รายงานการวิจัยฉบับสมบูรณ์

ผลของยาต้านมะเร็งซิสพลาตินต่อ ubiquitin ligase activity ของโปรตีนบีอาร์ซีเอวันที่ผ่าเหล่าชนิด D67E ที่พบในผู้ป่วยคนไทยที่เป็นมะเร็งเต้านม

The effect of the anticancer drug cisplatin on a ubiquitin ligase activity of the D67E BRCA1 mutation identified in Thai breast cancer patients

หัวหน้าโครงการวิจัย

รองศาสตราจารย์ ดร. อดิศร รัตนพันธ์

โครงการวิจัยนี้ได้รับทุนสนับสนุนการวิจัยจากเงินรายได้มหาวิทยาลัยสงขลานครินทร์ ประเภททั่วไป ประจำปีงบประมาณ 2554

ABSTRACT

BRCA1 is a tumor suppressor protein involved in maintaining genomic integrity through multiple functions in DNA damage repair, transcriptional regulation, cell cycle checkpoint, and protein ubiquitination. The BRCA1-BARD1 RING complex has an E3 ubiquitin ligase function that plays essential roles in response to DNA damage repair. BRCA1-associated cancers have been shown to confer a hypersensitivity to chemotherapeutic agents. Here, we have studied the effect of the anticancer drug cisplatin on a ubiquitin ligase activity of the D67E BRCA1 mutation identified in Thai breast cancer patients in comparison with the D67Y BRCA1 and the wild-type BRCA1. The D67Y BRCA1 RING domain protein exhibited the reduced ubiquitination function, and was more susceptible to the drug than the D67E or wild-type BRCA1 RING domain protein. Upon platination, both familial missense BRCA1 mutations showed similar changes in protein structure and folding, however, the D67Y BRCA1 RING domain protein exhibited the Pt binding approximately a 2-fold less than the D67E BRCA1 RING domain protein did. The difference in Pt binding might reflect an alteration in the microenvironment at the mutation site necessary for protein interactions. This evidence emphasized the potential of using the BRCA1 dysfunction as an important determinant of chemotherapy responses in breast cancer.

บทคัดย่อ

บีอาร์ซีเอวันเป็นโปรตีนกดมะเร็งที่ทำหน้าที่ในการรักษาเสถียรภาพของจีโนมโดยผ่านการซ่อมแซมดีเอ็นเอที่เสียหาย การควบคุมการถอดรหัส การควบคุมวงจรชีวิตของเซลล์ และ ubiquitination ของโปรตีน BRCA1-BARD1 RING complex ทำหน้าที่เป็น E3 ubiquitin ligase ซึ่งมีบทบาทที่สำคัญในการตอบสนองต่อการซ่อมแซมดีเอ็นเอที่เสียหาย ผู้ป่วยมะเร็งที่มีโปรตีนบีอาร์ซีเอวันผิดปกติจะตอบสนองต่อยาเคมีบำบัดได้ดี งานวิจัยชิ้นนี้ได้ศึกษาผลของยาต้านมะเร็งซิสพลาตินต่อ ubiquitin ligase activity ของโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67E ที่พบในผู้ป่วยคนไทยที่เป็นมะเร็งเต้านมโดยเปรียบเทียบกับโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67Y และโปรตีนบีอาร์ซีเอวันที่ปกติ พบว่าโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67Y ทำหน้าที่ ubiquitination ลดลงและไวต่อยาซิสพลาตินมากกว่าโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67E หรือโปรตีนบีอาร์ซีเอวันที่ปกติ เมื่อเกิดอันตรกิริยากับยาต้านมะเร็งซิสพลาตินโปรตีนบีอาร์ซีเอวันที่ผ่าเหล่าทั้งสองชนิดนี้มีการเปลี่ยนแปลงโครงสร้างทุติยภูมิที่คล้ายกันถึงแม้ว่าโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67Y มีความสามารถในการจับกับอะตอมพลาตินัมของยาซิสพลาตินได้น้อยกว่าโปรตีนบีอาร์ซีเอวันที่มีการผ่าเหล่าชนิด D67E ประมาณสองเท่า ความแตกต่างในการจับกับอะตอมพลาตินัมของยาซิสพลาตินของโปรตีนบีอาร์ซีเอวันที่ผ่าเหล่าทั้งสองชนิดนี้อาจสะท้อนถึงการเปลี่ยนแปลงสภาวะแวดล้อมภายในโครงสร้างของโปรตีนบีอาร์ซีเอวันในบริเวณที่มีการผ่าเหล่าซึ่งมีความจำเป็นต่อการเกิดอันตรกิริยากับโปรตีนอื่นในการทำหน้าที่รักษาเสถียรภาพของจีโนม ข้อมูลที่ได้จากการศึกษาครั้งนี้ได้เน้นถึงแนวโน้มของการใช้โปรตีนบีอาร์ซีเอวันที่ทำหน้าที่บกพร่องเป็นเป้าหมายในการตอบสนองต่อยาเคมีบำบัดในการรักษามะเร็งเต้านม

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Adisorn Ratanaphan

CONTENTS

INTRODUCTION

BRCA1 is a tumor suppressor gene composed of 24 exons, with an mRNA that is 7.8 kb in length, and 22 coding exons that translate into a protein of 1863 amino acids (Fig. 1) with a molecular weight of 220 kDa (Brzovic et al., 1998). It has 3 major domains, including (1) the N-terminal RING finger domain (BRCA1 RING domain), (2) the large central segment with the nuclear localization signal (NLS), and (3) the BRCA1 C-terminal domain (BRCT). The BRCA1 protein plays an essential role in maintaining genomic stability associated with a number of cellular processes, including DNA repair, a cell cycle checkpoint, transcriptional regulation, and protein ubiquitination (Huen et al., 2010; O'Donovan & Livingston, 2010).

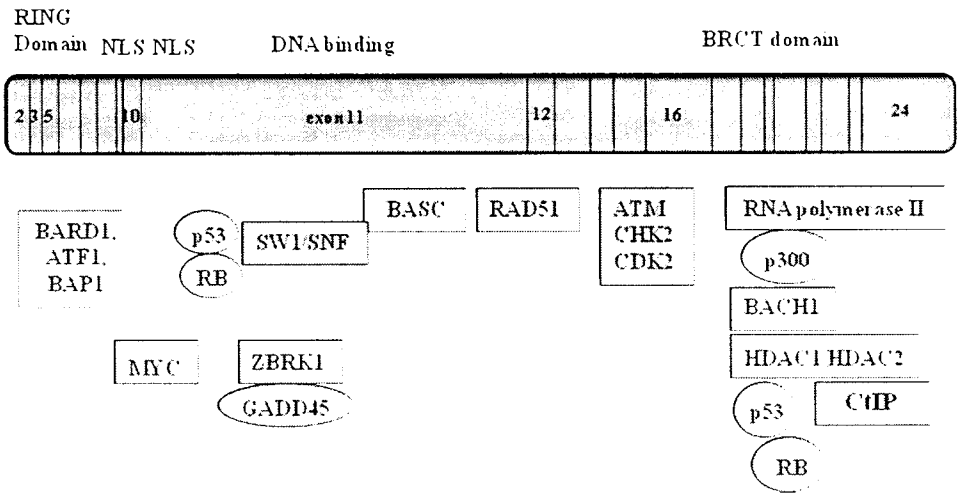


Figure 1. Scheme of BRCA1 mRNA and sites of protein interaction (Ratanaphan, 2011)

The BRCA1 protein contains an N-terminal RING finger domain. The N-terminal RING finger domain contains the conservative sequences of cysteine and histidine residues (C_3HC_4) necessary for specific coordination with two Zn^{2+} ions (Fig. 2). The first 109 amino acids of BRCA1 protein constitute a protease-resistance domain. The solution structure of the BRCA1 RING domain revealed the existence of antiparallel α-helices at both ends, flanking the central RING motif (residues 24-64) and was characterized by a short antiparallel three-stranded β-sheet, and two large Zn^{2+}-binding loops, and a central α-helix (Brzovic et al., 2001). The two Zn^{2+}-binding sites are formed in an interleaved fashion in which the first and

third pairs of cysteines (Cys24, Cys27, Cys44, and Cys47) form site I, and the second and fourth pairs of cysteines and a histidine (Cys39, His41, Cys61, and Cys64) form site II. It is an important domain since it might mediate a central role in macromolecular interactions to exert the tumor suppression functions.

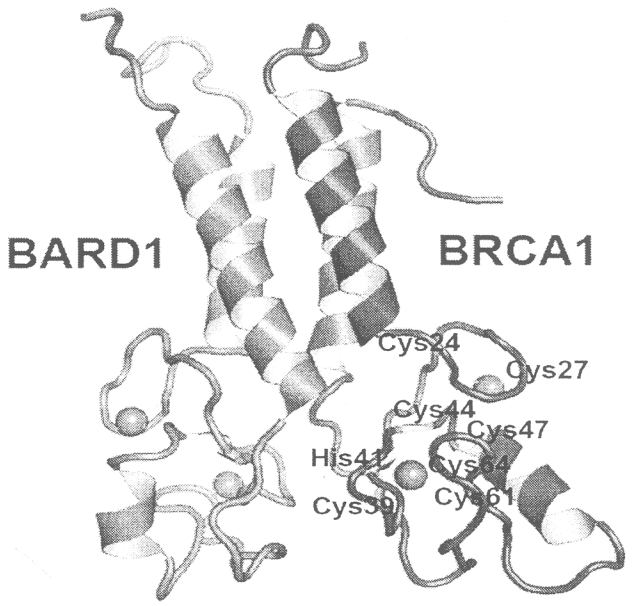


Figure 2. Solution structure of the BRCA1-BARD1 RING heterodimer. The BRCA1 subunit is shown in red, and the BARD1 subunit in blue. The grey spheres represent Zn^{2+} atoms. The dimerization interface is formed by the antiparallel α-helices, flanking the central RING motif of both BRCA1 and BARD1. The BRCA1 RING domain contains two Zn^{2+}-binding sites in an interleaved fashion, which the first and third pairs of cysteines (Cys24, Cys27, Cys44, and Cys47) form site I, and the second and fourth pairs of cysteines and histidine (Cys39, His41, Cys61, and Cys64) form site II. This structure was generated with PyMOL software (http://pymol.sourceforge.net) based on the protein databank (PDB: 1JM7) (Brzovic et al., 2001).

The solution structure together with yeast-two-hybrid studies revealed that the BRCA1 RING domain preferentially formed a heterodimeric complex with another RING domain BARD1 (BRCA1-associated RING domain 1) through an extensive four-helix-bundle interface (Brzovic et al., 2001; Wu et al., 1996). The binding interface is composed of residues 8-22 and 81-96 of BRCA1, and residues 36-48 and 101-116 of BARD1. The BRCA1-BARD1 complex requires each other for their mutual stabilities, and they are co-

localized in nuclear dots during S phase but not the G phase of the cell cycle and in nuclear foci (Hashizume et al., 2001). The progression to S phase by aggregation of nuclear BRCA1 and BARD1 implied the importance of both proteins for a DNA repair function (Jin et al., 1997). The BRCA1-BARD1 complex also exhibits enzymatic activity of an E3 ubiquitin ligase that specifically transfers ubiquitin to protein substrates that are essential for cellular viability (Hashizume et al., 2001; Xia et al., 2003). Ubiquitination is a form of post-translational modification responsible not only for traditionally targeting proteins for proteasome-dependent degradation but also for playing roles in diverse cellular processes, such as protein transport, and DNA repair (Bergink & Jentsch, 2009). Ubiquitination is a multistep process initiated by an ATP-dependent activation through the formation of a thioester bond between the C-terminal glycine of ubiquitin and a cysteine residue of a ubiquitin-activating enzyme (E1) (Fig. 3). The ubiquitin is then transferred to the active cysteine site of a ubiquitin-conjugating enzyme (E2) by transesterification. Finally, ubiquitin is specifically attached to the ε-amino group of a lysine on its protein substrates via an isopeptide bond mediated by a ubiquitin ligase (E3).

