Cisplatin Affects the Conformation of Apo Form, not Holo Form, of BRCA1 RING Finger Domain and Confers Thermal Stability

by **Apichart Atipairin**, **Bhutorn Canyuk**, and **Adisorn Ratanaphan***

Laboratory of Pharmaceutical Biotechnology, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand (phone: +66-0-7428-8867; fax: +66-0-7442-8239; e-mail: adisorn.r@psu.ac.th)

The breast cancer suppressor protein 1 (BRCA1) has been shown to participate in genomic integrity maintenance. Preclinical and clinical studies have recently revealed that the inactivation of BRCA1 in cancer cells leads to chemosensitivity. Approaching the BRCA1 RING protein as a potentially molecular target for a platinum-based drug might be of interest in cancer therapy. In the present study, the *in vitro* platination of the BRCA1 RING protein by the anticancer drug cisplatin was observed. The protein contained a preformed structure in the apo form with structural changes and resistance to limited proteolysis after Zn^{2+} binding. SDS-PAGE and mass-spectrometric analyses revealed that cisplatin preferentially formed monofunctional and bifunctional BRCA1 adducts. Tandem mass spectrometry (MS/MS) of the 656.29^{2+} ion indicated that the ion arose from $[Pt(NH_3)_2(OH)]^+$ bound to the BRCA1 peptide ^{111}ENNSPEHLK^{119}. The product-ion spectrum revealed the Pt-binding site on His117. Circular dichroism showed that the apo form, not holo form, of BRCA1 underwent more folded structural rearrangement upon cisplatin binding. Cisplatin-bound protein exhibited an enhanced thermostability by 13°, resulting from the favorably intermolecular cross-links driven by the free energy. Our findings demonstrated the first conformational and thermal evidences for a direct binding of cisplatin to the BRCA1 RING domain and could raise a possibility of selectively targeted treatment of cancer with less toxicity or improved response to conventional regimens.

Introduction. – Cisplatin (*cis*-diamminedichloroplatinum(II)), a Pt-based anticancer drug, is widely used for the treatment of human testicular, bladder, ovarian, and head and neck cancers [1]. Its anticancer activity potentially results from the modification of DNA through covalent cross-linkings or Pt–DNA adducts which interfere DNA replication and transcription, and ultimately leading to cancer cell death [2–4]. The effectiveness of the anticancer drug cisplatin depends on the drug uptake and its actual amount that reacts with the cellular targets. The physiological chloride concentration (100 mM) in blood and extracellular fluids is high enough to suppress cisplatin hydrolysis. Cisplatin reaches the surface of cells as a dichloro form. Passive diffusion is believed to be the main mechanism that enables it to enter the cells. The drug uptake in the breast cancer MCF-7 cells was 0.197 ng platinum per mg protein with a high accumulation ratio of 5.04 between the intracellular and extracellular platinum concentrations after a 24 h continuous treatment with cisplatin (0.1 μM) [5]. The intracellular activation of cisplatin is required before it plays an important role in cytotoxicity, facilitated by the low cellular concentration of Cl^- ions of *ca.* 2–3 mM. Chlorine groups of cisplatin are easily replaced by H_2O molecules to allow the

formation of aquated species in a stepwise manner. The hydration rate constant of monoaqua form was faster than that of diaqua form ($2.38 \times 10^{-5}\ s^{-1}$ *vs.* $1.4 \times 10^{-5}\ s^{-1}$) [6]. The aquated forms are more reactive to the cellular targets which contain nucleophilic groups such as DNA and RNA at N(7) of guanine and adenine bases and protein side chains of cysteine, methionine, and histidine at S and N moieties [7].

The interaction of cisplatin with proteins is of particular significance and believed to play an important role in drug distribution and inactivation responsible for determining its toxicity [8–15]. Intriguingly, its reaction is also implicated in some crucial aspects of protein structures and functions. For instance, cisplatin can cause the structural perturbation of a synthetic peptide containing a Zn^{2+} finger domain. The Pt coordination to Zn^{2+}-binding sites resulted in Zn^{2+} ejection and subsequently loss of protein tertiary structure, implying the inhibition of critically biological functions regulated by Zn^{2+} finger protein. Such a mechanism has been discussed in the apoptosis process mediated by the interaction of cisplatin and Pt-based compounds with Zn^{2+} finger transcriptional factors [16]. Likewise, the nucleocapsid Zn^{2+} finger NCp7 protein, a protein required for the recognition and packaging of viral RNA, was attracted by some Pt compounds, thereby inhibiting its nucleic acid binding and preventing the viral infectivity [17–19]. The Zn^{2+} finger protein, therefore, is a potential target for Pt compounds in medicinal application.

The breast cancer suppressor protein 1 (BRCA1) has been shown to play a vital role in genomic integrity maintenance through multiple functions in DNA damage repair, cell-cycle checkpoint, protein ubiquitination, and transcriptional regulation [20–22]. It contains 1863 amino acid residues that are characterized into three major domains, including the N-terminal Zn^{2+} finger RING domain (BRCA1 RING domain), the large central segment, and the BRCA1 C-terminal domain (BRCT). The BRCA1 RING domain contains the conservative sequences of cysteine and histidine (C_3HC_4) necessary for the specific coordination with two Zn^{2+} ions. Atomic structure of the BRCA1 RING domain have revealed that both ends of the domain are antiparallel α-helices, flanking the central RING motif characterized by a short antiparallel three-stranded β-sheets, two large Zn^{2+}-binding loops, and a central α-helix [23]. These two metal-binding sites are established 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. It is an important domain, since it can play a central role in BRCA1-mediated macromolecular interactions to exert its functions [24][25].

Recently, a new approach for cancer therapy is mediated by alteration in DNA repair process [26–28]. The cells with dysfunctional DNA repair accumulate the high level of DNA damage, eventually resulting in major genomic instability and cell death. Several lines of evidences demonstrated that cancerous cells with BRCA1 inactivation had a defect in DNA repair of double-strand breaks (DSB) through the mechanism of homologous recombination [29–31]. The DNA cross-linking agents that generate DSBs and require the homology-directed repair would be beneficial for treatment of such cancer cells. BRCA1-Deficient cells which impaired the BRCA1 function were shown to be hypersensitive to cisplatin, mitomycin C, and cyclophosphamide, and displayed the effectively clinical response for fighting BRCA1-associated breast and ovarian cancers, or even its aggressive forms of basal-like and triple negative subtypes

[32–34]. A clinical study assessed ten patients with BRCA1-positive breast cancer who were treated with cisplatin. An impressively pathologic complete response of 90% was observed in nine patients with excellent compliance [35]. Additionally, the Pt-based chemotherapy in patients with triple-negative breast cancer achieved a significantly higher complete response rate in comparison to non-triple-negative patients (88 and 51%, resp.) [36]. Reconstitution of BRCA1 in the cells *via* transfection undoubtedly gained the BRCA1 functions and resulted in a reduced level of cancer cell death, following treatment with cisplatin or other DNA-damaging agents [37]. Moreover, recent evidences revealed the implication of BRCA1 in cisplatin-resistant breast and ovarian cancer cell lines. These cells acquired resistance to DNA-damaging agents mediated by secondary mutation in BRCA1. This mutation restored BRCA1 protein expression and function in DNA repair, causing the cancer cells to become more tolerant to cisplatin [38][39].

The above data demonstrated the utilization of BRCA1 dysfunction as a clinically validated target for therapeutic application [40–47]. The interaction of cisplatin with the BRCA1 protein, particularly the BRCA1 RING domain, is of great interest. Alterations in Zn^{2+} coordination sites or some residues of the BRCA1 RING domain have been shown to perturb protein structure and ubiquitin ligase activity [48–50]. Therefore, targeting the BRCA1 RING domain through the disruption of Zn^{2+}-coordination sites by the Pt-based drugs might be effective for the eradication of cancers and recurrently Pt-resistant cancer with lesser adverse effects than the empirical and historical treatment. Here, we describe the first evidence for a direct binding of the anticancer drug cisplatin to the BRCA1 RING domain, particularly its conformation and thermal denaturation.

