draft manuscript by the co-authors.

Anita Hall

Established and passaged the PDX model as well as assisted with the experimental design of the pre-clinical drug studies.

Alyssa L. Smith

Performed the *BRCA2* mutation testing experiments.

Claire Bascuñana

Assisted with the acquisition of the clinical data presented in this manuscript.

Abba Malina

Assisted with shRNA protocol optimization and provided experimental design guidance.

Gulbeyaz Altinel

Assisted with the development of the PDX model, providing histological confirmation of the resected tumor tissue.

Sidong Huang

Assisted with the development of the long-term colony formation assays and provided the BRCA2-targeting shRNA constructs.

Jerry Pelletier

Assisted with the development the of shRNA-mediated *BRCA2* knockdown cell lines.

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ABSTRACT

BRCA2-associated pancreatic ductal adenocarcinoma (PDAC) may be uniquely sensitive to agents that target homology-directed DNA repair, such as DNA crosslinking agents (DCLs) and PARP inhibitors (PARPis). Here, we assessed the sensitivities of *BRCA2*-deficient (Capan-1) and –proficient (MIA PaCa-2) PDAC cell lines to a panel of DCLs and PARPis. Compared to MIA PaCa-2, Capan-1 was significantly more sensitive to all tested DCLs and PARPis, with comparable increased sensitivities to cisplatin and the PARPi, BMN 673 with respect to other DCLs and the PARPi, veliparib. We provide further support for this observation by showing that shRNA-mediated *BRCA2* knockdown in PANC-1, a *BRCA2*-proficient cell line, induces sensitivity to cisplatin and BMN 673 but not to veliparib. These findings were validated in a PDAC murine xenograft model derived from a patient carrying a *BRCA2* germline mutation. The trial shows 61% and 64% tumor growth inhibition and a marked reduction in cellular proliferation following BMN 673 and cisplatin treatments, respectively. Our findings support a personalized treatment approach for *BRCA*-associated PDAC and suggest that BMN 673 be considered for clinical trial in *BRCA*-associated PDAC.

Keywords: Pancreatic Ductal Adenocarcinoma, *BRCA2*, DNA Repair, PARP Inhibitors, BMN 673, Personalized Medicine

HIGHLIGHTS

- *BRCA2*-associated PDAC is sensitive to agents exploiting DNA repair defects
- BMN 673 inhibits tumor growth by 61% in a *BRCA2* PDAC xenograft model

1. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies worldwide¹. Approximately 80% of new cases continue to be diagnosed late, with advanced disease precluding curative resection². Unfortunately, the therapeutic options currently available for these patients are largely ineffective and even patients who present with operable disease have poor outcomes following resection due to early recurrences³. The challenges in identifying therapies with meaningful outcomes may reflect the genetic heterogeneity of PDAC. Therefore, research efforts focused on the genetic 'cataloguing' of PDAC⁴ may identify subsets of patients who will benefit from tailored treatment approaches⁵. Although the full spectrum of PDAC subtypes remains to be characterized, investigating PDAC associated with hereditary syndromes provides an opportunity to characterize the therapeutic sensitivities of PDAC arising from common genetic driver mutations.

Approximately 10% of PDAC cases are associated with strong family histories, with a fraction of these cases accounted by the tumor spectrums of recognized hereditary syndromes⁶. These syndromes include the hereditary breast and ovarian cancer (HBOC) syndrome, most often caused by germline mutations in the *BRCA1* and *BRCA2* genes⁷. Since BRCA-deficient tumors are impaired for homology-directed DNA repair (HDR), therapeutic strategies that exploit defects in HDR may represent an avenue to targeted therapy development for these PDAC cases⁸.

The hypothesis that BRCA-deficient cells are sensitive to agents that target DNA repair mechanisms is supported by a growing body of research suggesting increased

sensitivity of *BRCA*-associated breast and ovarian cancer to either DNA crosslinking agents (DCLs) or poly(ADP)-ribose polymerase inhibitors (PARPis)^{9,10}. Since DCLs cause double-strand DNA breaks (DSBs) that must be repaired by HDR, *BRCA*-deficient cells are vulnerable to these agents. Similarly, PARPis exploit the dependence of *BRCA*-deficient cells on alternative cellular DNA repair pathways by disrupting the base excision DNA repair (BER) pathway, creating a synthetic lethal interaction for cells with impaired HDR.

Despite these promising opportunities for personalized therapies, there is a lack of preclinical data comparing the various DCLs and PARPis to rationalize the selection of agents for clinical trial. In the present study, we present a PDAC case with a germline *BRCA2* mutation and a marked response to platinum-based chemotherapy (FOLFIRINOX¹¹). We investigated the *in vitro* cytotoxicities of a panel of DCLs and PARPis in *BRCA2*-deficient PDAC cell lines followed by *in vivo* validation of the two most efficacious agents, cisplatin and BMN 673 (a PARPi) in a xenograft model derived from our patient. Specifically, we evaluated the efficacy of the newest generation PARPi, BMN 673¹², in comparison to a panel of commonly used DCLs as well as veliparib, which is currently under clinical trial evaluation for *BRCA*-associated PDAC¹³. Our findings support a role for personalized therapeutic strategies for *BRCA2*-associated PDAC and suggest that BMN 673 be considered for clinical trial in this subset of PDAC.

2. METHODS

2.1 Cell culture: Capan-1 (HTB-79), MIA PaCa-2 (CRL-1420) and PANC-1 (CRL-1469) were obtained from ATCC (Manassas, USA) and cultured in DMEM (Wisent, St-Bruno, Canada) supplemented with 10% FBS, 5% glutamine and 5% penicillin-streptomycin.

2.2 Compounds: Gemcitabine (Enzo Life Sciences, Brockville, Canada), cisplatin (Enzo Life Sciences), oxaliplatin (Sigma Aldrich, Oakville, Canada), carboplatin (Sigma Aldrich), veliparib (Enzo Life Sciences) and BMN 673 (Abmole Biosciences, Hong Kong, China) were resuspended in water or DMSO.

2.3 Real-time cell analysis (xCELLigence): Compound-mediated *in vitro* cytotoxicity was monitored with the Real-Time Cell Analyzer (RTCA) dual-plate (DP) instrument, using the xCELLigence System (ACEA Biosciences, California, USA)¹⁴. Briefly, 10⁴ cells/well were plated and treated after 48 h. Experiments were performed in triplicates and IC₅₀ differences between Capan-1 and MIA PaCa-2 were evaluated using Student t-tests.

2.4 BRCA2-knockdown: Four *BRCA2*-targeting shRNAs in the pKLO.1 lentiviral vector (RNAi Consortium shRNA Library¹⁵; Supplementary Methods and Supplementary Table 1) were used to reduce *BRCA2* in PANC-1 and MIA PaCa-2. Cells were co-transfected with pKLO.1-*BRCA2*-shRNA (10 µg), pSPAX.2 packaging plasmid (7.5 µg) and pseudotyping plasmid pMDG.2 (3 µg) using calcium phosphate. Although *BRCA2* knockdown was tolerated by PANC-1 cells, it was lethal for MIA PaCa-2 cells (data not shown). Following puromycin selection (2 µg/mL) for 72 h, individual *BRCA2*-

knockdown PANC-1 clones were isolated and expanded. Two of these four TRC shRNA constructs (shRNA 2 [BRCA2], shRNA 3 [BRCA2]) provided adequate *BRCA2* knockdown, which was confirmed by Western blotting (Supplementary Methods). The empty pKLO.1 TRC cloning vector served as a control.

