PARP Inhibitor ABT-888 as Potentiating Agent for Topoisomerase Inhibitor SN-38

by

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Shridar Ganesan

and approved by

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ABSTRACT OF THE THESIS

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Thesis Director:

Shridar Ganesan

BRCA1 is a tumor suppressor gene that has been implicated as being involved in DNA repair through a process known as homologous recombination. Mutations in BRCA 1 have been linked to an increased risk of breast cancer, but recent studies have tried to use their knowledge of homologous recombination in order to find new ways to treat cancer. Most notably with poly(ADP-ribose) polymerase (PARP), it is an enzyme involved in the DNA repair of single strand breaks. If a cell is BRCA1 deficient it cannot repair its double strand breaks and if we inhibit PAR activity the cell cannot repair single strand breaks as a result the cell should accumulate damage and undergo apoptosis and die. The goal of the research is to investigate the role of PAR inhibition in BRCA1 deficient cells. WE hypothesized that BRCA1 deficient cells should die at a lower dosage of PAR inhibiting drugs in cytotoxic tests. We also tested the efficiency of cytotoxic therapies as an effective means of treatment and tested the maximum dose tolerable before the cells became changed. We first used immunofluroescence to check for PAR activity in different types of cells and to then see the effects of PAR inhibition. We then used PAR inhibitor ABT-888, topoisomerase inhibitor SN-38, and cisplatin, a DNA binding agent, to conduct cytotoxic tests in order to prove our hypothesis true.

Acknowledgement and/or Dedication

Instead of taking any short-cuts, I took the longer road. In retrospect I feel that I made the proper choice, so I have no regrets. Through my elongated journey I learned so much about myself that and I would not exchange this experience for any other.

The most important achievement I have earned is the right to call myself a scientist. I cannot thank the instructors at Rutgers and UMDNJ enough for helping me develop my intellectual potential. I had my doubts that I could become an able researcher worthy of being called a "scientist", but as a result of their teachings I have the confidence *and* desire to tackle any scientific conundrum. I never thought I would be one to seek greater challenges, but I have been transformed by the motivation of my instructors. However, one of the great ironies of my graduate education is that I learned how to ask more questions than answer them, but that is why biology is so exciting.

This thesis would not have been written without the support of Dr. Shridar Ganesan. There is no amount of gratitude that can convey how much I appreciate the opportunity and truly invaluable guidance that I received. I hope one day to develop as much insight and acumen as Dr. Ganesan.

There are so many people that also deserve individual gratitude for keeping on track and keeping me sane throughout this adventure. Atul: your disdain for tissue culture work, IP washing, and sonication is contagious, but you taught me tremendously. Ondrea: you gave me an opportunity to assess myself and gave me the privilege to become your mentor. "Don't disappoint". Will: I sort of, kind of, actually, like math thanks to you. Oh, and I sort of, kind of, actually like cubicles too. Dan: you have no idea how much you helped me refine my ideas. I'm not sure if you were aware or not,

but you gave me so much encouragement when I needed it most. And of course my JanJan, Ummi, Maria, Rayqa, Anna, Usma, Shadab, and Ayana: I know I tried your patience and I probably will not reciprocate, but I thank you anyway for not writing me off as a complete nobody.

How can I not thank the entire faculty and staff of CINJ? Everyone at CINJ taught me something unique and different about the world. I'd like to thank Yi Ting, Tulin, Pravin, Yanique, Kevin, Brij, Dimitri, Roman, Carl, Manish, Rob, and Wilberto. Thank you everyone for being such pleasant souls to work with and laughwith.

A special gratitude also belongs to the de Lame family. I am still amazed that you had faith in me when I didn't even know I had any potential. It was very endearing, and quite honestly, it was sometimes the only motivation I had to succeed. I owe you so much and what I have to offer does not even come close. Oh, and I'd like to thank Figgy for being my friend.

In short, I dedicate this thesis to everyone who put a smile on my face.

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Introduction

Breast cancer is the most common form of cancer afflicting American women with an estimated 1 in 8 American women developing a form of breast cancer within their lifetime. Of the various types of breast cancer, familiar breast cancer constitutes a small subset contributing to approximately 5 to 10% of all breast cancers. Among hereditary breast cancers approximately one third are due to mutations in the tumor suppressor gene *BRCA1*, and a third of the remaining hereditary breast cancers are due to mutations in the tumor suppressor gene *BRCA2*. [1] Although not conclusive, there is data to suggest that women who have a *BRCA1* or *BRCA2* mutation in their germline have a similar to worse prognosis for survival than those who do not harbor these mutations.[2] Fortunately clinical response to chemotherapy treatment of *BRCA1* breast cancers proves to be more beneficial than no adjuvant chemotherapy, suggesting further avenues of treatment for *BRCA1* derived breast cancer.[3]

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[1] Mskcc.org. 2 Aug. 2005. <http://www.mskcc.org/mskcc/html/8623.cfm>
[2] Baker, Mitzi. "Breast Cancer Prognosis in Women with BRCA1 and BRCA2 Mutations." GeneticHealth.com. 6 April 2001. <http://www.geneticheath.com/BROV_Prognosis_in_Women_With_BRCA1_or%20BRCA2_ Mutations.shtml>
[3] James, Colin R., Quinn, Jennifer.E., Mullan, Paul B., Johnston, Patrick G., Harkin, D.Paul. "BRCA1, a Potential Predictive Biomarker in the Treatment of Breast Cancer". The Oncologist. 12:142-150. (2007).

BRCA1 and Genomic Instability

Studies of *BRCA1* have revealed that deficiencies in *BRCA1* result in pleiotrophic phenotypes, including growth retardation, increased apoptosis, defective DNA damage repair, abnormal centrosome duplication, defective G2/M cell cycle checkpoint, impaired spindle checkpoint, chromosome damage, and aneuploidy. Consequently in addition to its role as a tumor suppressor, these phenotypes provide strong evidence for a prominent role of *BRCA1* in the maintenance of genomic stability.[4]

Genomic instability is the temporary or permanent alteration of the genome that may occur at a molecular level and/or at a chromosomal level. At the molecular level changes in the nucleotide may affect the structure of the gene or the expressed protein as a result of mutations, deletions, amplifications, microsatellite amplifications, and/or gene silencing via epigenetic modifications. Consequently alterations at the nucleotide level within any member of the DNA repair pathway may lead to a defective response to DNA damage and result in genetic instability. In addition instability on the chromosomal level is characterized by structural and numerical rearrangements which may result in the gain or loss of whole segments of a chromosome. Together these mechanisms of instability may lead to aneuploidy, the hallmark of many types of cancer.[5]

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[4] Deng, Chu-Xia, Wang, Rui-Hong. "Roles of BRCA1 in DNA damage repair: a link between development and cancer". Human Molecular Genetics. Vol. 12, Review Issue 1. (2003).

[5] Jefford, Charles Edward, Irminger-Finger, Irmgard. "Mechanisms of chromosome instability in cancers." Critical Reviews in Oncology Hematology59: 1-14. . (2006)

DNA Repair and *BRCA1*

Efficient and precise repair of DNA damage is the most important aspect in maintaining genomic stability. Upon single strand lesions the repair pathway may utilize various mechanisms to prevent further complications due to double strand breaks. Depending on the specific type of DNA lesion, the cells may undergo base excision repair (BER) (flipping the mutated base out of the DNA helix and repairing the base alone), nucleotide-excision repair (NER) (recognizing bulky distortions in the shape of the double helix and removing the short single-stranded DNA segment) or mismatch repair (MMR) pathways where the intact complementary strand to the lesion is utilized as a template for repair.

Exogenous insults to the genome from sources such as ionizing radiation (IR) or endogenous events such as collapse of replication forks upon encountering a SSB can lead to the more lethal double-strand breaks (DSBs). Their potential for harm is greater since there is no viable complementary strand to serve as a template. As a result the damaged cells have two options: non-homologous end joining (NHEJ) or homologous recombination (HR).

NHEJ is an error prone event that utilizes the Ku70/Ku80 heterodimer and DNA-dependent protein kinases (DNA-PKcs) to ligate the broken ends of DNA. Consequently, this results in minor changes to the genome, which by themselves may prove to be lethal to the cell. However, recent evidence suggests that alongside this repair pathway there exists a precise subpathway that utilizes Ku/DNA-PKCs and microhomologous

sequences flanking the lesion to result in minimal sequence modification. This sub-NHEJ pathway is believed to be dependent on the MRE11-RAD50-NBS1 (MRN) complex to anneal and religate the broken ends.

The HR repair pathway is regarded as an accurate repair process, utilizing the sister chromatid sequence as a template for the lesion. In mammalian species this process requires the recombinase activity of RAD51. RAD51 is so essential that homozygous loss of Rad51 in mice results in early embryonic lethality.[6]

It has been demonstrated in mammalian cells that RAD51 interacts with both *BRCA1/2*; both co-localize with RAD51 in vivo. Since both *BRCA1* and *BRCA2* have been shown to be involved in the HR-mediated DSB repair, the interaction with RAD51 is a means of preventing genomic instability and tumorigenesis. In the absence of *BRCA2*, cells are unable to form RAD51 foci

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[6] Tsuzuki, T. et al. "Targeted disruption of the Rad51 gene leads to lethality in embryonic mice". Procedings from. National. Academy of. Science. USA **93**, 6236-6240 (1996).

Poly(ADP-ribosyl)ation (PAR)

Poly(ADP-ribosyl)ation (PAR) is a post-translational modification that covalently links ADP-ribose units to multiple nuclear proteins. Although the exact role of PAR is still under investigation, it is involved in a variety of cellular processes including DNA repair, cell differentiation, regulation of chromatin structure, and gene regulation. The main catalysts for ADP-ribose polymer formation are poly(ADP-ribose) polymerase (PARP)-1 and 2 which have been shown to be instantly activated upon DNA damage. The process of PAR modification involves PARP-1 hydrolyzing NAD+ to nicotinamide, which is released, and ADP-ribose, which is polymerized onto substrate proteins. This process is repeated numerous times yielding polymers of various length from a few to 200 hundred ADP-ribose units. However, this process is also reversible with the endo- and exoglycosidic activity of PARG removing units from the substrate protein.

The functional consequence of a PARylated protein is unclear, but the increased affinity of PARP-1 to DNA strand interruptions and the increase in PAR synthesis implicates PARP and PARylation to DNA repair. Concomitantly there is enhanced catabolism of the PAR units that reduces the polymer half-life from several hours to a few seconds upon DNA damage. Together this PARylation-PARGylated cycle suggests a sensory role for recruitment and activation of the DNA repair machinery. Additionally the ADP-ribose units mimic the basic building blocks of DNA suggesting that PARylation of proteins, specifically histones, may be required to maintain and/or regulate chromatin structure.

PARylation of proteins has been identified in the repair of dsDNA and ssDNA breaks, implicating a role for PARP in various DNA repair pathways. One of the pathways, the base excision repair pathway, utilizes PARP-1 and PARylation to recruit XRCC1 to damaged sites. In the absence of PARP-1 enzymatic activity XRCC1 is not recruited to the break site, resulting in a delay of DNA repair. Consequently the defect in the BER pathway may be exploited to further sensitize tumor cells to therapeutic agents.

Irinotecan: Topoisomerase I Inhibitor

The double helical configuration of DNA poses a topological problem during transcription, chromatin remodeling, and replication: torsional strain is generated with the winding and unwinding of DNA. To eliminate this problem the topoisomerase class of enzymes catalyzes and guides the unknotting of DNA by creating transient, single-strand breaks in the DNA generating a TOP1 cleavage complex. This allows the DNA to relax from its supercoiled configuration allowing normal cellular processes to take place. Drugs such as irinotecan inhibit the religation of this nick, which eventually lead to double stranded beaks and induce cells to undergo apoptosis.