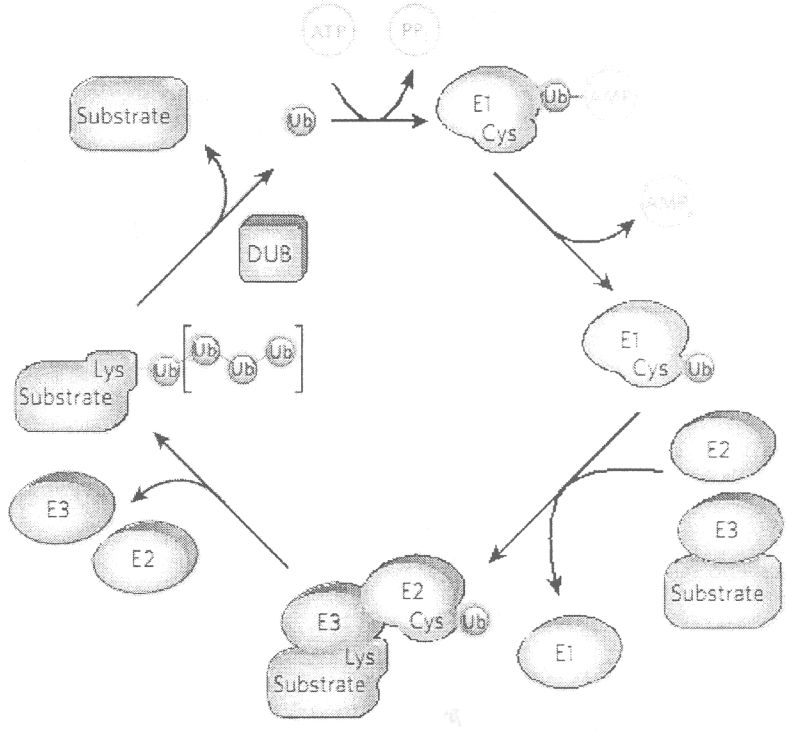


Figure 3. Overview of the ubiquitination pathway (Hochstrasser, 2009)

Putative substrates for the BRCA1-BARD1 RING complex identified at present are histones, that have recently emerged from in vitro studies, such as for BRCA1 itself, nucleosomal histones, γ-tubulin, the largest subunit of RNA polymerase II (RPB1), estrogen receptor α, nucleophosmin/B23 (NPM1), CtIP, progesterone receptor-A (PR-A), and the transcriptional factor TFIIE (Chen et al., 2002; Eakin et al., 2007; Horwitz et al., 2007; Kleiman et al., 2005; Mallery et al., 2002; Sato et al., 2004; Starita et al., 2004, 2005; Wu et al., 2007; Wu-Baer et al., 2010; Yu et al., 2006). BRCA1-dependent ubiquitination is probably responsible for modifying many cellular activities. Recently, extensive investigations have examined the relevance of the BRCA1-mediated E3 ubiquitin ligase activity to its tumor suppression function. After a DSB (Fig. 2), the BRCA1 complex can repair DNA lesions as a result of its co-localization with another DNA repair protein, MDC1, to the ubiquitinated histone variant γ-H2AX at the sites of DSBs through the interaction with RAP80 and phosphorylated Abraxas (Morris, 2010). Poly-ubiquitylated histones recruit RAP80, which promotes the accumulation of Abraxas/BRCA1/BARD1 at DSBs. It has been reported that RAP80 targets BRCA1 to K63-Ub at DSBs (Zhao et al. 2007). The Ubc13 E2 enzyme is responsible for K63-Ub synthesis. Ubc13 deficiency reduces HR and inhibits conjugated ubiquitin formation at DSBs and BRCA1 IRIF (Zhao et al. 2007). In addition, it has been recently indicated that Ubc13 functions in concert with the Ring Finger 8 (RNF8) E3 ligase to ubiquitinate substrates at DSBs (Huen et al. 2007). Both RNF8 and Ubc13 are required for RAP80-mediated BRCA1 DSB localization. In response to DSBs, RNF8 can form a complex with HERC2, a giant protein (4834 amino acids) which is an E3 ubiquitin ligase and is implicated in DNA damage repair through its C-terminal HECT-containing motif (Bekker-Jensen et al., 2010). The HERC2 protein could ubiquitinate and target BRCA1 for degradation, and its effect was blocked by BARD1 binding. It indicates that the stability of the BRCA1 protein was tightly regulated by at least two proteins (BARD1 and HERC2), and that this regulation was important for the BRCA1 tumor suppression function especially through the G_2-M cell cycle checkpoint. HERC2 is also needed for RNF8 to promote the Ubc13-dependent polyubiquitylation of histones (Wu et al., 2010). The hyperphosphorylated forms of full-length RPB1 (RNAPIIO) can be polyubiquitinated by the N-terminal residues 1–304 of BRCA1 in vitro (Bekker-Jensen et al., 2010). NPM1 and RPB8 were also ubiquitinated by the N-terminal constructs of BRCA1 in vivo and in vitro (Sato et al., 2004; Wu et al., 2007). Fulllength BRCA1, including the C-terminal residues 1852–1863, were

necessary for γ-tubulin monoubiquitination, although the domains required for γ-tubulin binding reside in the 803 amino acid terminal of BRCA1 (Starita et al., 2004). CtIP interacts with BRCT and is polyubiquitinated by BRCA1-BARD1 in a phosphorylation dependent manner. DNA damage induces a BRCA1-dependent, CtIP chromatin association and IRIF formation. This indicates that the ubiquitinated form of CtIP is targeted to DNA damage sites, rather than to the proteasome for degradation (Yu et al. 2006). There were many cancer-predisposing mutations in the BRCA1 RING domain, that inhibited the E3 ligase activity and its ability to accumulate at DNA damaged sites, were defective in the homologous recombination that is critical for tumor suppression (Morris et al., 2009; Ransburgh et al., 2010). However, the ubiquitination process is not only regulated by E3 ubiquitin ligase but also by deubiquitinating enzymes. A recently identified BRCA1-associated protein 1 (BAP1) has been shown to interfere with the BRCA1-BARD1 RING heterodimer and inhibits its activity (Nishikawa et al., 2009), and indicates that BAP1 and BRCA1-BARD1 regulate the ubiquitination process during the DNA damage response. The loss of the BRCA1 E3 ligase activity resulted in hypersensitivity of the cancer cells to ionizing radiations to indicate that ubiquitination had a significant role in the DNA damage response (Ruffner et al., 2001).

Cisplatin [(*cis*-diamminedichloroplatinum(II)] (Fig. 4), an alkylating agent and anticancer drug, is widely used for the treatment of human testicular cancer, bladder cancer and head and neck cancer (Zwelling & Korn, 1982; Loehrer & Einhorn, 1984; Pratt et al, 1994). The activity of cisplatin was found in the early sixties (Rosenberg et al., 1965, 1967, 1969) and was approved for use as an anticancer drug in 1978.

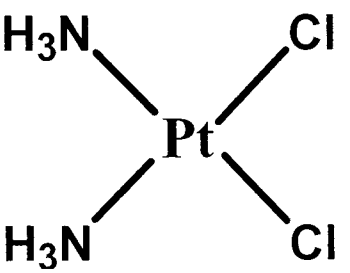


Figure 4. Structure of *cis*-diamminedichloroplatinum(II) or cisplatin

Following intravenous injection of cisplatin, the neutral, relatively unreactive complex is spread in the serum and when uncharged diffuses passively across cell membranes. Activation of cisplatin then occurs by hydrolysis and loss of the chloride ions to form a cationic species that reacts with DNA, proteins, and other intracellular components (Coley &

Martin, 1972). In the blood plasma, the high chloride concentration at approximately 100 mM keeps cisplatin predominately in the uncharged and relatively unreactive dichloro form. This form may react to some degree with the sulfhydryl groups of plasma proteins. The free dichloro form enters cells by passive diffusion. Cisplatin is a highly polar molecule that does not readily diffuse across lipid membranes (Hall et al., 2008). Although several lines of evidence have addressed the uptake of cisplatin through ion pumps and transporters (Hall et al., 2008), the copper influx transporter or copper transporter 1 (CTR1) has currently been shown to control the entry to cells of platinum-containing cancer drugs including cisplatin (Fig. 5)(Holzer & Howell, 2006; Holzer et al., 2004a, 2004b, 2006a, 2006b; Howell et al., 2010; Jandial et al., 2009; Larson et al., 2009; Pabla et al., 2009; Safaei, 2006; Safaei et al., 2004, 2008; Wu et al., 2009). In the cytoplasm, the relatively low chloride concentration of approximately 2-30 mM would favor the aquation reaction. The aquation reaction is driven by the high concentration of water and low concentration of chloride in the tissues. The rate constant for the hydrolytic replacement of the first chloro ligand at 37°C is $7x\ 10^{-3}\ min^{-1}$ and this corresponds to a half-life of 2 h (Coley & Martin, 1972). The hydrolysis of the first chloro group is about twice as fast as for the second chloro group (Cubo et al., 2009). The aquated platinum complex can then react rapidly with a variety of strong binding sites (Pratt et al., 1994).

The cytotoxicity of cisplatin to the cancer cells is directly related to how much drug enters the cell, and its cellular accumulation. It is generally accepted that the cytotoxicity of cisplatin results from the interaction of the drug with DNA through forming a covalent bond between a platinum atom and guanine or adenine. DNA is attacked by an activated platinum(II) complex at the guanine residues in position N7, in double stranded DNA from the side of the major groove. The attack is apparently preceeded by an electrostatic attraction between the positively charged platinum(II) complex and the negatively charged phosphodeoxyribose DNA backbone and facilitated by a bidirectional diffusion along the backbone (Holler 1993). The initial attack of DNA by activated cisplatin is followed by the replacement of the remaining chloro ligand before the DNA adduct performs an intramolecular attack on a second purine residue (either guanine or adenine)(Bernges & Holler, 1991). The formation of crosslinks is followed by a local, partial unwinding of double stranded DNA together with a second wave of crosslink formation. The formation of the crosslinks can be separated from the release of the second chloro group by employing the diaqua complex of cisplatin.

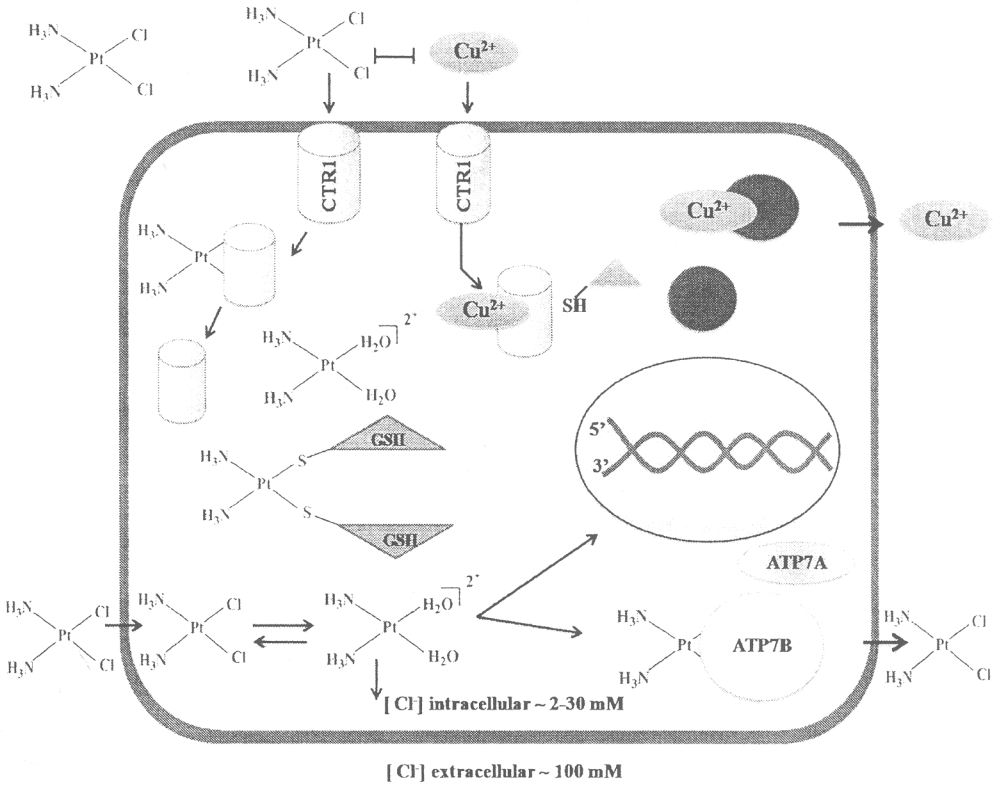


Figure 5. Schematic representation of the cellular uptake, accumulation and efflux of cisplatin in a mammalian cell. The free, neutral dichloro form of cisplatin can enter the cell by passive diffusion across the lipid bilayer or the copper influx transporter, copper transporter 1 (CTR1). In the cell, cisplatin can be inactivated by binding to glutathione (GSH), thiol-containing molecules in the cytoplasm or nuclear DNA. Cisplatin can be effluxed from the cell through the copper exporters ATP7B (copper-transporting ATPase β) or ATP7A (copper-transporting ATPase α)(Ratanaphan, 2012).