2.5 Immunofluorescence: 10^5 cells were seeded and allowed to grow overnight on glass cover slips in 24-well tissue culture plates, before being exposed to 8.5 Gy (^{137}Cs source biological irradiator calibrated at 1.98 Gy/min, RS 2000; Radsources, Brentwood, USA). Six hours following irradiation, cells were fixed and stained according to the manufacturer's instructions, using primary antibodies against γ -H2AX (Ser139) (1:2000, JBW301; Millipore) and Rad51 (1:2000, PC130; Calbiochem), secondary antibodies (AlexaFluor 488-conjugated AffiniPure or AlexaFluor 594-conjugated AffiniPure, 1:5000, Jackson ImmunoResearch), and DAPI (Life Technologies, Carlsbad, USA). Fixed cells were analyzed with the Zeiss LSM 700 Laser Scanning Confocal Microscope System (Zeiss, Toronto, Canada). HDR capacity was estimated by quantifying RAD51 nuclear focus formation in 150 cells in randomly chosen fields, excluding cells with fewer than 10 γ -H2AX nuclear foci¹⁶. Images were processed using a Carl Zeiss ZEN 2011 (Zeiss, Supplementary Table 2).

2.6 Long-term colony formation assays: Cells were plated in 6-well plates at 2×10^4 cells/well and treated 24 h later with veliparib, cisplatin or BMN 673. Following 10 days of treatment, cells were fixed and stained with 0.1% crystal violet. Images were taken on a Carl Zeiss Axio Zoom V16 (Zeiss) and processed using ImageJ (National Institutes of Health, Bethesda, USA). Mean pixel intensity was calculated (black = 0, white = 255).

Values were normalized to the DMSO control using the following formula:

$\frac{x-DMSO\ value}{255-DMSO\ value} * 100 = \% \text{ cell death.}$ Prism 6 (GraphPad, La Jolla, USA) was used to calculate percent cell death as a function of drug concentration and to determine IC₅₀ values.

2.7 Establishment of a mouse patient-derived xenograft (PDX) model: PDAC tissue was obtained from a 47-year old male patient carrying a germline *BRCA2* French Canadian founder mutation (*BRCA2*:c.3170_3174delAGAAA, Figure 1)¹⁷ who underwent resection and was enrolled in the Quebec Pancreas Cancer Study¹⁸. Following histological confirmation of PDAC, 1 mm³ tumor pieces were implanted subcutaneously on both flanks of two 5-week old female SCID/Beige mice, grown to an 8 mm diameter prior to passaging, and frozen in 10% FBS/DMSO following the third passage. Once preclinical trial test drugs had been selected, third passage tumor pieces were thawed and regrown to an 8 mm diameter in 5-week old female SCID/Beige mice. Retention of the *BRCA2* mutation following four passages was confirmed (Supplementary Materials) before mincing the tumors into 1 mm³ pieces and implanting them subcutaneously into both flanks of fifteen 5-week old female SCID/Beige mice. Protocols were approved by the McGill University Faculty of Medicine Animal Care Committee and the McGill Institutional Research Board.

2.8 In vivo cisplatin and BMN 673 efficacy studies: Cisplatin and BMN 673 were solubilized in DMSO and diluted with PBS containing 10% dimethylacetamide (Sigma-Aldrich) and 6% Solutol (Sigma-Aldrich). Once tumors reached an average volume of 120 mm³ [$V = \frac{4}{3}(\pi \times (\frac{length}{2}) \times (\frac{width}{2})^2)$],¹⁹ the mice were randomized into cisplatin,

BMN 673, and vehicle control arms (3-5 mice/group; 2 tumors/mouse) and treated for four weeks. Cisplatin was administered (4 mg/kg, 0.1 cc, i.p.) once weekly, while BMN 673 (0.33 mg/kg, 0.05 cc) and the vehicle (0.05 cc of 10% dimethylacetamide, 6% Solutol in PBS) were administered by oral gavage once daily. Mice were weighed and tumor volumes were determined twice weekly. Tumors were collected on day 29, 24 h following the final treatment dose. Relative tumor volume (RTV) and percentage tumor growth inhibition (% TGI) were calculated as previously described²⁰.

2.9 Immunohistochemistry: Hematoxylin and eosin (H&E) staining was performed on PDX tumor tissue sections. The Ki-67 tumor proliferation indices for each PDX treatment arm were determined by immunostaining using a rabbit polyclonal Ki-67 antibody (1:1000, ab15580; Abcam, Ontario, Canada) and scoring five randomly selected fields (200x magnification), avoiding tissue areas with extensive necrosis. Images were taken using Aperio AT2 (Leica Biosystems, Concord, Canada) and analyzed using Aperio ImageScope (Leica Biosystems, Supplementary Table 2).

3. RESULTS

3.1 Clinical response to platinum-based therapy in a PDAC case with a *BRCA2* germline mutation. Figure 1A shows partial and complete radiological responses of the patient's primary tumor and liver metastasis, respectively. We confirmed the *BRCA2* germline mutation carrier status in the patient (Figure 1B) and retention of the mutation in the PDX established from this case (Figure 1C), by Sanger sequencing.

3.2 *BRCA2*-deficient cells manifest increased sensitivity to DCLs and PARPis. We compared the *in vitro* sensitivities of *BRCA2*-deficient Capan-1 and *BRCA2*-proficient

MIA PaCa-2 cell lines to gemcitabine, a panel of DCLs (cisplatin, oxaliplatin, and carboplatin) and PARPis (veliparib, BMN 673). As anticipated, Capan-1 cells showed increased sensitivity to all tested DCLs and PARPis compared to MIA PaCa-2 cells, but not to gemcitabine treatment (Figure 2A). Mean IC_{50} values were $11.4 \text{ mM} \pm 1.4 \text{ mM}$ versus $12.7 \text{ mM} \pm 3.6 \text{ mM}$ for gemcitabine (NS), $38.3 \text{ } \mu\text{M} \pm 7.3 \text{ } \mu\text{M}$ versus $10.2 \pm 1.5 \text{ } \mu\text{M}$ ($p = 0.0150$) for cisplatin, $96.5 \text{ } \mu\text{M} \pm 22.7 \text{ } \mu\text{M}$ versus $24.9 \text{ } \mu\text{M} \pm 8.3 \text{ } \mu\text{M}$ ($p = 0.0287$) for oxaliplatin, $700.3 \text{ } \mu\text{M} \pm 70.7 \text{ } \mu\text{M}$ versus $99.4 \text{ } \mu\text{M} \pm 4.6 \mu\text{M}$ ($p = 0.0015$) for veliparib, $152.7 \text{ } \mu\text{M} \pm 3.0 \text{ } \mu\text{M}$ versus $89.7 \text{ } \mu\text{M} \pm 10.5 \text{ } \mu\text{M}$ ($p = 0.0001$) for carboplatin and $58.23 \pm 8.1 \text{ } \mu\text{M}$ versus $16.0 \pm 5.4 \text{ } \mu\text{M}$ ($p = 0.0105$) for BMN 673 in MIA PaCa-2 versus Capan-1 cells, respectively (Figure 2A). Considering the treatment cytotoxicity differences in Capan-1 versus MIA PaCa-2 cells together with the resultant IC_{50} values among the various DCLs, the data suggest that, of the agents tested, cisplatin is the most advantageous DCL in BRCA2-deficient cells. Similarly, these *in vitro* results favour BMN 673 over veliparib in the treatment of BRCA2-associated PDAC. Moreover, the cytotoxicity fold-differences and IC_{50} values of cisplatin and BMN 673 appeared comparable, suggesting that the efficacy of cisplatin and BMN 673 may be similar and that BMN 673 may be a less toxic alternative to cisplatin^{21,22}.

3.3 Capan-1 cells exhibit reduced HDR capacity. We confirmed that HDR is impaired in the BRCA2-deficient Capan-1 cells and intact in the BRCA2-proficient MIA PaCa-2 cells by evaluating the HDR response following DNA damage induction by irradiation. DNA damage was assessed by γ -H2AX immunostaining, while RAD51 foci formation was used to evaluate HDR activity. Phosphorylation of H2AX at Ser-139 serves as a marker for DSB damage²³. RAD51 directs the critical strand invasion step of HDR and,

thus, can be used as a marker for HDR competence²⁴. As expected, in BRCA2-proficient MIA PaCa-2 cells, there was colocalization of γ -H2AX and RAD51 foci (Figure 2B, top panel). In contrast, Capan-1 cells exhibit high cytoplasmic levels of RAD51 in the presence of nuclear γ -H2AX staining (Figure 2B, bottom panel). This suggests defective HDR in Capan-1 but not in MIA PaCa-2, providing a mechanism for the increased sensitivity of Capan-1 cells to DCL agents and PARP inhibitors.