ABT-888 as a Topoisomerase I Potentiating Agent

Because PARP activity is essential for repair of ssDNA breaks, it is postulated that inhibition of PARP may sensitize tumor cells to cytotoxic agents that are capable of inducing DNA damage normally repaired through the base excision repair pathway. In fact earlier studies have shown that inhibition of PARP sensitizes tumor cells to therapeutic compounds such as temozolomide, platinums, topoisomerase I inhibitors, and radiation.[7,8] These results provide an opportunity to exploit the DNA repair-deficient pathway of *BRCA1/2* null cells.

BRCA1 and *BRCA2* deficient cells have been shown to be highly sensitive to various PARP inhibition compounds compared to wild-type cells.[9,10] Consequently by employing the topoisomerase I inhibitor irinotecan in a *BRCA1/2* null background, the tumor cells are targeted by exploiting defects in two repair pathways which may prove to be extensively lethal to the cancer cells.

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[7] Curtin, NJ, Wang LZ, Liakouvaki A, et al. "Novel poly-(ADP-ribose) polymerase-1 inhibitor, AG14361, restores sensitivity to temozolomide in mismatc repair-deficient cells," Clinical Cancer Research; 10:881-9. 2004

[8] Miknyoczki, SJ, Jones-Bolin S, Pritchard S et al. "Chemopotentiation of temozolomide, irinotecan, and cisplatin activity by CEP-6800, a poly (ADP-ribose) polymerase inhibitor". Molecular Cancer Therapy;2:371-82. 2003

[9] Farmer, H. McCabe N, Lord CJ, et. Al. "Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy". Nature; 434;917-21. 2005

[10] Bryant, HE, Schultz N, Thomas HD, et al. "Specific killing of BRCA2-deficient tumors with inhibitors of poly-(ADP-ribose) polymerase". Nature; 434 913-7. 2005

Materials & Methods

Compound: Pure ABT-888 was synthesized by Abbott Cancer Research and Process Chemistry. The synthesis and analysis of the compound is published elsewhere.[11] ABT-888 was dissolved in DMSO (Sigma) for a final concentration of 20mM.

Poly(ADP-ribose) polymer immunoblot: The entire procedure was performed on ice. Media was aspirated from plates and rinsed twice with cold PBS. In each plate 200 uL of NETN buffer (0.5% Nonidet P-40/1 mM EDTA/20 mM Tris·HCl, pH 8.0/100 mM NaCl) supplemented with protease inhibitor (Sigma Aldrich) was added and distributed over plate. Plates were incubated over ice for 15 minutes after which they were scraped and transferred to microfuge tubes. Cells were further incubated on ice for an additional 10 minutes. Following incubation, lysates were sonicated 3x (6 pulses each time) and then centrifuged for 20 minutes at 2000 rcf. Supernatant was collected and stored in $-80^{O}C$ for further analysis.

For gel electrophoresis lysates were quantitated using Bio-Rad Protein Determination Assay. Briefly, a 1x working solution was made and protein lysates were diluted 1:500 for quantification. A standard curve was prepared using BSA prepared from lyophilized powder. Standard concentrations were prepared in concentrations of 0, 2, 4, 6, and 8 ug/uL and samples were read using a Beckman Spectophotometer at 590 nM. Following quantification 50 ug of protein lysates were loaded onto a Ready Gel Tris-HCl gel 4%-

[11] Zhu, GD, Gong J, Gandhi V, Penning TD, Giranda VL. 1H-Benzimidazole-4-carboxamides substituted with a quaternary carbon at the 2-position are potent parp inhibitors. United States patent application 2006/ 02292890. 2006.

15% (Bio-Rad) diluted with Sample Buffer, Laemmli 2× Concentrate (Sigma). Gel electrophoresis was carried out at 80V until sample surpassed the stacking gel, at which point the voltage was increased to 150V. Following electrophoresis the gel was released from the cassette and incubated in ice-cold
semi-dry transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 10 minutes on ice. Simultaneously a PVDF membrane (Millipore) was dipped in 100% methanol and also incubated in the ice-cold semi-dry transfer buffer for 10 minutes. Proteins from gel were transferred to membrane in Trans-Blot Semi-Dry Transfer Cell apparatus for 1 hour at 20V.

Following transfer the membrane was blocked in 5% milk-PBS-0.1% Tween overnight at $4^{O}C$. For incubation with primary antibody, anti-PAR antibody (Trevigen) was diluted 1:400 in 5% milk-PBS-0.1% Tween, and the blot was incubated for one hour at room temperature. After 3x – 5 minute washes in PBS-0.1% Tween, the blot was incubated with 1:4000 anti-mouse-HRP (Pierce) in 5% milk-PBS-0.1% Tween for one hour at room temperature. After 3x – 5 minute washes in PBS-0.1% Tween, the blot was developed using ECL (Amersham) and developed.

MTS assay: To determinate appropriate cell density in 96-well plate, cells were first serially seeded in 1:2 dilutions. After 96 hours, cells were examined under microscope. Appropriate cell density was the number of cells seeded on day one that reached ~ 80% - 90% confluency 96 hours later.. See Table 1 for summary of cell density. For MTS assay, cells were trypsinized, counted, and seeded at appropriate density. Drugs (SN-38,

cisplatin, ABT-888) were serially diluted and added to cells the following day. Cells were incubated with drug for 96 hours at which point 20 uL of MTS + PMS (20:1) ratio was added cells. Cells were incubated with MTS reagent until O.D. readings at 490 nM were between 0.8-1.2. Softmax Software was used to analyze and plot data.

Cell Type	Cells Seeded per Well
V79	500
VC8	1000
114-6	500
114-2	1000
MCF-7	2000
MDA-MB-436	5000
Sum1459pt	5000

Table 1: Cell density for MTS assay

***Immunofluorescence*:** Cells were seeded onto either chamber slides or sterile coverslips and allowed to attach overnight. After treatment (IR, ABT-888, control), media was aspirated, and cells were washed once with PBS, followed by 10 minute incubation with 4% paraformaldehyde. After incubation, cells were washed twice with PBS, 5 minutes per wash, and then treated with 0.5% TritonX-100 for 10 minutes. After incubation cells were again rinsed twice with PBS. Primary antibody dilutions were prepared in 5% goat serum, and cells were incubated for 45 minutes. Following primary antibody treatment, cells were washed twice, 10 minutes per wash, with PBS. Cells were then incubated with secondary antibody dilutions prepared in 5% goat serum for 30-45 minutes. Following antibody incubation, cells were again washed 2x in 10 minute washes in PBS. When mounting coverslips to glass slides, DAPI was added for nucleic acid staining, and subsequently slides were sealed with nail polish.

Laser-induced, localized DNA double strand breaks: Cells were seeded on Lab-Tek Chamber slides with 10 uM IDU for 24 hours. For micro-irradiation cells were mounted on the stage of an Axiovert 200M microscope integrated with the Palm microlaser Workstation (P.A.L.M) software.

Results:

Initial establishment of *BRCA1* status in cell lines

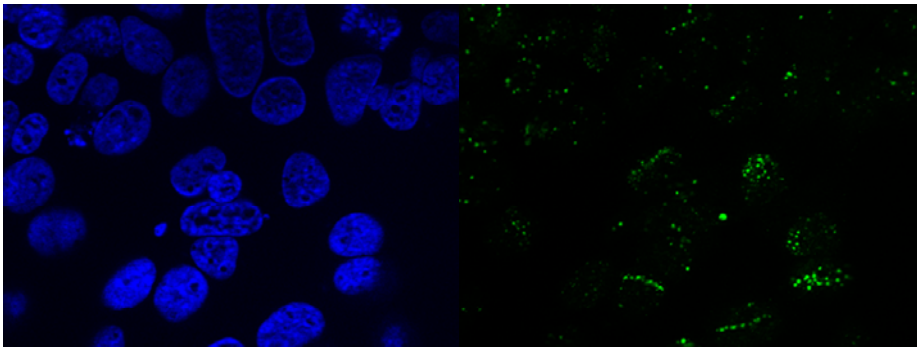
BRCA1 has been implicated in DNA repair via its requirement for HR. Therefore to test whether the cells have a functionally active *BRCA1* protein, local DNA damage via a laser microbeam was induced in various cells lines. Cells were assayed for the localization of *BRCA1* 6 hours after induction of DNA damage via immunofluorescence. Positive control for DNA damage was assessed by the presence of γ-H2AX, a marker for double-strand breaks.

The co-localization of *BRCA1* and γ-H2AX in cells exposed to the microbeam is readily visible in MCF-7 cell line, indicating a functionally active *BRCA1*. In contrast basal activity of *BRCA1* confers a nuclear dot formation through the nucleus in S-phase cells (Scully Science 5 April 1996). Upon microbeam induction, *BRCA1* was seen only in a fine line traversing the nucleus, confirming the validity of the microbeam results (**Fig 1).**

However in MDA-MB-436, there is a clear absence of *BRCA1* response to the site of DNA damage (as indicated by the presence of γ-H2AX), indicating a non-functional *BRCA1* protein. Similarly the lack of *BRCA1* response in the Sum149 pt cells shows either a complete absence of *BRCA1* localization with γ-H2AX or a very weak response, implying that Sum149 is not completely devoid of *BRCA1* function. (**Fig 1).**

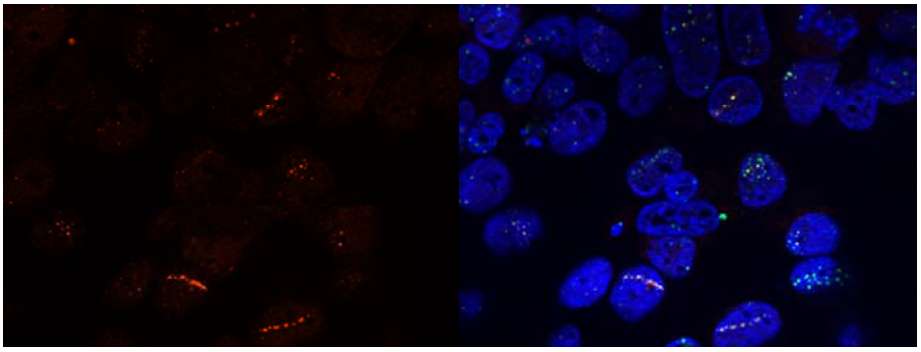
MCF-7 – A BRCA-1 positive cell line

DAPI



pH2AX

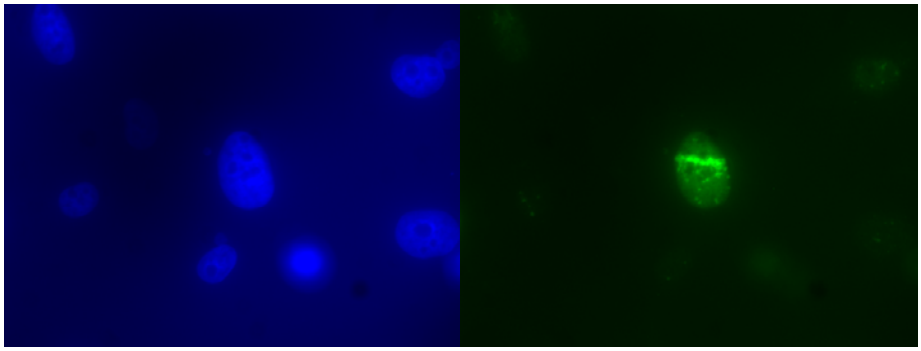
BRCA1



Merged

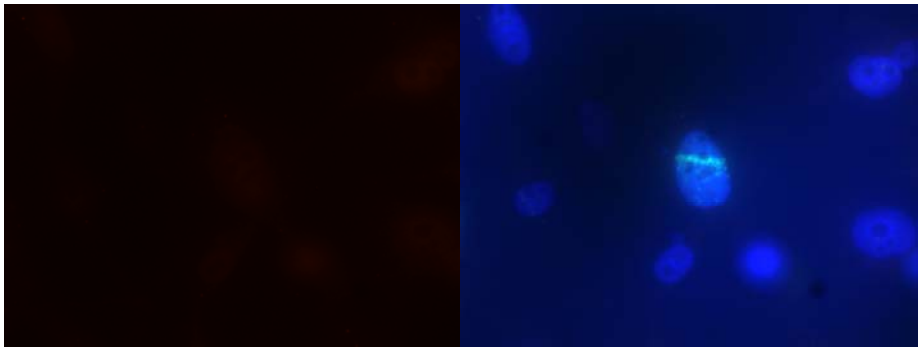
MDA MB 436 – A BRCA-1 deficient cell line

