Examining the association between BRCA1 and topoisomerase I in cancer cells in response to camptothecin treatment

Godley IV, Frederick Augustus
EXAMINING THE ASSOCIATION BETWEEN BRCA1 AND TOPOISOMERASE I IN CANCER CELLS IN RESPONSE TO CAMPTOTHECIN TREATMENT

by

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EXAMINING THE ASSOCIATION BETWEEN BRCA1 AND TOPOISOMERASE I IN CANCER CELLS IN RESPONSE TO CAMPTOTHECIN TREATMENT

FREDERICK AUGUSTUS GODLEY IV

ABSTRACT

DNA topoisomerase I (TopoI) is an essential enzyme involved in the relief of DNA supercoiling during replication. TopoI plays important role in various DNA events, however the recognition that it is the target of anticancer drug camptothecins (CPTs) led to the rapid growth in this field. CPTs inhibit TopoI during S phase and cause double stranded DNA lesions in rapidly dividing cells. This class of drug is used extensively in oncology clinical settings worldwide. However, resistance to this type of therapy has been found in approximately 70% of the patient population. Current evidence supports that degradation of TopoI by the Ubiquitin Proteasomal Pathway (UPP), and consequent compensation by Topoisomerase II expression may be involved in imparting drug resistance, but this mechanism requires much greater understanding. Protein-protein interaction studies have indicated that, BRCA1 is the E3 ligase for TopoI ubiquitination in response to CPT. BRCA1 impaired cells fail to ubiquitinate and degrade TopoI and are sensitive to CPTs. It is important to note that triple negative breast cancer patients have impaired BRCA1 function, higher mutation rate and/or a lower expression of BRCA1. The Bharti lab has shown that TopoI associates with BRCA1. Our work attempts to elucidate the nature of the interaction between BRCA1 and TopoI in the hope of better understanding the mechanism of resistance to camptothecin therapy in TNBC.
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LIST OF ABBREVIATIONS

BACH1 ......................................................... BRCA1 Associated Helicase
BARD1 .......................................................... BRCA1 Associated RING Domain
BRCA1 .......................................................... Breast Cancer Type 1 Susceptibility Protein
BRCT ............................................................. BRCA1 C-Terminus
CtIP ............................................................. CtBP Interacting Protein
CPT ............................................................. Camptothecin
DNA .............................................................. Deoxyribonucleic acid
DNA-PK .......................................................... DNA Protein Kinase
EDTA ............................................................. Ethylene diamine tetraacetic acid
EGTA ............................................................. ethylene glycol tetraacetic acid
ER ................................................................. Estrogen Receptor
GST .............................................................. Glutathione-S-Transferase
HR ................................................................. Homologous Repair
HER2 ............................................................. Human Epidermal Growth Factor Receptor 2
IPTG ............................................................. Isopropyl β-D-1-thiogalactopyranoside
MG132 .......................................................... Reversible, cell permeable protease inhibitor
NCI-60 ............................................................ NCI developed sixty cell line panel
NHEJ ............................................................. Non-Homologous End Joining
PBS .............................................................. Phosphate-buffered saline
PBS-T ............................................................ Phosphate-buffered saline/Tween-20
Progesterone Receptor

Really interesting New Gene

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Active metabolite of Irinotecan

Human Topoisomerase I

Triple Negative Breast Cancer

Ubiquitin Proteasomal Pathway
INTRODUCTION

BRCA1 and predisposition to cancer

The breast cancer type 1 susceptibility protein (BRCA1) has been identified as a gene linked to the development of ovarian and breast cancer. BRCA1 is a ubiquitous protein that is involved in a wide range of processes, including DNA repair, protein ubiquitination, and cell cycle regulation. Cloning of this gene has not only enhanced our understanding of cancer pathogenesis but it also has changed the perception of cancer inheritance and has moved the discussion into the public domain. Studies have shown an 80% increased lifetime risk of inherited breast and ovarian cancer when carrying a germ line mutation in BRCA1, including a 45% increased risk of inherited breast cancer alone. (Yarden et al, 2006). The N-terminal ‘Really Interesting New Gene’ (RING) domain and C-terminal BRCA1 C-terminus (BRCT) repeats found within BRCA1 are often mutated in hereditary breast or ovarian cancers. For example, a C61G or C64G mutation in the RING domain or R1699W, R1699L, and R1699Q missense mutations in the BRCT domain have been linked to breast and ovarian cancer (Bouwman et al, 2013). Our understanding of the mutant BRCA1 is limited; a large number of mutations have been reported, but their diagnostic and prognostic value is not completely understood. For example, some hypothesize that BRCA1 mutations may actually result in better short-term survival rates for epithelial ovarian cancer (Dos Reis et al, 2014).

Developments in breast cancer tumor pathogenesis have led to the identification of four different patient populations: Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor (HER2) positive and the patient
population who is negative for all three of these markers, are put into the triple negative breast cancer (TNBC) category. Studies have further divided TNBC into six potential distinct groups based on their genomic signature. Consistently included in the genomic characteristics of TNBC is the impaired function of BRCA1, either by mutation or protein expression (Kurebayashi). The BRCA1 promoter has been reported to be silenced via hypermethylation in over 60% of medullary or metaplastic breast cancers (Kurebayashi, 2009). BRCA1 promoter methylation and consequential silencing may occur early in the pathogenesis of sporadic breast cancer, and these cancer cells share phenotypic similarities with breast cancer cells resulting from a somatic mutation of BRCA1. As a potential way to identify silencing of the BRCA1 gene and to predict the appearance of basal-like cancer, the hypermethylation pattern of the BRCA1 promoter has been shown to be significantly present in the peripheral blood cells of patients with breast cancer (Iwamoto et al, 2014). Patients whose phenotype falls within the ER, PR, or HER2 categories are treated with a targeted therapy such as trastuzumab or hormone therapy, while TNBC patients are treated by chemotherapy due to a lack of a specific target. Recent work in the Bharti lab and a clinical trial by NEKTAR Pharmaceutical indicates that Topoisomerase I inhibitors might be the most suitable chemotherapeutic agent to treat TNBC patients (Shah et al, 2012).

The role of BRCA1 in the cellular response to DNA Damage

In recent years, BRCA1 has become a focus of great interest to the scientific community. Although its functions are not fully understood, it has been demonstrated that
the presence of functional BRCA1 is necessary for genomic integrity (Silver et al, 2012). BRCA1 has been linked to important DNA repair mechanisms, such as non-homologous end joining, single strand annealing, and homologous recombination (Coleman and Greenberg, 2011), particularly at the checkpoint between the S and G2 phases of the cell cycle (Ohta et al, 2009). Without the BRCA1 protein, cells undergo apoptosis after acquiring numerous DNA lesions without proper repair (Carvalho et al, 2014). BRCA1 can interact with Rap80, which may play a role in “selecting” the type of DNA repair that the cellular machinery will conduct (Coleman and Greenberg, 2011). In the presence of a DNA double stranded break, the Nibrin repair complex (composed of MRE nuclease, Rad50, and Nbs1) is recruited to the site of DNA damage as a primary sensor. CtIP (CtBP interacting protein), an endonuclease important in 5’ to 3’ resection of the DNA, then associates with the Nibrin complex, and this is critical for homologous recombination mediated by DNA double-stranded break repair. (Coleman and Greenberg, 2011).