3.4 shRNA-mediated *BRCA2* knockdown impairs HDR in PANC-1 cells. We determined if HDR impairment and, consequently increased sensitivity to DCLs and PARPis, can be induced in BRCA2-proficient PDAC cells by *BRCA2* knockdown. *BRCA2* knockdown with targeted shRNAs but not with control shRNAs was confirmed by Western blotting (Figure 3A). Prior to characterizing if *BRCA2* knockdown resulted in increased sensitivity of PANC-1 cells to DCLs and PARPis, we evaluated the effect of *BRCA2* knockdown on HDR. The cell lines were irradiated to induce DNA damage and probed for nuclear RAD51 and γ -H2AX foci formation. Mean RAD51 foci values were 327 ± 105.2 (SD) foci/150 cells ($p = 0.03$) and 296 ± 110 (SD) foci/150 cells ($p=0.03$) in PANC-1_shRNA 2 [*BRCA2*] and PANC-1_shRNA 3 [*BRCA2*], respectively, versus 588 ± 45.2 (SD) foci/150 cells in PANC-1_shRNA [Control] cells. The reduction in nuclear RAD51 and γ -H2AX foci co-localization in *BRCA2* knockdown versus control clones indicates that HDR impairment is induced upon *BRCA2* knockdown (Figure 3B). However, shRNA-mediated *BRCA2* knockdown does not appear to fully inactivate HDR since residual nuclear RAD51 foci were still present in the knockdown cells (Figure 3B). This finding suggests that the residual *BRCA2* expression with shRNA-mediated knockdown is sufficient to direct nuclear localization of RAD51 to sites of DSBs. This

differed from what was observed in Capan-1, in which the PDAC phenotype likely arose from a driver germline *BRCA2* mutation. The complete cytoplasmic RAD51 confinement in Capan-1 suggests full HDR inactivation (Figure 2B, bottom panel), whereas reduced nuclear localization of RAD51 in response to DNA damage following shRNA-mediated *BRCA2* knockdown in PANC-1 (Figure 3B) suggests decreased, but not fully impaired, HDR. This provided us with additional cell lines harboring intermediate HDR activity with which to further characterize the *in vitro* effectiveness of BMN 673 compared to veliparib and cisplatin prior to undertaking a BMN 673 preclinical PDX trial.

3.5 *In vitro* shRNA-mediated *BRCA2* knockdown sensitizes PANC-1 cells to BMN 673 and cisplatin. To further evaluate the efficacy of BMN 673 compared to veliparib and cisplatin, we performed long-term colony formation assays. We evaluated the *in vitro* sensitivities of our PANC-1 *BRCA2*-knockdown cell lines to cisplatin, veliparib and BMN 673 (Figure 3C). Based on our cytotoxicity results in Capan-1 cells, we hypothesized that the PANC-1 *BRCA2*-knockdown cell lines, harboring reduced but not fully impaired HDR, would be sensitive to cisplatin and BMN 673 but not to veliparib. As predicted, veliparib treatment did not result in a significant shift in the IC_{50} values in *BRCA2*-knockdown cells, with IC_{50} values of $52.86 \mu M \pm 4.52 \mu M$ ($p = 0.53$) and $29.37 \pm 1.96 \mu M$ ($p < 0.0001$) μM , in PANC-1_shRNA 2 [BRCA2] and PANC-1_shRNA 3 cell lines, respectively, compared to $55.41 \mu M \pm 5.72 \mu M$ in the control cell line (Figure 3C, Supplementary Figure 1). However, with BMN 673 treatment, the IC_{50} values were $0.57 \mu M \pm 0.16 \mu M$ ($p < 0.0001$) and $0.53 \mu M \pm 0.13 \mu M$ ($p < 0.0001$) in PANC-1_shRNA 2 [BRCA2] and PANC-1_shRNA 3 [BRCA2] cell lines compared to $1.22 \mu M \pm 0.25 \mu M$ in the control cell line (Figure 3C, Supplementary Figure 2). In cisplatin-treated cells, we

observed IC_{50} values of $1.30 \mu M \pm 0.23 \mu M$ ($p < 0.0001$) and $0.63 \mu M \pm 0.12 \mu M$ ($p < 0.0001$) in PANC-1_shRNA 2 [BRCA2] and PANC-1_shRNA 3 [BRCA2] compared to $2.70 \mu M \pm 0.41 \mu M$ in the control cell line (Figure 3C, Supplementary Figure 3). This increased sensitivity to cisplatin and BMN 673 but not to veliparib in PDAC cells with incomplete HDR inactivation, suggests that BMN 673 is a more effective PARPi for *BRCA2*-associated PDAC compared to the earlier-generation PARPis such as veliparib, and that BMN 673, rather than veliparib, should be selected for our preclinical PDX trial evaluation.

3.6 Cisplatin and BMN 673 demonstrate equivalent growth inhibition in a preclinical trial. To validate our *in vitro* findings, we undertook a preclinical trial using a PDX model. Following randomization to vehicle (control), cisplatin and BMN 673 trial arms, mice were treated for 4 weeks with the respective agents and monitored for tumor growth inhibition before being sacrificed. Figure 4A demonstrates marked growth inhibition with cisplatin and BMN 673 treatments. End-point tumor volumes correlated with the growth curve observations. Cisplatin treatment (6 tumors) resulted in significant growth inhibition (GI) compared to vehicle-treated controls (8 tumors) ($189.24 \text{ mm}^3 \pm 31.65 \text{ mm}^3$ (SD) versus $520.55 \text{ mm}^3 \pm 62.68 \text{ mm}^3$ (SD); $p = 0.0004$). In support of our *in vitro* findings, treatment with BMN 673 (10 tumors) also resulted in significant GI compared with vehicle-treated controls (8 tumors) ($195.05 \text{ mm}^3 \pm 95.21 \text{ mm}^3$ (SD) versus $520.55 \text{ mm}^3 \pm 62.68 \text{ mm}^3$ (SD); $p = 0.0006$). In fact, the mean RTVs was reduced from 4.27 (range 3.81-4.78) in control mice to 1.53 (range 1.34-1.61; $p < 0.0001$) in cisplatin-treated mice and 1.53 (0.80-2.50; $P = 0.0003$) in BMN 673-treated mice. Moreover, we did not find a significant difference in GI between the cisplatin and BMN 673 groups,

with 64% and 61% tumor GI, respectively, compared to control tumors (Figure 4B). These data suggest that cisplatin and BMN 673 have similar efficacies in *BRCA2*-associated PDAC. There were three deaths during the trial (two vehicle- and one BMN 673-treated mouse) related to drug administration by oral gavage. Six mice had a weight loss of greater than 10% body weight (Figure 4C). Two of these mice were treated with cisplatin, three were treated with BMN 673, and one was a control animal. We did not observe a statistical difference in weight loss between treatment arms (vehicle $6.2\% \pm 5.1\%$ (SD); cisplatin $11.7\% \pm 9.1\%$ (SD); BMN 673 $12.3\% \pm 4.6\%$ (SD)).

Histological evaluation of the tumors revealed microscopic differences in vehicle- versus cisplatin- and BMN-treated tumors. Although there were equivalent number of necrotic areas among treatment groups, the vehicle-treated tumor cells had more mitotic features (smaller cells with less prominent nucleoli) compared to the cisplatin- and BMN-treated tumors (larger cells with enlarged nuclei) (Figure 5A). These findings are consistent with a decrease in the proliferation index of the treated tumors.

To quantify the proliferation index of the treated versus control tumors, the post-treatment tumors were analyzed by Ki-67 immunostaining. Cisplatin- and BMN673-treated tumors showed a significantly lower number of proliferating cells versus vehicle-treated tumors (Figure 5B). On average, the percentage of proliferating cells per high-power field was 5.9-fold lower in the BMN673- treated cells [$1.6\% \pm 1.3\%$ (SD) versus $9.1\% \pm 1.4\%$ (SD); $p = 0.0024$] and 6.1-fold lower in the cisplatin-treated cells [$1.5\% \pm 0.2\%$ (SD) versus $9.1\% \pm 1.4\%$ (SD); $p = 0.0008$]. These data suggest that the GI effects observed in the cisplatin and BMN 673 treatment arms are due to the anti-proliferative

effects of these agents, and that these two drugs have equivalent anti-proliferative effects on a PDAC arising from germline *BRCA2* mutation carriers.