The distribution of bifunctional adducts formed by cisplatin has been studied extensively by mapping with restriction endonucleases and exonucleases (Cohen et al., 1980; Kelman & Buchbinder, 1978; Royer-Pokora et al., 1981; Tullius & Lippard, 1981), by replication mapping with prokaryotic and eukaryotic DNA polymerases (Fichtinger-Schepman et al., 1982; Hemminki & Thilly, 1988; Pinto & Lippard, 1985), by enzymatic digestion analysis (Eastman, 1983, 1986, 1987, Fichtinger-Schepman et al., 1985, 1987a, 1987b) and by NMR spectroscopic studies of platinated oligonucleotides (Sherman & Lippard, 1987; Berners-Price et al., 1992). The predominant adducts formed by cisplatin on DNA *in vitro* are 1,2-intrastrand crosslinks between the purine N7 positions in d(GpG) (>65%

of the total adducts) and d(ApG) sequences (20%-25%), while longer range intrastrand adducts and interstrand crosslinks are minor products (~4%) (Fichtinger-Schepman et al., 1985). Immunochemical detection of adducts (Bedford et al., 1988) has been employed to show that the intrastrand d(GpG) crosslink represents 50%-75% of the total platinum-DNA adducts formed in human cells in culture, as well as being the predominant adduct detected in the white blood cells of cancer patients undergoing cisplatin chemotherapy. The presence of d(GpG) adducts in the white blood cells of patients is associated with a favorable response to chemotherapy (Reed et al., 1987), leading researchers to seek a correlation between a tumor response and the cellular removal of such adducts.

Although the interstrand crosslinks represent a minor portion and are generally regarded as too rare to account for the cytotoxicity of cisplatin, DNA interstrand crosslinks occur predominantly between two guanine N7 atoms on opposite strands (Eastman, 1987). A distance of ~3 angstrom is required for the cisplatin crosslinking reaction. Thus, two adjacent guanine residues on the opposite strands, either in the 5'-CG-3' or 5'-GC-3' sequences, are the most probable reaction sites on DNA. However, in both cases, formation of the crosslink in the B-DNA implies a large distortion of the double helix since the two N7 atoms in d(CG) and d(GC) sequences are separated by about 9 and 7 angstrom, respectively. Manipulation of three-dimensional molecular models inferred that the d(CG) sequence is more able to match the interstrand cross-linking requirement (Eastman, 1987). An *in vitro* study has shown that RNA synthesis by a bacteriophage RNA polymerase acting on platinated DNA is blocked at the level of the adducts. The ability of adducts to terminate transcription has been used to map the cisplatin binding sites on a double-stranded DNA. Analysis of the modification patterns shows that the interstrand crosslinks are preferentially formed at the d(GC) sequences. This has been confirmed by a chemical study of the interstrand adduct within the d(CGAGC) sequence. The guanine residues at the d(GC) site are not reactive with DMS, which indicates that platination occurs at the N7 position of these residues (Lemaire et al., 1991).

The interaction of cisplatin with proteins is of particular significance, and it is believed to play an important role in the distribution of the drug and inactivation responsible for determining its efficacy and toxicity (Casini et al., 2008; Sun et al., 2009; Timerbaev et al., 2006). It has been reported that albumin is not the major binding partner of cisplatin and that other proteins also have important roles in the pharmacokinetics of the drug (Timerbaev et al., 2006). It is intriguing, that protein adducts affect some crucial aspects of protein structures and functions (Table 1). For instance, the platination of human serum albumin caused the partial unfolding of protein structure at a high drug concentration, and induced intermolecular

crosslinks possibly at Cys34 and/or Met298 via bifunctional adducts or via NH_3 release (Ivanov et al., 1998; Neault & Tajmir-Riahi, 1998). Myoglobin, a small protein, that contains a heme group necessary for oxygen transport in skeletal muscles and myocardial cells, intramolecularly formed mono- and bi-functional adducts with cisplatin. Its putative platinum-binding sites were His116 and His119 (Zhao & King, 2010). A number of intramolecular crosslinks also occurred in ubiquitin adducts (Casini et al., 2009). The loss of activity by protein aggregation and prevention of the C-terminal heat shock protein 90 was reported as the consequence of cisplatin binding but the protein did not exhibit any conformational change (Ishidaa et al., 2008).

Again it is intriguing that cisplatin can cause a structural perturbation of a synthetic peptide containing a Zn^{2+} finger domain. The platinum links to the Zn^{2+}-binding sites by ejection of the Zn^{2+} and this led to a subsequent loss of the protein tertiary structure, and implied the inhibition of critical biological functions regulated by the Zn^{2+} finger protein. Such a mechanism has been discussed for the apoptosis process mediated by the interaction of cisplatin and other platinum-based compounds with the Zn^{2+} finger transcriptional factors (Bose et al., 2005). Likewise, the nucleocapsid Zn^{2+} finger NCp7 protein, a protein required for the recognition and packaging of viral RNA, became attached to some platinum compounds that inhibited its nucleic acid binding and prevented viral infectivity (de Paula et al., 2009; Musah, 2004). The Zn^{2+} finger protein, therefore, is a potential target for platinum compounds in medicinal applications. Recently, the interaction of cisplatin with the BRCA1 DNA repair protein has been studied. It revealed that the apo-form, not the holo-form, of BRCA1 underwent more folded structural rearrangement upon cisplatin binding at His 117, and this inactivated an E3 ubiquitin ligase activity (Atipairin et al., 2010; 2011b).

Recently, evidence from several preclinical and clinical studies have identified the possibility of utilizing DNA damaging agents such as platinum-based drugs in patients with a BRCA1 mutation (Narod, 2010; Pal et al., 2011). It revealed that the response to cisplatin treatment was a dose-dependent manner in human breast cancer cells *in vitro* (Tassone et al., 2003; Wong et al., 2011). After a 24 h exposure to the drug, cisplatin concentration of 60-100 μM was required for a half inhibition in cell proliferation of the BRCA1-competent MCF-7 and MDA-MB-231 cells while cisplatin at 20 μM led to a 50% reduction in cell viability of the BRCA1-defective HCC1937 cells (BRCA1 5382insC mutation)(Yde & Issinger, 2006. Furthermore, a clinical study showed that nine out of 10 (90%) breast cancer patients who carried the common BRCA1 C61G and 5382insC mutations achieved a complete pathological

response in cisplatin-based chemotherapy (Byrski et al., 2009). In a retrospective study with 102 BRCA1 mutation carriers, ten out of 12 (83%) patients with the presence of BRCA1 C61G and 5328insC founder mutations who were treated with cisplatin also experienced a high rate of a pathological complete response while the remaining 90 patients who were treated by other regimens obtained a much lower response rate (16%)(Byrski et al., 2010). These were consistent with previous studies, demonstrating that BRCA1 mutations which disrupted the E3 ligase activity and the homologous recombination repair of RING domain (C61G mutation) and BRCT domain (5382insC mutation) caused the significant cytoplasmic mislocalization of BRCA1 and altered the formation of DNA repair-associated nuclear foci in response to DNA damage (Au & Henderson, 2007; Ohta et al., 2011; Ransburgh et al., 2010; Rodriquez et al., 2004). This contributes to the inhibition of nuclear DNA repair and transcription function. Therefore, the increased cisplatin sensitivity in the BRCA1-mutated breast cancers might be related to an impaired BRCA1 function normally responsible for repairing DNA adducts produced by cisplatin, and ultimately results in cell death (Alli et al., 2011; Price & Monteiro, 2010; Tassone et al., 2009). It suggests that the BRCA1 gene product acts as a key modulator of drug sensitivity in breast cancer cells (Quinn et al., 2003). This was consistent with previous studies, showing that cisplatin-based chemotherapy achieved an increased response rate for triple-negative breast cancer (Silver et al., 2010; Sirohi et al., 2008). This evidence has emphasized the potential of using the BRCA1 dysfunction as an important determinant of chemotherapy responses in breast cancer (Mullan et al., 2006). Interestingly, an unprecedented D67E BRCA1 mutation (substitution of aspartic acid with glutamic acid at position 67) has only been identified in three unrelated Thai breast cancer patients (Patmasiriwat et al., 2002). This mutation is assumed to be a founder mutation in Thais. According to the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/), the D67Y BRCA1 mutation (substitution of aspartic acid with tyrosine at position 67) identified in eight European patients has been observed in the same protein residue. These mutations are classified as variants of unknown clinical significance. However, they are located in the second Zn^{2+} binding loop (residue 58-68) that forms a recognition interface with an E2 ubiquitin-conjugating enzyme (Brzovic et al., 2006). It is postulated that these substitutions might interfere at the E2 binding interface and consequently the ubiquitin ligase function. In this study, we have investigated the functional consequences of the familial D67E and D67Y mutations in the BRCA1 RING domain on the ubiquitin ligase activity, together with their respective responses to cisplatin *in vitro*. The

findings could provide additional insights into the BRCA1-dependent ubiquitination inactivated by cisplatin and be of interest for molecular-targeted cancer therapy.

Table 1. Some consequences of protein adducts by cisplatin

Protein	Consequences
BRCA1 (Atipairin et al., 2010)	Conformational change of the apo-form of the RING domain and confers thermal stability of a platinum-adduct at His117.
hCtr1 (Crider et al., 2010)	Intramolecular crosslinks through replacing all platinum ligands by three Mets and an amide nitrogen of a peptide backbone
Ubiquitin (Casini et al., 2009)	Four distinct adducts: $[Pt(Ub)(NH_3)_2Cl]$, $[Pt(Ub)(NH_3)_2H_2O]$, $[Pt(Ub)(NH_3)_2]$, and $[Pt(Ub)(NH_3)]$
Cytochrome c (Zhao & King, 2009)	Monoadduct at Met 65
Hsp90 (Ishidaa et al., 2008)	Dimer formation, inhibition of aggregation prevention of HSP90C, altered secondary structure of HSP90N, Increased protease resistance of HSP90N
Lysozyme (Casini et al., 2007)	Monofunctional adduct at His 15
C4 zinc-finger module (Bose et al., 2005)	Structural perturbation of a synthetic peptide containing a Zn^{2+} finger domain
Na, K-ATPase (Neault et al., 2001)	Binding constant $1.93x10^4$ M^{-1}, led to secondary structural changes
Transferrin (Cox et al., 1999)	Monofunctional adduct at Met 256 or Met 499, no observed conformational change
Urease (Du et al., 1999)	Secondary structural change, reduced denaturation temperature, urease inactived
Albumin (Neault & Tajmir-Riahi, 1998)	Binding constant $8.52x10^2$ M^{-1}, led to secondary structural changes
γ-Globulin (Chen et al., 1994)	12.4 mol platinum per protein molecule (30-fold excess of cisplatin), protein precipitation

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), isopropyl-β-D-thiogalactoside (IPTG), iodoacetamide, sodium cacodylate trihydrate, *t*-octylphenoxypolyethoxyethanol (triton X-100), phenylmethylsulphonylfluoride (PMSF), glutaraldehyde, and *cis*-diamminedichloroplatinum(II)(cisplatin) were purchased from *Sigma Chemicals Co.* (USA). Agarose power, tris(hydroxymethyl)aminometane (molecular biology grade), elastase and trypsin (sequencing grade) and dATP, dCTP, dGTP and dTTP were from *Promega Corporation* (USA). Restriction enzymes *BamH* I and *Xho* I were obtained from *New England BioLabs Inc.* (USA). Nonidet P-40 (NP40) was purchased from *Bio Basic Inc.* (Canada). Acetonitrile (HPLC grade) and kanamycin were obtained from *Roth* (Germany). Bacto™ tryptone, Bacto™ yeast extract and Bacto™ agar were from *Becton, Dickson and company* (USA). Dithiothreitol, ethylenediaminetetraacetic acid disodium salt (EDTA), guanidine hydrochloride, trifluoroacetic acid and zinc(II) chloride were obtained from *Fluka* (Switzerland). Human His_6-E1 enzyme was purchased from Enzo Life Science (Switzerland).

Methods

1. Preparation of the complementary DNA fragments of the *BRCA1* gene (GenBank no. U14860)

1.1 Total RNA isolation

Total RNA from human leukocytes was isolated using QIAamp® RNA Blood Kit (QIAGEN) by mixing 1 volume (1.5 ml) of the whole human blood with 5 volumes (7.5ml) of the hypotonic solution for erythrocyte lysis. The mixture was vortexed briefly 2 times during incubation on ice for 10-15 min, and subsequently centrifuged at 400xg for 10 min at 4 °C. After the supernatant was discarded, the cell pellet mainly containing leukocytes was resuspended with the hypotonic solution (3 ml per 1.5 ml of the whole blood), and centrifuged at 400xg for 10 min at 4 °C for complete removal of erythrocytes. The pelleted leukocytes

was resuspended with the leukocyte lysis buffer (600 µl), and loaded onto the QIAshredder spin column for efficient disruption of the cells and homogenization of the lysate, resulting in the optimal yield and purity. The QIAshredder spin column was centrifuged for 2 min at maximum speed to homogenize. The homogenized lysate was mixed with 70% ethanol (600 µl), and transferred to the QIAamp spin column, which allows RNAs longer than 200 bases to bind to the membrane. Small RNAs such as 5.8S RNA, 5S RNA, and tRNA (approximately 160, 120, and 70-90 nucleotides in length, respectively) do not bind in quantity under the conditions used. The QIAamp spin column was centrifuged at ≥ 8000xg for 15 s, and transferred into a new 2-ml collection tube. The QIAamp spin column was applied with the washing buffer 1, and centrifuged at ≥ 8000xg for 15 s to wash the RNA sample. The QIAamp spin column was placed in a new 2-ml collection tube, added with the washing buffer 2 (500 µl), and centrifuged at ≥ 8000xg for 15 s. After the flow-through was discarded, the washing buffer 2 (500 µl) was added on the QIAamp spin column, and the column was centrifuged at full speed for 3 min. RNase-free water (50 µl) was directly added onto the membrane of the QIAamp spin column, sitting in a new 1.5-ml microcentrifuge tube, and finally centrifuged at ≥8000xg for 1 min to elute total RNA.