**BRCA1: An E3 Ubiquitin Ligase**

In the eukaryotic cell, ubiquitination occurs through the concerted action of an activating enzyme (E1), an ubiquitin conjugating enzyme (E2), as well as an ubiquitin-protein ligase (E3). An ubiquitin molecule is joined to a cysteine of an E1 enzyme via an ATP-dependent process. From an E1 enzyme, the carboxy-terminal glycine of ubiquitin is first attached to a cysteine on an E2 enzyme via a thioester bond. The ubiquitin is then joined to a specific lysine on a target protein by the E3 enzyme, forming an isopeptide
bond. BRCA1 is an enzyme that forms a heterodimer with BARD1 and acquires E3 ligase function (Baer and Thomas, 2002). This discovery provided a direct glimpse of the role of BRCA1 in various cellular events, including DNA damage repair (Baer, 2013). The E3 ubiquitin ligase function seems to be the most significant of the BRCA1 protein when in complex with BARD1 (Iwamoto et al., 2014). While the number of E1 and E2 enzymes is fairly limited (two and approximately 50, respectively), there are over 600 E3 enzymes, and thus each one of these ligases are only required in specific circumstances. The BRCA1/BARD1 heterodimer has various E3 ligase substrates that play an important role in DNA double stranded break repair, including the H2AX histone protein, CtIP, and BACH1 (Kalb et al., 2014). Additionally, the Bharti lab has identified TopoI as a target for the BRCA1/BARD1 E3 ligase. Lysine 48 ubiquitination has been shown to cause degradation of TopoI via the 26S proteasomal pathway (Shah et al., 2012). Based on these findings it has been proposed that the rate of TopoI degradation might provide an insight into TopoI inhibitor resistance mechanism.

**BRCA1 and BARD1 Structure**

The canonical isoform of BRCA1, corresponding to the full-length product of the human BRCA1 gene, is a 1,863 amino acid protein with a mass of approximately 220 kDa. It contains two nuclear localization sequences located at residues 503-508 and 607-614. It also contains a coiled-coil domain between amino acids 1364-1437. This coiled-coil domain contains a serine-cluster region phosphorylated by the ATM/ATR kinases in response to DNA damage. The BRCA1 protein contains an N-terminal zinc finger
‘Really Interesting New Gene’ (RING) domain that acts as the E3 Ubiquitin ligase when in complex with BARD1 (Silver et. al, 2012). The BARD1 protein also contains an N-terminal RING domain that is homologous to the RING domain in BRCA1. The BRCA1 and BARD1 proteins heterodimerize via their RING domains. This can be seen in the crystal structure shown in Figure 1.
Figure 1: Representation of the crystal and primary structures of BRCA1 and BARD1: A) Showing the linear structure and location of the RING domains in the BRCA1 and BARD1 proteins that allow for heterodimerization, as well as the BRCT domains of both proteins. B) Shows the structure of the BRCA1/BARD1 heterodimer, which acts as an E3 ubiquitin ligase within the cell.

Although both can exist as homodimers (Meza et al, 1999), neither the BRCA1 protein nor the BARD1 protein is particularly stable when not heterodimerized (Baer and Ludwig, 2002). A C61G mutation in the RING domain is responsible for destabilizing
the BRCA1/BARD1 dimer, which disrupts the E3 ligase activity (Silver et al, 2012). When the RING domain is mutated, some capability for DNA repair by BRCA1 is present, but the ability for BRCA1 to act as a tumor suppressor is absent (van de Groep et al, 2011, Silver et al, 2012). The BRCA1 protein also contains two carboxy-terminal BRCT domains between amino acids 1650-1863. These domains are phosphopeptide-binding domains (Silver et. al, 2012), and are essential for the tumor suppressor function of BRCA1 (Silver et al, 2012).

![BRCA1 Structure](image)

**Figure 2: BRCA1 Structure**

**Topoisomerase I Structure and Function**

Topoisomerase I is a 765 amino acid (91 kDa) protein. Its N-terminal domain from amino acid 1 through 215 is variable, but contains its Nuclear Localization Sequences. Despite being not well conserved, this region is consistently made up of charged amino acids. Topol contains a nonessential linker domain that is also not well conserved, and is found between amino acids 637-713 (Champoux, 2001). The carboxy terminus contains a tyrosine at position 723 that is a part of its active site and is necessary for the enzyme activity (Champoux, 2001).
Human Topoisomerase I (TopoI) is an enzyme highly involved in various genomic processes, including DNA replication. Its most important function in this process is the relaxation of DNA supercoils caused by the replication process.

Topoisomerases relax DNA by cutting it, performing a controlled rotation, and religating...
the strand. This enzyme is absolutely essential to the maintenance of genomic integrity. It performs its function by first binding to DNA through its Tyrosine723 residue, forming a phosphodiester bond with a 3’ phosphate group, which causes a nick in the DNA. The reverse transesterification reaction religates the DNA. It is important to mention that in the presence of TopoI inhibitors, religation is inhibited and the consequent collision of the replication fork leads to DNA double stranded breaks, and a stalled replication fork. It has been demonstrated that TopoI is ubiquitinated and degraded to resolve the create stalled replication forks and initiate DNA double stranded break repair (Desai et al, 2001). The Bharti lab has shown BRCA1 is the E3 ligase for TopoI ubiquitination. We asked if BRCA1 associated with TopoI, and also investigated the nature of this association.

TopoI was extensively studied in transcription and DNA replication, however identification of TopoI as the target of a class of drugs known as camptothecins (Hsiang et al, 1985) led to the rapid growth of our understanding of this enzyme. Camptothecin and its analogues (such as Irinotecan and Topotecan) are used extensively in the oncology clinic to treat colorectal cancer, small cell lung carcinoma, ovarian, breast, and pancreatic cancer (Pommier Review in Nature, 2013). However, only 13 to 30% of patients respond to this class of drug. The mechanism of this drug resistance is not well understood. It is important to note that TopoI is ubiquitinated and degraded by the Ubiquitin Proteasomal Pathway (UPP) in response to camptothecin therapy. The rate of degradation in the cell varies in different cancer cell lines (Shah et al, 2012). The cells that degrade TopoI rapidly in response to camptothecin treatment are resistant and the
cells that fail to rapidly degrade TopoI are sensitive to the drug (Desai et al, 2001). However, this mechanism of degradation is not understood. The Bharti lab has identified the molecular determinants of TopoI degradation by UPP and has identified BRCA1 as the E3 ligase. They have also shown that TopoI association with a DNA-PK complex including the proteins Ku70/80 allows DNA-PK to phosphorylate TopoI at S10. The phosphorylation is critical for the ubiquitination and proteasomal degradation of TopoI. As previously discussed, TopoI is ubiquitinated by the BRCA1 E3 ligase, and cells deficient in BRCA1 function fail to ubiquitinate and degrade TopoI. Importantly, cells with impaired BRCA1 E3 ligase activity are not sensitive to DNA double stranded breaks induced by mitomycin C or x-RT, however they are sensitive to camptothecins (Sato et al, 2012). This supports Bharti lab work that BRCA1 is the E3 ligase for TopoI. However, the lab has not been able to demonstrate direct association between TopoI and BRCA1. Since BRCA1 binds to phosphorylated proteins via its BRCT domains, we hypothesize that phosphorylation of TopoI mediates the interaction between BRCA1 and TopoI. In this work we aimed at understanding the nature of this interaction.