DAPI



pH2AX

BRCA1



Merged

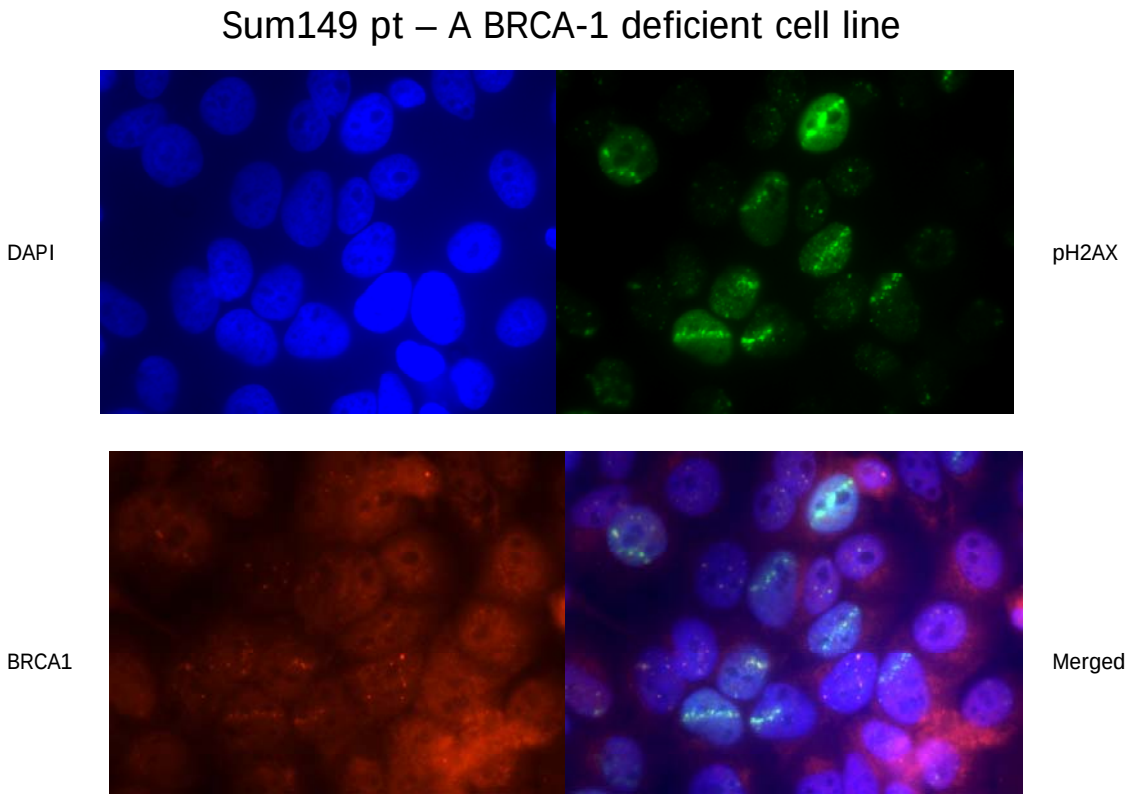


Figure 1: Establishment of BRCA1 status in wildtype (MCF-7) and BRCA1 null (MDA-MB-436 and Sum149 pt) cell lines.

Establishment of PAR inhibition by ABT-888

To assess whether the novel PAR inhibitor is effective in preventing PAR activation, MCF7 cells were titrated with various concentrations of the inhibitor for 1 hour and then exposed to IR to induce DNA damage and elicit a PAR response. Results assessed by immunofluorescence demonstrate that at 50 uM and above, the PAR signal is completely ablated, whereas at concentrations below 50 uM, weak to strong PAR signal can still be detected (Fig 2).

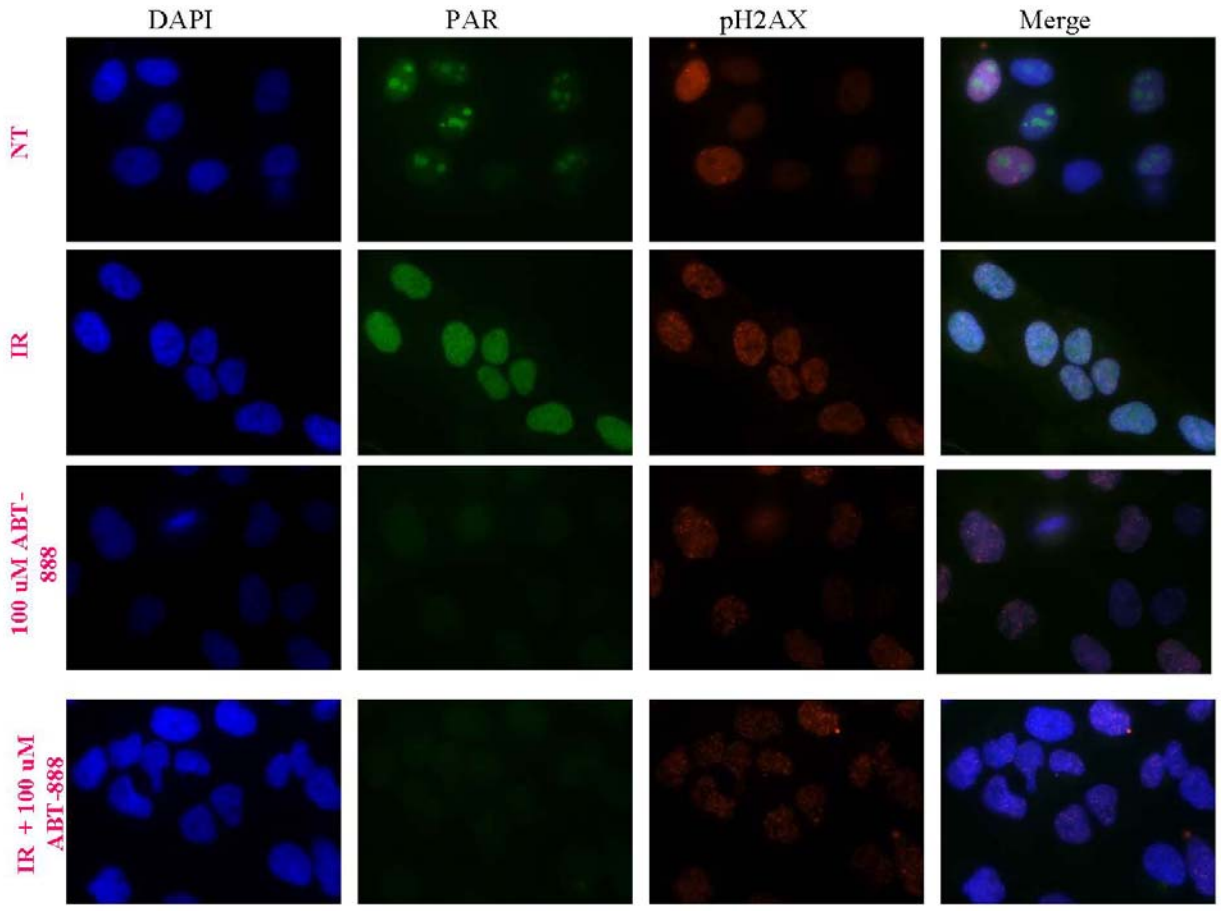


Figure 2: Titration of ABT-888 with and without exposure to IR.

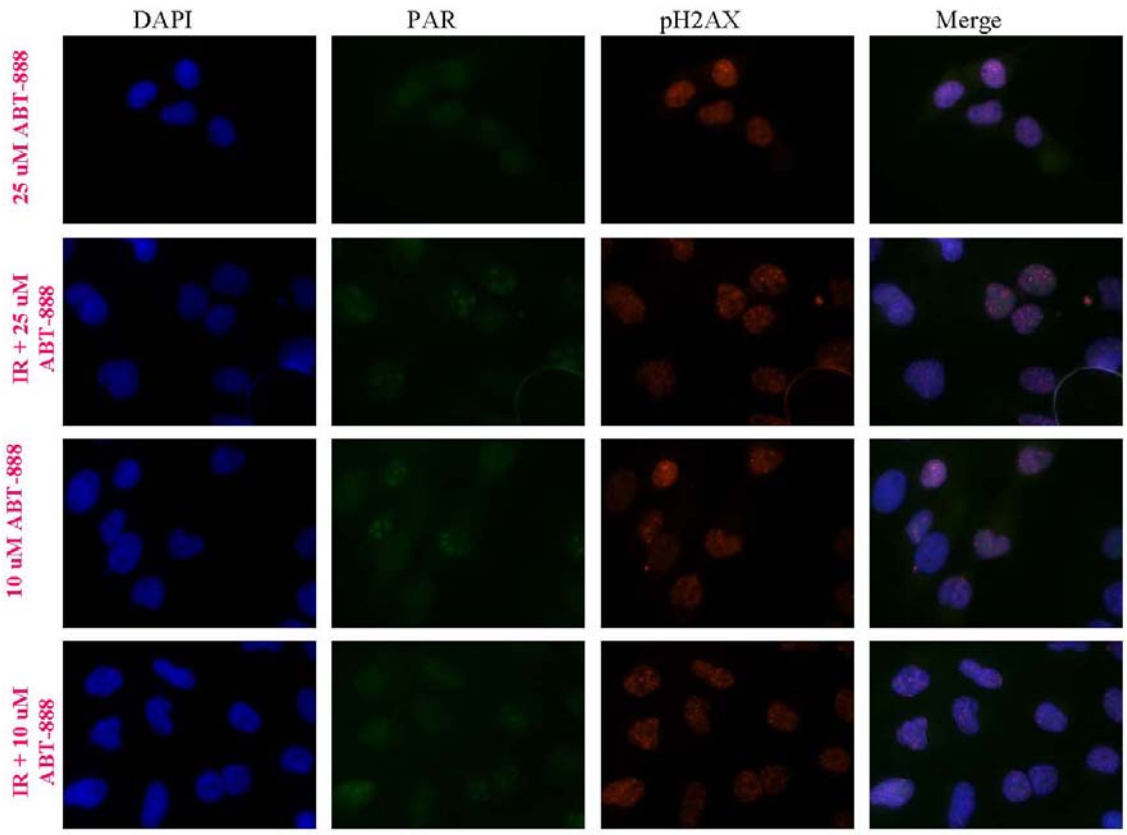


Figure 2 (continued): Titration of ABT-888 with and without exposure to IR.

