

SHORT REPORT

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## In Vitro Enhanced Sensitivity to Cisplatin in D67Y BRCA1 RING Domain Protein

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**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 functional consequence of the in vitro E3 ubiquitin ligase activity and cisplatin sensitivity of the missense mutation D67Y BRCA1 RING domain. 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. This evidence emphasized the potential of using the BRCA1 dysfunction as an important determinant of chemotherapy responses in breast cancer.

**Keywords:** BRCA1, cisplatin, ubiquitination, cancer chemotherapy

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## Introduction

The *BRCA1* gene encodes a 1,863-residue protein that participates in the maintenance of genomic stability through DNA repair, cell cycle checkpoint control, transcriptional regulation and protein ubiquitination.<sup>1,2</sup> The N-terminus of the BRCA1 protein contains a RING domain, both ends of which adopt antiparallel  $\alpha$ -helices, that flank the central RING motif characterized by a short antiparallel three-stranded  $\beta$ -sheets, two large  $Zn^{2+}$  binding loops and a central  $\alpha$ -helix.<sup>3</sup> 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. This domain is essential for mediating macromolecular interactions to exert tumor suppression functions.<sup>4,5</sup> The BRCA1 RING domain preferentially forms a heterodimeric complex with another RING domain of BARD1 (BRCA1-associated RING domain 1) through an extensive four-helix-bundle interface.<sup>3</sup> The interaction between the BRCA1 and BARD1 RING domains markedly exhibits an enzymatic activity of an E3 ubiquitin ligase.<sup>6–8</sup> The RING heterodimer BRCA1-BARD1 can mediate *auto*-ubiquitination of BRCA1 and *trans* ubiquitination of other protein substrates.<sup>9,10</sup> Many cancer-predisposing mutations in the BRCA1 RING domain that inhibit the E3 ligase activity and the accumulation at damaged sites are defective in DNA double-strand break (DSB) repair pathways, and render cancerous cells hypersensitive to ionizing radiations and alkylating agents.<sup>11–14</sup> Therefore, the BRCA1-dependent ubiquitination has recently been linked to tumor suppression by its participations in DNA repair and transcription.<sup>15,16</sup>

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.<sup>17,18</sup> It revealed that the response to cisplatin treatment was a dose-dependent manner in human breast cancer cells *in vitro*.<sup>19,20</sup> After a 24 h exposure to the drug, cisplatin concentration of 60–100  $\mu$ M was required for a half inhibition in cell proliferation of the BRCA1-competent MCF-7 and MDA-MB-231 cells while cisplatin at 20  $\mu$ M led to a 50% reduction in cell viability of the BRCA1-defective HCC1937 cells (BRCA1 5382insC mutation).<sup>21</sup>