1.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolated total RNA was used as the template for complementary DNA (cDNA) synthesis, using QIAGEN OneStep RT-PCR Kit®. The combination of the Omniscript and Sensiscript reverse transcriptases was included in the enzyme mix, which provides the highly efficient and specific reverse transcription for the first strand cDNA synthesis from RNA templates with as little amount as 1 pg. The second strand cDNA synthesis was obtained by the function of HotStarTaq DNA polymerase. The reactions were heated at 95 °C for 15 min to activate the DNA polymerase, and simultaneously to inactivate the reverse transcriptase. The reaction mixture was prepared by mixing template RNA (<2 µg/reaction), gene-specific primers for the cDNA synthesis of the *BRCA1* gene fragment (nucleotides 1-912, Fig. 6) (forward: 5′-ATGGATTTATCTGCTCTTCGC-3′, reverse: 5′-GAATTCAGCCTTTTCTAC-3′), dNTP mix, QIAGEN OneStep RT-PCR buffer, and QIAGEN OneStep RT-PCR enzyme (Table 2). Reactions were performed on a thermal cycler, according to the thermal cycling conditions (Table 3).

```
1     ATGGATTTAT CTGCTCTTCG CGTTGAAGAA GTACAAAATG TCATTAATGC TATGCAGAAA
61    ATCTTAGAGT GTCCCATCTG TCTGGAGTTG ATCAAGGAAC CTGTCTCCAC AAAGTGTGAC
121   CACATATTTT GCAAATTTTG CATGCTGAAA CTTCTCAACC AGAAGAAAGG GCCTTCACAG
181   TGTCCTTTAT GTAAGAATGA TATAACCAAA AGGAGCCTAC AAGAAAGTAC GAGATTTAGT
241   CAACTTGTTG AAGAGCTATT GAAAATCATT TGTGCTTTTC AGCTTGACAC AGGTTTGGAG
301   TATGCAAACA GCTATAATTT TGCAAAAAAG GAAAATAACT CTCCTGAACA TCTAAAAGAT
361   GAAGTTTCTA TCATCCAAAG TATGGGCTAC AGAAACCGTG CCAAAAGACT TCTACAGAGT
421   GAACCCGAAA ATCCTTCCTT GCAGGAAACC AGTCTCAGTG TCCAACTCTC TAACCTTGGA
481   ACTGTGAGAA CTCTGAGGAC AAAGCAGCGG ATACAACCTC AAAAGACGTC TGTCTACATT
541   GAATTGGGAT CTGATTCTTC TGAAGATACC GTTAATAAGG CAACTTATTG CAGTGTGGGA
601   GATCAAGAAT TGTTACAAAT CACCCCTCAA GGAACCAGGG ATGAAATCAG TTTGGATTCT
661   GCAAAAAAGG CTGCTTGTGA ATTTTCTGAG ACGGATGTAA CAAATACTGA ACATCATCAA
721   CCCAGTAATA ATGATTTGAA CACCACTGAG AAGCGTGCAG CTGAGAGGCA TCCAGAAAAG
781   TATCAGGGTA GTTCTGTTTC AAACTTGCAT GTGGAGCCAT GTGGCACAAA TACTCATGCC
841   AGCTCATTAC AGCATGAGAA CAGCAGTTTA TTACTCACTA AAGACAGAAT GAATGTAGAA
901   AAGGCTGAAT TC
```

Figure 6. Nucleotide sequence of the *BRCA1* gene fragment (nucleotide 1-912).

Table 2. Reaction components for the One-step RT-PCR.

Components	Volume (µl)	Final concentration
RNase-free water	27	-
5x QIAGEN One-step RT-PCR buffer	10	1x
dNTP mix (10 mM of each dNTP)	2	400 µM of each
10 µM forward primer	3	0.6 µM
10 µM reverse primer	3	0.6 µM
QIAGEN One-step RT-PCR enzyme mix	2	-
Template RNA	3	1 pg -2 µg / reaction
Total volume	50	-

Table 3. Thermal cycling conditions for the One-step RT-PCR.

Reverse transcription	50 °C, 30 min
Initial PCR activation	95 °C, 15 min
3-step cycling denaturation annealing extension	 94 °C, 30 s 55 °C, 45 s 72 °C, 1 min
Number of cycles	30 cycles
Final extension	72 °C, 10 min

2. DNA amplification of the *BRCA1*, *BARD1*, *ubiquitin*, and *UbcH5c* genes

The *BRCA1* gene fragments, containing the nucleotides 1-912, were amplified by the polymerase chain reaction (PCR) using the *BRCA1* cDNA as the templates. The *BARD1* gene fragment (nucleotides 76-981), and the full-length *ubiquitin* (*Ub*) and *UbcH5c* genes were amplified by PCR using the Addgene plasmids 12646, 12647, and 12643 as the templates, respectively. The reaction mixtures were prepared by mixing template DNA, primer solutions (Table 4), dNTP mix, 10X ProofStart PCR buffer, and water followed by the addition of the ProofStart DNA polymease (QIAGEN) to the individual PCR tubes. The reactions (Table 5) were performed on a thermal cycler, according to the thermal cycling conditions (Table 6). The PCR products were electrophoresed on 1% agarose gel, and then extracted by QIAquick® gel extraction kit (QIAGEN).

Table 4. The oligonucleotide primers for the amplifications of the *BRCA1*, *BARD1*, *ubiquitin* (*ub*), and *UbcH5c* genes.

Construct name	Protein residue	Primer	Direction (5′ → 3′)
BRCA1 (1-304-wt)	1-304	Forward	GACACGCGGATCCATGGATTTATCTGCTCTTCG
		Reverse	GACACCGCTCGAGTCAGAATTCAGCCTTTTCTACATTC
BARD1 (26-327)	26-327	Forward	GACACGCGGATCCATGGAACCGGATGGTCGCGG
		Reverse	GACACCGCTCGAGTCATCTATTGTGATGGCCACGTTTTC
Ubiquitin (Ub)	1-76	Forward	GACACGCGGATCCATGCAGATCTTCGTCAAGAC
		Reverse	GACACCGGCGGCCGCTCAACCACCTCTTAGTCTTAAG
UbcH5c	1-147	Forward	GACACGCGGATCCATGGCGCTGAAACGGATTAATAAG
		reverse	GACACCGCTCGAGTCACATGGCATACTTCTGAG

Table 5. Reaction components for PCR amplification.

Components	Volume (µl)	Final concentration
Double distilled water	variable	-
10X ProofStart PCR buffer	10	1x
dNTP mix (10 mM of each dNTP)	3	300 µM of each
10 µM forward primer	10	1 µM
10 µM reverse primer	10	1 µM
ProofStart DNA polymerase	2	5 units
Template DNA	variable	100 ng - 1 µg / reaction
Total volume	100	-

Table 6. Thermal cycling conditions for PCR.

Initial denauration	95 °C, 5 min
3-step cycling	
denaturation	95 °C, 30 s
annealing	55-63 °C, 45 s
Extension	72°C, 1 min
Number of cycles	35 cycles
Final extension	72 °C, 10 min

3. Extraction and purification of the PCR products

The PCR products (from 2.) were electrophoresed on 1% agarose gel. DNA fragments were detected by staining with ethidium bromide, and visualizing by illumination with 300 nm UV light. Targeted DNA fragments were sliced from the agarose gel with a clean and sharp scalpel, and extracted by QIAquick® gel extraction kit (QIAGEN). Three volumes of the solubilizing buffer were added to a volume of gel (100 mg ~ 100 µl) to dissolve the agarose gel slice, and to provide the appropriate conditions (high salt and pH ≤ 7.5) for binding of DNA to the silica gel membrane while the contaminants pass through the column. The mixture was incubated at 50 °C for 10 min, then loaded onto a QIAquick spin column, and centrifuged at 10000xg for 1 min. The flow-through was discarded, and the washing

buffer (750 μl) was added to the QIAquick spin column that was subsequently centrifuged at 10000xg for 1 min. The flow-through was discarded, and the QIAquick spin column was additionally centrifuged at 10000xg for 1 min to completely eliminate the washing buffer. Water (50 μl) was directly added onto the membrane of the QIAquick spin column, sitting in a new 1.5-ml microcentrifuge tube, and finally centrifuged at full speed for 1 min to elute purified DNA.

4. Plasmid constructions

The BRCA1 protein amino acid residues 1-304 (the *BRCA1* gene nucleotides 1-912), and those of the BARD1 protein amino acid residues 26-327 (the *BARD1* gene nucleotides 76-981) were produced as a GST fusion by cloning the respective genes into the *Bam*HI/*Xho*I sites of pGEX-4T1 (Amersham Biosciences)(Fig. 7). Full-length ubiquitin (*Ub*) and *UbcH5c* genes were inserted into the *Bam*HI/*Not*I and the *Bam*HI/*Xho*I sites, respectively, of the pET28a(+) derivative for expression of His_6-tagged proteins. The digestion reactions (50 μl) contained 5 μl of 10x enzyme buffer, 0.5 μl of 100x BSA, DNA fragments or 10 μg of plasmids (pGEX4T1 and pET28a(+) derivative), 1 μl of each endonuclease (20 units), and water. The reactions were then incubated at 37 °C for 6 h, and subsequently electrophoresed on 1% agarose gel. The digested DNA fragments were purified using QIAquick® gel extraction kit. The digested DNA fragments were ligated into the respective endonuclease sites of the plasmids by T4 DNA ligase. The ligated constructs were transformed to *E. coli* DH5α by mixing the ligation reactions with 100 μl of competent cells, and then the cells were incubated on ice for 30 min. The cells were shocked at 42 °C for 90 s, and immediately placed on ice for 5 min. LB broth (800 μl) was added to the cells, and further incubated at 37°C for 1 h. The cells were centrifuged at 5000xg for 1 min. The medium was discarded, and the cell pellet was resuspended in 100 μl of the fresh LB broth. The cells were grown on LB agar, containing 100 μg/ml ampicillin (for pGEX-4T1) or 50 μg/ml kanamycin [for pET28a(+) derivative] for clone selection. All Recombinant plasmids were isolated, and characterized by restriction analysis and DNA sequencing.

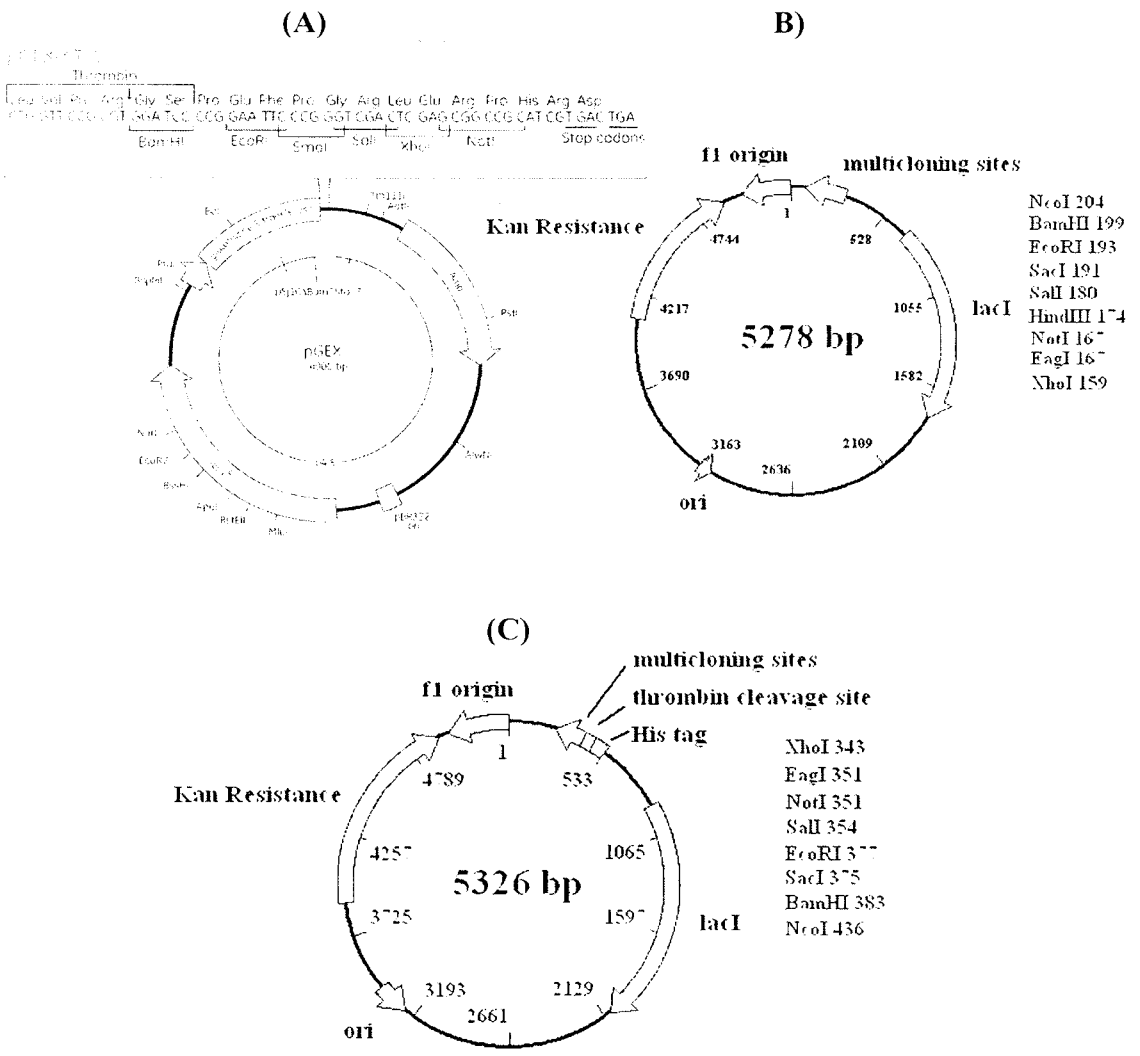


Figure 7. Schematic representations of the bacterial expression plasmids. (A) pGEX-4T1, (B) pET28a(+) derivative without the His_6 fusion tag, and (C) pET28a(+) derivatives with the His_6 fusion tag.