BRCA1 and Topoisomerase I

Phosphorylation of serine residues within a specific amino acid motif is necessary for the interaction of BRCA1 with DNA repair proteins (Clark et al, 2012). TopoI can be phosphorylated by DNA-PK within the cell and it has been demonstrated that this phosphorylation at S10 of TopoI is important for BRCA1 interaction and ubiquitination
of TopoI (Shah et al, 2012). It has been proposed that the phosphorylation allows for the interaction of BRCA1 and TopoI in response to DNA damage.

BRCT domains in BRCA1 may be able to interact specifically with regions of proteins with a pSer-X-X-Phe motif (Campbell et al, 2010). Certain residues within the BRCT domain themselves allow for interactions with the phosphorylated serine at position 10, while the phenylalanine binds within a pocket at the interface of the BRCT repeats. The ability of the +3 residue to interact with BRCA1 in DNA repair may be possible through formation of salt bridges between the carboxylate groups of phenylalanine and an arginine at position 1699 (Campbell et al, 2010). This is demonstrated by the missense variants R1699W, R1699L, and R1699Q of BRCA1, which are significantly associated with breast cancer, and do not function to maintain genomic integrity (Zhou et al, 2000). Of particular interest is the finding that the BRCT domains mediate BRCA1 association with the BACH1 complex. The BACH1 complex contains a helicase, and is able to interact with BRCA1 via phosphorylated S990 on BACH1 (Yu et al, 2003). Proteins that have been phosphorylated by ATM/ATR kinase, or DNA Protein Kinase (DNA-PK), in response to DNA damage are able to bind to the BRCT domains as well, supporting the evidence that TopoI requires phosphorylation before interacting with BRCA1/BARD1. It has been shown that TopoI is phosphorylated by DNA-PK in response to DNA double stranded breaks following camptothecin treatment, and is a binding target for BRCA1 (Shah et. al, 2012).

This assessment shows the significance of understanding the interaction of Topoisomerase I and BRCA1. We aim to demonstrate that phosphorylation of TopoI in
the first 139 amino acids is required for binding to the BRCA1/BARD1 heterodimer, and that BRCA1 is present at high levels in cells where DNA damage is caused by camptothecin treatment. However, different cell lines display different levels of binding between TopoI and the BRCA1/BARD1 heterodimer despite these proteins being intact, suggesting that the previously described interaction of TopoI and BRCA1/BARD1 following phosphorylation of S10 is not the only requirement for the association between these proteins (Shah et al, 2012).
SPECIFIC AIMS

The primary objective of our project was to characterize the interaction of Topoisomerase I and BRCA1 in cancerous cells.

Specifically:

1. We wished to visualize the differential association of Topoisomerase I and BRCA1 in the cell (specifically the nuclei) in response to treatment with camptothecins.

2. To examine the effect of phosphorylation in the direct binding of the BRCA1/BARD1 heterodimer to the first 139 amino acids of Topoisomerase I using in vitro phosphorylation and phosphopeptide competition assays.
MATERIALS AND METHODS

NaOH Cell Lysis

Approximately 4 million cells were plated on 10 mm tissue culture treated plates. Following incubation at 37°C for between 10-12 hours, cells were treated with 1 µM MG132 for 30 minutes. MG132 is important to inhibit proteasomal function within the cells. The time of incubation was dependent on the cell cycle analysis for various cell lines, previously determined (Shah et al, 2012). Following initial treatment with MG132, media was removed. Cells were then treated with new media with 1 µM SN38, which is the active metabolite of the camptothecin Irinotecan, for 30 minutes. Following treatment, 1 mL of NaOH solution (containing 0.2 M NaOH and 2 mM EDTA) was added to cells for harvesting. Cell lysate was recovered and incubated for 5 minutes on ice. Cell lysate was sonicated for 30 seconds. Lysate was then neutralized with 100 µL of 2 M HCl. Following neutralization, 100 µL of buffer containing 10% NP-40, 1 M Tris (pH 7.4), 0.1 M MgCl₂, 0.1 M CaCl₂, 10 mM DTT, 1% NaV, 1% NaF, 1% PMSF, 2% Aprotonin, 5% Leupeptin, and 5% Pepstatin was added.

Micrococcal Nuclease Digestion

100 units/mL of micrococcal nuclease (Wharton, Inc.) was added to cell lysates, and the lysate was incubated at 37°C for 30 minutes. Following incubation, 1 mM EGTA as a Calcium chelating agent was added to lysate to stop the reaction.
**Immunoprecipitation**

For immunoprecipitation, 4 µg (40 µL) of Santa Cruz α-BRCA1 C-20 antibody was added to 300 µL of prepared lysate in an Eppendorff tube. The solution was incubated overnight at 4°C. Following incubation, 50 µL of Protein A/G sepharose beads were added to the lysate. The lysate was then incubated for 1 hour at 4°C, after which the beads were washed three times with cold phosphate-buffered saline (PBS). Samples were then boiled to elute the protein and loaded in a SDS-PAGE gel.

**Western Blot/Immunoblot**

Cell lysates were analyzed on SDS-PAGE gel, and the proteins were transferred to a nitrocellulose membrane. The membrane was placed in small plates, and 5% fat free milk blocking solution was added. The membrane was blocked for 60 minutes, and membrane was washed three times with PBS-T, for 20 minutes each. The primary antibody was added to membranes. The membranes with primary antibody were placed at 4°C for rocking at 90 rpm overnight. Following overnight incubation, membrane was washed three times with PBS-T, for 20 minutes each. After washing, secondary antibody was added to membrane, diluted appropriately in 5% blocking solution, and rocked for 1 hour. Following incubation with secondary antibody at room temperature, membrane was washed three times with PBS-T, for 20 minutes each. The membrane was then placed in 6 mL of equal parts Western Lightning Oxidizing Reagent and Enhanced Luminol Reagent. The signal was detected on X-ray film.
GST-Topoisomerase I Transformation and Purification