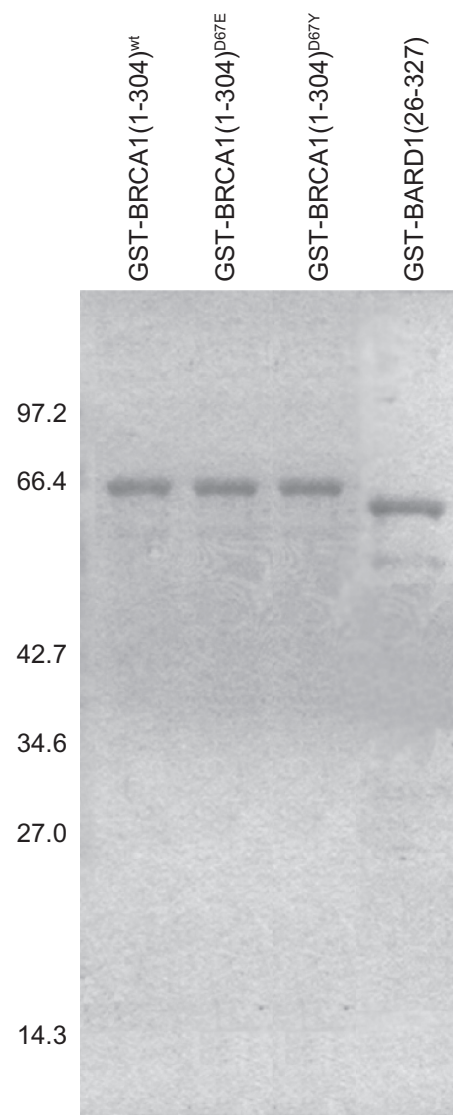
Furthermore, a clinical study showed that nine out of 10 (90%) breast cancer patients who carried the common BRCA1 C61G and 5382insC mutations achieved a complete pathological response in cisplatin-based chemotherapy.<sup>22</sup> In a retrospective study with 102 BRCA1 mutation carriers, ten out of 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%).<sup>23</sup> 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.<sup>11,12,24,25</sup> 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.<sup>26–28</sup> It suggests that the BRCA1 gene product acts as a key modulator of drug sensitivity in breast cancer cells.<sup>29</sup> This was consistent with previous studies, showing that cisplatin-based chemotherapy achieved an increased response rate for triple-negative breast cancer.<sup>30,31</sup> This evidence has emphasized the potential of using the BRCA1 dysfunction as an important determinant of chemotherapy responses in breast cancer.<sup>32</sup> 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.<sup>33</sup> 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.<sup>34</sup> 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.

## Materials and Methods

### Plasmid construction and protein purification

The short N-terminal fragment of the BRCA1 protein amino acid residues 1–304 was produced as a glutathione *S*-transferase (GST) fusion by cloning the respective gene into pGEX-4T1 (Amersham Biosciences). BRCA1 point mutations were constructed by the QuikChange Lightning site-directed mutagenesis kit (Stratagene). The mutagenic primers were as follow: forward: 5'-CCTTTATGTAAGAATGAGATAACCAAAAGG-3' and reverse: 5'-CCTTTTGGTTATCTCATTCTTACATAAAGG-3' for D67E; and forward: 5'-CCTTTATGTAAGAATTATATAACCAAAAGGAG-3' and reverse: 5'-CTCCTTTTGGTTATATAATCTTACATAAAGG-3' for D67Y. The base changes are underlined in the sequence. The *BARD1* gene that encodes the protein residues 26–327 was amplified by the polymerase chain reaction from a *BARD1* gene template (Addgene plasmid 12646),<sup>35</sup> and was cloned into pGEX-4T1. Full-length ubiquitin (*Ub*) (Addgene plasmid 12647) and *UbcH5c* (Addgene plasmid 12643) genes were inserted into the pET28a(+) derivative for expression of His<sub>6</sub>-tagged proteins. All recombinant plasmids were verified by DNA sequencing, and transformed into *Escherichia coli* BL21(DE3) for production of the protein. Protein expression was induced with 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 12 h at 25 °C. Cell pellets were resuspended in a lysis buffer of 50 mM Tris (pH 7.4), 50 mM NaCl, 10% glycerol, 10 mM  $\beta$ -mercaptoethanol, 1% Triton X-100, 0.5% NP-40 and 1 mM PMSF, and then lysed by sonication. GST-tagged proteins were freshly prepared using a glutathione-agarose column (Amersham Biosciences) (Fig. 1). The purified proteins were extensively dialyzed against deionized water. His<sub>6</sub>-Ub and



**Figure 1.** Affinity purification of GST-BRCA1(1-304)<sup>wt</sup>, GST-BRCA1(1-304)<sup>D67E</sup>, GST-BRCA1(1-304)<sup>D67Y</sup>, and GST-BARD1(26-327) proteins (2  $\mu$ g) 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.

His<sub>6</sub>-UbcH5c proteins were purified using nickel beads (Qiagen), and then dialyzed against a buffer, containing 50 mM Tris (pH 7.0), 10 mM  $\beta$ -mercaptoethanol and 10% glycerol. Human His<sub>6</sub>-E1 enzyme was purchased from Enzo Life Sciences.