4. DISCUSSION

The poor outcome of patients with PDAC reflects the desperate need for improved treatment strategies²⁵. In this study, we assessed the efficacy of DCLs and PARPis in *BRCA2*-associated PDAC. As predicted, we observed increased *in vitro* sensitivity of *BRCA2*-deficient (Capan-1) PDAC cells to all agents tested. We also showed that shRNA-mediated reduction of *BRCA2* expression in PANC-1 induces sensitivity to cisplatin and BMN 673 but not to veliparib, highlighting the increased potential efficacy of BMN 673 in *BRCA2*-associated PDAC, compared to the older generation PARPis. These observations were subsequently validated in a PDX model by demonstrating 61% and 64% tumor growth inhibition with BMN 673 and cisplatin treatment, respectively.

Our findings are consistent with recent retrospective case series reports suggesting that DCL and PARPi treatment is beneficial in *BRCA1*- and *BRCA2*-associated PDAC. In a case series of PDACs harboring germline *BRCA1* or *BRCA2* mutations, partial and complete radiologic responses were reported in ten patients treated with either a combination of a PARPi and gemcitabine, a PARPi alone or with DCLs²⁶. In a larger series of 71 cases, superior overall survival for *BRCA1*- and *BRCA2*-associated PDAC was observed with DCL treatment²⁷.

PDAC associated with germline mutations in *PALB2* may also be sensitive to agents that target DNA repair defects since *PALB2* is involved in HDR²⁸. In fact, Villarroel *et al.*²⁹ observed a marked mitomycin C treatment response in a PDAC case

with biallelic *PALB2* inactivation. More recently, Smith *et al.*³⁰ observed a sustained complete response following BMN 673 treatment of a Wilms tumor PDX carrying a *PALB2* mutation.

Although DCL exposure may result in superior tumor responses in *BRCA1*-, *BRCA2*- and *PALB2*-associated PDAC, these agents have debilitating toxicities^{21,22}. Therefore, since PARPis selectively target *BRCA/PALB2*-associated tumors without serious side effects³¹, there is strong motivation to evaluate the efficacy of these agents either as monotherapy or in combination with reduced DCL dosing to limit toxicity. In fact, sub-analysis of *BRCA1*- and *BRCA2*-associated PDAC, in a recent phase II study of olaparib monotherapy across different tumor types associated with germline *BRCA1* or *BRCA2* mutations, demonstrated complete or partial responses (21.7%), six-month progression-free survival (36%), and one year overall survival (41%) without major adverse events³².

BMN 673 has potentially advantageous features over other agents in its drug class. Compared to earlier generation PARPis, BMN 673 functions both by inhibiting PARP catalytic activity and by tightly trapping PARP to DNA at sites of single-strand DNA breaks, resulting in increased potency¹². These features suggest that BMN 673 may be the agent of choice in its drug class for the treatment of tumors with HDR deficiency. Its potentially increased efficacy may allow it to be used in monotherapy regimens without cytotoxic agents or in combination therapies with lower DCL dosing to maintain manageable toxicity. In fact, our observations support this notion. We show that PDAC cells with *BRCA2* deficiencies are sensitive to BMN 673 at low dosages. In addition,

BMN 673 displayed similar *in vitro* and *in vivo* efficacy to cisplatin, our most efficacious DCL tested.

Although our study is the first to evaluate BMN 673 and compare the cytotoxicities of a panel of DCLs and PARPis in PDAC cells with HDR deficiencies, our investigation was limited by the availability of a single PDAC cell line with a germline *BRCA2* mutation (Capan-1). Therefore, we used shRNA technology to develop additional cell lines expressing reduced *BRCA2*. Although these cell lines provide supporting evidence for the efficacy of BMN 673, they likely do not fully recapitulate the *BRCA2*-deficiency of PDAC cells derived from patients harboring germline *BRCA2* mutations. Also, our preclinical trial validation was limited by the availability of a single PDX model and we cannot exclude the possibility of variable responses of BMN 673 across the spectrum of *BRCA1*-, *BRCA2*- and *PALB2*-associated PDAC. Despite these resource limitations, our observations are striking and provide enthusiasm and rationalization to evaluate BMN 673 in clinical trial as single-agent therapy and in combination with DCLs, particularly cisplatin.

The patient from whom the xenograft was established presented with a pancreatic tail PDAC and limited metastatic liver disease. Following marked response of the primary tumor and complete radiologic response of the liver metastasis with platinum-based therapy (FOLFIRINOX, Figure 1A), he underwent a distal pancreatectomy and splenectomy. In addition, intraoperative ultrasonography revealed limited residual liver metastatic disease, which was ablated. Although patients with metastatic PDAC are not typically resected, our patient's performance and response to FOLFIRINOX, and his

inability to continue on FOLFIRINOX due to thrombocytopenia, together with data suggesting improved outcomes in *BRCA2*-associated PDAC²⁸, provided motivation for surgical intervention. Unfortunately, although our patient remains alive 26 months following resection, he has recently recurred. However, our preclinical trial results suggest that his recurrence may be effectively controlled with BMN 673 therapy.

Our results provide rationale to evaluate BMN 673, either alone or in combination with DCLs, in a clinical trial of *BRCA2*-associated and alike (*BRCA1* and *PALB2*) PDAC. In addition, this investigation highlights the value of PDX models in delineating personalized treatment strategies for difficult to treat and rare malignancies.

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facilitating rapid genetic testing and confirmation of the mutation in our patient.

6. CONFLICT OF INTEREST DISCLOSURES

The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1. 47 year-old male with PDAC carrying the French Canadian founder *BRCA2*:c.3170_3174delAGAAA germline mutation. A) Computed tomography images showing response to platinum-based therapy (FOLFIRINOX). Left, at presentation. Right, following 3 cycles of gemcitabine and 12 cycles of FOLFIRINOX. Arrows indicate complete radiological response of the liver metastasis and marked partial response of primary tumor (converted from a solid to cystic lesion with treatment). B) Sequencing chromatogram of the patient's lymphocyte DNA showing a 5 base-pair heterozygous deletion of the *BRCA2* gene. C) Sequencing chromatogram of DNA extracted from a fourth passage PDX tumor showing the same *BRCA2* 5 base-pair heterozygous deletion.

Figure 2. *In vitro* sensitivity to DCLs and PARPis and HDR characterization of MIA PaCa-2 and Capan-1. A) IC₅₀ values of DCLs and PARPis in MIA PaCa-2 and Capan-1

were determined using an impedance-based assay (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). B) γ -H2AX and RAD51 nuclear foci formation following irradiation with 8.5 Gy was determined by immunofluorescent staining for MIA PaCa-2 (upper panels) and Capan-1 (lower panels). DAPI, γ -H2AX, RAD51 and composite immunostaining results are shown for both cell lines.

Figure 3. *In vitro* characterization of PANC-1 cell lines with *BRCA2* knockdown. A) Immunoblotting results probing for *BRCA2* and κ EF2 in the indicated cell lines. B) shRNA-mediated knockdown of *BRCA2* reduces RAD51 foci formation following irradiation with 8.5 Gy (* $p \leq 0.05$). C) Percent (%) cell death as a function of compound concentration is shown for *BRCA2* knockdown and parental PANC-1 cells exposed to cisplatin, veliparib and BMN 673 (dotted line: 50% cell death).

Figure 4. Preclinical PDX trial results comparing BMN 673 and cisplatin response. A) PDX tumor volume (mm^3) growth curves of BMN 673-, cisplatin- and vehicle-treated mice. Arrow indicates the start of treatment. B) Plot comparing tumor growth inhibition at the end of cisplatin and BMN 673 treatment versus vehicle treatment (* $p < 0.05$). TV1: average tumor volume on first treatment day = 121.78 mm^3 . C) Plot monitoring weight loss for BMN 673-, cisplatin- and vehicle-treated mice.