Two 14 mL round bottom tubes were placed on ice. 100 µL of BL21DE3 bacteria were placed into each tube, and kept on ice. 2 µL of GST TopoI 1-139 vector was placed in one tube, while 2 µL of pGEX vector was placed in the other tube (control). Tubes were incubated on ice for 30 minutes. Following incubation, tubes were placed in 42°C for 45 seconds exactly. Tubes were removed from heat, and placed on ice for 2 minutes. Following cooling, bacteria were pipetted on agar plate (with ampicillin), and spread with a sterile loop. The agar plates were then incubated overnight at 37°C. Following overnight incubation, one isolated colony was added to 3 mL of LB broth in a 14 mL round-bottomed tube. 3 µL of ampicillin (final concentration [100 mg/mL]) was added to the LB broth. Tubes were incubated at 37°C overnight. Following overnight incubation, 3 mL of broth with visual bacterial growth (TopoI 1-139 Positive) was added to 1 Liter of LB broth (with ampicillin) at room temperature. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression, and bacteria were centrifuged at 10,000 rpm. The bacterial pellets were resuspended in PBS, and incubated on ice for 20 minutes. The pellets were then frozen with liquid nitrogen for 5 minutes, and then run under warm water. Triton X-100 was added, and suspended bacterial pellets were sonicated on ice. 1M NaCl and 1% PEG was then added to solution, and the suspended bacteria were centrifuged at 12,000 rpm for 15 minutes at 4°C. Supernatant was collected following centrifugation. 150 µL of suspended beads were placed in an Eppendorff tube. Beads were spun at 2000 rpm for 2 minutes. Following centrifugation, supernatant fluid was removed from beads. Beads were washed three times with 60 µL of PBS to equilibrate
beads. Bacterial supernatant was added to 150µL bed volume of beads, and rotated for one hour at 4°C. Beads were washed three times with PBS-T. Following washing, 250 µL of cold PBS was then used to resuspend beads. Beads were run on SDS-PAGE gel, followed by Western blot (see above protocol) to visualize Topoisomerase I expression.

Nuclear Isolation

Four 225 mm² flasks were plated with cells from selected cell lines and incubated for 10-12 hours. As in the NaOH cell lysis, the time of incubation was dependent on the cell cycle analysis for various cell lines. Cells were treated with 1 µM of a proteasomal inhibitor, MG132, for thirty minutes. Following the first treatment, the flasks inhibited for experimental conditions were treated with 1 µM of SN38, for 30 minutes. Flasks not treated with SN38 were the negative control in the experiment. Following treatments, cells were harvested with trypsin, and spun at 1500 rpm for 5 minutes at 4°C. Cell pellets were resuspended in 500 µL of hypotonic buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 2 mM Beta-mercaptoethanol, 0.2% NP40, and protease/phosphatase inhibitors). Nuclei were then dounced with 15 strokes from Type B pestle. Following homogenation, nuclei were spun at 2300 rpm for 15 minutes at 4°C. Supernatant was removed, and the nuclear pellet was resuspended in 300 µL of Nuclear Extraction buffer (10 mM HEPES, 420 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 100 µL NP40, 1 µL NaV, 10 µL NaF, 10 µL PMSF, 2 µL Aprotinin, 5 µL Leupeptin, 5 µL Pepstatin, 1 M CaCl₂, 1 M MgCl₂, 10 µL DTT, 10 µL Tris (pH 7.5), 647 µL dH₂O). Lysates were then transferred to Eppendorff tubes and rotated at 4°C for 1 hour. Following rotation, left
over nuclei were spun at 12,000 rpm for 15 minutes. Supernatant was transferred to clean Eppendorff tube, and diluted times four with cold PBS. Following dilution, optical density readings were taken. Immunoprecipitation was then performed with appropriate antibody depending on the purpose of the experiment (see Immunoprecipitation protocol).

**Binding Experiment – TopoI 1-139**

12 µL of TopoI 1-139 GST protein, 2 µL ATP (final concentration 1 mM), 2 µL ssDNA, 59 µL of DNA-PK buffer (25 mM HEPES, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 20% glycerol, 1% NP-40, dH₂O), and 5 µL of DNA-PK (from Invitrogen) was added to Eppendorff tube. Kinase reaction was allowed to proceed for 30 minutes at 30°C. Following reaction, solution was diluted to 300 µL total volume with cold PBS. Following dilution, 50 µL bed volume of GST sepharose beads was equilibrated with cold PBS, and added to Eppendorff tube containing kinase reaction. The beads with the TopoI 1-139 protein were rotated at 4°C for 1 hour. Following rotation, beads were washed with 100 µL of PBS, 1 M NaCl, and 1% PGE, and rotated at 4°C for 10 minutes. Following rotation, beads were washed two times with 300 µL of cold PBS. 5 µL of purified BRCA1/BARD1 were added to the beads, and the beads were rotated overnight at 4°C. Following overnight rotation, beads were washed three times with cold PBS. Beads were diluted with equal volume of Lamelli dye (10% DTT), and run on SDS-PAGE gel. A Western blot was performed to visualize results of binding experiment (see protocol above).
Cell Culture

The triple negative breast cancer (TNBC) cell line HCC1937-BRCA1 and colorectal cancer lines HCT15 and HCT116 were maintained in Roswell Park Memorial Institute Medium (RPMI) with 10% fetal bovine serum, 100 units/mL of Streptomycin and Penicillin, and 0.1% Mycoplasm prophylactic. Previous work in the Bharti lab led to the development of the cell line HCC1937-BRCA1, which contains an expression lentiviral vector for BRCA1 as well a gene for blasticidin resistance under the rsv promoter, so that the cells could be selected for the presence of the BRCA1 expression (Shah et al). These HCC1937-BRCA1 cells were grown with 5µg/ml of blasticidin. The cell lines were grown in a humidified incubator at 5% CO₂ and at 37°C.

<table>
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<th>Cell Type</th>
<th>BRCA1 Status</th>
<th>Topol Status</th>
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<tbody>
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<td>HCT15</td>
<td>Human Colorectal Adenocarcinoma</td>
<td>Wild Type</td>
<td>Point mutation in linker region</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human Colorectal Carcinoma</td>
<td>Wild Type</td>
<td>Wild Type</td>
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<td>TNBC Basal A</td>
<td>Inserted via lentivirus</td>
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Table 1: Characteristics of Experimentally Utilized Cell Lines

Fluorescent Labeling of Purified Protein

(Courtesy Molecular Probe) An aliquot of purified BRCA1/BARD1 was dialyzed to remove primary amines in the buffer, and was concentrated to 200 µM in PBS (pH 7.6). 1 M sodium bicarbonate solution was prepared using kit from AlexaFluor. 100 µL
of protein was added to a reaction tube, followed by $1/10^{th}$ volume (10 µL) of 1 M sodium bicarbonate solution. This solution was vortexed briefly to mix contents of the reaction. 10 µL of dH$_2$O was added to one vial of Alexa Fluor 647 succinimidyl ester, resulting in a dye concentration of 7.94 nmol/µL. An appropriate amount of dye was added to the purified protein, which dependent on the desired molar ratio of the reaction. The reaction tube was incubated for 15 minutes at room temperature. To separate the conjugate from unreacted dye, the suspended gel resin from the AlexaFluor kit was suspended in a spin column. The resin was packed using a microcentrifuge at 16,000 rpm, and fluorescently labeled protein was added in 50 µL aliquots per spin column. The columns containing labeled protein and unconjugated dye were again spun at 16,000 rpm. The fluorescently labeled protein was removed, and the amount was measured using a Nanodrop machine.