5. Site-directed mutagenesis of the BRCA1 RING domain

The missense mutations (D67A, D67E, D67Y, and C61G) were introduced to the BRCA1 RING domain using the QuikChange® site-directed mutagenesis kit (Stratagene). The basic procedure utilized a supercoiled double stranded DNA plasmid with an insert of interest [pGEX-BRCA1(1-304)], and two synthetic oligonucleotide primers containing the desired mutation. The primers (Table 7), each complementary to the opposite strands of the plasmid,

are extended during temperature cycling by using *PfuTurbo*® DNA polymerase that replicates both plasmid strands with high fidelity (Tables 8 and 9). Incorporation of the primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the PCR products were treated with *Dpn* I. The *Dpn* I endonuclease is specific for the methylated and hemimethylated DNA at the target sequence (5′-Gm^{6}ATC-3′), and thus is used to digest the parental DNA template for selecting the synthesized mutant DNA. DNA isolated from almost all *E. coli* strains is dam-methylated, and therefore susceptible to *Dpn* I digestion. The nicked plasmid DNA, incorporating the desired mutations, is then transformed into *E. coli* XL1-Blue supercompetent cells that can repair the nicks. Briefly, 1 μl of the *Dpn* I-treated DNA was mixed with 50 μl of the supercompetant cells. The transformation reactions were heated at 42 °C for 45 s, and then placed on ice for 2 min. After adding 0.5 ml of NZY^{+} broth preheated to 42 °C, the reactions were incubated at 37 °C for 1 h with shaking. The reactions (250 μl) were poured on the LB-kanamycin agar plates, and then the transformation plates were incubated at 37 °C for 16 h. The desired mutant plasmids were isolated, and verified by DNA sequencing.

Table 7. The oligonucleotide primers for site-directed mutagenesis.

Construct	Primer	Primer design (5′ → 3′)
BRCA1-D67A	forward	GTCCTTTATGTAAGAATGCTATAACCAAAAGGAGCCTACA
	reverse	TGTAGGCTCCTTTTGGTTATAGCATTCTTACATAAAGGAC
BRCA1-D67E	forward	GTCCTTTATGTAAGAATGAGATAACCAAAAGGAGCCTACA
	reverse	TGTAGGCTCCTTTTGGTTATCTCATTCTTACATAAAGGAC
BRCA1-D67Y	forward	CCTTTATGTAAGAATTATATAACCAAAAGGAG
	reverse	CTCCTTTTGGTTATATAATTCTTACATAAAGG
BRCA1-C61G	forward	GAAAGGGCCTTCACAGGGTCCTTTATGTAAGAATG
	reverse	CATTCTTACATAAAGGACCCTGTGAAGGCCCTTTC

Table 8. The sample reaction for site-directed mutagenesis.

Components	Volume (µl)
10× reaction buffer	5
dsDNA template (5-50 ng)	0.5
oligonucleotide primer #1 (125 ng)	1.25
oligonucleotide primer #2 (125 ng)	1.25
dNTP mix	1
Quik Solution reagent	1.5
PfuTurbo DNA polymerase (2.5 U/µl)	1
ddH_2O	to 51

Table 9. Thermal cycling condition for site-directed mutagenesis.

Segment	Cycles	Temperature	Time
1	1	95 °C	30 sec
2	18	95 °C	30 sec
		55 °C	1 min
		68 °C	1 min/kb of plasmid length

6. Verification of the recombinant plasmids by automated DNA sequencing

The inserted *BRCA1*, *BARD1*, *ubiquitin*, and *UbcH5c* DNA fragments were verified by DNA sequencing using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem). The reaction mixtures consisted of Big dye Terminator ready reaction mix (8 µl), double strand DNA template (200-500 ng), and primer (3.2 pmol) in 20 µl of total volume, and were placed in a thermal cycler, and run for 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min with an initial denaturation at 96 °C for 1 min. The unincorporated Big Dye Terminator was removed by the addition of 75% isopropanol (80 µl) that was subsequently left at room temperature for 15 min. The mixtures were spun at 14000xg for 20 min at room temperature, and the supernatant was discarded. Ethanol (70%) (250 µl) was added, and the samples were spun at 14000xg for 5 min. After the supernatant was discarded, the samples were dried in a heat-block at 90 °C for 1 min. The samples were

resuspended in 6 μl of the loading dye (deionized formamide and Blue dextran at the ratio of 1:5), heated at 90 °C for 2 min, and then placed on ice immediately. One microliter of each sample was loaded into a separate lane of polyacrylamide gel, and run in a DNA sequencer. The sequence data were aligned to *BRCA1* (GenBank no. U14860), *BARD1* (GenBank no. NM000465), *ubiquitin* (GenBank no. NM021009), and *UbcH5c* (GenBank no. U39318) databases.

7. Expression and purification of the recombinant proteins

All recombinant plasmids were transformed into *E. coli* BL21(DE3) for productions of the proteins. Transformed bacterial cells were grown in 1000 ml of Luria Broth medium, containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml) with shaking at 37 °C. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM to induce the expression when the $A_{600\ nm}$ of the culture reached 0.5-0.6. Cells were allowed to grow for 12 h at 25 °C after the induction, and harvested by centrifugation. Cell pellets were resuspended in 20 ml of lysis buffer of 50 mM Tris (pH 7.4), 50 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% NP-40, 10 mM β-merceptoethanol, and 1 mM PMSF, and then lysed by sonication (10 min with 60% amplitude, 9 s pulse on, and 4 s pulse off). GST-tagged proteins were freshly prepared using a glutathione-agarose column (Amersham Biosciences) by loading the soluble portions of cell lysates into the glutathione-agarose GSTrap column (1 ml) with the slow rate of 0.3 ml/min. The bound proteins were washed with a 10 fold column volumn of the washing buffer [50 mM Tris (pH 7.4), 50 mM NaCl, and 10 mM β-merceptoethanol), and eluted with a buffer, containing 50 mM Tris (pH 7.4), 10 mM β-mercaptoethanol, and 20 mM reduced glutathione. The purified GST-tagged protein was extensively dialyzed at 4 °C against deionized water. His_6-tagged proteins (His_6-Ub and His_6-UbcH5c) were purified using 1 ml of Ni^{2+}-NTA beads (QIAGEN) by which the bound proteins were first washed with a 10 fold column volumn of the binding buffer [50 mM Tris (pH 7.4), 50 mM NaCl, and 10 mM imidazole] before being eluted with the binding buffer containing 300 mM imidazole. Purified His_6-Ub and His_6-UbcH5c proteins were then dialyzed at 4 °C against a buffer, containing 50 mM Tris (pH 7.0), 10 mM β-mercaptoethanol, and 10% glycerol. The amount of proteins was quantitated by the Bradford assay, using BSA as a standard.

8. Preparation of the platinated BRCA1

Cisplatin was prepared as a stock solution (1 mM) in deionized water. Purified wild-type and mutant BRCA1 RING domain proteins (1.67 μM) were mixed with cisplatin at concentration of between 0-100 μM. The reaction mixtures were incubated at 4 °C in the dark for 24 h, and subjected to extensive ultrafiltration using Macrosep centrifugal devices (Pall Life Sciences) to remove any unbound platinum. The amount of protein was then carefully determined by the Bradford assay, using BSA as a standard.

9. *In vitro* ubiquitin ligase activity assay

The ubiquitin ligase reactions (20 μl) contained 20 μM Ub, 300 nM E1, 5 μM E2/UbcH5c, 2 μg mutant BRCA1 or mutant BRCA1 adducts and 2 μg BARD1 in a buffer, containing 50 mM Tris (pH 7.5), 0.5 mM DTT, 5 mM ATP, 2.5 mM $MgCl_2$, and 5 μM $ZnCl_2$. Two separate reactions were incubated at 37 °C for 3 h, and then terminated by adding an equal volume of SDS-loading dye before electrophoresis on 8% SDS-PAGE and visualization of the protein bands using silver-staining. The relative E3 ligase activity of the mutants and their platinated BRCA1s was quantified by normalizing the density of an apparent band of the ubiquitinated-protein conjugates to that of the control untreated BRCA1, using a Bio-Rad GS-700 imaging densitometer.

10. Circular dichroism

Wild-type and mutant BRCA1 RING proteins (10 μM) were prepared in 10 mM cacodylate buffer pH 6.8. Metal-dependent folding of the protein was monitored by acquiring CD spectra over a range of 200-260 nm, using a Jasco J720 spectropolarimeter (Japan Spectroscopic Co. Ltd.). Measurements of metal-protein interaction were carried out at 20 °C using a 0.1 cm quartz cuvette. The spectrum was the average of five separate spectra with a step size of 0.1 nm, a 2 s response time, and a 1 nm bandwidth. The data were baseline-corrected by subtraction of cacodylate buffer. The effects of metal-binding on protein stability were determined in the presence of three molar equivalent ratios of Zn^{2+} to protein. CD experiments were performed in three separate scans in the range from 25 to 95 °C at 208 nm with a heat rate of 1 °C/min. Thermal renaturation (25 °C after being heated at 95 °C) was

also observed after the same length of time course for denaturation. The binding constant was determined by

$$\theta_{obs} = \theta_{max}[(1+(KC/N)+KP)/(2KP) - \sqrt{(((1+(KC/N)+KP)/(2KP))^2 - C/(NP))}]$$

in which θ_{obs} is the observed ellipticity change at any concentration of metal, θ_{max} is the ellipticity change when all of the proteins bind metal, K is the binding constant, P is the protein concentration, C is the concentration of metal added, and N is the number of binding sites (Engel, 1974). The free energy of binding was given by

$$\Delta G = -RT \ln K$$

in which ΔG is the free energy, R is the gas constant of 1.987 cal mol^{-1}, T is the temperature in Kelvin, and K is the binding constant.

RESULTS

Protein purification

All recombinant plasmids were verified by DNA sequencing, and transformed into *Escherichia coli* BL21(DE3) for production of the protein. GST-tagged proteins were freshly prepared using a glutathione-agarose column (Fig. 8).

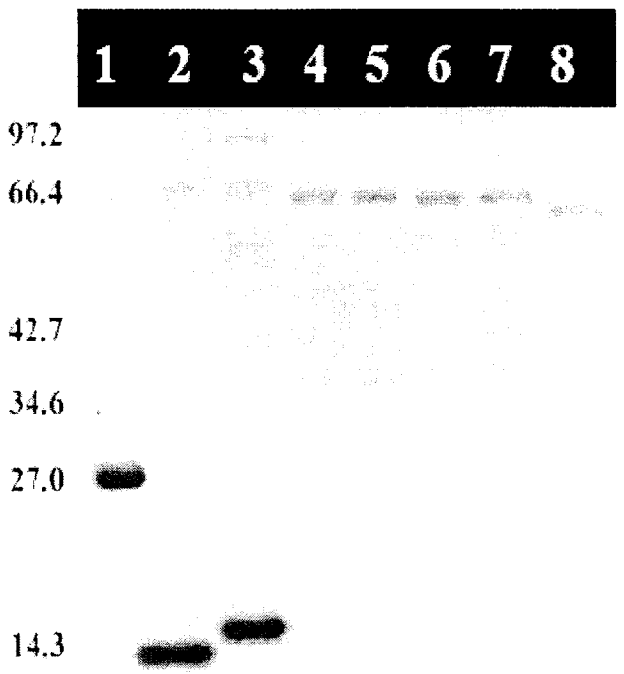


Figure 8. Expression and purification of the mutant and wild-type BRCA1(1-304) RING proteins. Transformed *E. coli* BL21(DE3) cells were induced by 0.5 mM IPTG, and analyzed by 15% SDS-PAGE with Coomassie blue staining. Affinity purification of glutathione *S*-transferase (GST)(*Lane 1*), His_6-ubiquitin (Ub)(*Lane 2*), His_6-UbcH5c (*Lane 3*), GST-BRCA1(1-304)^{wt} (*Lane 4*), GST-BRCA1(1-304)^{D67E} (*Lane 5*), GST-BRCA1(1-304)^{D67Y} (*Lane 6*), GST-BRCA1(1-304)^{D67A} (*Lane 7*), and GST-BARD1(26-327) (*Lane 8*). Proteins (2 μg) that were used for the in vitro ubiquitin ligase assay, was identified by 12% SDS-PAGE with Coomassie blue staining. The molecular mass marker (kDa) was positioned.