Results. – *Expression and Purification of the BRCA1 RING Domain.* The BRCA1 RING domain consisting of residues 1–139 was expressed in *E. coli* BL21(DE3) with the regulation of the inducible T7 promoter of a pET28a (+) derivative. Because of the restriction sites used, BRCA1 contained the leading MGS residues derived from the plasmid. It was purified to an apparent homogeneity by reversed-phase (RP) chromatography (*Fig. 1*). Partial amino acid sequences of BRCA1 were verified by analyzing the peptides from a tryptic digestion with mass spectrometry (*Fig. 2*). The experimentally obtained sequences corresponded to the N-terminal region of BRCA1. Purified BRCA1 proteins were further used to characterize some properties in the following experiments.

Protein Secondary Structure and BRCA1 Dimerization. CD Spectra of the BRCA1 RING domain with and without Zn^{2+} bound showed similar profiles in shape with some differences in their amplitudes (*Fig. 3*). This indicated the potential preformation of the BRCA1 structure in the absence of Zn^{2+} or apo form, and the BRCA1 RING domain gained additionally folded structure in the holo form after Zn^{2+} binding. A nonlinear least-squares fit of the BRCA1–Zn^{2+} binding isotherm with a simple 1:2 coordination model yielded a binding constant of $2.79 \pm 0.24 \times 10^6$ M^{-1} and provided the free energy of binding (ΔG) of -8.64 kcal mol^{-1} (*Fig. 4*). Furthermore, gel-filtration chromatography revealed that BRCA1 was eluted as a single peak with the molecular mass of a dimer (data not shown). The result from glutaraldehyde cross-linking also confirmed that the BRCA1 RING domain predominantly formed a dimer, although

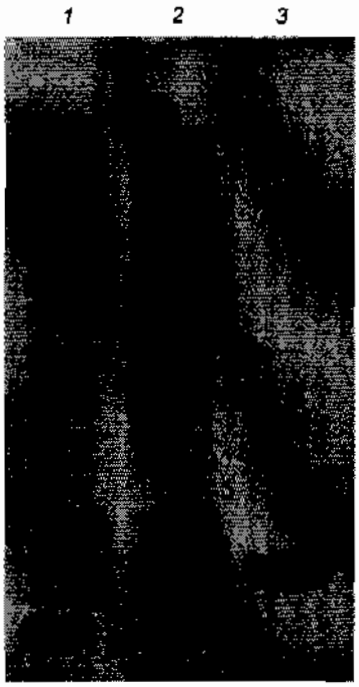

Fig. 1. *Expression and purification of the BRCA1 RING domain.* Transformed *E. coli* BL21(DE3) cells were induced with 0.5 mM IPTG and analyzed by 15% SDS-PAGE with Coomassie blue staining. *Lane 1*: whole-cell lysate uninduced; *Lane 2*: whole-cell lysate after a 4-h induction; *Lane 3*: purified BRCA1 protein after reversed-phase chromatography.

1 MDLSALR***VEE VQNVINAMQK ILECPICLEL IKEPVSTKCD HIFCK***FCMLK 50

51 LLNQK***KGPSQ CPLCK***NDITK R***SLQESTRFS QLVEELLKII CAFQLDTGLE*** 100

101 ***YANSYNFAKK ENNSPEHLKD EVSIIQSMGY R***NRAKRLLQ 139

Fig. 2. *Amino acid sequence of the peptides after tryptic digestion.* BRCA1 Protein band from Coomassie blue-stained SDS-PAGE was excised, alkylated with iodoacetamide, in-gel digested with trypsin, and then analyzed by LC/ESI-MS/MS. Amino acid sequences of each tryptic peptide were indicated in italic bold letters. These corresponded to the N-terminal region of BRCA1. The Zn^{2+}-binding sites were identified with the asterisk.

the higher order of oligomerization was also observed at higher glutaraldehyde concentration and prolonged incubation (data not shown).

Moreover, limited proteolysis was used to probe the structural consequence upon Zn^{2+} binding. BRCA1 without Zn^{2+} or in excess of ethylenediaminetetraacetic acid (EDTA) was rapidly degraded after the addition of elastase (*Fig. 5,a*). Two residual fragments were apparent, and the examination by in-gel tryptic digestion with mass spectrometry revealed the identity of the residues 1–88 and 8–38 which possessed only the minimum RING domain. Similar results were obtained with trypsin digestion (*Fig. 5,b*). The results suggested that BRCA1 without Zn^{2+} was considerably flexible

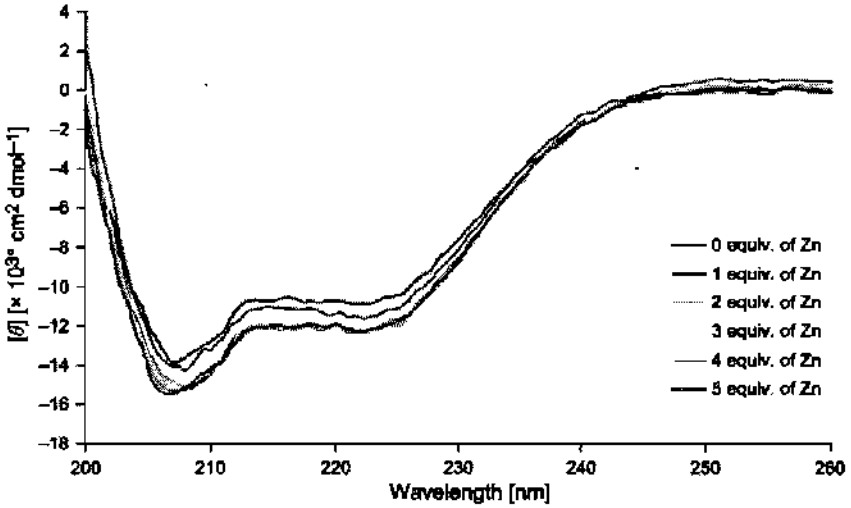

Fig. 3. *The CD spectra of the BRCA1 RING domain.* Samples (0–5 mol-equiv. ratio of Zn^{2+} to protein) were used to monitor Zn-dependent folding property of the BRCA1 RING domain. Values were given as the mean residue ellipticity. Samples were incubated with Zn^{2+} at 4° for 24 h before CD measurement. The measurements were performed at 20° with the scanning rate of 50 nm/min.

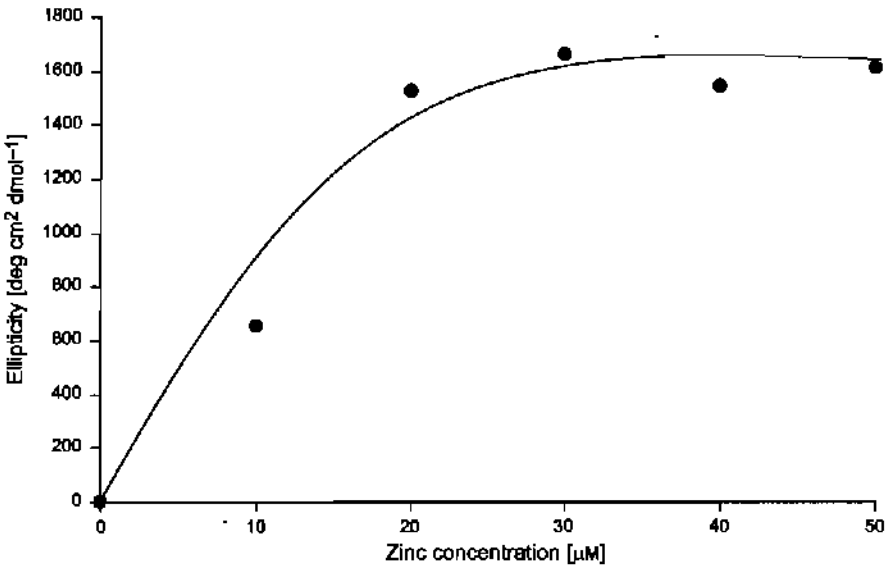

Fig. 4. *Titration curve of Zn^{2+} binding to BRCA1.* Changes in ellipticity of protein at 208 nm with increasing Zn^{2+} concentration were plotted, and the binding constant calculated using *Eqn. 1* was $2.79 \pm 0.24 \times 10^6\ M^{-1}$.

with slightly or no protease resistanee. On the other hand, BRCA1 with Zn^{2+} was rather resistant to proteolysis throughout the course of time (*Fig. 5,c* and *d*). It indicated that Zn^{2+} causes the structnre of the BRCA1 protein to bc more folded or rigid with reduced proteolytic susceptibility throughout its C-terminal portion.