**Purification of BRCA1/BARD1**

(Courtesy Jeff Parwin Lab) Flasks of SF9 insect cells grown in suspension culture were infected with either 4 mL of BRCA1 virus or 4 mL of BARD1 virus. Cells were incubated for at least 3 days. The media and cells from the flasks were collected in 50 mL tubes. Cells were then spun at 2000 rpm for 8 minutes at room temperature in a swinging rotor centrifuge. Cells were then washed once with room temperature PBS, and again rotated at 2000 rpm for 8 minutes at room temperature. Lysis buffer (20% glycerol, 20mM Tris-HCl (pH 7.9), 500 mM NaCl, 4 mM MgCl$_2$, 0.4 mM EDTA, 20 mM β-glycerophosphate, 2 mM DTT, 0.2 M PMSF, Protease Inhibitor Tablet) was added to the
packed cells in 4 times their volume. Cells were dounced with a type A pestle, by 3 series of 10 strokes during a 30 minute period, being kept on ice. The lysate was then diluted with a dilution buffer (10% glycerol, 20 mM Tris-HCl (pH 7.9), 0.02% NP-40). The lysates were placed in an oak ridge tube, and an SS34 rotor was used to centrifuge the lysate at 11,000 rpm for 10 minutes at 4°C. The cleared supernatant was collected, and 100 µL was set aside for gel-loading use. Following removal of Tris from beads, IgG beads were used at 1/10th of the packed insect cell volume. IgG was recycled following use by washing in 0.1 M glycine (pH 3) and then by washing in 0.1 Tris (pH 7.5). The tube was checked for complete filling, and lack of air bubbles. Binding with IgG was performed for 3 hours at 4°C. Following binding, the bead pellet was resuspended by tapping the tube. Wash buffer (15% glycerol, 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 0.01% NP-40, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM PMSF, Protease Inhibitor Tablet) was used to wash the bead pellet four times. Tubes were then centrifuged at 4°C. A 27 gauge needle was used to remove the wash buffer following the final wash. Another wash was added in with Tobacco Etch Virus nuclear inclusion a endopeptidase (TEV protease) buffer (5% glycerol, 10 mM Tris-HCl (pH 8), 0.15 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 0.5 mM DTT). Supernatant buffer was removed to the top of the column using a 27-gauge needle. 2 column volumes of TEV buffer were added to the column. The beads were incubated for 30 minutes at room temperature, with occasional agitation for mixing. The beads were then spun down, and the supernatant was collected using a 27-gauge needle, and stored in chilled eppendorff tubes. To test for uncleaved BRCA1/BARD1, two column volumes of
0.1 M glycine (pH 3) were added to the beads. The beads were then mixed, spun down, and the supernatant was collected. 20 µL of 1 M Tris (pH 8.0) was added to neutralize the solution. BRCA1 and BARD1 preparations were snap frozen and stored at -80°C. Samples were run on SDS-PAGE gel for further analysis.

**Enzyme-Linked Immunosorbent Assay**

GST-Topoisomerase I 1-139 dissolved in PBS was incubated overnight at 4°C in a Glutathione-coated 96-well plate. As a control, GST protein was also incubated overnight in Glutathione-coated wells. Following overnight incubation, all wells were washed with cold PBS three times. Predetermined amounts of fluorescently labeled BRCA1/BARD1 (See fluorescent labeling protocol above) were added to wells containing GST and wells containing GST-TopoI 1-139. The plate was then incubated at 4°C for 2 hours to allow for binding. Following incubation, wells were again washed three times with cold PBS. Binding detection signal was read using a Tecan machine.
RESULTS

Topoisomerase I associates with BRCA1: Sodium Hydroxide Cell Lysis and Nuclear Isolation:

We first asked if TopoI and BRCA1 are in the same complex. For this we performed immunoprecipitation experiments with anti-TopoI antibodies, and to determine direct association, GST pull downs were performed. HCT15 and HCT116 cells were treated with camptothecins and harvested after designated time points. Cells were lysed and lysates were subjected to immunoprecipitation with anti-TopoI. Immunoprecipitates were analyzed by immunoblot analysis with anti-BRCA1 antibody (Figure 5). We observed that full-length BRCA1 associates with the full-length TopoI enzyme. In addition other fragments of BRCA1 were also observed indicating the association of various fragments of this protein. These results affirmed that BRCA1 and TopoI are in complex within the cell. Full-length BRCA1 protein was visualized at 220 kDa, as well as bands representing the degradation products of BRCA1 at approximately 150 kDa, and 100 kDa. In HCT15 lysates (lanes 1-2, 5-6 in Figure 5), we asked if the association between the proteins increased in response to camptothecins. We were not able to visualize any difference in the association of TopoI and full-length BRCA1 in the HCT15, however, BRCA1 fragment and full length association was significantly higher in HCT116 lysates (lanes 3-4, 7-8 in Figure 5). We visualized greater levels of association between BRCA1 and TopoI in response to camptothecin treatment. This experiment allowed us to confirm that TopoI and BRCA1 associate at greater levels in response to DNA damage caused by camptothecin treatment.
Previous work in the Bharti lab has demonstrated the co-localization of BRCA1 and Topoisomerase I in the nuclei of HCC1937-BRCA1 cells following camptothecin treatment. To assess the difference in association of BRCA1 and Topoisomerase I in nuclei, we performed immunoprecipitations with nuclear extracts from the HCC1937-BRCA1 cell line (see Materials and Methods section). In this cell line, a functional BRCA1 gene had been inserted via lentivirus to replace a truncated version of the BRCA1 protein. In HCC1937-BRCA1 cells both control and treated with SN38, TopoI-BRCA1 complexes were pulled down with anti-Topoisomerase I, and precipitated with Protein A/G agarose beads. The results were visualized by immunoblot with anti-BRCA1 antibody. We used nuclear extracts as input and purified BRCA1/BARD1 for comparison of results. As seen in Figure 6, bands were visualized that confirmed the presence of degradation products of BRCA1 within the nuclei. These results showed that SN38 treated cells had greater association of BRCA1 and Topol. These results reinforced the results from the sodium hydroxide lysis, that TopoI associates with BRCA1 in complex and in certain cell lines this association is enhanced in response to camptothecin treatment.