Time-dependence of ubiquitination reaction

To study whether the in vitro ubiquitination is time-dependent, ubiquitin ligase reactions, containing 20 μM His_6-Ub, 300 nM His_6-E1, 5 μM His_6-UbcH5c, 2 μg GST-BRCA1(1-304), and 2 μg GST-BARD1(26-327), were incubated at various time intervals (0, 1, 2, and 3 h) at 37 °C. The result revealed that the optimal reaction incubation of 3 h apparently showed the ubiquitinated products (Fig. 9).

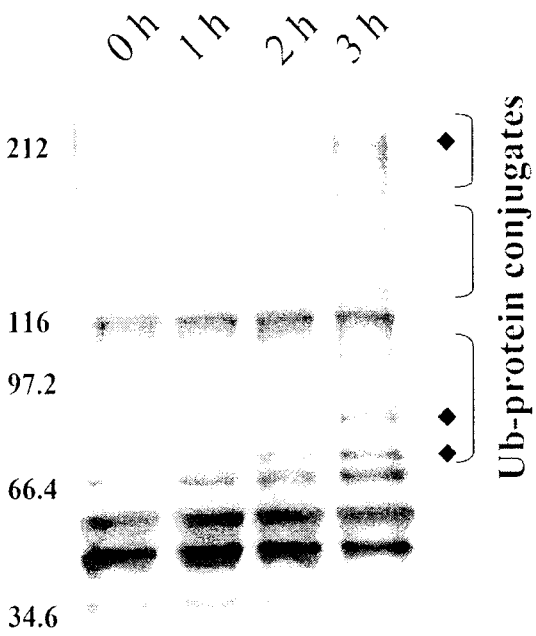


Figure 9. Time-dependence of ubiquitination reaction. Ubiquitin ligase reactions, containing 20 μM His_6-Ub, 300 nM His_6-E1, 5 μM His_6-UbcH5c, 2 μg GST-BRCA1(1-304), and 2 μg GST-BARD1(26-327), were incubated at various time intervals (0, 1, 2, and 3 h) at 37 °C. The samples were terminated by adding an equal volume of SDS-loading dye, and heating at 95 °C for 5 min. The samples were then resolved on 8% silver-stained SDS-PAGE.

D67E BRCA1 and D67A BRCA1 retain ubiquitin ligase activity

BRCA1 has been shown to form a stable heterodimeric complex with BARD1 through their RING domains (Brzovic et al., 2001). This complex showed an enzymatic activity of E3 ubiquitin ligase, producing high molecular weight ubiquitinated products. GST protein as a negative control did not contribute to the activity. The familial, deleterious C61G mutation of

the second Zn^{2+}-binding residue in BRCA1 that was used as a negative control completely abolished the ubiquitin ligase activity. However, the familial D67E and the designed D67A BRCA1 mutations maintained the ubiquitination activity identically to the wild-type protein, whereas the D67Y mutation partially abrogated this activity (Fig. 10 and 11).

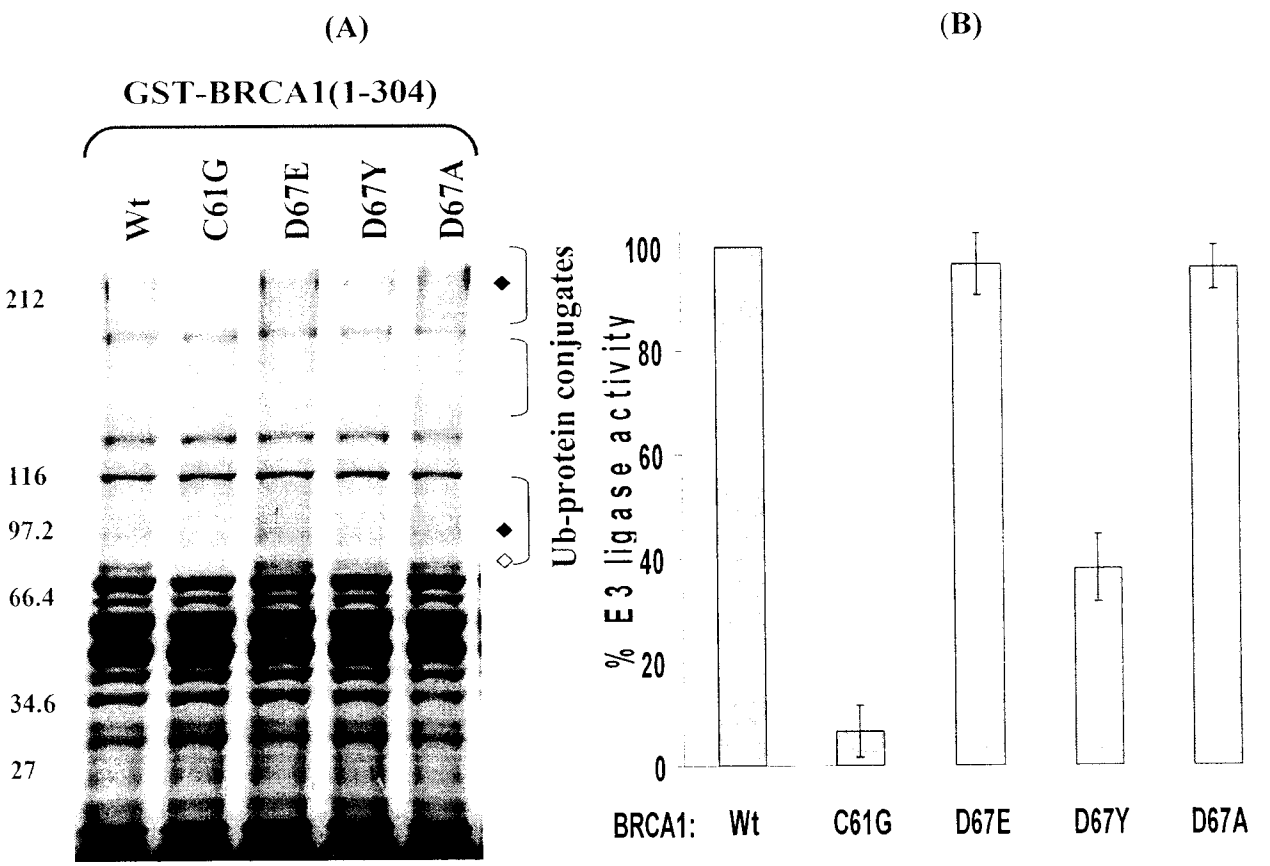


Figure 10. (A) *In vitro* ubiquitin ligase activity of the mutant BRCA1 RING proteins. The mutant BRCA1 proteins (C61G, D67E, D67Y, and D67A) were assayed for the ubiquitin ligase activitiy compared to the wild-type protein. Ubiquitin ligase reactions, containing 20 μM His_6-Ub, 300 nM His_6-E1, 5 μM His_6-UbcH5c, 2 μg GST-BRCA1(1-304), and 2 μg GST-BARD1(26-327), were incubated at 37 °C for 3 h. The reactions were terminated by adding an equal volume of SDS-loading dye, and heating at 95°C for 5 min. The samples were then resolved on 8% silver-stained SDS-PAGE. (B) An apparent ubiquitinated product as indicated by the open diamond (◊) from the two separate reactions was quantified by Bio-Rad GS-700 Imaging Densitometer. The relative E3 ligase activity (%) was plotted against each BRCA1 RING mutant protein.

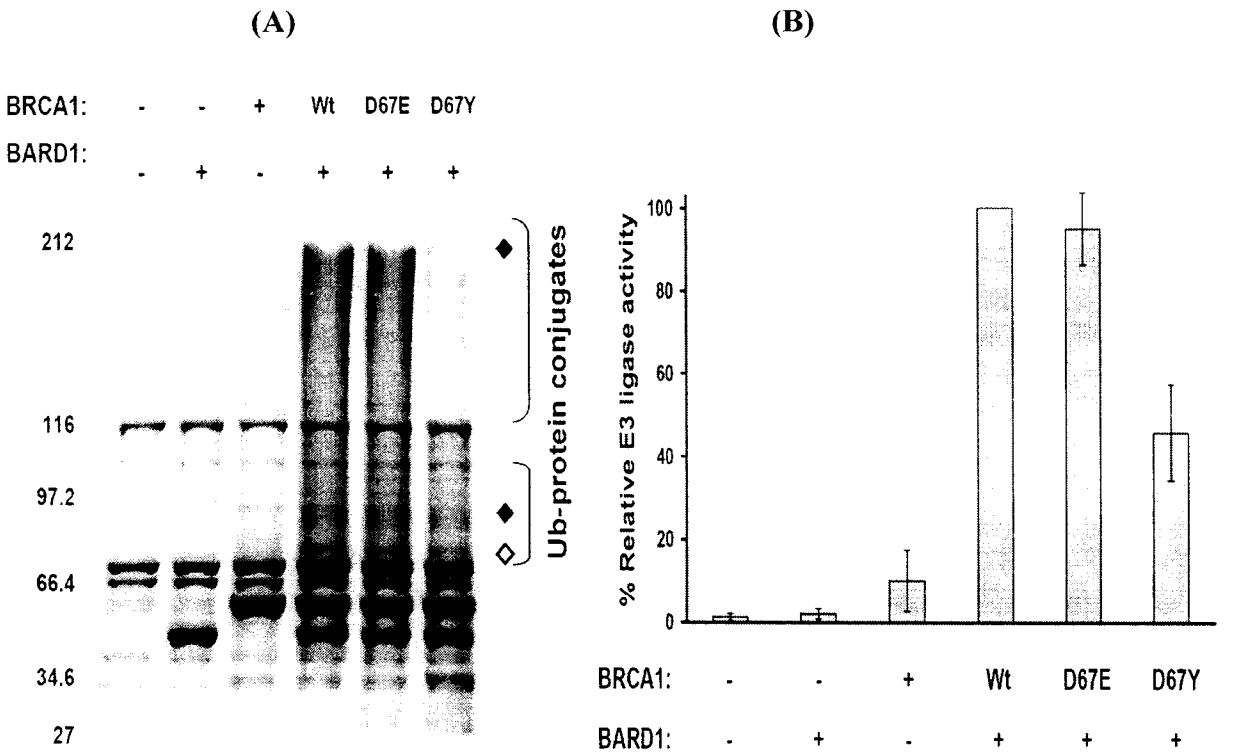
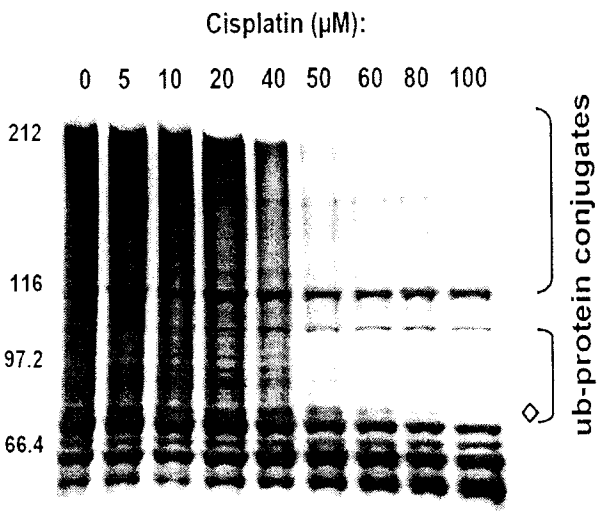


Figure 11. (A) *In vitro* ubiquitin ligase activity of the mutant BRCA1 RING proteins. The mutant D67E and D67Y BRCA1 RING domain proteins were assayed for ubiquitin ligase activity for comparison to the wild-type protein. Complete reaction mixtures, containing 20 μM Ub, 300 nM E1, 5 μM UbcH5c, 2 μg BRCA1 (residues 1-304) and 2 μg BARD1 (residues 26-327), were incubated at 37 °C for 3 h. Reactions were terminated by adding an equal volume of SDS-loading dye and heating at 95°C for 5 min before resolving by 8% SDS-PAGE and staining with silver. The ubiquitinated products are indicated by a diamond. (B) An ubiquitinated product as indicated by the open diamond (◊) in (A) and the two separate reactions were quantified by a Bio-Rad GS-700 imaging densitometer. The relative E3 ligase activity is shown for each BRCA1 RING variant.