Figure 5. H&E (40x and 400x magnification) and Ki-67 staining (200x magnification) of tumor sections. A) Top row: H&E staining (40x) showing equivalent areas of necrosis (arrows) among treatment groups. Bottom row: H&E staining (400x) of tumor sections showing atypical cells with enlarged nuclear structures (arrows) in cisplatin- and BMN-treated cells and increased mitotic cells (arrows) in vehicle-treated

tumors. B) Ki-67 staining (200x) of tumor sections showing decreased Ki-67 staining in cisplatin- and BMN 673-treated xenografts.

Figures

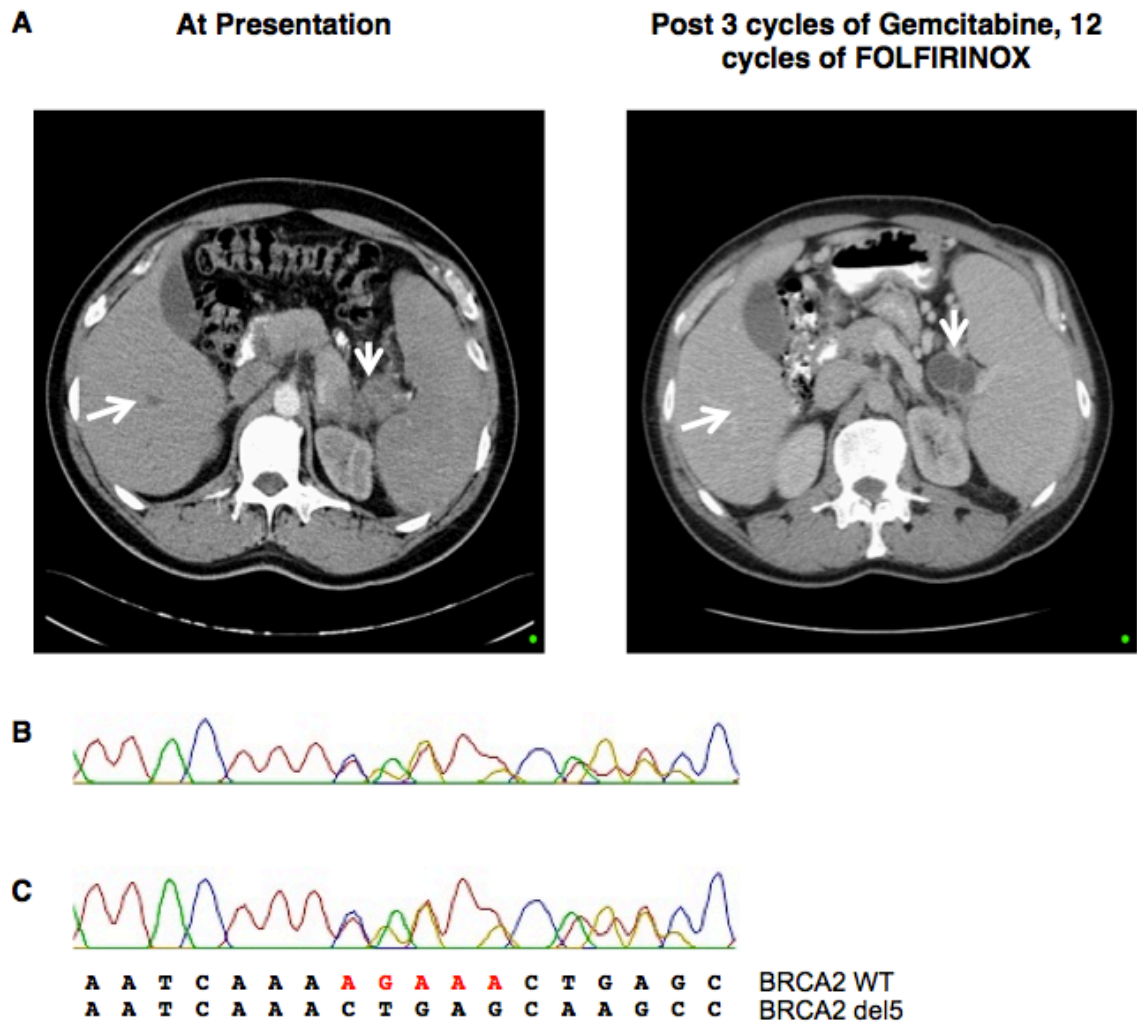


Figure 1.

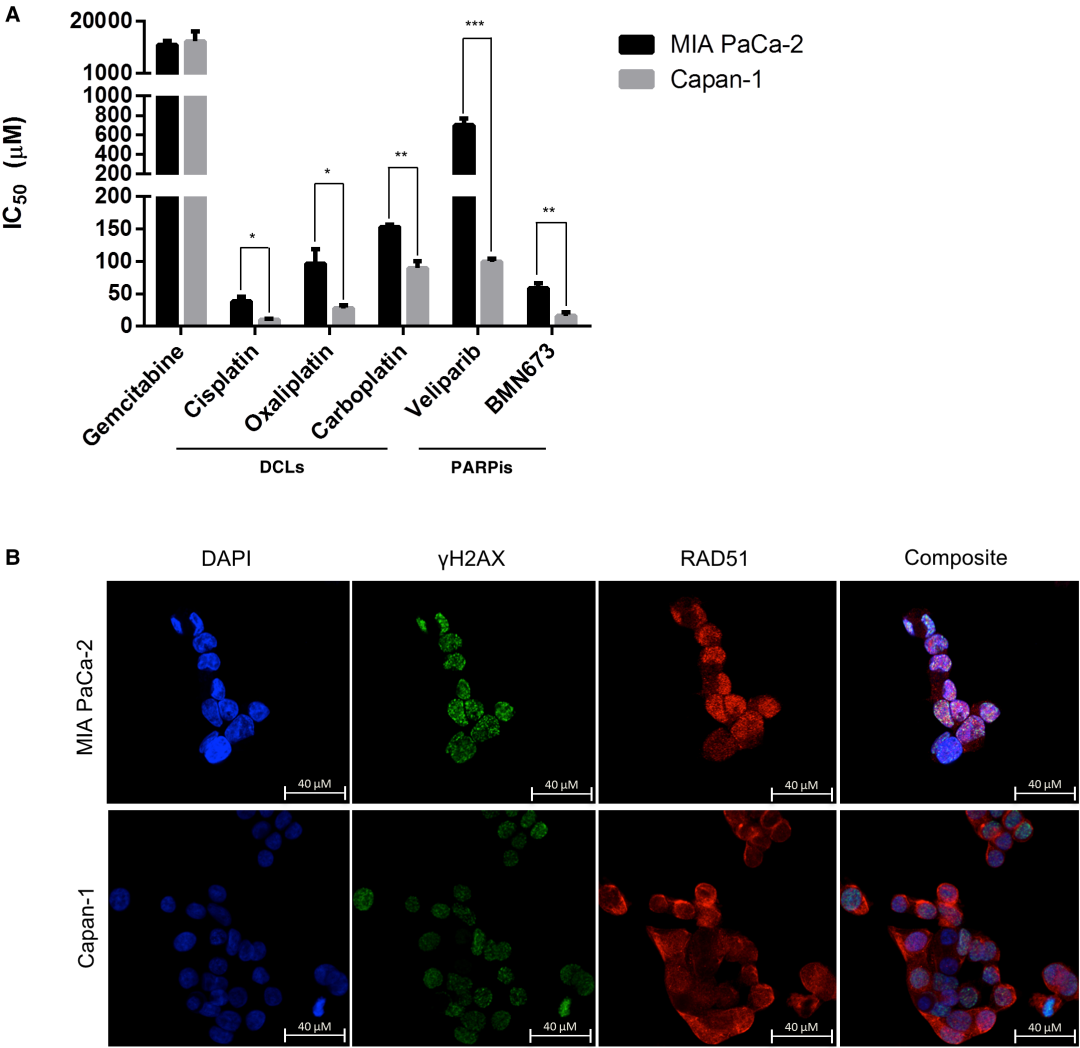


Figure 2.