Direct Binding Experiments:

i) Protein Expression of GST, GST-TopoI 1-139:

BL21DE3 bacteria were transformed with PGEx-4T vectors for GST, and GST-TopoI 1-139 expression (See Materials and Methods). Four hours after IPTG induction, bacteria were harvested and GST and GST-TopoI 1-139 proteins were purified. A part of
purified protein was analyzed by SDS-PAGE gel and immunoblotting with anti-TopoI antibody to demonstrate expression of the vectors (Figure 8). As seen in Figures 7 and 8, bands were visualized at 45 kDa, which indicated the presence of the TopoI 1-139. The band at 60 kDa has previously been shown to be due to background associated with purifying GST-proteins. GST and GST-TopoI 1-139 proteins attached to glutathione sepharose beads were used for pull down experiments.

**ii) Purification of BRCA1/BARD1:**

SF9 cells were plated on 100mm tissue culture plates and grown until confluent. Equal amounts of plates were infected with BRCA1 and BARD1 baculovirus to produce protein. Cells were harvested after 3 days, lysed, and purified with IgG agarose beads (See Materials and Methods). We visualized the presence of the BRCA1 protein by using silver staining (Figure 9). Bands at 220 kDa indicated the presence of the full-length BRCA1 protein, as well as degradation fragments of BRCA1. Similarly, BARD1 protein was also purified.

**iii) Characterizing the binding of BRCA1/BARD1 to Topoisomerase I:**

The TopoI amino terminus (amino acids 1-210) is the protein-protein interacting domain. TopoI is phosphorylated at S10 by DNA-PK and this is critical for its ubiquitination and proteasomal degradation (Shah et al, 2012). It is also known that the BRCT domain of BRCA1 associates with phosphorylated proteins and peptides
(Campbell et al, 2010). We asked if the TopoI amino-terminus binds directly with BRCA1, and if phosphorylation of S10 is critical for this association.

BRCA1/BARD1 forms a heterodimer to become a potent E3 ligase (Baer and Ludwig, 2002). We performed a direct binding assay with purified BRCA1/BARD1 complexes and GST TopoI 1-139 fusion protein. For experimental conditions, GST-TopoI 1-139 was phosphorylated with DNA-PK (see Materials and Methods section) and incubated with glutathione sepharose beads, while in the control condition, only GST protein was added with DNA-PK. Purified BRCA1/BARD1 was incubated with GST or GST-TopoI 1-139 bound to sepharose beads. Following washing of the beads to remove unbound BRCA1/BARD1, protein complexes were eluted and run on SDS-PAGE gel. We visualized the binding by immunoblotting with anti-BRCA1 antibody. As seen in Figure 10, we were able to confirm that the BRCA1/BARD1 heterodimer was bound to TopoI 1-139. In contrast, no association of BRCA1/BARD1 with GST protein was observed.

In a follow-up experiment, we examined the significance of the phosphorylation of TopoI 1-139 in the binding of BRCA1/BARD1. We incubated both non-phosphorylated (control) and phosphorylated GST TopoI 1-139 bound to glutathione sepharose beads. Purified BRCA1/BARD1 was then incubated with the beads, and after extensive washing, the complexes were eluted and visualized by immunoblotting with anti-BRCA1 antibody. As seen in Figure 11, we did not observe any noticeable difference in binding of BRCA1/BARD1 between the non-phosphorylated TopoI 1-139 and the phosphorylated TopoI 1-139. This indicated that the phosphorylation of TopoI 1-
139 by DNA-PK might not be absolutely necessary for the interaction of BRCA1 and TopoI, or that TopoI 1-139 has a basal level phosphorylation during expression of the protein in bacteria.

To further examine potential significance of the phosphorylation of TopoI 1-139, we prepared a competition assay with a phosphopeptide to determine if the BRCA1/BARD1 heterodimer could be competed off of phosphorylated GST TopoI 1-139. The phosphopeptide used (topoI-phospho S10) has the sequence MSGDHLHNDpSQIEADFRLND, and was synthesized at Molecular Biology core facility (Dana-Farber Cancer Institute, Boston, MA). As in previous experiments, we prepared three different samples of phosphorylated GST TopoI 1-139. We then incubated the samples with BRCA1/BARD1, as well as with various concentrations of phosphopeptide (500 nM, 1µM, and 1.5 µM). After washing, the adsorbates were analyzed by immunoblotting with anti-BRCA1 antibody. As in previous experiments, we ran an input of BRCA1/BARD1 to allow for comparison. Results demonstrate the TopoI 1-139 association with BRCA1, but the phosphopeptide did not compete with the binding, except for a minor difference when we used lower concentrations of phosphopeptide (Figure 12).
**Figure 5: BRCA1 associated with TopoI in Cells:** HCT15 and HCT116 lysates that were control or treated with SN38 were harvested and incubated with anti-TopoI antibody and immunoprecipitated with 40µL of protein A/G agarose beads. Following immunoprecipitation, the complexes were eluted and run on 7.5% SDS-PAGE gel. Immunoblotting with anti-BRCA1 antibody allowed for visualization and analysis of BRCA1 protein in complex with TopoI in both cell lines.
Figure 6: Visualizing enhanced association of BRCA1 with Topoisomerase I in Nuclear Isolates: HCC1937-BRCA1 nuclear isolates both control and treated with SN38 were incubated with anti-TopoI antibody and precipitated with Protein A/G agarose beads. Following immunoprecipitation of TopoI-BRCA1 complexes, complexes were eluted and run on SDS-PAGE gel. Visualization of bands was performed by immunoblot with anti-BRCA1.
Coomassie Stain

Figure 7: TopoI 1-139 Expression in Bacteria: BL21DE3 E. coli bacteria were transformed with GST TopoI 1-139 vector, and grown in LB agar media. GST TopoI protein was collected and purified with glutathione sepharose beads in two separate tubes following expression of these vectors with IPTG. Protein was eluted from beads and run on SDS-PAGE gel alongside previously purified TopoI 1-139 for comparison. Coomassie Blue stain visualized the purified GST-TopoI 1-139 at approximately 45 kDa.
Figure 8: Topol expression in Bacteria: BL21DE3 E. coli bacteria were transformed with GST Topol 1-139 vector, and grown in LB agar media. GST Topol protein was collected and purified with glutathione sepharose beads in two separate tubes following expression of these vectors with IPTG. Protein was eluted from beads and run on SDS-PAGE gel alongside previously purified Topol 1-139 for comparison. Bands for Topol 1-139 were visualized at 45 kDa.
Figure 9: Protein Expression of BRCA1: SF9 cells were infected with a BRCA1 baculovirus to initiate protein expression. Following infection, cells were lysed, and BRCA1 protein was purified using IgG agarose beads (see Materials and Methods). Silver staining visualized BRCA1 at 220 kDa purified from the SF9 lysate.
Figure 10: Direct binding of TopoI 1-139 and BRCA1/BARD1 heterodimer: GST-TopoI 1-139 were phosphorylated with DNA-PK. Purified BRCA1/BARD1 was added to the beads, control and experimental with TopoI 1-139, and samples were rotated to allow binding. Following washing of unbound BRCA1/BARD1, protein complexes were eluted and run on 7.5% SDS-PAGE gel, immunoblotting with anti-BRCA1 antibody was performed to allow for analysis.
Figure 11: Effect of TopoI phosphorylation on BRCA1/BARD1 Association: GST TopoI 1-139, both non-phosphorylated and phosphorylated with DNA-PK were incubated with Glutathione Sepharose beads. Purified BRCA1/BARD1 was added to the beads, control and experimental with TopoI 1-139, and samples were rotated to allow binding. Glutathione sepharose beads were washed and associated proteins were analyzed by immunoblot analysis with anti-BRCA1.
Figure 12: Competition Assay with phosphopeptide following BRCA1/BARD1 binding to Topol 1-139: GST Topol 1-139 were phosphorylated with DNA-PK and incubated with glutathione sepharose beads. Purified BRCA1/BARD1 was added to the beads along with three different concentrations: 500 nm, 1 µM, and 1.5 µM phosphopeptide for competition, and samples were rotated to allow binding. Following washing, protein complexes were eluted and analyzed by immunoblot analysis with anti-BRCA1.
DISCUSSION