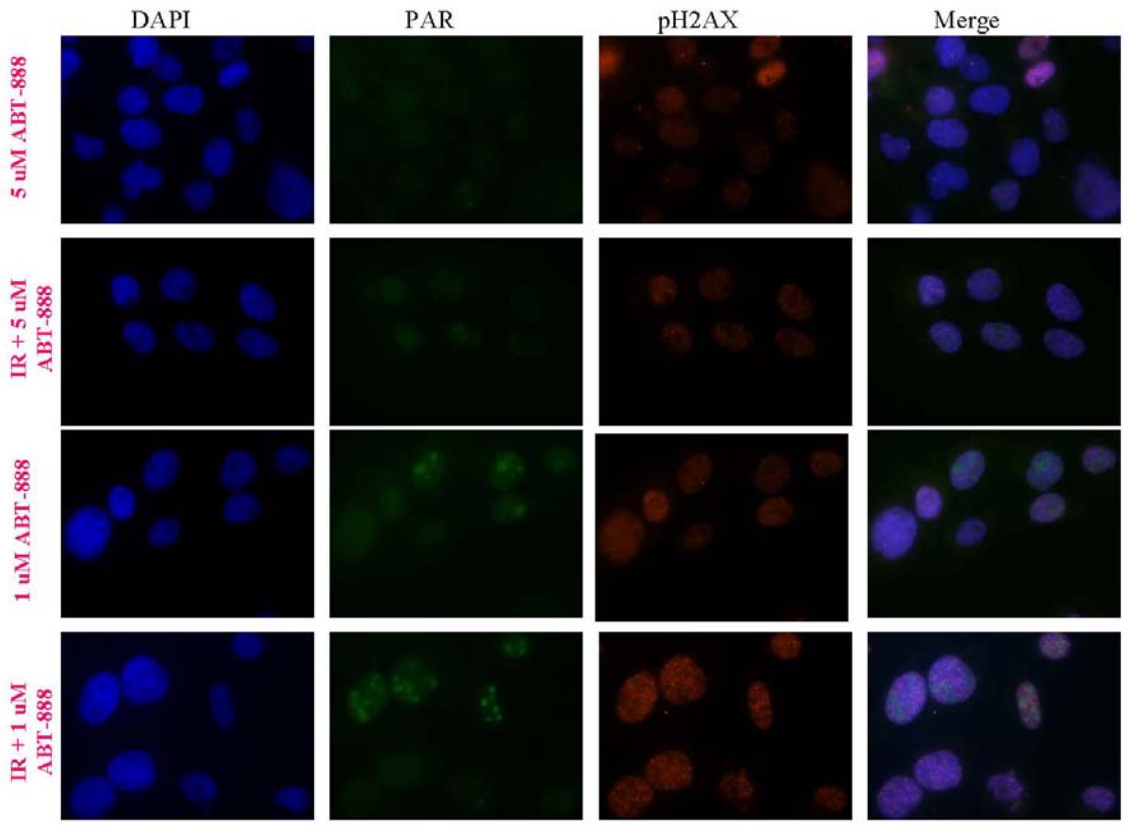


Figure 2 (continued): Titration of ABT-888 with and without exposure to IR.

The previous assay was performed in various other cells lines, but due to high background an effective PAR inhibition dose could not be determined (data not shown). As an alternate method protein lysates of the various cell lines with and without ABT-888 were assayed for PAR by Western using an anti-PAR antibody (Trevigen). This method proved to be very useful since a semi-quantitive analysis for total PAR could be assessed.

Initially it was necessary to determine if PAR activity could be detected via Western analysis. MCF7 cells were treated with 100 uM of ABT-888 for 24 hours and then harvested for protein. Analysis by Western showed PAR activity mostly as an aggregate of high molecular weight proteins **(Fig 3**). With the addition of ABT-888, the high molecular weight aggregate is completely abolished. This indicates the suitability of the assay for PAR detection.

The cytotoxicity experiments are to be performed over a four day period, so various concentrations of ABT-888 were titrated in MCF7, MDA-MB-436, V79, and VC8 for a total of 96 hours. The purposes of this assay are to (1) determine what is the minimum effective concentration of ABT-888 in the different cell lines and (2) to assess if PAR is still inhibited over a 4-day period. Western analysis clearly shows that for the MCF7 cell line at 10 uM there is complete inhibition of PAR. Lower concentrations of ABT-888 show weak signaling of PAR down to 0.1 uM. These weak signals may either be PAR events that are still able to occur despite the presence of the inhibitor or PARylated proteins that have a greater than expected half life and stability and have not been degraded by PARG. At 10 nM PAR activity is comparable to non-treated cells. In

MDA-MB-436 cells Western analysis reveals that there is 100% complete inhibition of PAR at concentrations as low as 100 nM. Ten-folds lower at 10 nM there is approximately 50% the level of PAR activity as compared to the non-treated cells. This is a rather dramatic recuperation of PAR activity compared to MCF7 cells. Similar results as MDA-MB-436 were obtained in VC8 cells (**Figure 4).**

Western Analysis with Anti-PAR antibody

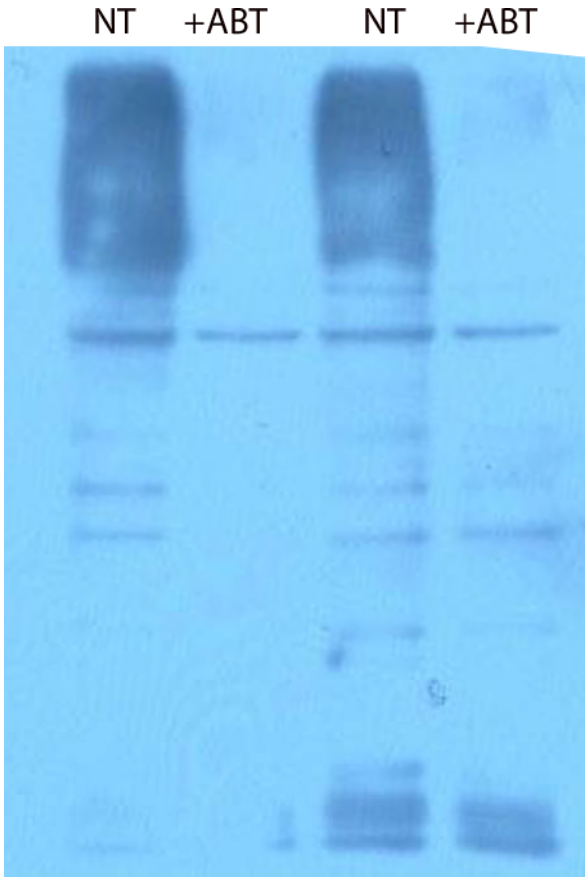


Figure 3: MCF7 cells with/without 100 uM ABT-888. Cells were treated for 24 hours before being harvested for protein.

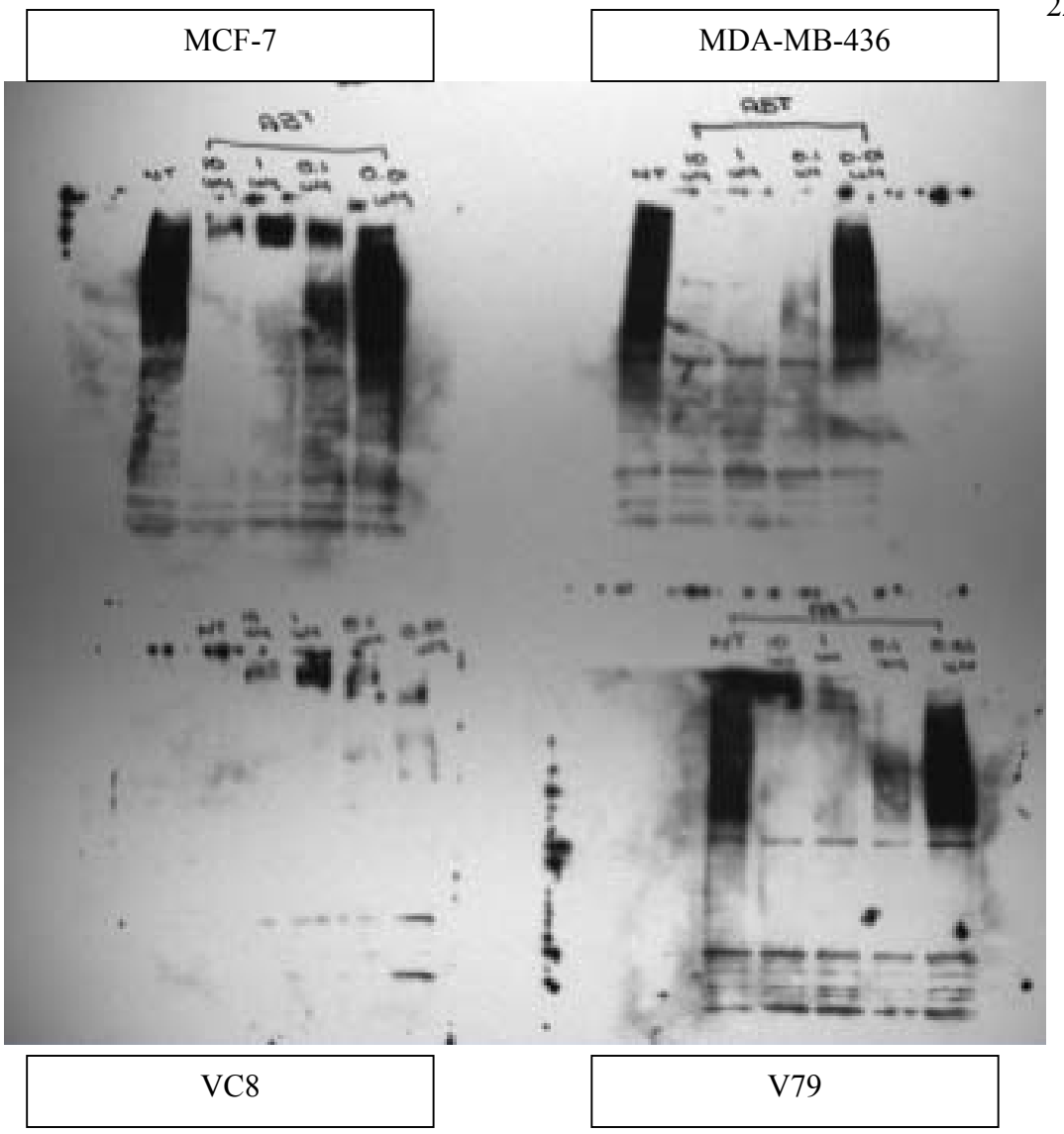


Figure 4: ABT-888 Titration – Relative abundance of PAR activity 96 hours after treatment.

Assessment of Cell Death due to Cisplatin

In order to determine the reliability of the MTS cytotoxicity assay, the cell lines were treated with cisplatin. Cisplatin is platinum-based chemotherapy drug that has been shown to be more sensitive in cells with a defective HR pathway (Powell) [12]. This observation was confirmed by comparing the IC_{50} value (concentration of drug that confers 50% cell death) of the various cells lines. (See Fig 5,Table 1). In the human cells, MDA-MB-436 and Sum149pt showed approximately 25 times more sensitivity to cisplatin compared to MCF-7 cells. Similarly when comparing the hamster cell lines, *BRCA2* deficient cells VC8 were approximately 30 times more sensitive to cisplatin than their wild-type counterpart V79 cells. Since the cisplatin cytotoxicity results are consistent with published data, the MTS assay is a suitable assay for assessing cell toxicity for various drug agents.