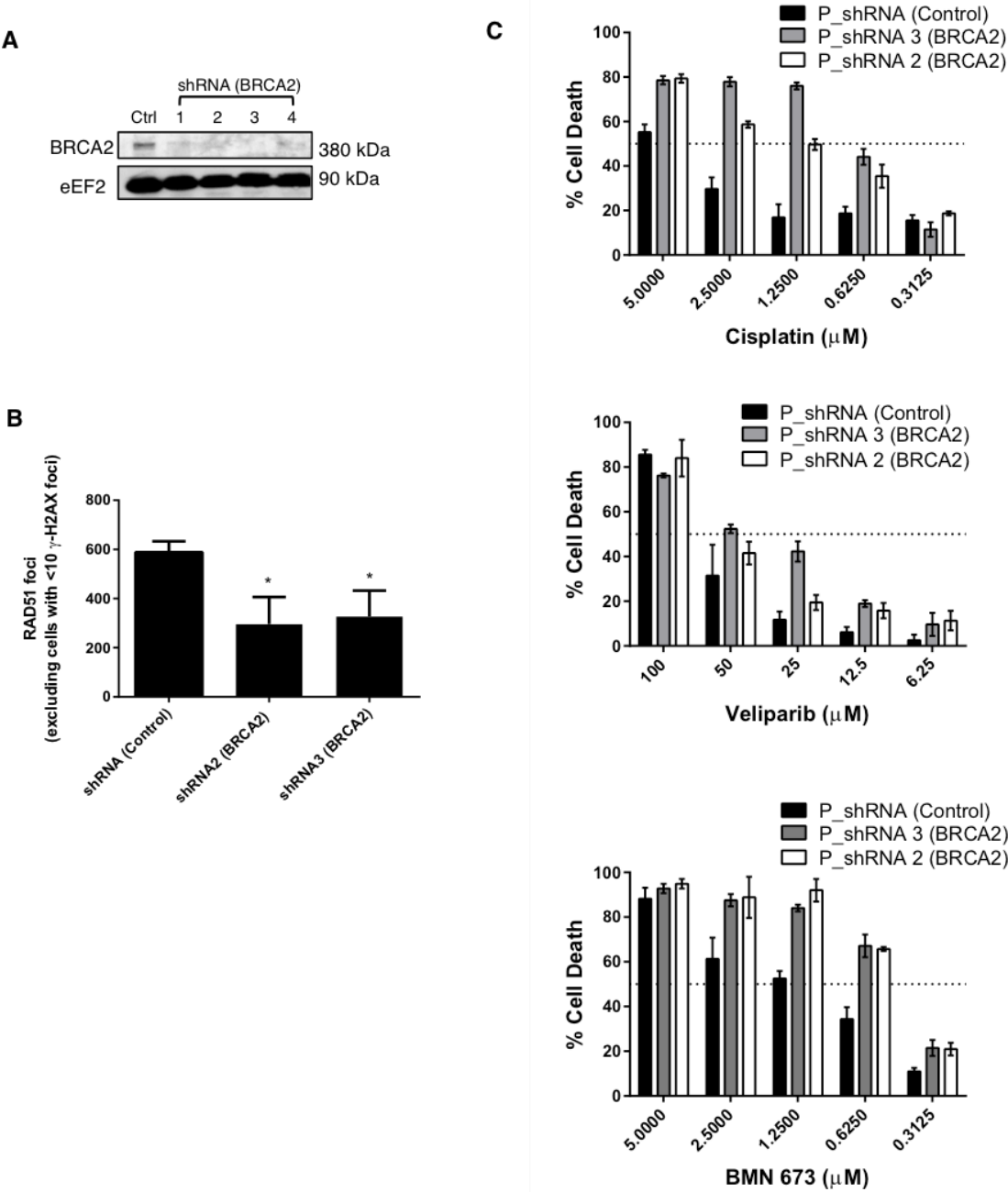


Figure 3.

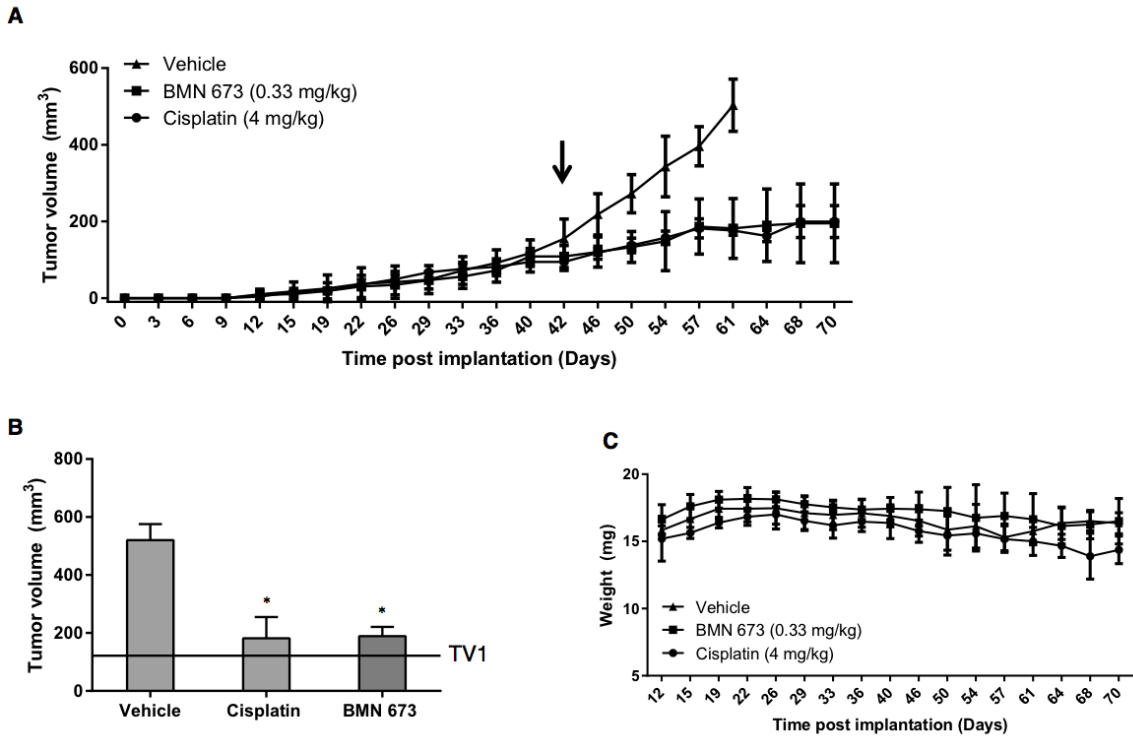


Figure 4.