Human DNA topoisomerase I (TopoI) is a target of anticancer drug camptothecin and their analogues. First and second generation Topoisomerase I inhibitors, like Topotecan and Irinotecan, are currently being used in the clinical setting (Pommier review in Nature, 2013). The majority of third generation drugs are nano-linked, and have proven to be promising in clinical trials (Michael et al, 2015). The benefit of nano-linked treatment has been the reduction of high toxicity levels found with previous TopoI inhibitor therapy. However, the rate of response to the drugs are still similar to the second generation drugs used in the clinic, suggesting that the resistance mechanism to this class of drug remains present and needs to be addressed to improve the efficacy of therapy (Michael et al, 2015). We will describe the nature of this resistance, and the recent findings that have shed light onto the most probable mechanism of this resistance.

Secondly, we will describe the previous work done in the Bharti lab that highlighted the lack of understanding about TopoI and BRCA1 that this project attempts to describe. Finally, we will describe our work on the examination of BRCA1 and TopoI interaction in National Cancer Institute (NCI-60) cell lines, as well as our study of the direct interaction of BRCA1 and TopoI, and conclude with the future directions of this topic.

Camptothecin Resistance:

CPTs represent one of the most potent classes of anticancer drug but depending of tumor type patient response is only 13-30% (Arakawa et al, 2006). There have been
three possible mechanisms of resistance proposed: lack of drug accretion in the cell due to ABC transporters, TopoI mutations that disrupt the interaction of the enzyme with DNA/camptothecins, and the degradation rate of TopoI via the ubiquitin proteosomal pathway (UPP) following treatment with camptothecins (Shah et al, 2012).

Recent work examining all 49 known ABC transporters/MDR genes in comparison to camptothecin response has concluded that camptothecins are not a substrate for ABC transporters (Szakacs et al Cancer Cell, 2004). Mutations in the TopoI gene are rare. The only report of a TopoI mutation that has affected enzyme activity has been in three plant species: *camptotheca acuminata, ophiorrhiza pumila,* and *ophiorrhiza liukuensis* plants (Moisan et al, 2006, Sirikantaramase et al, 2008). The TopoI genes of more than 200 small cell lung cancer patients were sequenced and no mutation was found (Takatani et al, 1997). Similarly, in NCI-60 cell lines, a panel of 60 human cancer cell lines with 9 different types of tumors, only one mutation was detected in the TopoI gene. This mutation in the HCT15 cell line was found in the linker region not critical for enzyme function or DNA-binding indicating that this mutation is not important for CPT resistance. It is important to mention that NCI 60 cell lines, there are CPT sensitive and CPT resistant cell lines (Weinstein et al, 2002).

Topoisomerase I degradation via the Ubiquitin Proteasomal Pathway (UPP) provides the most promising theory for a mechanism of resistance. CPT-induced TopoI degradation was first reported by Rubin et al and later confirmed by Chen lab (Chang et al, 2002). The D’Arpa lab first demonstrated that the degradation of TopoI in the response to CPT is caused by ubiquitin proteasomal pathway (Desai et al, 2001). Later,
Liu lab demonstrated that differential rates of degradation of Topoisomerase I determine the response of cancerous cells to camptothecin treatment. (Desai et al, 2001). However, the mechanism of UPP mediated TopoI degradation is not understood. Using a functional proteomic approach, the Bharti lab isolated an interacting protein complex and found that BRCA1 is the E3 ligase of TopoI. We asked if TopoI associates with BRCA1, and is this association direct?

**BRCA1/BARD1 association with TopoI within the cell**

UPP mediated proteasomal degradation is a complex and multi step pathway and the specificity of this pathway is determined by E3 ligase (Nalepa et al, 2006). Since the resistance to camptothecin treatment has been shown to be associated with rapid degradation of TopoI, greater knowledge of the interaction between the E3 ubiquitin ligase and TopoI will define this pathway and may be helpful to improve current therapy. The Bharti lab has previously proposed a mechanism for the downregulation of TopoI. Following the covalent binding of the TopoI-camptothecin-DNA complex, TopoI is phosphorylated by DNA-PK in complex with the single stranded DNA binding proteins Ku70/80. This phosphorylation is essential for the association of BRCA1/BARD1. BRCA1/BARD1, an E3 ligase, then ubiquitimates TopoI, causing it to be degraded by the proteasome. The BRCT domains of BRCA1 have been shown to preferentially bind to phosphorylated residues within specific consensus sequences. These phosphoresidues bind to the pocket formed by two BRCT domain of BRCA1.

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To understand and dissect the association between TopoI-BRCA1 we performed immunoprecipitation experiments, GST pull down experiments, and phosphopeptide competition assays. TopoI immunoprecipitation experiments with HCC1937-BRCA1, HCT15, and HCT116 nuclear extracts followed by BRCA1 as well as BARD1 immunoblotting were used to demonstrate that TopoI and BRCA1 associate in the cell. In a reciprocal immunoprecipitation we used BRCA1 antibody for immunoprecipitation and immunoprecipitates were analyzed by immunoblotting with anti-TopoI. These experiments clearly demonstrated that TopoI and BRCA1-BARD1 are in a protein complex. BRCA1 associates with large number of proteins, and three distinct protein complexes have been identified (Harper and Elledge, 2007). In these studies, the association between BRCA1 and TopoI was not reported. In our experiments we have found this association even with the basal level of BRCA1 in HCT15 and HCT116 cells. Most, if not all, of the previous reports have tagged and overexpressed BRCA1 to isolate and identify BRCA1-associated proteins.