Cisplatin Binding to BRCA1 and Protein Conformation. It was well-established that cisplatin induced the bifunctional adducts through the intermolecular cross-links of some proteins [11]. The mono- and trifunctional protein adducts also occurred by the intramolecular cross-links [13]. Thus, the types of adduct formation hy cisplatin are distinctive and dependent on the accessibility of Pt-center and protein side chains. The BRCA1 RING domain formed intermolecular cross-links caused by cisplatin, and the high amount of cross-links was accompanied by an increase in cisplatin concentration (*Fig. 6*). This result was further verified by mass spectrometric (MS) analysis, suggesting favorable monofunctional and bifunctional BRCA1 adducts (*Fig. 7*).

Although cisplatin has been demonstrated to induce protein dimerization and perturbed some protein structures, the secondary structure of the BRCA1 RING domain in the apo form was maintained and nnderwent more folded structural rearrangement after increasing cisplatin concentrations as judged by an increase in negative CD spectra at 208 and 220 nm (*Fig. 8,a*). It was possible that cisplatin might bind to the unoccupied Zn^{2+}-binding sites and cause the structural changes from 50% α-helix, 9% β-sheets, 14% turn, and 26% disordered element to 60% α-helix, 2% β-sheets, 15% turn, and 24% disordered element. The binding constant of the *in vitro* platination was $3.00 \pm 0.11 \times 10^6\ M^{-1}$, and the free energy of binding (ΔG) was -8.68 kcal mol^{-1} (*Fig. 9*). In addition, CD spectra of BRCA1 pre-incubated with Zn^{2+} gave the identical profilcs, suggesting that cisplatin could interact with other residues rather than the Zn^{2+}-binding sites, and barely affected the overall conformation of Zn^{2+}-bound BRCA1 (*Fig. 8,b*).

To locate the binding site of cisplatin on BRCA1, in-gel tryptic digestion of free BRCA1 and cisplatin–BRCA1 adducts (molar ratio 1:1) were subjected to LC/MS. The result revealed a unique fragment ion (+2) with a peak at *m/z* 656.29 obtained only from cisplatin–BRCA1 adduct digests (*Fig. 10*). Tandem MS analyses (MS/MS) of the 656.29^{2+} ion (measured mass 1310.57 Da) indicated that the ion arose from $[Pt(NH_3)_2(OH)]^+$ (theoretical mass 245.99 Da) which was attached to a BRCA1 peptide ^{111}ENNSPEHLK^{119} (theoretical mass 1066.44 Da) with a mass difference of 0.86. Coordination of H_2O to cisplatin lowers its pK_a (pK_{a1} 5.37 and pK_{a2} 7.21) to give hydroxo forms [51]. This product potentially reacted with BRCA1 and yielded BRCA1-$Pt(NH_3)_2(OH)$ as described by the following reactions.

$$Pt(NH_3)_2Cl_2 + 2\,H_2O \rightleftharpoons [Pt(NH_3)_2(H_2O)_2]^{2+} + 2\,Cl^-$$

$$[Pt(NH_3)_2(H_2O)_2]^{2+} \rightleftharpoons [Pt(NH_3)_2(H_2O)(OH)]^+ + H^+$$

$$BRCA1 + [Pt(NH_3)_2(H_2O)(OH)]^+ \rightleftharpoons BRCA1\text{–}Pt(NH_3)_2(OH) + H_3O^+$$

The product-ion spectrum of the ion (+2) with the peak at *m/z* 656.29 revealed the sequence ions (b_2^+, $[b_3 - H_2O]^+$, $[b_4 - H_2O]^+$, $[b_6 - H_2O]^+$, $[b_7 - H_2O]^+$, b_7^+, $[b_8 - H_2O]^+$, b_8^+, and y_2^+, which corresponded to the peptide Glu111–Lys119 of BRCA1

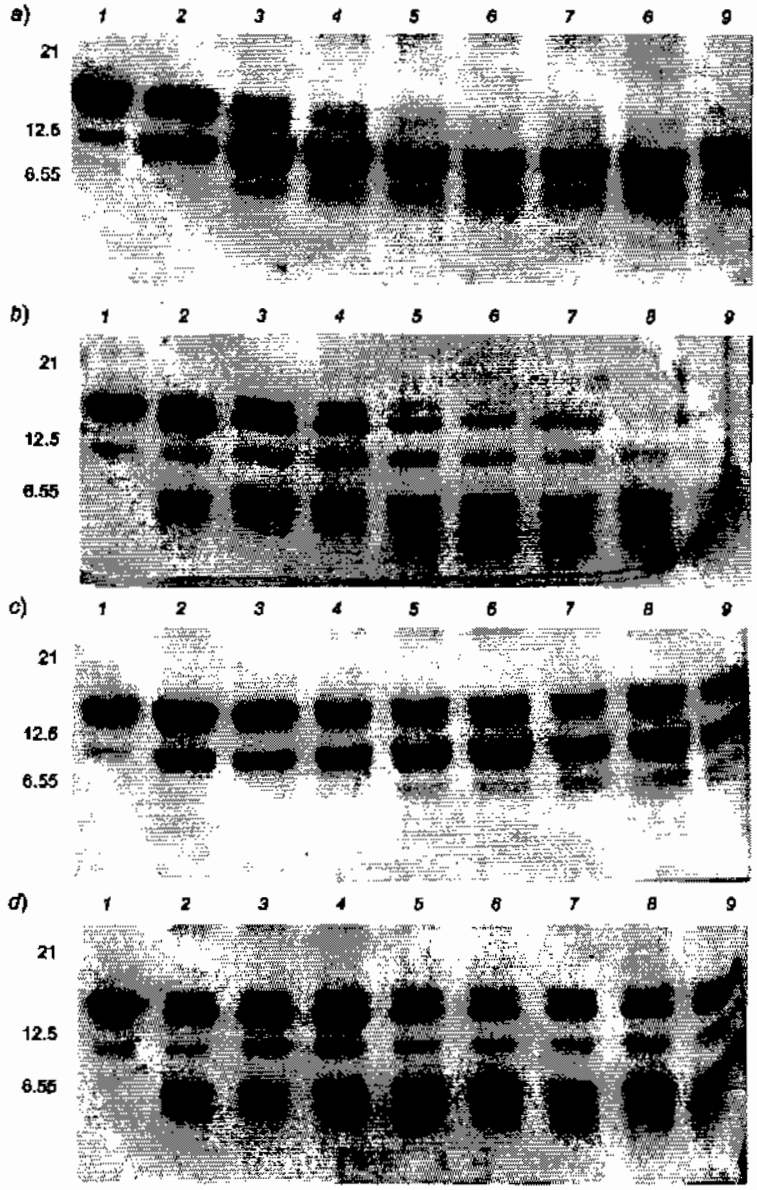

Fig. 5. *Limited proteolysis of the BRCA1 RING domain.* Purified BRCA1 proteins without (*a* and *b*) and with (*c* and *d*) Zn^{2+} were incubated with elastase (*a* and *c*) or trypsin (*b* and *d*) at the protein/protease ratio of 100–200 : 1 (*w/w*). Reaction aliquots were removed at 0, 0.25, 0.5, 1, 2, 3, 6, 12, and 24 h after the addition of protease (*Lanes 1–9*, resp.) and then identified on 15% Coomassie blue-stained SDS-PAGE. The molecular mass marker (kDa) was positioned.