### Preparation of the platinated BRCA1

Cisplatin (Sigma-Aldrich) was prepared as a stock solution (1 mM) in deionized water. Purified wild-type and mutant BRCA1 RING domain proteins (1.67  $\mu$ M) were mixed with cisplatin at concentration of between 0–100  $\mu$ M. 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.

### In vitro ubiquitin ligase activity assay

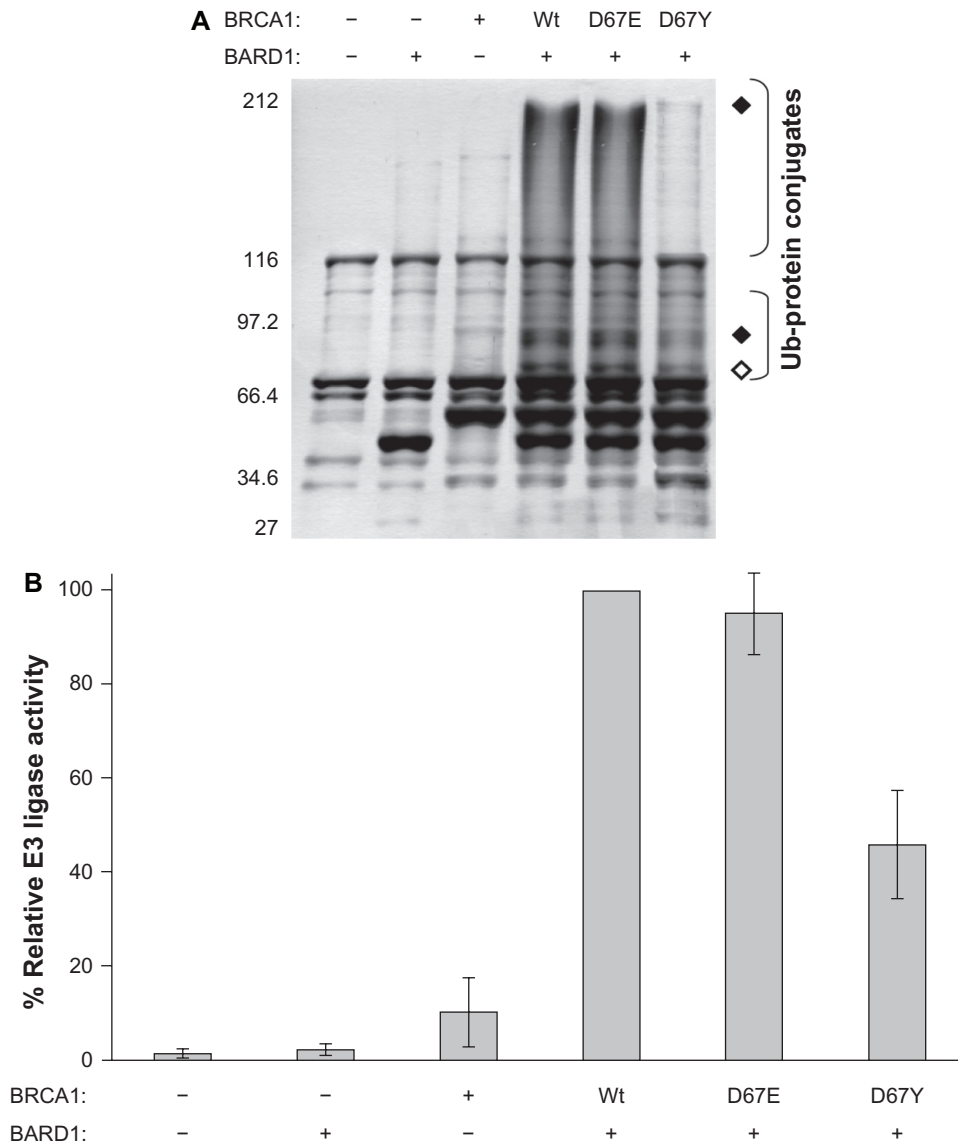
The ubiquitin ligase reactions (20  $\mu$ l) contained 20  $\mu$ M Ub, 300 nM E1, 5  $\mu$ M E2/UbcH5c, 2  $\mu$ g BRCA1 or BRCA1 adducts and 2  $\mu$ g BARD1 in a buffer, containing 50 mM Tris (pH 7.5), 0.5 mM DTT, 5 mM ATP, 2.5 mM MgCl<sub>2</sub> and 5  $\mu$ M ZnCl<sub>2</sub>. Two separate reactions 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 mutant 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.