12 http://www.ncbi.nlm.nih.gov/pubmed/18473729?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum

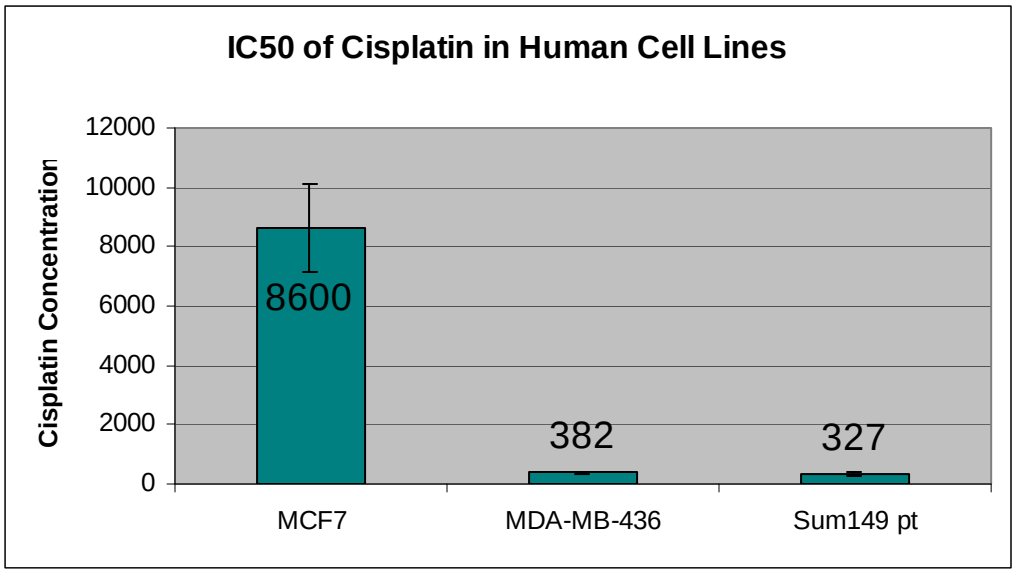
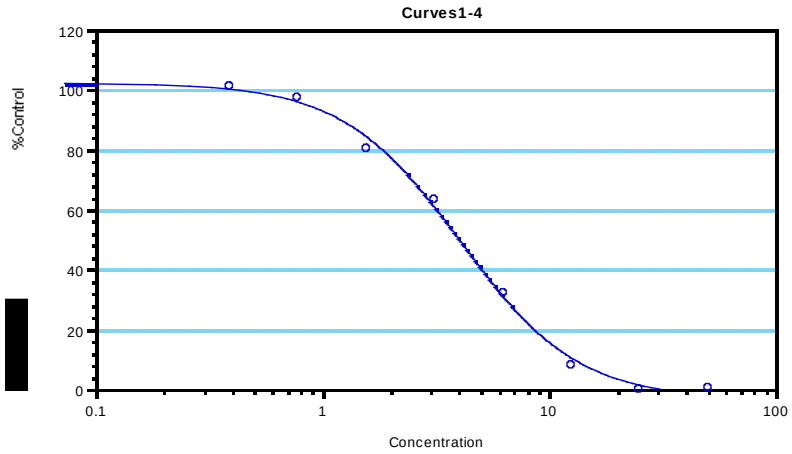


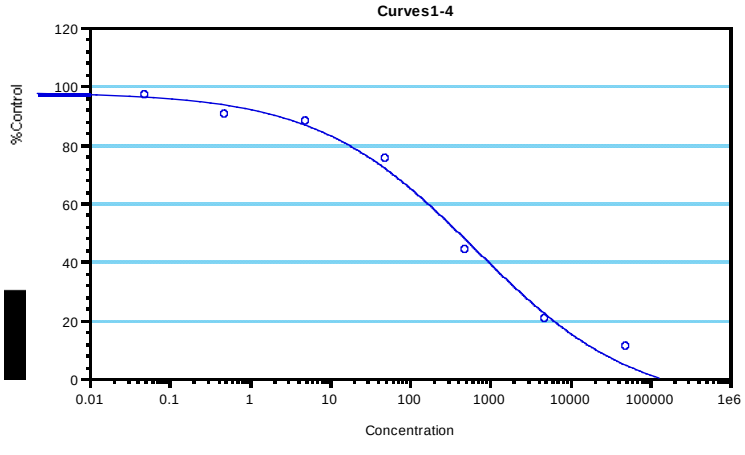
Figure 5: Summary of Cisplatin Sensitivity in Human cell lines ***Concentration at nM***

MCF7 Cisplatin 50 uM



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Plot#1 (Drug1: Concentration vs %Control)		102.174	1.671	4.064	-3.488	0.997

MDA-MB436 Cisplatin 500uM



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Plot#1 (Drug1: Concentration vs %Control)		97.754	0.437	676.73	-9.732	0.992

Sum149 pt Cisplatin 20 uM

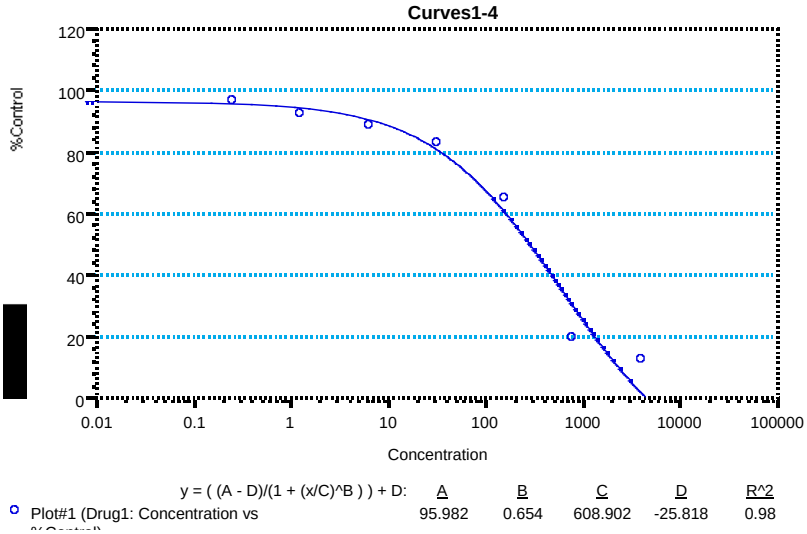


Figure 6: Cell Sensitivity assay of different cell lines against Cisplatin.

Cell Sensitivity to ABT-888

Cell viability curves were determined for ABT-888 within the various cell lines **(Figure 6)**. Across human cell lines ABT-888 has been consistently more potent in the *BRCA1* deficient cell lines than the wild-type cells. More specificially a three-fold sensitivity was observed in MDA-MB-436 and Sum149 pt cells compared to MCF7. These results lead to a strong indication that the *BRCA1* deficient cells are unable to repair the presumably single-stranded breaks (see discussion).

The results of ABT-888 toxicity in the human cell line, though relevant in assessing role of *BRCA1* in DNA damage and repair, may be difficult to interpret since all three cell lines are of different origins. This may raise legitimate doubts as to whether enhanced toxicity in *BRCA1* deficient cell lines are due truly to lack of *BRCA1* or are there other factors that are contributing to increased sensitivity. To address this issue toxicity profiles for ABT-888 were assayed in mouse cell line 114-6 and its derivative 114-2, which lacks functional *BRCA1*. Similarly profiles were also calculated for the *BRCA2* deficient VC8 and its progenitor V79. Hence the difference within each couple is based solely the status of *BRCA1* or *BRCA2*.

Once again there is enhanced sensitivity in *BRCA1* and *BRCA2* deficient cell lines to ABT-888 compared to their wildtype counterpart. The difference in 114-2 is approximately 4-fold sensitivity, similar to results in the human cell line **(Figure 7)**. . However a more dramatic effect was seen in *BRCA2* deficient cell lines where there was an approximately 40 fold difference in sensitivity **(Figure 8)**.

Cell Sensitivity to Ku

Now that the sensitivity of ABT-888 in different cells has been established, the relative potency of the drug was compared to Ku, another PARP inhibitor. Since the IC50 values of ABT-888 in the human cell lines was in the micromolar range, but published reports of the Ku class compounds is in nanomolar ranges, an upper limit of 100 uM of Ku compound was chosen and the drug was serially diluted 1:5 to cover a range from 1 nM to 100 uM. From the cytotoxicity profiles it can be seen that at 100 uM of ABT-888 and 100 uM of Ku there is 70% survival of MCF7. This suggests that Ku compound and ABT-888 have similar potency in the cell line.

However, the Ku compound displayed more sensitivity in the Sum149pt cell line. Since an acceptable sigmoidal curve was achieved with Ku compound in Sum149 pt cells, by comparing the IC50 values it is evident is that Ku is approximately 10 times more potent than ABT-888 (5 uM vs. 50 uM). This indicates that the Ku compound may have additional targets in addition to PARP that may induce cell death. However in MDA-MB-436 cell lines at 100 uM of Ku there is approximately 80% survival of MDA-MB-436 cells while at the same concentration there is 40% survival with ABT-888, suggesting greater potency of ABT-888 in this particular cell line. These conflicting data may be a result of differences in metabolism of the drug and/or differences in the molecular profile of cell lines.

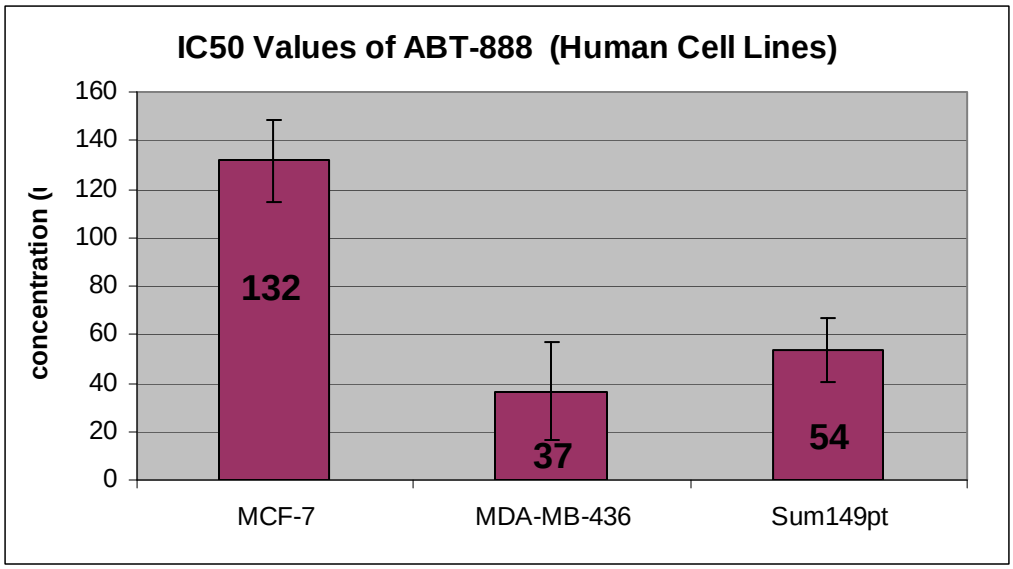


Figure 7: Cell sensitivity profile as assessed by MTS assay in human cell lines.