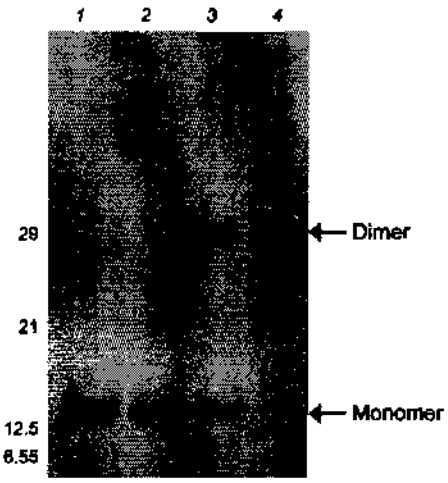

Fig. 6. *15% SDS-PAGE of BRCA1 intermolecular cross-linking by cisplatin.* BRCA1 Proteins (10 μM) were incubated with a number of cisplatin concentrations in the dark at 37° for 24 h. *Lane 1*: protein without cisplatin; *Lanes 2–4*: proteins with 10, 100, and 1000 μM cisplatin, respectively.

(*Fig. 11*). The ions with peaks at 1165.53 and 1293.57 were the Pt-bound b_6^+ (theoretical *m/z* 1166.39) and b_9^+ (theoretical *m/z* 1294.43), respeetively, whereas the ion corresponding to the peak at *m/z* 656.28 was assigned as the Pt-free b_6^+ ion with losing a H_2O molecule (theoretical *m/z* 653.26). It is specnlated that cisplatin interacts with the eounterpart of the b_6^+ ion (His117–Lys119). The ion with the peak at *m/z* 641.32 was the Pt-containing y_3^+ ion (theoretical *m/z* 642.24), and the ion with the peak at *m/z* 260.19 was the Pt-frce y_2^+ ion (theoretical *m/z* 260.20). The difference in *m/z* (381.13 Da) indicated the binding of eisplatin to His117 (theoretieal *m/z* 382.05 Da).

Thermal Denaturation of Cisplatin-BRCA1 Adducts. Thermal denaturation was monitored by CD to follow heat-induced unfolding which determined the effect of cisplatin binding on the stability of the BRCA1 RING domain. BRCA1 pre-incubated with or without Zn^{2+} was incubated with cisplatin, and CD spectra showed the identical changes with an increase in ellipticity when the temperatnre was raised from 15° to 95° (*Fig. 12*). It indicated that the folded proteins gradually lost the contents of the ordered struetures. When cooling to 20° after being heated at 95°, 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. Furthermore, the thermal denaturation curves were used to compare the stabilities among platinated proteins, and the melting temperatures (T_m) were collected in the inset (*Fig. 13*). The results showed that the melting temperatures of BRCA1 were *ca.* 74° and 83° in the absence and presenee of Zn^{2+}, respeetively. This suggested that the BRCA1 RING domain was more thermostable by *ca.* 9° upon Zn^{2+} binding. Thus, it supported the important role of Zn^{2+} in the determination and stabilization of the local secondary structure in the RING domain. It was notable that cisplatin at the coneentration of 10 μM exhibited similar melting temperatures as those observed for Zn^{2+} binding to the BRCA1 RING domain. However, higher melting temperatnres were observed at a tenfold concentration of cisplatin. These data suggested that cisplatin binding to the BRCA1 RING domain conferred an enchanced thermostability

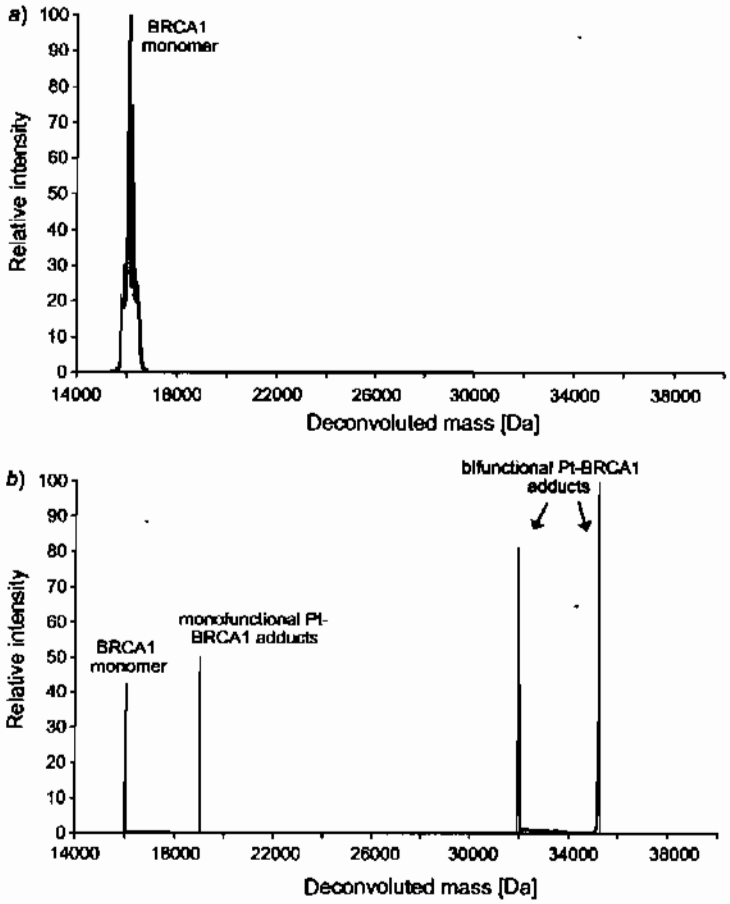

Fig. 7. *MS Analyses of cisplatin–BRCA1 adducts. a*) BRCA1 Proteins (15 μM) or *b*) cisplatin–BRCA1 adducts (1 : 1) were incubated in the dark at 37° for 24 h. Samples were directly subjected to an ESI mass spectrometer, and the deconvoluted spectra were given.

by 13°. Resistance to thermal denaturation of cisplatin-modifed BRCA1 RING domain might result from the favorable intermolecular cross-links driven by the free energy.

Discussion. – The interactions of some proteins with cisplatin have extensively beeu investigated, and the cisplatin–protcin adducts are divergent in the formations and functions. For instance, thc platination of human serum albumin caused a partial unfolding of the protein structure at high drug concentration and induced intermolecular cross-links [8][11]. A few types of intramolecular cross-links occurred also iu ubiquitin adducts [13]. The loss of activity in protein aggregation prevention of the C-terminal heat shock protein 90 was reported as the consequencc of cisplatin binding, but it did not show any conformational change [52].