## Results and Discussion

The BRCA1 and BARD1 RING domains preferentially form a stable heterodimeric complex through an extensive four-helix-bundle interface.<sup>3,36</sup> This interaction provides the proper contact surface on BRCA1 in the first and second Zn<sup>2+</sup> 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. 2A).<sup>37,38</sup> The familial D67E BRCA1 mutation still maintained the E3 ligase activity that was identical to the wild-type protein (Fig. 2B). A 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.<sup>8</sup> 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.<sup>38</sup>

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.<sup>39</sup> Interestingly, the substitution of aspartic acid with tyrosine at this position exhibited only partial E3 ligase activity (Fig. 2B). The bulky hydrophobic side-chain of tyrosine possibly disrupts the second Zn<sup>2+</sup> binding loop and weakens the association with E2/UbcH5c, resulting in the reduced ubiquitination function. Recently, this substitution mutant has been tested for a function in the homologous recombinant pathway.<sup>12</sup> 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.<sup>40</sup>

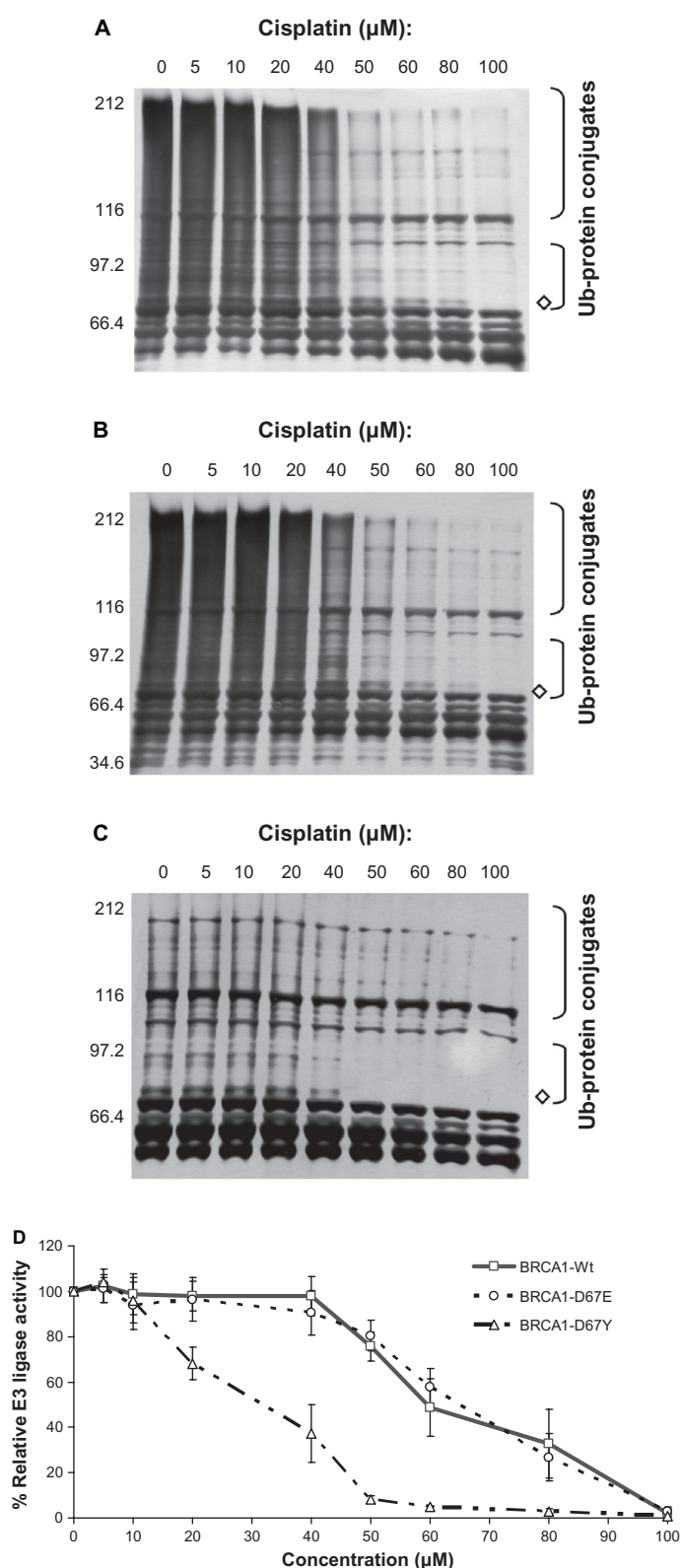
To determine the functional consequence of the BRCA1 mutation on the response to cisplatin, the wild-type and mutant BRCA1 RING proteins were treated with cisplatin *in vitro* at a number of concentrations between 0–100  $\mu$ M. The BRCA1 E3 ligase function was inactivated in a platinum concentration dependent manner (Fig. 3). Both wild-type and D67E BRCA1 had an identical response to the drug with an effective concentration of 100  $\mu$ M that completely inhibited the activity (Fig. 3A and B). 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  $\mu$ M (Fig. 3C). The IC<sub>50</sub> value for the E3 ligase activity was approximately 60  $\mu$ M for the wild-type and mutant D67E BRCA1, and 32  $\mu$ M for the D67Y BRCA1 RING domain proteins, respectively (Fig. 3D). 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,<sup>21</sup> 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.