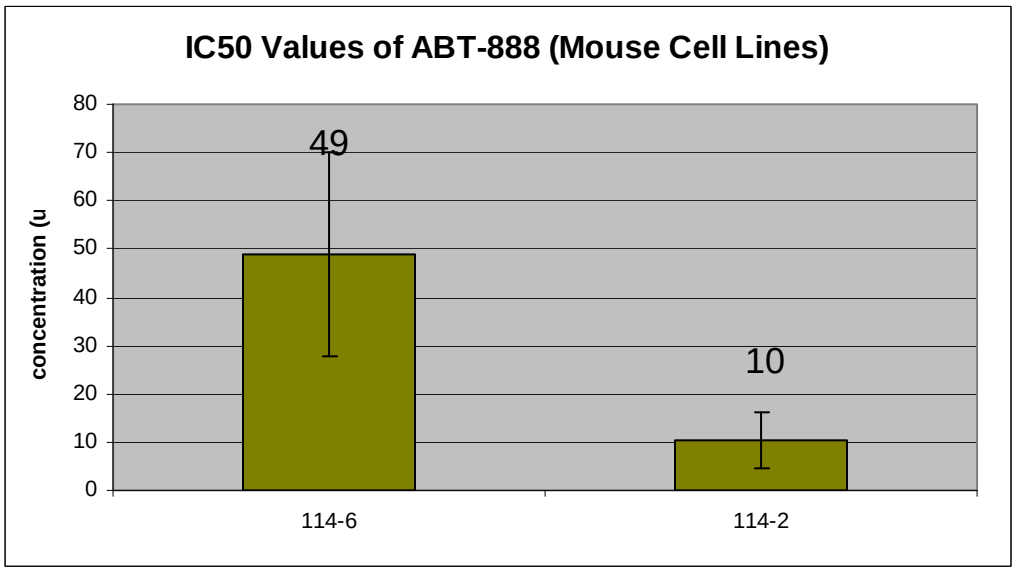


Figure 8: Cell sensitivity profile as assessed by MTS assay in mouse cell lines

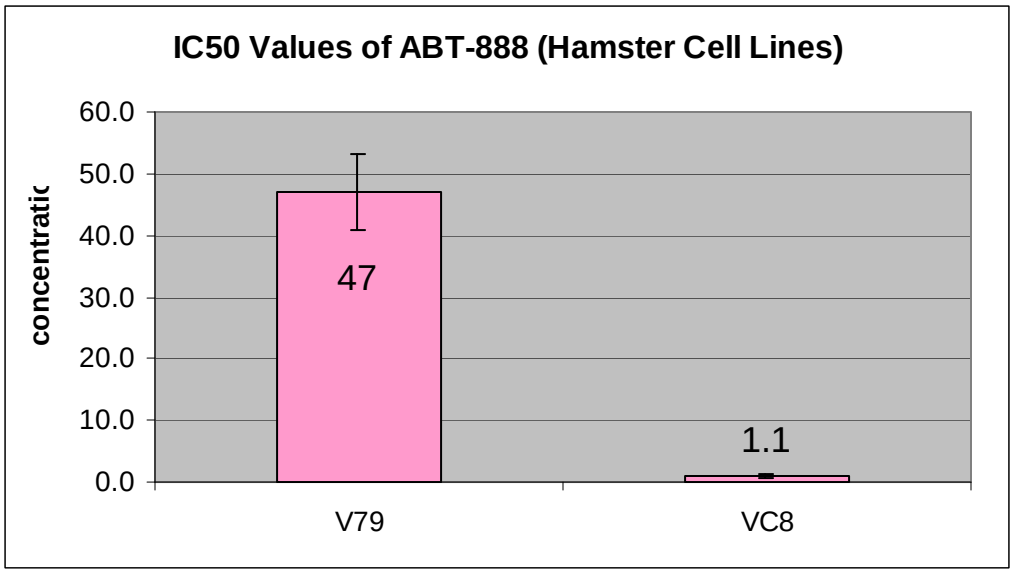


Figure 9: Cell sensitivity profile as assessed by MTS assay in mouse cell lines

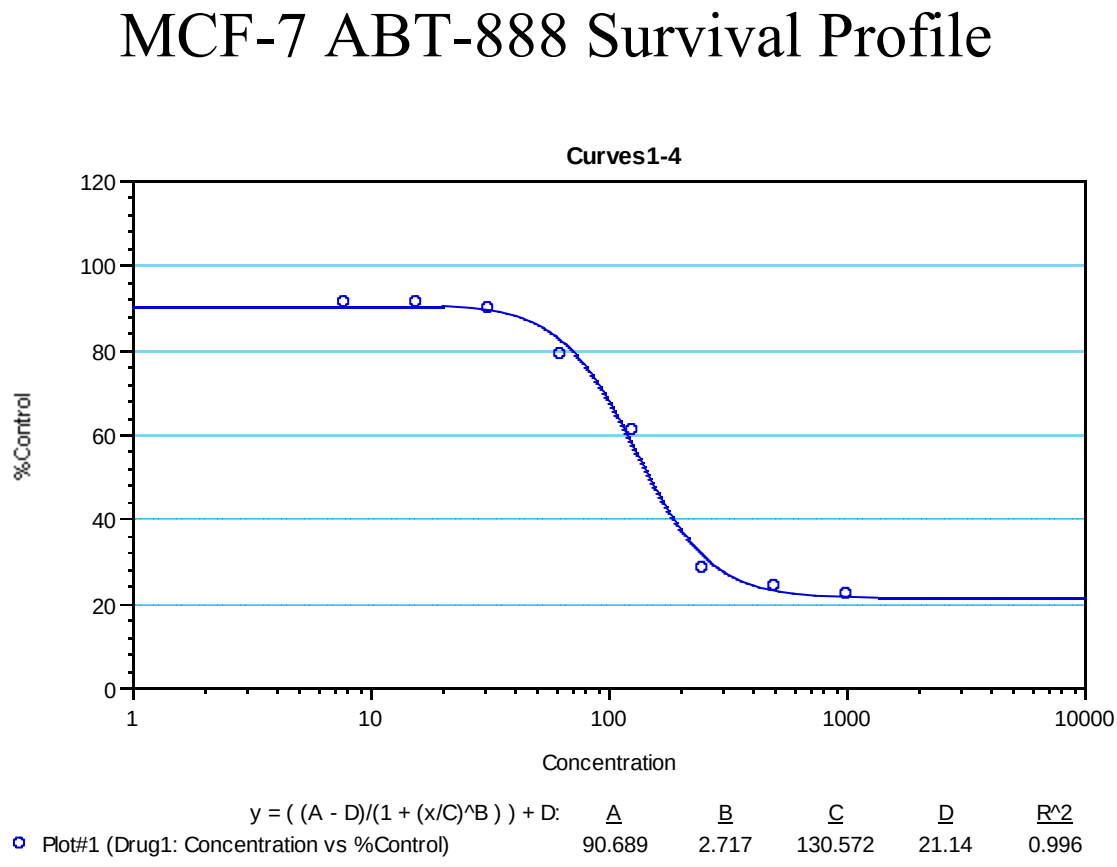


Figure 10

MDA-MB-436 ABT-888 Survival Profile

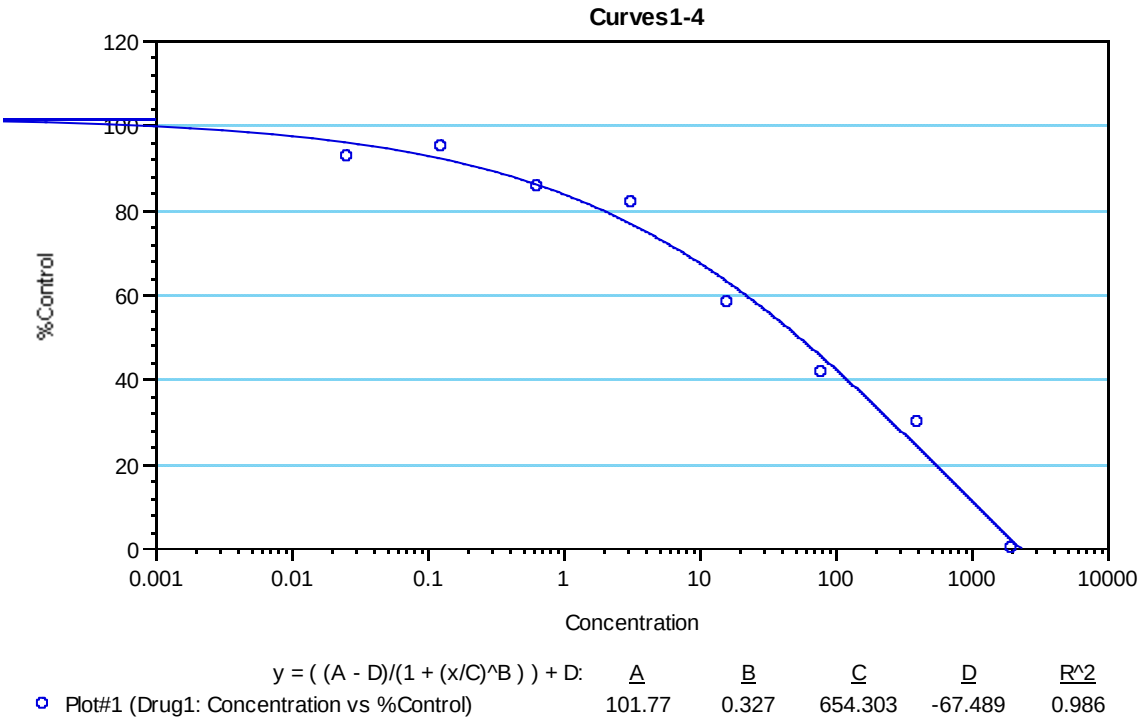


Figure 11

Sum149 pt ABT-888 Survival Profile

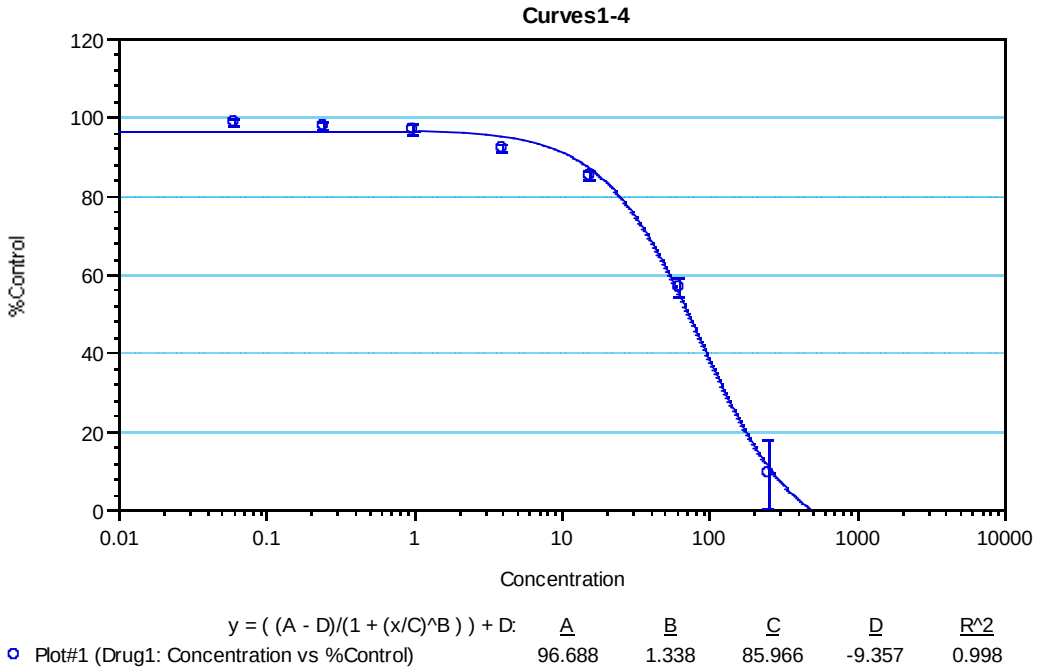


Figure 12

Drug Combination Assay

The aim of this project is to assess if PAR inhibition may potentiate the effects of SN-38 and cisplatin in cells with non-functional *BRCA1/2*; therefore in determining the appropriate dose for the combination assays the lowest concentration of ABT-888 that inhibits PAR is to be used. For all the tested cell lines the lowest inhibiting dose is 1 uM of ABT-888. Consequently to determine if the addition of ABT-888 enhances the toxic effects of SN-38 or cisplatin, 1 uM of ABT-888 was used as the putative potentiating dose. According to the time course assay effects of ABT-888 can be detected 1 hour after treatment to cells. Therefore ABT-888 was treated ~1-3 hours before addition of SN-38 or cisplatin.