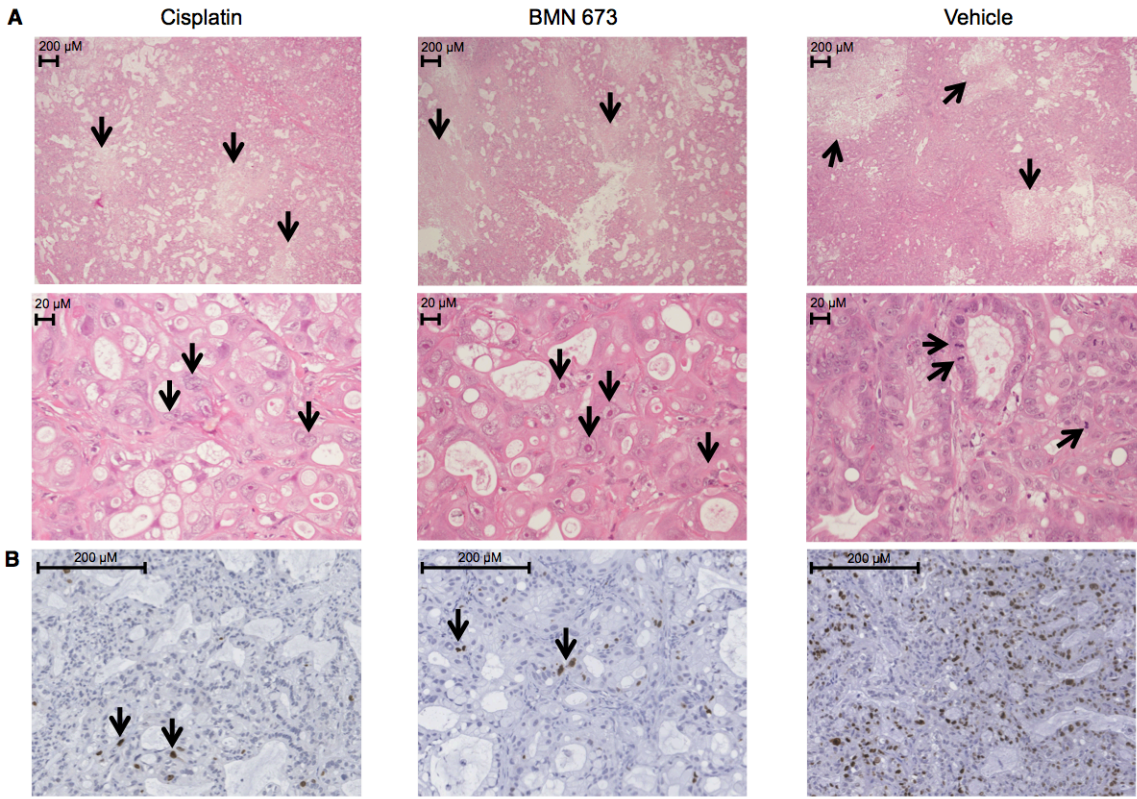


Figure 5.

Supplementary Material

1. Methods

1.1 Western-blotting: Protein extracts were solubilized in NuPAGE LDS sample buffer (106 mM Tris-HCl, 141 mM Tris Base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 SERVA Blue G250, 0.175 mM Phenol red, pH 8.5). Lysates (100 ug) were resolved in 3-8% Tris-Acetate gels and transferred to a nitrocellulose membrane (GE Life Sciences, Quebec, Canada) at 200 mA for 2 hours using NuPAGE Transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2). After blocking and subsequent washing, the membrane was exposed to the BRCA2 primary antibody (1:500, CA1033; Calbiochem, Etobicoke, Canada) or the eEF2 primary antibody (loading control, 1:1000, 2332S; Cell Signaling, Danvers, USA). The membrane was then exposed to a horseradish peroxidase-conjugated secondary antibody (1:5000, Peroxidase-conjugated AffiniPure; Jackson ImmunoResearch, West Grove, USA). Blot analyses were visualized using Western Lightning Plus ECL (Perkin Elmer, Waltham, USA).

1.2 BRCA2 mutation analysis: PDX DNA was extracted using ONE-4-ALL Genomic DNA Mini-Preps Kit (Bio Basic Canada Inc., Markham, Canada). The *BRCA2*:c.3170_3174delAGAAA mutation was confirmed in the PDX tumor tissues by Sanger sequencing. The patient's lymphocyte DNA was isolated according to manufacturing protocols for Ficoll-Paque (GE Healthcare Life Sciences) and sequenced in parallel. The primer sequences used for the tumor DNA were AGGAAATCAAGCTCTCTAACA (forward) and CCTGCTTGAAAATAACATCTG

(reverse), whereas the GGAGGTAGCTTCAGAACAGCTT (forward) and TTTCTGCCTTTTGGCTAGGTGT (reverse) primer sequences were used for the lymphocyte DNA.

2. Tables

2.1 Supplemental Table 1: TRCN numbers and shRNA sequences of *BRCA2*-targeting shRNAs.

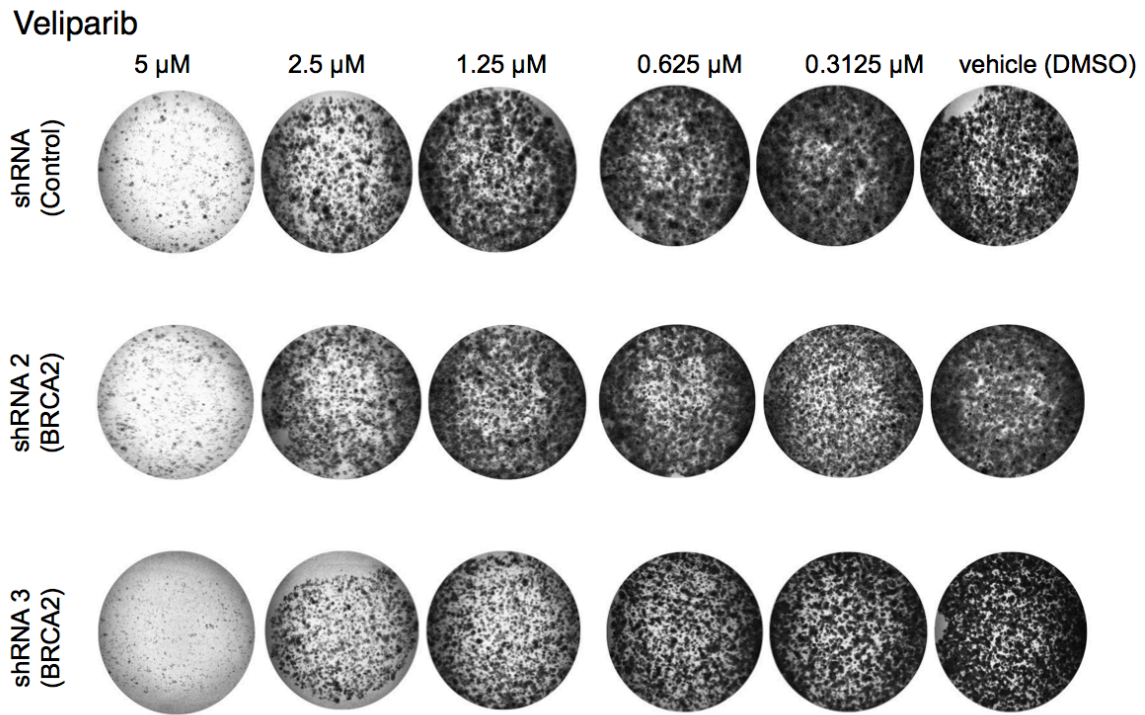
	TRCN Number	shRNA Sequence
shRNA 1 (BRCA2)	TRCN0000040193	CCGGCGCTTAACCTTTCCAGTTTATCTCGAGATAAACTGGAAAGGTTAAGCGTTTTG
shRNA 2 (BRCA2)	TRCN0000040194	CCGGGCAGCCATTAAATTGTCCATACTCGAGTATGGACAATTTAATGGCTGCTTTTTG
shRNA 3 (BRCA2)	TRCN0000040195	CCGGGCGTTTCTAAACATTGCATAACTCGAGTTATGCAATGTTTAGAAACGCTTTTTG
shRNA 4 (BRCA2)	TRCN0000040196	CCGGCCTCTGAAAGTGGAAGTGGAACTCGAGTTTCCAGTCCACTTTCAGAGGTTTTTG