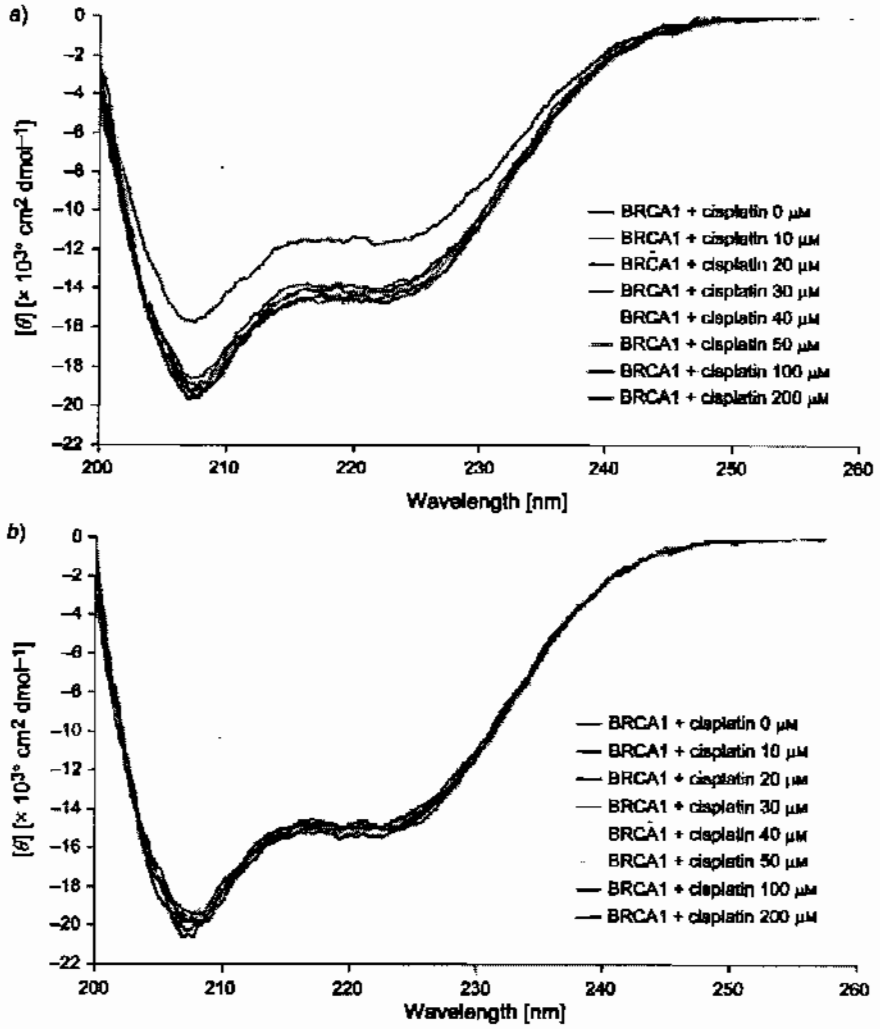

Fig. 8. *The CD spectra of cisplatin–BRCA1 adducts.* BRCA1 Proteins (10 μM) without Zn^{2+} (*a*) and with pre-incubation of 3 mol-equiv. ratio of Zn^{2+} to protein (*b*) were mixed by a number of cisplatin concentrations. Samples were incubated in the dark at ambient temperature for 24 h before CD measurement at 20° with the scanning rate of 50 nm/min. The mean residue ellipticity and wavelength ranging from 200 to 260 nm were plotted.

Numerous studies of BRCA1 have revealed its involvement in DNA repair whose functional loss results in increased anticancer activities of some DNA-damaging chemotherapeutics [35–38]. Targeting cancer cells specifically by utilizing the advantage of BRCA1 inactivation could provide an effective clinical response with less adverse effects for treatment of BRCA1-associated cancers and their aeqnired

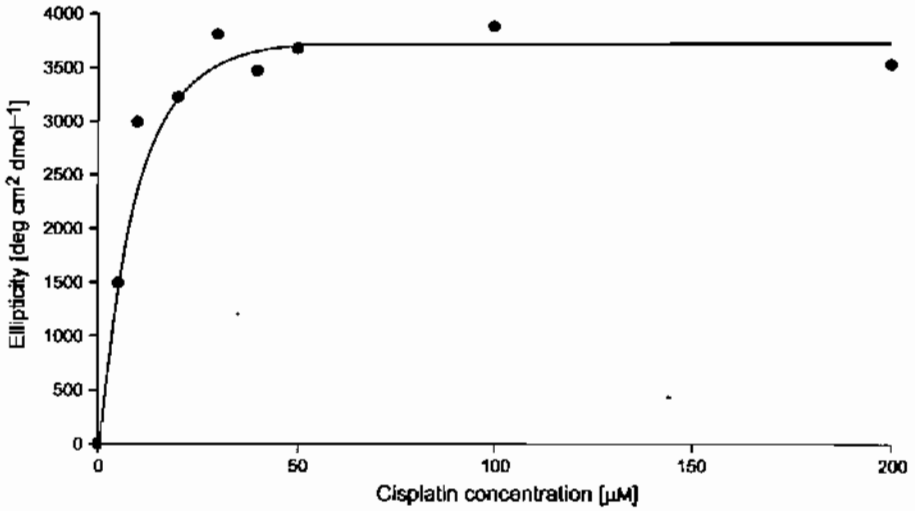

Fig. 9. *Titration curve of cisplatin binding to BRCA1.* Changes in ellipticity of protein at 208 nm with increasing cisplatin concentration were plotted. The binding constant calculated using *Eqn. 1* was 3.00 ± 0.11 × 10^6 M^{-1}.

resistance [35][53]. In the present study, the BRCA1 RING domain was targeted by the antieaneer drug eisplatin, aud its structural eonsequences of protein conformation and thermal denaturation were observed. The RING protein revealed some structural elements iu its apo form and additional folded strueture in the holo form. Not only was the structure more folded or compaeted upon metal binding, but the protein coordinating with Zn^{2+} appeared to be resistant to proteolysis. This was in eorrelation to other Zn^{2+} finger proteins such as transcription factor IIIA, exhibiting the metal-dependent foldiug reçognized in the RING domain family to provide the proper conformation for interactions with other macroinolecules [54].

Cisplatin-modified RING protein presented herein revealed favourable mono- and bifunetional BRCA1 adducts. Binding of cisplatin to the apo form of BRCA1 underwent more folded structural rearrangements potentially at the vacant Zn^{2+}-binding sites. However, cisplatin did not pertnrb the global conformation of the holo form of the BRCA1 RING protein. It implied that the drug interacted with other residues beyond the Zn^{2+}-binding sites. Tandem mass spectrometric analyses (MS/MS) indicated the formation of monofunctional adduct with $[Pt(NH_3)_2(OH)]^+$ on the BRCA1 peptide ^{111}ENNSPEHLK^{119}. The protonation of Lys119 at neutral pH and the preference of aqnated cisplatin for His based on a dipeptide His-Ser model also supported our reanlt that His117 was the Pt-binding site [55]. Although the hydroxo form in the adduct complex is generally less reactive than the aqua form, its existence may be essential for interaction with other nucleophiles as it shows the significant reactivity towards thiol groups [56].

The Pt binding to BRCA1 had the binding constant of 3.00 × 10^6 M^{-1}, cquivalent to that of Zn^{2+} binding (2.79 × 10^6 M^{-1}). The calculated free energy of cisplatin and Zn^{2+}