Analysis of the cytotoxic effects of drug combinations was assessed by Calcusyn Software, which yields a combination index (CI) for quantification of synergism or antagonism for two drugs. The combination index is calculated as follows:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

In the denominator (D_x) is for D "alone" (drug concentration) that inhibits a system by x % as assessed by the MTS assay. From a pharmacological standpoint the goal is to use less drug to achieve a certain effect. In a hypothetical situation, one may suppose there is

only one drug available for an assay and this drug kills 10% of the cells at 10 uM. Then the CI = 10/10 = 1. Therefore CI = 1 for any single agent effect since both the numerator and denominator will be the same. If there are two drugs in a system and in combination less drug is required to have the same effect, then the numerator value will be smaller than the denominator value. As an example if both Drug 1and Drug 2 individually killed 10% of the cells at 10 uM and in combination only 2 uM of each drug was required to achieve the same effect, then

$$CI = \frac{2\ uM}{10\ uM} + \frac{2\ uM}{10\ uM} = 0.4$$

The CI value 0.4 indicates that less drug is needed in combination to achieve a certain effect then by each drug itself. On the other hand if more drug was needed to achieve the same effect, then the CI value will be greater than 1. Thus combination index values are grouped into a trichotomy of CI < 1, C=1, C > 1 representing synergism, additive effect, and antagonism.

Combination Effects of Human Breast Cancer Cell Lines

In all human cell lines the inhibiting dose of 1 uM ABT-888 without additional drugs did not result in any lethality. Therefore any cytotoxic effect is a result of the combination of the drugs. In MCF-7 the combination index profiles indicate synergism (CI<1) particularly in the lower concentration ranges of both SN-38 and cisplatin. However, when comparing the cell viability profiles, there is an insignificant shift in the curve with the addition of the PAR-inhibitor; the difference between the two curves falls within the error bar. Thus in this particular cell line with functioning *BRCA1/2* the inhibition of PAR does not potentiate effects of SN-38 nor cisplatin **(Figure 9a/b)**.

Immunofluorescence analysis of Sum149pt shows the presence of non-functioning *BRCA1*, as indicated by its failure to localize to the laser-induced DNA damage. Profiles of this cell line suggest enhanced sensitivity to both cisplatin and SN-38 compared to MCF-7 cells. With the addition of PAR inhibitor no potentiating effect was recognized with cisplatin. The combination index profiles reveals predominantly antagonist activity between the two drug points. However there is some indication of synergistic effects at two concentrations points of cisplatin: 6.4 nM and 20 uM. However the general trend indicates that similar to MCF-7 cells there is no evidence of synergy.

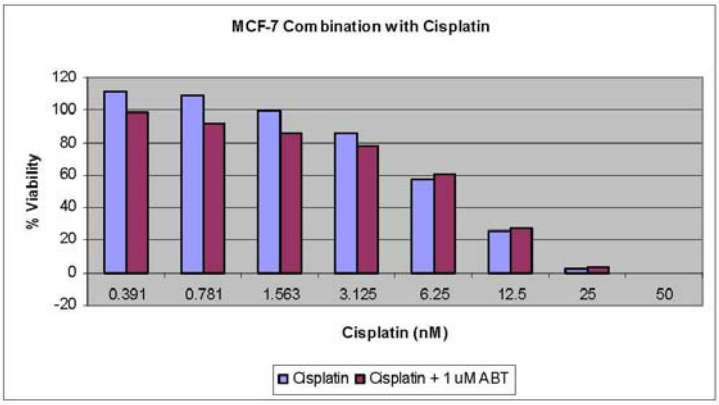
Conversely, according to the drug sensitivity profile of Sum149pt treated with SN-38, there appears to be a significant shift in cell viability with addition of ABT-888. As mentioned earlier 1 uM ABT-888 does not inflict any toxicity by itself, but pretreatment

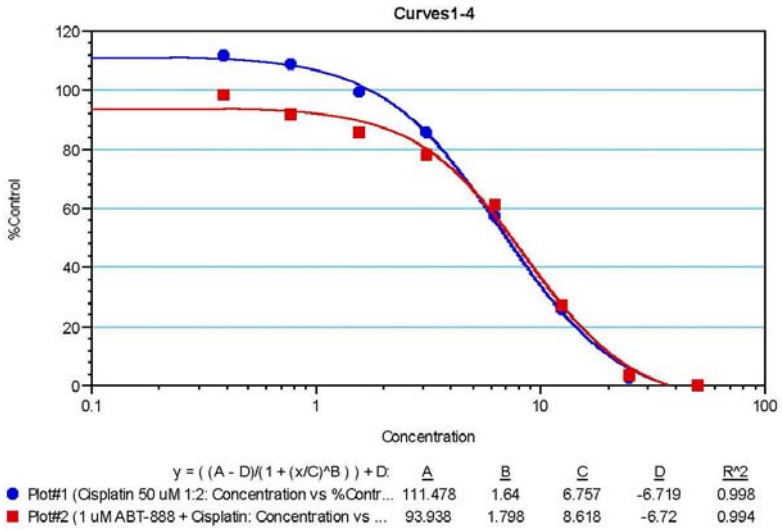
with the PAR inhibitor kills approximately 50% of the population at the lower SN-38 concentrations. These results are confirmed with the combination index values where almost every concentration point indicated some synergy.

Similar to Sum149pt MDA-MB-436, a cell line with nonfunctional *BRCA1*, synergy is observed with ABT-888 and SN-38 while the combination assay with cisplatin did not display any indications for drug potentiation. These results are corroborated with the drug sensitivity curves and combination index values.

Overall the evidence presented provides strong support for the potentiation of SN-38 with ABT-888 within a *BRCA1* null background while the potentiating effects on cisplatin are doubtful.

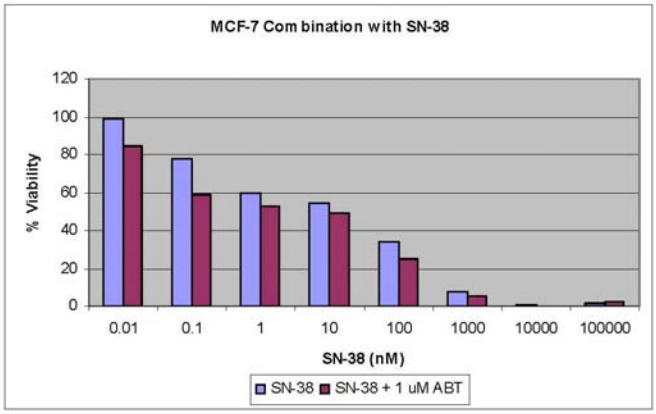
It is expected that similar results be seen in a *BRCA2* negative environment, and so the cytotoxicity profiles of hamster *BRCA2* cells were determined. Although the toxicity profiles display an enhanced potentiating effect on both SN-38 and cisplatin, the results are spurious. As with previous experiments 1 uM ABT-888 was used as a pretreatment for the cytotoxicity assay. However unlike previous experiments both hamster cell lines V79 and VC8 displayed considerable cell death at this concentration. Thus in comparing the curve with the CI values, no synergy is detected in these cell lines. A concentration lower than 1 um ABT-888 would not completely inhibit the effects of PARylation and therefore further analysis cannot be carried out with these cell lines.

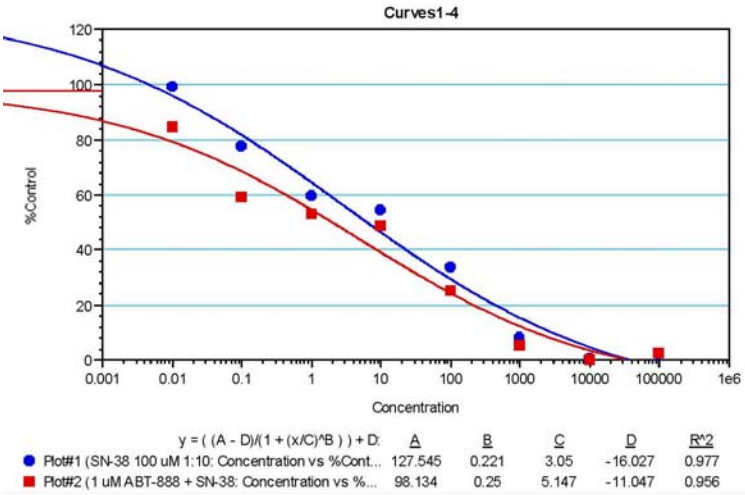




Cisplatin (nM)	Combination Index
0.391	0.326
0.781	0.273
1.563	0.402
3.125	0.65
6.25	0.989
12.5	1.259
25	1.201
50	0.839

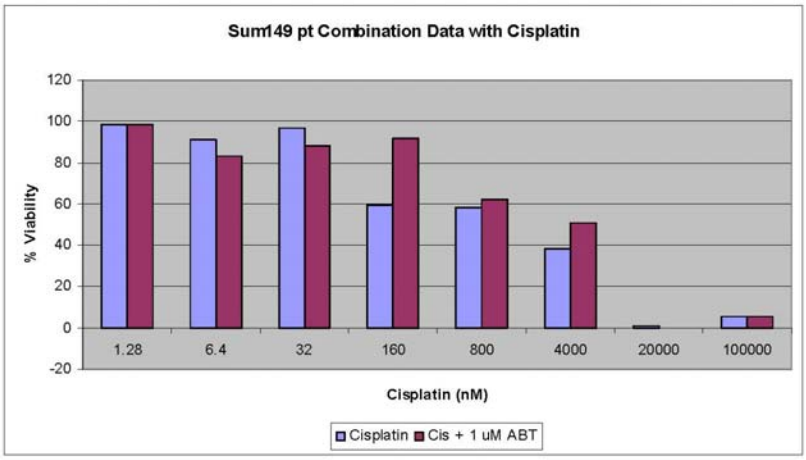
Figure 13

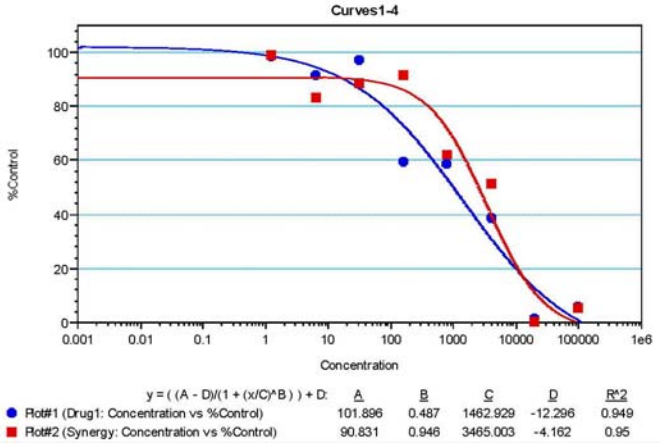




SN-38 (nM)	Combination Index
0.01	0.069
0.1	0.041
1	0.166
10	1.102
100	1.485
1000	0.495
10000	0.003
100000	9.645