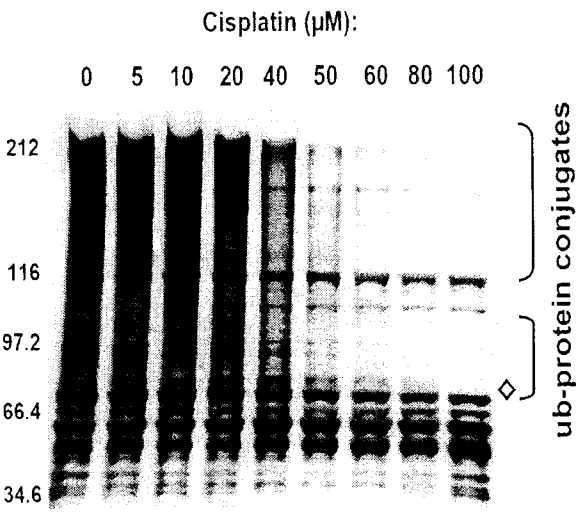
Due to completely loss of E3 ligase activity, the C61G BRCA1 was, therefore, not assayed for cisplatin sensitivity *in vitro* ubiquitination reaction. In addition, the designed D67A BRCA1 that retained *in vitro* ubiquitin ligase activity, similar to the D67E BRCA1, was not further evaluated for drug sensitivity. Interesting the D67Y BRCA1 exhibited a partially defective E3 ubiquitin ligase activity. Consequently, the D67Y BRCA1 was further assayed for chemotherapeutic response in comparison with the D67E BRCA1 and the wild-

type BRCA1 protein. The wild-type and mutant proteins were treated with cisplatin *in vitro* at a number of concentrations between 0-100 μM. The BRCA1 E3 ligase function was inactivated in a platinum concentration-dependent manner (Fig. 12). However, the D67E BRCA1 had an identical response to the drug, when compared with the wild-type protein, with an effective concentration of 100 μM that completely inhibited the activity (Fig. 12a and 12b). It was consistent with our previous result that the D67E mutation barely affected the native structure and function of the protein. Surprisingly, the D67Y BRCA1 that was a partially defective E3 ligase showed a promising outcome with an effective dose of 50 μM (Fig. 12c). The IC_{50} value for the E3 ligase activity was approximately 60 μM for the wild-type and mutant D67E BRCA1, and 32 μM for the D67Y BRCA1 RING domain proteins, respectively (Fig. 12d).

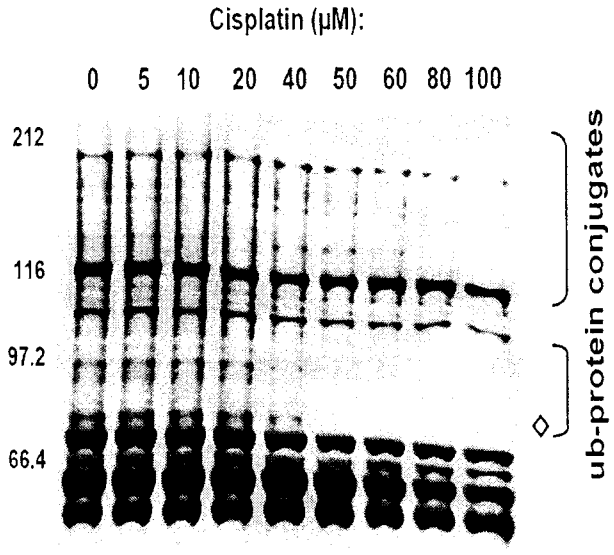
(A)



(B)



(C)



(D)

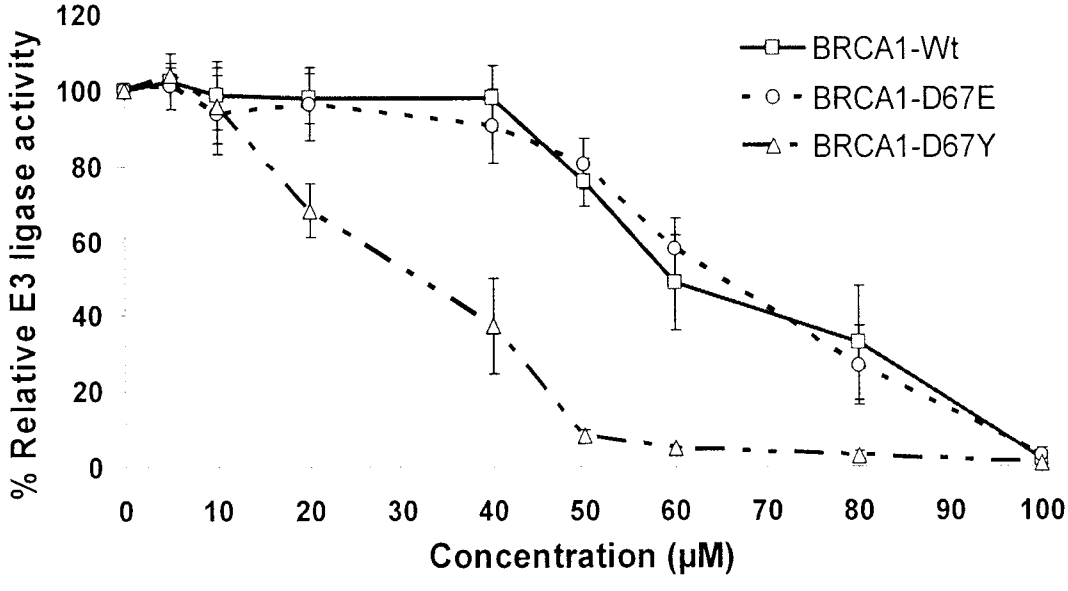


Figure 12. In vitro E3 ubiquitin ligase activity of the cisplatin-BRCA1 adducts. Two μg of the wild-type (A), D67E (B) or D67Y (C) BRCA1 RING domain protein was incubated with a number of cisplatin concentrations between 0-100 μM, and assayed for the ubiquitin ligase activity. An apparent ubiquitinated product (as indicated by the open diamond) in gels was markedly reduced as the cisplatin concentration increased. The relative E3 ligase activity of BRCA1 adducts (%) was plotted as a function of the cisplatin concentrations (D).

Cisplatin binding to mutant BRCA1 and protein conformation

The secondary structure of the wild-type BRCA1 underwent more folded structural rearrangement after increasing cisplatin concentrations as judged by an increase in negative CD spectra at 208 and 220 nm (Fig. 13). It was possible that cisplatin might bind to the unoccupied Zn^{2+}-binding sites and cause the structural changes, using the CONTIN program, from 19% α-helix, 30% β-sheets, 16% turn, and 35% disordered element to 24% α-helix, 25% β-sheets, 15% turn, and 35% disordered element. The binding constant of the *in vitro* platination was 4.85±0.23 x 10^4 M^{-1}, and the free energy of binding (ΔG) was 1.79 kcal mol^{-1}.

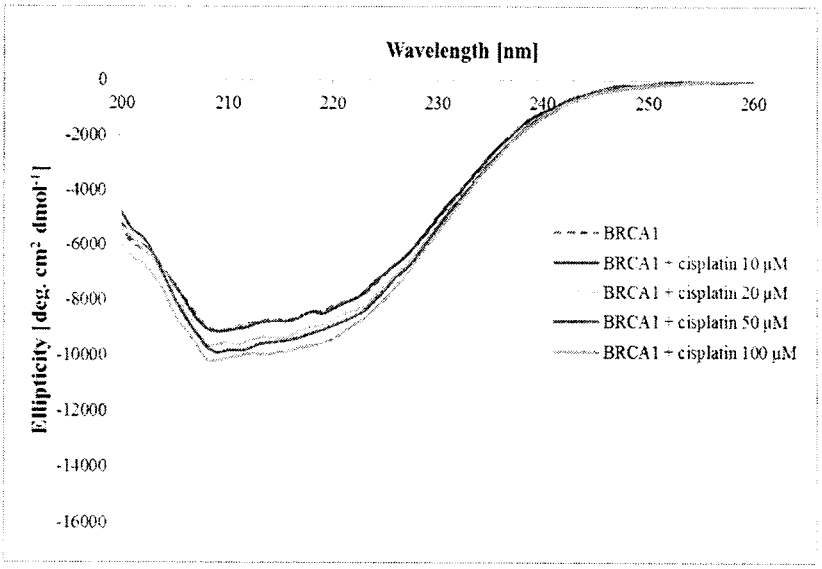


Figure 13. The CD spectra of the wild-type BRCA1 upon platination. Ten micromolar of BRCA1 (1-304) protein was pre-incubated with 30 μM of $ZnCl_2$ at 4°C for 8 h. Samples were then incubated with cisplatin in the dark at ambient temperature for 16 h before CD measurement at 25 °C with the scanning rate 50 nm/min. The mean residues ellipticity and wavelength ranging from 200 to 260 nm were plotted.

The mutant D67E BRCA1 and D67Y BRCA1 also exhibited a CD spectral change upon platination by cisplatin (Fig. 14A and 14B). With use of the CONTIN program, the secondary structures of both mutant proteins were predicted (Greenfield 2006; Provencher & Glöckner, 1981). Structural changes were observed with 18% and 8% α-helices, 32% and 43 % β-sheets, 11% and 18% β-turns, and 39% and 31% disordered elements for D67E BRCA1

and D67Y BRCA1, respectively. This indicated that cisplatin perturbed the structure of mutant BRCA1 proteins compared with the wild-type BRCA1 protein. The binding constants of the *in vitro* platination for D67E and D67Y BRCA1 were 6.11±0.44 x 10^5 M^{-1} and 2.45±0.45 x 10^5 M^{-1}, and the free energy of binding (ΔG) were 0.29 and 0.83 kcal mol^{-1}, respectively.

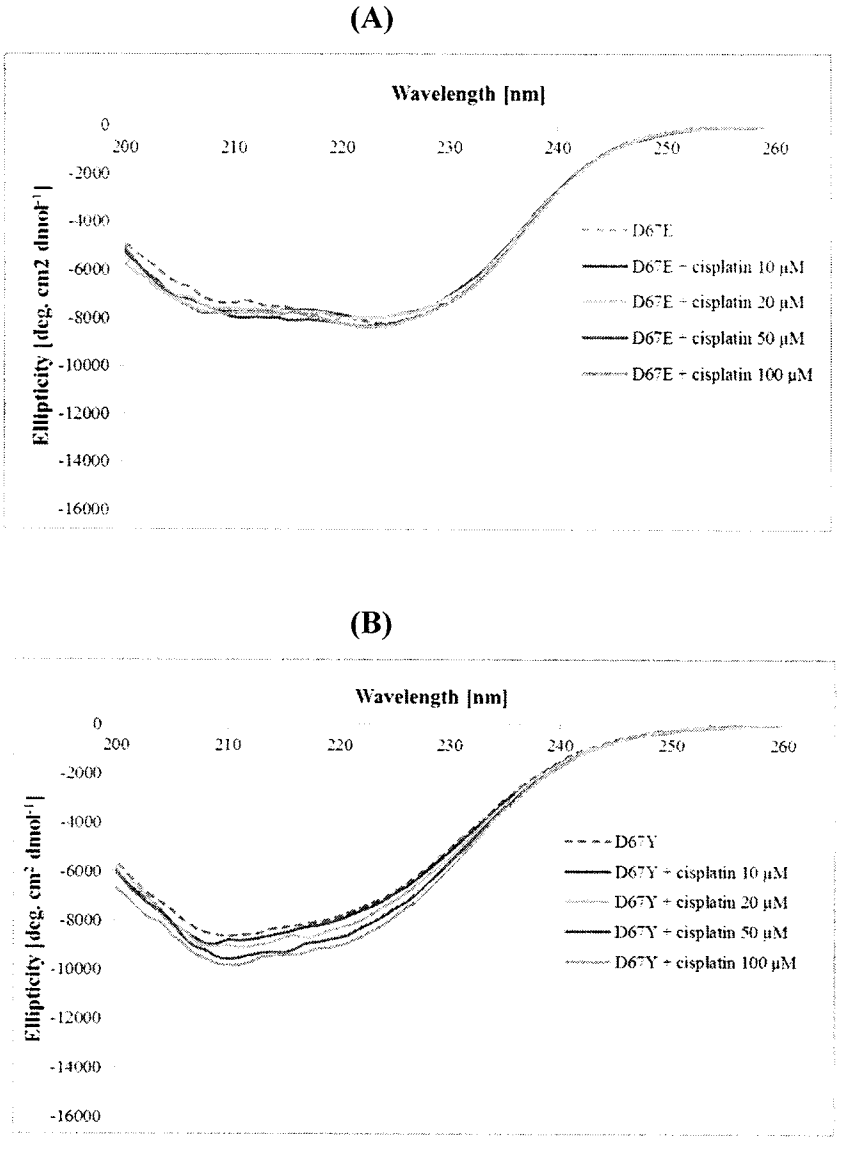
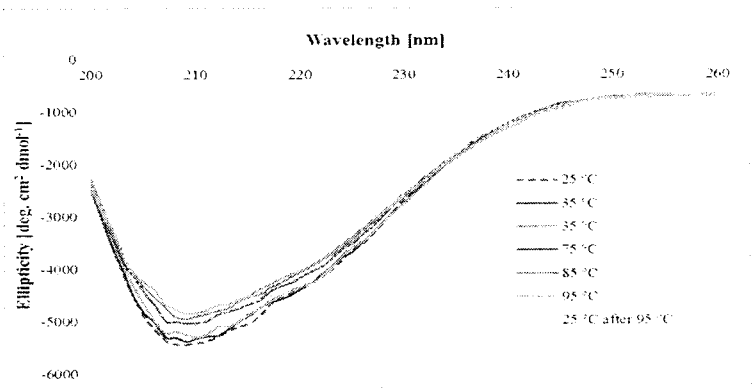


Figure 14. The CD spectra of the cisplatin-mutant BRCA1 adducts. Ten µM of D67E (A) or D67Y (B) protein was pre-incubated with 30 µM of $ZnCl_2$ at 4 °C for 8 h. Samples were then further incubated with various concentration of cisplatin in the dark at ambient temperature for 16 h before CD measurement at 25 °C with the scanning rate 50 nm/min. The mean residues ellipticity and wavelength ranging from 200 to 260 nm were plotted.