2.2 Supplemental Table 2: Acquisition data for immunofluorescence images.

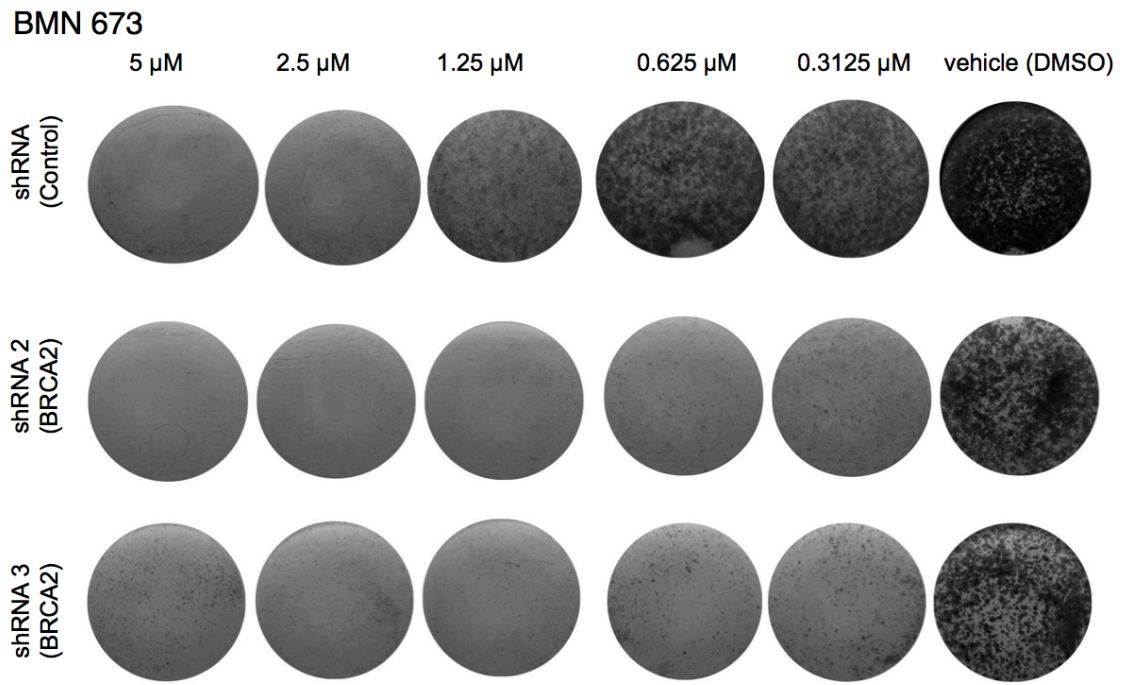
Microscope	LSM700, AxioObserver
Objective	Plan-Apochromat 40x/1.4 Oil DIC
Scaling (per pixel)	0.16 μm x 0.16 μm
Image size (pixels)	1024 x 1024
Image size (scaled)	160.04 μm x 160.04 μm
Image format	8 bit
Scanning mode	Frame
Scanner zoom	X:1, Y:1
Pixel time	1.58 μs
Line time	15.49 s
Averaging	4

3. Figures

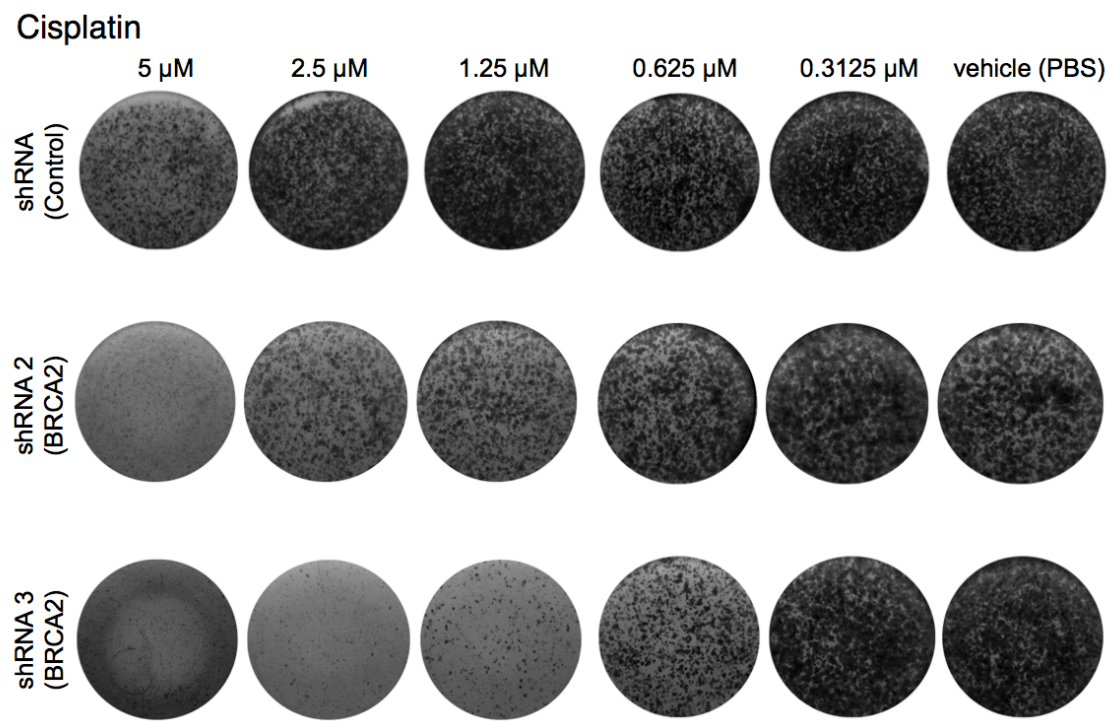
3.1 Supplemental Figure 1: Control [shRNA (Control)] and BRCA2 knockdown [shRNA 2 (BRCA2) and shRNA 3 (BRCA2)] cells were seeded in 6-well plates, exposed to a 10-day veliparib treatment course, fixed and stained with 0.1% crystal violet for visualization. Veliparib doses (left to right, top to bottom): 100, 50, 25, 12.6, 0.625 μ M, vehicle (DMSO).



3.2 Supplemental Figure 2: Control [shRNA (Control)] and BRCA2 knockdown [shRNA 2 (BRCA2) and shRNA 3 (BRCA2)] cells were seeded in 6-well plates, exposed to a 10-day BMN 673 treatment course, fixed and stained with 0.1% crystal violet for visualization. BMN 673 doses (left to right, top to bottom): 5, 2.5, 1.25, 0.625, 0.3125 μ M, vehicle (DMSO).



3.3 Supplemental Figure 3: Control [shRNA (Control)] and BRCA2 knockdown [shRNA 2 (BRCA2) and shRNA 3 (BRCA2)] cells were seeded in 6-well plates, exposed to a 10-day cisplatin treatment course, fixed and stained with 0.1% crystal violet for visualization. Cisplatin doses (left to right, top to bottom): 5, 2.5, 1.25, 0.625, 0.3125 μM , vehicle (PBS).



CHAPTER III:

General Discussion and Original Contributions

General Discussion and Original Contributions

The results of my dissertation provide compelling data for BMN 673 as a promising treatment agent in *BRCA2*-associated PDAC. I found BMN 673 and cisplatin to have increased *in vitro* cytotoxicities in *BRCA2*-deficient PDAC cells (Capan-1) and validated these observations in a preclinical trial using a PDX model. Specifically, I observed equivalent tumor growth inhibition capacities of BMN 673 and cisplatin in the preclinical trial. Although these findings are striking and suggest that BMN 673 be tested in a phase II clinical trial of *BRCA1*- *BRCA2*-, and *PALB2* –associated PDAC, further preclinical trials, using PDXs from additional PDAC cases with *BRCA1*, *BRCA2* and *PALB2* germline mutations, to validate my findings would strengthen the rationale for a clinical trial. In addition, preclinical trials evaluating combinations of BMN 673 with clinically relevant DCL agents would further rationalize the design of a clinical trial. Since *BRCA1*-, *BRCA2*- and *PALB2*-associated PDACs are rare cases, designing a phase 3, and even a phase 2, trial is enormously challenging. Thus, the use of preclinical PDX trials to rationalize the choice and combination of agents for clinical trial is of particular importance for this subtype of PDAC, and highlights the utility of PDXs in designing rationale clinical trials for rare and difficult to treat cancers.

My original contributions are the comparison of the *in vitro* cytotoxicities to a panel of DCLs and PARPi in *BRCA2*-proficient versus *BRCA2*-deficient PDAC cell lines, the sensitization to cisplatin and the PARPi BMN 673 using shRNA technology to reduce *BRCA2* expression in a *BRCA2*-proficient PDAC cell lines, and finally, the *in vivo* response of a PDX tumor model derived from a *BRCA2*-associated PDAC to the PARPi BMN 673, with comparable sensitivity to cisplatin.

CHAPTER IV:

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