TopoI is ubiquitinated by BRCA1 in the response to CPT, so we asked if the association between these two proteins is enhanced in drug treated cells. We were able to visualize differential association of these proteins when the cells were treated with camptothecins (see Results section, Figures 5 and 6), which supported our hypothesis that the BRCA1/BARD1 heterodimer is involved in the ubiquitination of TopoI and its degradation, leading to resistance to camptothecin treatment. Consistent with this hypothesis is the observation that camptothecin therapy can be more effective in TNBC patients with a BRCA1 mutation or degradation, as they would not have the fully
functional E3 ubiquitin ligase (Michael et al, 2015) and consequently would be less resistant to camptothecin treatment. We attempted to highlight these differential rates of degradation and the consequential amount of interaction between the E3 Ubiquitin ligase BRCA1/BARD1 heterodimer and TopoI.

Direct Interaction of Purified TopoI 1-139 and BRCA1/BARD1

Camptothecin treatment has shown promise in the treatment of triple-negative breast cancer patients, as well as patients with ovarian cancer, highlighting the need for greater understanding of the interaction between TopoI and BRCA1/BARD1. In our work on this project, we were able to reproducibly show that there is direct interaction between purified BRCA1/BARD1 heterodimer and purified TopoI 1-139 (see Results section, Figure 10). Our work has shown that a specific region of TopoI is sufficient for binding to the BRCA1/BARD1 heterodimer. It is the hope that future work may be able to take advantage of this direct interaction in the interest of more effective therapy.

To follow up these findings, we tried to compete the BRCA1/BARD1 heterodimer off from the direct binding to TopoI. Previous work in the Bharti lab has demonstrated that the phosphorylation of the S10 residue of TopoI is essential for the degradation of TopoI by the UPP. Contrary to our expectation, we did not see any significant difference in binding (Figures 11 and 12) at any of the three phosphopeptide concentrations used (500 nM, 1 µM, and 1.5 µM). These results further supported that phosphorylation of TopoI 1-139 may not be absolutely necessary for the direct binding with the BRCA1/BARD1 heterodimer. However, because of the relatively high
concentration of phosphopeptide used in the competition assay, there is a possibility that
the formation of phosphopeptide oligomers did not allow for any significant competition.
Despite attempting the assay with lower concentrations of phosphopeptide (100 nm, 250
nm, 500 nm), we still did not see any difference in the amount of binding between
BRCA1/BARD1 and TopoI. We also attempted to create a competition assay using GST-
TopoI 1-139 attached to a 96-well GST plate, fluorescently labeled BRCA1/BARD1, and
phosphopeptide. Our early results have shown that a more specific method of
determining binding levels is required to qualify the nature of the BRCA1/BARD1
heterodimer with TopoI. Our future work will focus on optimizing the use of this more
specific assay. The finding that the S10 phosphorylation of TopoI is a key component of
the interaction between BRCA1/BARD1 and TopoI begs further work on this subject,
and supports our evidence that a phosphopeptide may be able to compete off the
heterodimer.
CONCLUSION

We have observed that TNBC cells with deficiencies in BRCA1 amount or function fail to ubiquitinate and degrade Topoisomerase I. This highlights the possibility that these cells are susceptible to treatment by camptothecins, a finding that has major implications for their continued use of these drugs in the clinic. We were able to show through immunoprecipitation experiments that in the HCC1937 and HCT15 cell lines TopoI and BRCA1 association increased in response to treatment with camptothecins. Although we were able to demonstrate that phosphorylated TopoI 1-139 was able to bind with BRCA1 in a direct binding assay, we were not able to show significant competition with phosphopeptide. However, we have identified and continue to improve a more specific assay to continue to examine this possibility. Because of our understanding of the UPP, we expect that any disruption in binding between TopoI and BRCA1 will sensitize cancer cells to camptothecin treatment. As BRCA1 acts as the E3 Ubiquitin ligase for TopoI during its degradation, this supports the current understanding that the UPP pathway may be a reasonable topic of continued investigation to combat the prevalent resistance to camptothecins. If we understand this and confirm our findings, they will provide the basis for treating TNBC cells where BRCA1 is mutated or down-regulated with TopoI inhibitors.
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CURRICULUM VITAE

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OBJECTIVES

To obtain opportunities focused in medicine, especially focused in Orthopedics, Trauma Surgery, Cardiology, and Oncology.

COMPETENCIES

- Strong analytical abilities as well as ability to problem solve
- High work ethic
- Rapid learner
- Ability to work positively with all ages and genders
- Solid understanding of medicine, with eagerness to learn

EDUCATION

- Graduated Boston College, Chestnut Hill, MA
  Major: Biology (Pre-Medical Program Concentration), Minor: History
  Overall GPA: 3.437 Pre-Medical GPA: 3.316
  Graduated: May 2013

- Currently attending Boston University School of Graduate Medical Sciences
  Began: September 2013
  Anticipated Graduation: May 2015
  Overall GPA: 3.79

HONORS

- Pedro Arrupe Award (Eagle EMS) – “For selfless dedication to Eagle EMS, and the safety of our campus-wide community.”
- Kevin M. Eidt Award (Eagle EMS) – “The highest honor given to a member of Eagle EMS - For outstanding commitment and leadership to the development of our organization.”
EXTRA CURRICULARS

- Founded and participated in an intramural soccer team in the spring of 2010-2011
- Member of the Boston College Campus School Marathon Team, 2009-2013 (did not run the marathon 2012, 2013)
  - Raised 2,435 dollars for The Campus School at Boston College
- Greater Boston Food Bank Volunteer (February 2014-August 2014)
- Boston University School of Medicine - Outreach Van Project member (September 2014-Present)

EXPERIENCE

Alliance ENT
*Medical Records Employee (Summer 2009)*
Worked in the office of Dr. Godley, Dr. Christu, and Dr. Mehta, working in the medical records department. Was responsible for organizing, recording, and filing patient information as well as observing coding of medical procedures.

South County Hospital Emergency Room
*Volunteer (Summer 2010)*

The ENT Center of Rhode Island
*Intern (Summer 2011, Summer 2012)*
A surgical center where my responsibilities included assisting in the Operating Room, sterilizing instruments, PACU aide, and Office Assistant. 32 hours/week.

Eagle EMS
Over 30 hours a week of volunteer EMS service at Boston College, includes continuing education courses as well as opportunities to provide Basic Life Support on campus. I was also a BLS and First Aid instructor for Boston College and the surrounding community. My position as Member Services Coordinator gave me valuable experience working with over 110 members to address issues within the organization and create an atmosphere of professionalism and trust. As Director of Operations, I learned much about the business side of an organization, as well as gained skill at working with people and improving quality of patient care through leading the QAQI program for this organization. Through this program I was able to gain experience in the hospital as well, working with other doctors and nurses to provide superior patient care.
**Bharti Lab**

*Lab Technician (July 2014-present)*

I am currently working in Dr. Bharti’s lab while pursuing a Master’s degree. The lab focuses on a class of chemotherapy drugs known as camptothecins, which are human Topoisomerase I inhibitors. My work and the topic of my Master’s thesis focused on the interaction of BRCA1 and Topoisomerase I in response to camptothecin treatment. My responsibilities in the lab included running experiments in alignment with the main objectives of the lab, maintaining the supplies in the lab, as well as the cleaning and maintenance of our equipment.