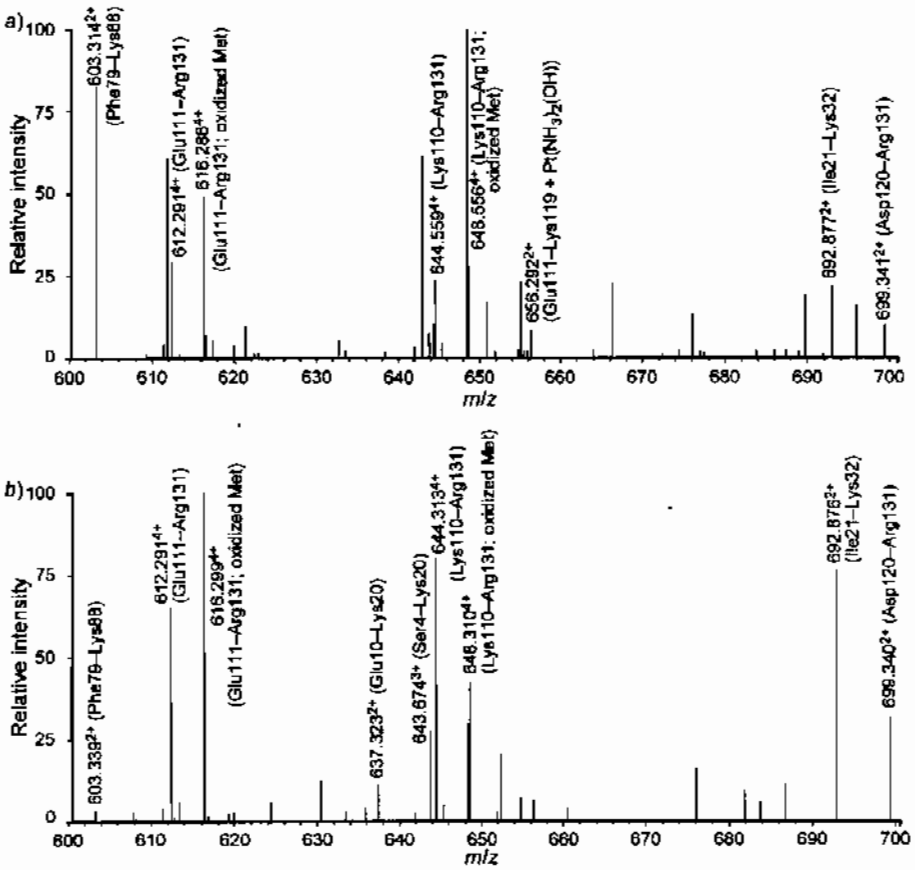

Fig. 10. *MS Analyses of the cisplatin–BRCA1 adduct digests.* In-gel tryptic digestion of *a*) the cisplatin–BRCA1 adducts (molar ratio 1:1), and *b*) free BRCA1 were subjected to analysis by LC/MS. A unique fragment ion of 656.29^{4+} only derived from the cisplatin-BRCA1 adduct digests presented a Pt-containing peptide.

bindings were *ca.* -8.68 and -8.64 kcal mol^{-1}, respectively, suggesting the thermodynamic contribution of metal-induced protein folding in the RING domain to drive protein folding, dimerization, and thermostability of BRCA1. The comparison of these two binding constants was not straightforward as described in a previous study, demonstrating the affinity of Zn^{2+} and cisplatin to a short Zn^{2+} finger peptide of 31-mers in a different fashion [16]. Zn^{2+} Binding to such a peptide employed the stepwise coordination by four cysteines with the binding constant of 3.91×10^4 M^{-1}, whilst the Pt binding involved the coordination by two cysteines with the affinity of 8.80×10^4 M^{-1}. However, the binding constant of the Pt-atom to a larger Zn^{2+} finger protein is suggested to be much higher due to other favorable binding sites beyond the Zn^{2+}-binding residues of protein. Moreover, a short synthetic peptide containing a minimal

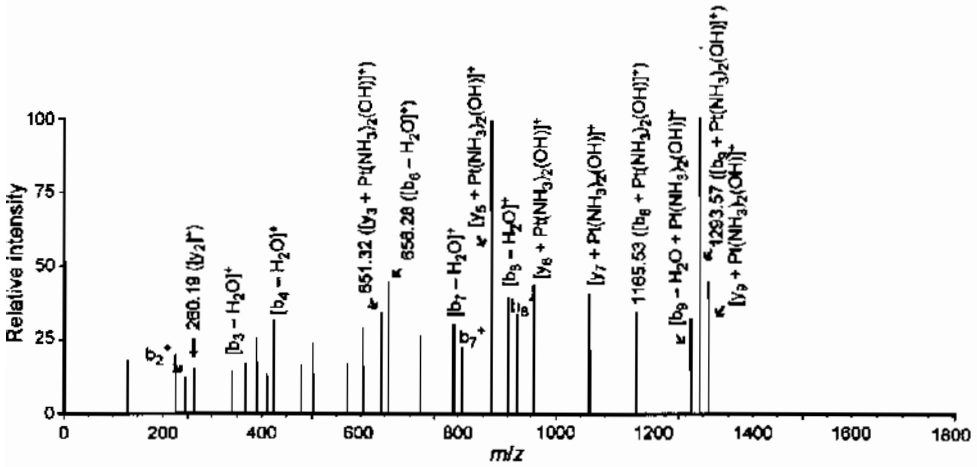

Fig. 11. *The product-ion spectrum of the MS/MS analysis for the 656.29²⁺ ion.* It indicated that $[Pt(NH_3)_2(OH)]^+$ was attached to a peptide ^{111}ENNSPEHLK^{119} of BRCA1.

BRCA1 RING domain exhibited the Co^{2+} binding constants ranging from 1.26×10^5 to 3.85×10^7 M^{-1} [57]. Generally, the binding specificity of a Zn^{2+}-binding peptide for Co^{2+} was 2–4 orders of magnitude lower than that for Zn^{2+} [58][59]. Our observed binding constants are, therefore, consistent with these studies and showed a *ca.* 34-fold higher Pt affinity than that observed for the short Zn^{2+} finger peptide of 31-mers (3.00×10^6 *vs.* 8.80×10^4 M^{-1}), implying the overall influence of the adjacent residues of the RING protein on the metal affinity [16]. Additionally, the Zn^{2+}-bound BRCA1 RING domain was more thermostable by 9° than the Zn^{2+}-free protein. The increased stability was apparently provided by the coordinating Zn^{2+}, which mostly contributed to the proper folding of BRCA1. Although the melting temperature of the BRCA1 RING domain was high and far from the physiological condition (*ca.* 74–83°), it was consistent with the previous study, showing that the Zn^{2+} finger domain formed a thermostable structure [53][60]. Furthermore, the increased thermostability of cisplatin–BRCA1 adducts by 13° probably resulted from the thermodynamically stabilizing contribution of intermolecular cross-links [61].

Cisplatin can interact nonspecifically with cellular proteins. Nucleophilic thiol proteins such as glutathione and metallothioneins are capable of binding to cisplatin before reaching the cellular targets. The intracellular concentration of glutathione is as high as 10 mM, and it correlates to cisplatin resistance in which 1 mol of Pt binds to 2 mol of glutathione with the rate constant of 8.45×10^{-2} M^{-1} s^{-1} [62][63]. Moreover, the increased levels of metallothioneins have been found in some cisplatin-resistant cells [64]. The stoichiometry of cisplatin–metallothionein (7:1) complex is established with a significantly high association constant of 2.3×10^{23} M^{-1} [65]. The high abundance and affinity to Pt of both two proteins in cells can compete with the BRCA1 RING protein for cisplatin binding. To avoid cisplatin inactivation, some specific enzyme inhibitors for biosynthesis of glutathione and metallothioneins have been used [66].

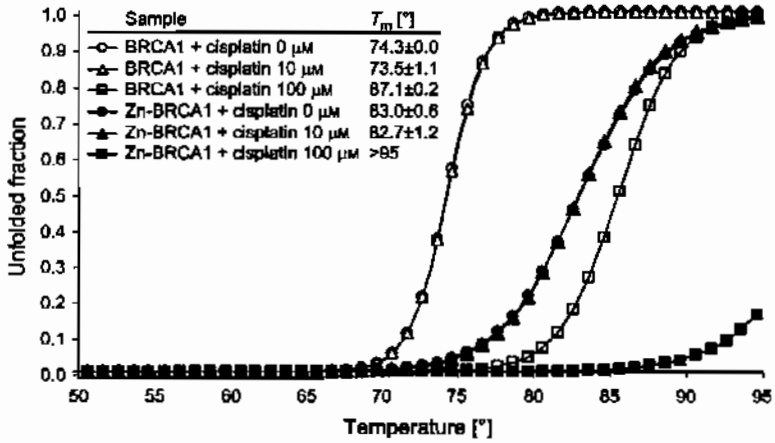

Fig. 13. *Thermal denaturation curves of cisplatin–BRCA1 adducts.* BRCA1 Protein (10 μM) without Zn^{2+} and with pre-incubation of 3 mol-equiv. ratio of Zn^{2+} to protein were mixed with various concentrations of cisplatin (0, 10, and 100 μM). Samples were incubated in the dark at ambient temperature for 24 h before CD measurement. The CD signals at 208 nm were measured, and the unfolded fraction as a function of temperature was plotted.

ligase activity which is closely associated with the DNA repair pathways [50]. The complete information would be beneficial for future therapeutic strategy of utilizing the BRCA1 RING domain as a potentially molecular target for Pt-based agents in the treatment of BRCA1-associated cancer and its aggressively basal-like and triple negative subtypes with higher survival rate [35][36].