**Figure 2. (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  $\mu$ M Ub, 300 nM E1, 5  $\mu$ M UbcH5c, 2  $\mu$ g BRCA1 (residues 1-304) and 2  $\mu$ g BARD1 (residues 26-327), were incubated at 37  $^{\circ}$ C for 3 h. Reactions were terminated by adding an equal volume of SDS-loading dye and heating at 95  $^{\circ}$ 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.

It has recently been shown that cisplatin affects the conformation of the apo form of the BRCA1 RING domain, forming intramolecular and intermolecular adducts.<sup>41</sup> 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.<sup>35</sup> 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.<sup>42,43</sup> It revealed that the BRCA1-deficient cells displayed a defective DNA repair and a 100-fold increased



**Figure 3.** In vitro E3 ubiquitin ligase activity of the cisplatin-BRCA1 adducts. Two  $\mu\text{g}$  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  $\mu\text{M}$ , 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).

sensitivity to cisplatin than those containing the wild-type BRCA1.<sup>44</sup> Inhibition of endogenous BRCA1 expression also promoted the hypersensitivity to cisplatin that was associated with decreased DNA repair and increased apoptosis.<sup>45</sup> It indicates that the reduced BRCA1 expression observed in sporadic cancers might be exploited for DNA damage-based chemotherapy.<sup>46</sup> 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.<sup>47</sup> 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.<sup>31</sup> 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 chemosensitivity.<sup>48–52</sup> 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). This could raise the possibility of utilizing the BRCA1 mutations as a potentially molecular target for platinum-based drugs in cancer chemotherapy.<sup>53–57</sup>

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## Disclosures

This manuscript has been read and approved by all authors. The authors have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have permission to reproduce any copyrighted material. The authors declare no conflicts of interest.



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