Thermal stability of cisplatin-modified mutant BRCA1

Mutant BRCA1 proteins were pre-incubated with Zn^{2+}, and then further incubated with cisplatin. CD spectra of the mutant proteins showed similar changes with an increase in ellipticity when the temperature was raised from 25 °C to 95 °C (Fig. 15). This indicated that the folded proteins gradually lost the contents of the ordered structures. When cooling to 25 °C after being heated at 95 °C, CD spectrum was partially recovered, indicating an incomplete reversibility of the unfolding/refolding process. The irreversibility was probably caused by the aggregation of the heat-unfolded protein.

(A)



(B)

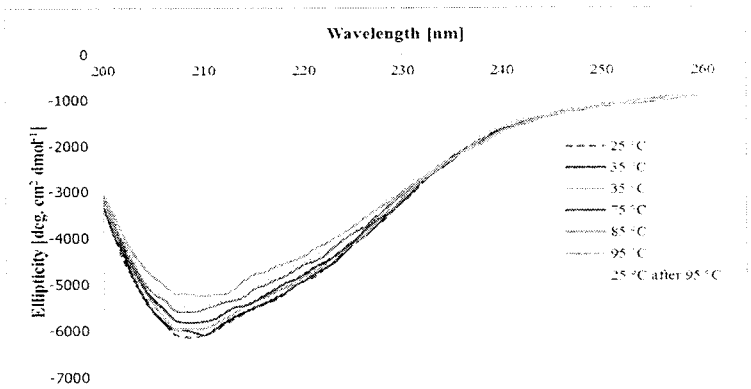


Figure 15. Thermal transition of cisplatin-mutant BRCA1 adducts; D67E (A) and D67Y (B). Ten μM of each mutant BRCA1 protein was pre-incubated with 30 μM of $ZnCl_2$ at 4°C for 8 h. Samples were incubated with 50 μM of cisplatin in the dark at ambient temperature for 16 h. The measurements were performed from 25 °C to 95 °C. After heating at 95 °C, CD measurement at 25 °C was performed. The CD spectra were plotted between mean residues ellipticity and wavelength.

DISCUSSION

The BRCA1 and BARD1 RING domains preferentially form a stable heterodimeric complex through an extensive four-helix-bundle interface (Brzovic et al., 2001; Brzovic et al., 2003). This interaction provides the proper contact surface on BRCA1 in the first and second Zn^{2+} binding loops and in the central helix of the RING for binding E2/UbcH5c. This RING heterodimer BRCA1-BARD1 contained the E3 ubiquitin ligase activity, that promoted the formation of high molecular weight polyubiquitin species, that was obviously greater than those produced by the individual BRCA1 or BARD1 RING domains (Fig. 11a and 11b)(Mallery et al., 2002; Morris et al., 2006). The familial D67E BRCA1 mutation still maintained the E3 ligase activity that was identical to the wild-type protein. Our previous study demonstrated that this conservative missense mutation was shown to be slightly less thermostable, to suggest that a slight conformational change was present and this produced a proposed surface modification (Atipairin et al., 2011a). However, the mutation barely perturbed the native global structure of the BRCA1 RING domain that was consistent with a study, revealing that the D67E mutation could interact with its partners BARD1 and E2, and thus retained the ubiquitin ligase activity (Morris et al., 2006). The mutation has recently been shown to inhibit estrogen signaling similar to the wild-type BRCA1, to indicate that it might be a neutral or mild cancer-risk modifier of the other defective mechanisms, underlying BRCA1 mutation-related breast cancer (Pongsavee et al., 2009). Interestingly, the substitution of aspartic acid with tyrosine at this position exhibited only partial E3 ligase activity (Fig. 11b). The bulky hydrophobic side-chain of tyrosine possibly disrupts the second Zn^{2+} binding loop and weakens the association with E2/UbcH5c, resulting in the reduced ubiquitination function. This substitution mutant has been tested for a function in the homologous recombinant pathway (Ransburgh et al., 2010). It was shown that the D67Y BRCA1 still preserved DNA recombinant activity similar to the wild-type protein. However, it was identified as a variant of uncertain clinical significance based on the Myriad Genetic Laboratories database (Judkins et al., 2005). In addition, the D67Y BRCA1 had an intermediate effect on centrosome duplication, a critical step in the maintenance of genetic stability of mammary epithelial cells (Kais et al., 2012).

To determine the functional consequence of the BRCA1 mutation on the response to cisplatin, the wild-type and mutant BRCA1 RING proteins were treated with cisplatin *in vitro*

at a number of concentrations between 0-100 μM. The BRCA1 E3 ligase function was inactivated in a platinum concentration dependent manner (Fig. 12). Both wild-type and D67E BRCA1 had an identical response to the drug with an effective concentration of 100 μM that completely inhibited the activity (Fig. 12a and 12b). It was consistent with our previous result that showed the D67E mutation barely affected the native structure and function of the protein. Surprisingly, the D67Y BRCA1 that was a partially defective E3 ligase showed a promising outcome with an effective dose of 50 μM (Fig. 12c). The IC_{50} value for the E3 ligase activity was approximately 60 μM for the wild-type and mutant D67E BRCA1, and 32 μM for the D67Y BRCA1 RING domain proteins, respectively (Fig. 12d). It indicated that this partial defective E3 ligase D67Y BRCA1 exhibited susceptible to the anticancer drug cisplatin. Although the cisplatin concentration used in the present study is comparable to that for inhibiting breast cancer cell proliferation (Yde & Issinger, 2006), further investigations should be performed with respect to the BRCA1 subcellular localization and chemosensitivity of cells harbouring the D67E and D67Y mutations, together with the status of BRCA1 proteins being platinated and BRCA1 E3 ligase activity upon cisplatin treatment *in vivo* for clinical relevance. In the present study, the secondary structure of the mutant D67E BRCA1 and D67Y BRCA1 underwent more folded structural rearrangement after increasing cisplatin concentrations. Interestingly, the D67Y BRCA1 revealed a decrease in α-helices, in turn, an increase in β-sheets when compared with the D67E or wild-type BRCA1. This indicated that cisplatin perturbed the secondary structure of both mutant BRCA1 proteins compared with the wild-type BRCA1 protein. The binding constants of the *in vitro* platination for D67E and D67Y BRCA1 were $6.11\pm0.44 \times 10^5\ M^{-1}$ and $2.45\pm0.45 \times 10^5\ M^{-1}$, and the free energy of binding (ΔG) were 0.29 and 0.83 kcal mol^{-1}, respectively, suggesting the thermodynamic contribution of Pt-induced mutant BRCA1 folding in the RING domain. Although both mutant proteins showed similar changes in folding upon thermal denaturation, the D67Y BRCA1 exhibited the Pt binding approximately a 2-fold less than the D67E BRCA1 did. The difference in Pt binding might reflect an alteration in the microenvironment at the mutation site necessary for protein interactions. However, the Pt binding to these mutant BRCA1 RING domains did not show any association with their E3 ubiquitin ligase activities.

It has recently been shown that cisplatin affects the conformation of the apo form of the BRCA1 RING domain, forming intramolecular and intermolecular adducts (Atipairin et al., 2010). A preferential platinum-binding site was located on the BRCA1 histidine 117, and an enhanced thermostability was observed after the protein was treated with cisplatin.

Furthermore, the functional consequence of the platinated BRCA1 on the specificity of the ubiquitin ligase was that it inhibited activity with: transplatin > cisplatin > oxaliplatin > carboplatin (Atipairin et al., 2011b). The geometry and the properties of the leaving and non-leaving groups of the platinum complexes played an important role in controlling the reactivity towards BRCA1. It implies that the platinum-BRCA1 adducts can affect the RING structure and the ubiquitination function. Recently, preclinical and clinical studies have attempted to exploit an advantage of the inherent weakness of BRCA1 dysfunction in DSB repair for an improved outcome in breast cancer treatment (Lee & McLeod, 2011; Vollebergh et al., 2011). It revealed that the BRCA1-deficient cells displayed a defective DNA repair and a 100-fold increased sensitivity to cisplatin than those containing the wild-type BRCA1 (Bhattacharyya et al., 2000). Inhibition of endogenous BRCA1 expression also promoted the hypersensitivity to cisplatin that was associated with decreased DNA repair and increased apoptosis (James et al., 2007). It indicates that the reduced BRCA1 expression observed in sporadic cancers might be exploited for DNA damage-based chemotherapy (Quinn et al., 2009). This sensitivity was found to be reversed upon the correction of the open reading frames of the mutated BRCA1 by secondary intragenic mutations that restored the BRCA1 protein expression and function in DNA repair (Dhillon et al., 2011). Factors associated with a good cisplatin response also included young age, low *BRCA1* mRNA expression, *BRCA1* promoter methylation, p53 mutations, and a gene expression signature of the activity of E2F3 (Silver et al., 2010). The significant benefits of cisplatin treatment in the improved response and overall survival rate have been observed in the BRCA1-associated head and neck, bladder, ovarian and non-small cell lung (NSCL) cancer patients as a result of which larger-scale prospective clinical trials have to be designed for determining the clinical relevance of chemosensivity (Burkitt & Ljungman, 2007; Font et al., 2011; Gallagher et al., 2011; Taron et al., 2004; Vencken et al., 2011). Therefore, further investigation of the BRCA1 response to cisplatin in a large number of defective BRCA1 mutations is needed, particularly a relationship between the BRCA1-mediated ubiquitination and selective chemosensitivity (in *BRCA1* carriers)(Ratanaphan, 2012). This could raise the possibility of utilizing the BRCA1 mutations as a potentially molecular target for platinum-based drugs in cancer chemotherapy Berrada et al., 2010; Cho et al., 2011; Hastak et al., 2010; Moiseyenko et al., 2010; Rocca et al., 2008).

CONCLUSION

Mutation of the breast cancer susceptibility gene *(BRCA)* is linked to a subset of hereditary breast cancers which account for 5-10% of all breast cancers. Germline mutations in the *BRCA1* gene account for 50% of hereditary breast cancers, and 80% of both hereditary breast and ovarian cancers. *BRCA1* encodes a 1,863-residue protein that participates in genomic integrity maintenance through DNA repair, cell cycle checkpoint, protein ubiquitination, and transcriptional regulation. The BRCA1-BARD1 RING complex has an E3 ubiquitin ligase function that plays essential roles in response to DNA damage repair. BRCA1-associated cancers have been shown to confer a hypersensitivity to chemotherapeutic agents. Cisplatin sensitivity of the missense mutation D67E and D67Y BRCA1 RING domains on the *in vitro* E3 ubiquitin ligase activity revealed that the D67Y BRCA1 protein exhibited the reduced ubiquitination function, and was more susceptible to the drug than the D67E or wild-type BRCA1 protein. Both mutant proteins showed similar changes in folding upon thermal denaturation, however, the D67Y BRCA1 exhibited the Pt binding approximately a 2-fold less than the D67E BRCA1 did. The difference in Pt binding might reflect an alteration in the microenvironment at the mutation site necessary for protein interactions. This evidence emphasized the potential of using the BRCA1 dysfunction as an important determinant of chemotherapy responses in breast cancer.

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