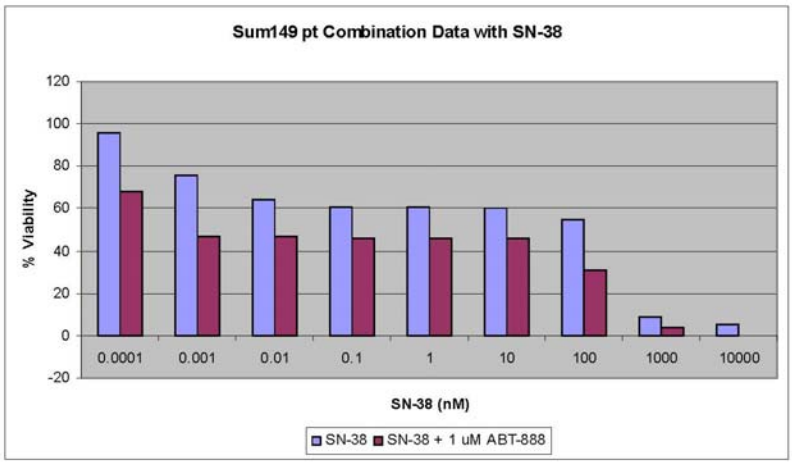
Figure 14

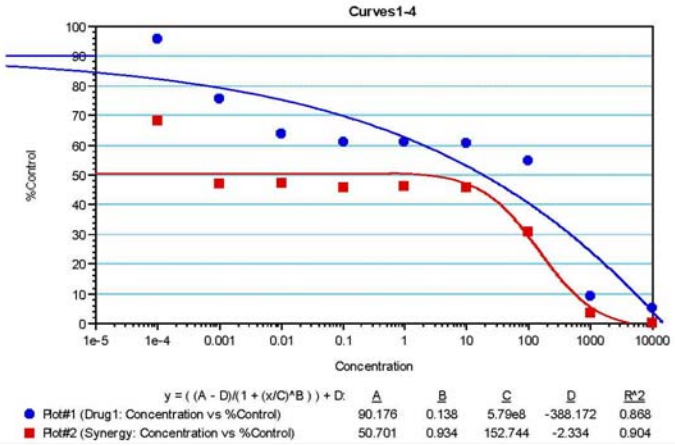




Cisplatin (nM)	Combination Index
1.28	4.598
6.4	0.399
32	1.539
160	9.996
800	3.575
4000	9.396
20000	0.003
100000	4.175

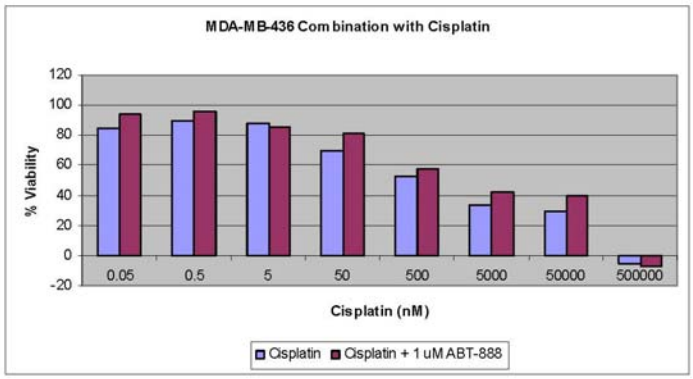
Figure 15

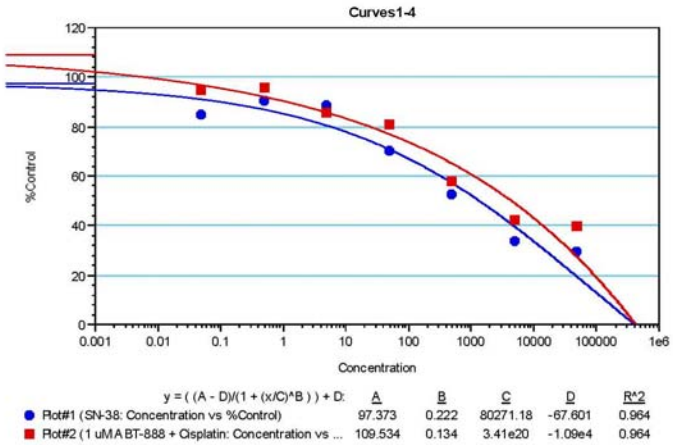




SN-38 (nM)	Combination Index
0.0001	0.117
0.001	0.049
0.01	0.054
0.1	0.079
1	0.372
10	3.139
100	2.602
1000	0.004
10000	6.54E-05

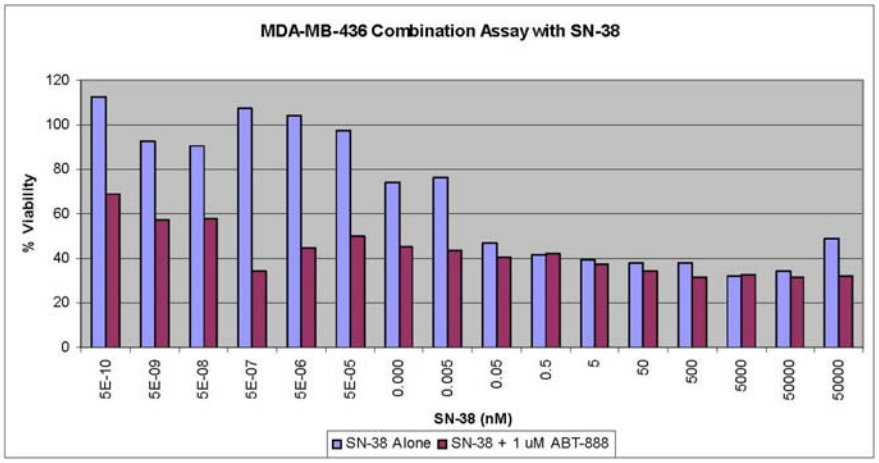
Figure 16

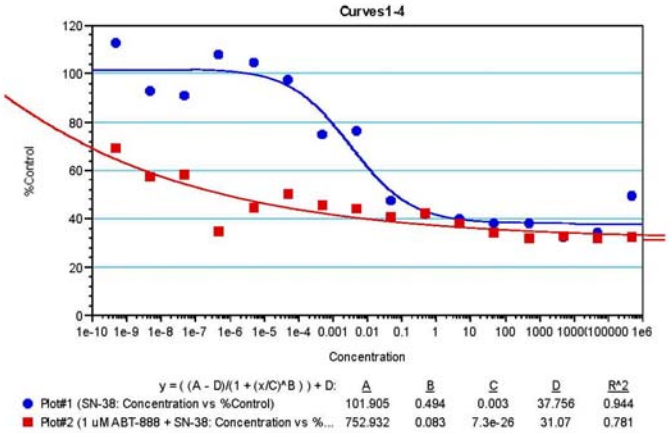




Cisplatin (nM)	Combination Index
0.05	1.45E+06
0.5	1.07E+04
5	578.791
50	273.141
500	20.744
5000	4.947
50000	4.465
500000	0.008

Figure 17





SN-38 (nM)	Combination Index
5.00E-10	0.012
5.00E-09	0.01
5.00E-08	0.011
5.00E-07	0.009
5.00E-06	0.008
5.00E-05	0.007
0.0005	0.007
0.005	0.007
0.05	0.04
0.5	0.034
5	0.174
50	0.091
500	3.009
5000	58.464
50000	329.914
500000	598.509

Figure 18

Discussion:

The cell's ability to poly(ADP-ribosyl)-ate protein substrates has been implicated as a sensor mechanism for both single- and double- stranded DNA breaks. This suggests that this post-translation modification of proteins is a critical component of maintaining genomic stability and regulating DNA repair. In cells lacking BRCA1 there is a limited capacity for repair of the genome. Therefore it has been proposed that by inducing DNA strand breakage with a chemotherapeutic drug and by inhibiting one of the cell's surveillance mechanism for such breaks, the effects would be more pronounced in cells with defective BRCA1.

ABT-888 as a Single and Potentiating Agent

The efficacy ABT-888 in BRCA1 null cells is significantly more potent than in cells with BRCA1 wildtype. This effect has been demonstrated across a variety of mammalian species, and it has also been validated using another PAR inhibitor, Kudos. However, when comparing the IC50 value of both ABT-888 and Kudos, the later compound appears to be more potent in cell lethality than the ABT-888 compound. This suggests that either the Kudos compound targets additional substrates and/or the Kudos compound inhibits the poly(ADP-ribosyl)-ation more effectively than ABT-888. The majority of poly(ADP-ribosyl)-ation is performed by PARP1 with some activity contributed by PARP2. It is worth investigating if each PAR inhibitor is targeting either one or both enzymes.

As a potentiating agent, ABT-888 offers some positive evidence that it may facilitate cell lethality by inhibiting DNA single-stranded repair. It is postulated that with inhibition of single-stranded repair, these breaks eventually result in double stranded breaks that are unable to be repaired in a BRCA1 null background. However potentiating effects were not demonstrated in cells treated with cisplatin. Although PARP is reported to detect both single- and double-stranded breaks, the double stranded breaks from cisplatin are induced by the disruption of the replication machinery. It is possible that the surveillance capacity of PARP is cell cycle dependent (and so poly(ADP-ribosyl)ation is not utilized in S-phase cells).

Using as assay that indirectly measures cell viability based on cell metabolism may not be ideal for certain cell lines, especially those harboring a deficient BRCA1 gene. The assay cannot distinguish between quiescent, growth arrested cells and those that have been undergone cell death. In addition slow growing cells, like the BRCA1 negative cell lines, are not suitable for growth in 96-well assay. These factors limit the utility of the assay As a result a better assay such as colony formation

Definition of PAR Inhibition

Since DAPI stains DNA, areas of no staining are indicative of the nucleolus region. It can be clearly seen that for cells in tissue culture, basal PAR activity is limited to the nucleolus region of the cell. Upon induction of DNA damage by irradiation PAR activity extends beyond the nucleolus throughout the entire nucleus of the cell. This increase in PAR activity as a result of DNA damage is corroborated by the increase in pH2AX foci in the cells.

It is worth nothing that even though PAR activity was ablated with ABT-888 at concentrations about 50 uM, the presence of pH2AX foci was still prominent. This indicates that mechanism of PAR activation is not upstream of pH2AX response pathway; it can either be downstream of pH2AX or in a different pathway. pH2AX is a downstream response of the ATM/ATR pathway. Generally in response to irradiation, which induces double strand breaks, ATM is autophosphorylated at serine 1981. (Kurz, Miller DNA Repair). The presence of pH2AX foci indicates that occlusion of the ATM pathway was not achieved by ABT-888. The appearance of 53BP1 foci, another downstream effector of ATM, in the presence of ABT-888 further corroborate this observation.

Another point of significant interest is the localization of PAR in the presence of ABT-888 at lower concentrations. It has been pointed out that there is high PAR activity throughout the nucleus upon IR, and this activity is completely ablated in the presence of

the inhibitor at 50 uM. However, at lower concentrations of the inhibitor where PAR activity is still evident, upon IR PAR activity is contained within the nucleous and has not dissipated throughout the nucleus. This complicates the meaning of "PAR inhibition". Clearly PAR activity is still present at lower concentrations, but it is restricted from expanding the nucleolus. This could indicate that at lower concentrations, the enzyme PARP may be able to auto-PARylate itself, and/or the mechanism of expanding the PAR process outside the nucleous has been inhibited. This raises the issue of what is the effective ABT-888 concentration that would cause the cells to default to other repair pathways. Is the effective concentration the one where complete ablation is required to default to another pathway or is a partial inhibition of PAR sufficient to achieve the same result. Further research is required to look into this phenomenon.

Mechanisms of Cell Death by ABT-888

Cell toxicity due to ABT-888 may be independent of PAR inhibition. Western Analysis of PAR activity indicates complete inhibition by ABT-888 at 10 uM with low residual activity down to 0.1 uM in MCF7 cells. However based on the cell viability curve of ABT-888 in MCF7 cells there is no cell death at these concentrations even though PAR activity is abolished at these activities. In fact the IC50 value of ABT-888 in MCF7 cells is 132 uM. This gives strong indication that inhibition of PAR activity by itself may not be sufficient to kill cells.

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