This work was supported by the *National Synchrotron Research Center of Thailand* (1-2548/LS01), and, in part, by the *National Research Council of Thailand* (PHA5111990041S), and Prince of Songkla University (PHA530188S). We would like to thank the Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, for research facilities. Prof. Dr. *Udo Heinemann*, and Drs. *Eva-Christina Müller* and *Albrecht Otto* at Max-Delbrück Center for Molecular Medicine (MDC), Berlin, Germany, are acknowledged for kind assistance and discussions.

Experimental Part

General. cis-Diamminedichloroplatinum(II) (cisplatin), bovine serum albumin (BSA), isopropyl-β-D-thiogalactoside (IPTG), iodoacetamide, sodium cacodylate trihydrate, *t*-octylphenoxypolyethoxyethanol (*Triton X-100*), phenylmethylsulfonyl fluoride (PMSF), and glutaraldehyde were purchased from *Sigma Chemicals Co.* (USA). Agarose power, (amino)tris(hydroxymethyl)methane (molecular-biology grade), elastase, and trypsin (sequencing grade), and dATP, dCTP, dGTP, and dTTP were from *Promega Corporation* (USA). Restriction enzymes *Bam*HI and *Xho*I were obtained from *New England BioLabs Inc.* (USA). *Nonidet P-40* (*NP40*) was purchased from *Bio Basic Inc.* (Canada). MeCN (HPLC grade) and kanamycin were obtained from *Roth* (Germany). *Bacto*™ tryptone, *Bacto*™ yeast extract, and *Bacto*™ agar were from *Becton, Dickson & Co.* (USA). Dithiothreitol, ethylenediaminetetraacetic acid disodium salt (EDTA), guanidine hydrochloride, CF_3COOH, and $ZnCl_2$ were obtained from *Fluka* (Switzerland).

Cloning of the BRCA1 RING Domain. The BRCA1 RING domain containing the first 139 amino acid residues was prepared by RT-PCR. Total RNA was extracted from human white blood cells using the

Total RNA Isolation Kit (*QIAGEN*, D-Hilden), which was further used for cDNA synthesis of the *BRCA1* gene fragment using the *QIAGEN OneStep RT-PCR Kit*. The *BRCA1* gene fragment was amplified by PCR. Primers used were synthesized to incorporate the 5′ *Bam*HI and 3′ *Xho*I endonuclease restriction sites on the PCR products (forward primer: 5′-GACACGCGGATCCATGGATT-TATCTGCTCTTCG-3′, reverse primer: 5′-GACACCGCTCGAGTCACTGTAGAAGTCTTTTGG-CAC-3′). PCR Products were digested with *Bam*HI and *Xho*I, cloned into a plasmid pET28a(+) derivative, and subsequently verified by DNA sequencing. The recombinant plasmids were transformed into *Escherichia coli* BL21(DE3) for protein synthesis.

Expression and Purification. Transformed *E. coli* BL21(DE3) cells were grown in *Luria Broth* medium with 30 μg/ml kanamycin at 37°. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM to induce the expression, when the $A_{600\,nm}$ reached 0.5–0.6. Cells were allowed to grow for 4 h after induction and harvested by centrifugation. Cell pellets were resuspended in lysis buffer (50 mM *Tris*, pH 7.6, 50 mM NaCl, 10 mM DTT, 1% *Triton X-100*, 0.5% *NP-40*, and 1 mM PMSF) and then lysed with a French press. Lysate was purified using a Vydac protein C4 RP column (*Grace*, Deerfield, IL, USA) with a MeCN gradient, containing 0.1% CF_3COOH. Purified protein was identified on 15% Coomassie blue-stained SDS-PAGE and subsequently confirmed by sequencing the tryptic digested peptides.

Gel-Filtration Chromatography. Purified BRCA1 proteins from RP chromatography were lyophilized and resuspended in 2M guanidine HCl with 3 mol-equiv. ratio of Zn^{2+} to protein. Proteins (0.3 mM) were applied on an anal. *Superose 12 HR 10/30* column (*Amersham Biosciences*, Piscataway, NJ, USA). The column was pre-equilibrated with 25 mM *Tris*, pH 7.0, 150 mM NaCl, and 10 μM $ZnCl_2$, and the elution profiles were monitored at 212 nm.

Glutaraldehyde Cross-Linking. Peak fractions from gel filtration column (typically 2 μM) were subjected to the cross-linking reaction in the presence of 0.001–0.05% glutaraldehyde (*w/v*) at ambient temp. Reaction aliquots were removed at 0, 15, 30, and 60 min after the addition of glutaraldehyde, quenched with an equal volume of SDS-loading dye, and visualized on 15% SDS-PAGE by silver staining.

Limited Proteolysis and Mass Spectrometry. Protein samples (30 μM) in the absence and presence of 3 mol-equiv. ratio of Zn^{2+} to protein were prepared in 10 mM cacodylate buffer pH 6.8 and mixed with either elastase or trypsin at the protein/protease ratio of 100–500 : 1 (*w/w*) at 37°. Reaction aliquots at different time intervals were quenched by adding an equal volume of SDS-loading dye. Samples were visualized on 15% SDS-PAGE by Coomassie blue staining. Proteolysis of metal-free protein was also determined in the presence of 0.5 mM EDTA. To determine the constituents of the digested products, the protein bands of interest from the SDS-PAGE gel were excised, in-gel alkylated with iodoacetamide, and digested with sequencing-grade trypsin (*Promega*). In-gel digestions of free BRCA1 and cisplatin-BRCA1 adducts for characterizing the binding sites of cisplatin were also performed with ignoring the modification by iodoacetamide. The peptide mixture was separated on a *PepMap C18* column (75 μm/150 mm dimensions with 3 μm particle size), using a gradient of 4–50% MeCN. Eluted peptides were ionized by electrospray ionization (ESI), and mass spectra were acquired with a *QTRAP 4000* Mass Spectrometer (*Applied Biosystems/MDS Sciex*). MS/MS Analyses were conducted using collision-energy profiles chosen on the basis of the *m/z* value and the charge state of the parent ion. The *Analyst/Bioanalyst* software (version 1.4.1, *Applied Biosystems*) was used to process and submit the data to the MASCOT server (version 2.2, *Matrix Science Ltd.*, London, UK) for in-house search against the SwissProt protein database. The mass tolerance of precursor ions and sequence ions was set to 0.4 Da. The searches included variable modifications of cysteine with propionamide and carbamidomethyl, and methionine oxidation.

Circular Dichroism. Protein samples (10 μM) were prepared in 10 mM cacodylate buffer pH 6.8, according to *Bradford* assay using BSA as standard. $ZnCl_2$ and cisplatin were prepared as 5 mM stock solns. in deionized H_2O. 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.*, Hachioji City, Japan). Measurements of Zn^{2+} and cisplatin binding were carried out at 20° 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. Data were baseline-corrected by the subtraction of cacodylate

buffer. The secondary structures of proteins were predicted by the CONTIN program [69]. The effect of Zn^{2+} and cisplatin bindings on the protein stability was determined in the absence and presence of 3 mol-equiv. ratio of Zn^{2+} to protein. CD Experiments, involving thermal denaturation, were performed in three separate scans in the range from 15° to 95° at 208 nm with a heat rate of 1°/min. Thermal renaturation (20° after being heated at 95°) was also observed. The binding constant was determined using *Eqn. 1* [70]:

$$\theta_{obs} = \theta_{max}\left((1 + (kC/n) + kP)/(2kP) - \sqrt{((1 + (kC/n) + kP)/(2kP))^2 - C/(nP)}\right) \quad (1)$$

in which θ_{obs} is the observed ellipticity change at any concentration of metal, θ_{max} is the ellipticity change, when all of the protein binds 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.

The free energy of binding was given by *Eqn. 2*.

$$\Delta G = -RT \ln k \quad (2)$$

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

REFERENCES

[1] F. Muggia, *Gynecol. Oncol.* **2009**, *112*, 275.
[2] D. Wang, S. J. Lippard, *Nat. Rev. Drug Discov.* **2005**, *4*, 307.
[3] G. E. Damsma, A. Alt, F. Brueckner, T. Carell, P. Cramer, *Nat. Struct. Mol. Biol.* **2007**, *14*, 1127.
[4] A. Ratanaphan, S. Wasiksiri, B. Canyuk, P. Prasertsan, *Cancer Biol. Ther.* **2009**, *8*, 890.
[5] A. Ghezzi, M. Aceto, C. Cassino, E. Gabano, D. Osella, *J. Inorg. Biochem.* **2004**, *98*, 73.
[6] L. Cubo, D. S. Thomas, J. Zhang, A. G. Quiroga, C. Navarro-Ranninger, S. J. Berners-Price, *Inorg. Chim. Acta* **2009**, *362*, 1022.
[7] P. Jordan, M. Carmo-Fonseca, *Cell. Mol. Life Sci.* **2000**, *57*, 1229.
[8] J. F. Neault, H. A. Tajmir-Riahi, *Biochim. Biophys. Acta, Prot. Struct. Mol. Enzymol.* **1998**, *1384*, 153.
[9] L. Trynda-Lemiesz, H. Kozlowski, B. K. Keppler, *J. Inorg. Biochem.* **1999**, *77*, 141.
[10] I. Khalaila, C. S. Allardyce, C. S. Verma, P. J. Dyson, *ChemBioChem* **2005**, *6*, 1788.
[11] A. I. Ivanov, J. Christodoolou, J. A. Parkinson, K. J. Barnham, A. Tucker, J. Woodrow, P. J. Sadler, *J. Biol. Chem.* **1998**, *273*, 14721.
[12] C. S. Allardyce, P. J. Dyson, J. Coffey, N. Johnson, *Rapid Commun. Mass Spectrom.* **2002**, *16*, 933.
[13] T. Peleg-Shulman, Y. Najajreh, D. Gibson, *J. Inorg. Biochem.* **2002**, *91*, 306.
[14] A. Casini, G. Mastrobuoni, C. Temperini, C. Gabbiani, S. Francese, G. Moneti, C. T. Supuran, A. Scozzafava, L. Messori, *Chem. Commun.* **2007**, 156.
[15] A. Casini, C. Gabbiani, G. Mastrobuoni, L. Messori, G. Moneti, G. Pieraccini, *ChemMedChem* **2006**, *1*, 413.
[16] R. N. Bose, W. W. Yang, F. Evanics, *Inorg. Chim. Acta* **2005**, *358*, 2844.
[17] R. A. Musah, *Curr. Top. Med. Chem.* **2004**, *4*, 1605.
[18] A. I. Anzellotti, Q. Liu, M. J. Bloemink, J. N. Scarsdale, N. Farrell, *Chem. Biol.* **2006**, *13*, 539.
[19] Q. A. de Paula, J. B. Mangrum, N. P. Farrell, *J. Inorg. Biochem.* **2009**, *103*, 1347.
[20] E. M. Rosen, S. Fan, R. G. Pestell, I. D. Goldberg, *J. Cell Physiol.* **2003**, *196*, 19.
[21] K. Gudmundsdottir, A. Ashworth, *Oncogene* **2006**, *25*, 5864.
[22] R. I. Yarden, M. Z. Papa, *Mol. Cancer Ther.* **2006**, *5*, 1396.
[23] P. S. Brzovic, P. Rajagopal, D. W. Hoyt, M.-C. King, R. E. Klevit, *Nat. Struct. Biol.* **2001**, *8*, 833.
[24] C.-X. Deng, S. G. Brodie, *BioEssays* **2000**, *22*, 728.
[25] U. K. Westermark, M. Reyngold, A. B. Olshen, R. Baer, M. Jasin, M. E. Moynahan, *Mol. Cell. Biol.* **2003**, *23*, 7926.
[26] N. Turner, A. Tutt, A. Ashworth, *Curr. Opin. Pharmacol.* **2005**, *5*, 388.

[27] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, R. A. Sharma, *Nat. Rev. Cancer* **2008**, *8*, 193.
[28] M. R. Kelley, M. L. Fishel, *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 417.
[29] M. E. Moynahan, J. W. Chiu, B. H. Koller, M. Jasin, *Mol. Cell* **1999**, *4*, 511.
[30] C.-X. Deng, R.-H. Wang, *Hum. Mol. Genet.* **2003**, *12*, R113.
[31] J. M. Stark, A. J. Pierce, J. Oh, A. Pastink, M. Jasin, *Mol. Cell. Biol.* **2004**, *24*, 9305.
[32] A. Bhattacharyya, U. S. Ear, B. H. Koller, R. R. Weichselbaum, D. K. Bishop, *J. Biol. Chem.* **2000**, *275*, 23899.
[33] P. O. Chappuis, J. Goffin, N. Wong, C. Perret, P. Ghadirian, P. N. Tonin, W. D. Foulkes, *J. Med. Genet.* **2002**, *39*, 608.
[34] P. Tassone, M. T. Di Martino, M. Ventura, A. Pietragalla, I. Cucinotto, T. Calimeri, P. Neri, M. Caraglia, P. Tagliaferri, A. Bulotta, *Cancer Biol. Ther.* **2009**, *8*, 648.
[35] T. Byrski, T. Huzarski, R. Dent, J. Gronwald, D. Zuziak, C. Cybulski, J. Kladny, R. Gorski, J. Lubinski, S. A. Narod, *Breast Cancer Res. Treat.* **2009**, *115*, 359.
[36] B. Sirohi, M. Arnedos, S. Popat, S. Ashley, A. Nerurkar, G. Walsh, S. Johnston, I. E. Smith, *Ann. Oncol.* **2008**, *19*, 1847.
[37] J. E. Quinn, R. D. Kennedy, P. B. Mullan, P. M. Gilmore, M. Carty, P. G. Johnston, D. P. Harkin, *Cancer Res.* **2003**, *63*, 6221.
[38] P. Tassone, P. Tagliaferri, A. Perricelli, S. Blotta, B. Quaresima, M. L. Martelli, A. Goel, V. Barbieri, F. Costanzo, C. R. Boland, S. Venuta, *Br. J. Cancer* **2003**, *88*, 1285.
[39] E. M. Swisher, W. Sakai, B. Y. Karlan, K. Wurz, N. Urban, T. Taniguchi, *Cancer Res.* **2008**, *68*, 2581.
[40] G. Damia, M. D'Incalci, *Eur. J. Cancer* **2007**, *43*, 1791.
[41] R. Litman, R. Gupta, R. M. Brush Jr., S. B. Cantor, *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 426.
[42] S. D. Cosimo, J. Baselga, *Eur. J. Cancer* **2008**, *44*, 2781.
[43] S. Yi, J. Uhm, E. Cho, S. Lee, M. Park, H. Jun, Y. Park, J. Ahn, Y. Im, W. Kang, K. Park, *J. Clin. Oncol.* **2008**, *26 (Suppl)*, Abstr. 1008.
[44] J. E. Jaspers, S. Rottenberg, J. Jonkers, *Biochim. Biophys. Acta, Rev. Cancer* **2009**, *1796*, 266.
[45] K. Rhiem, B. Wappenschmidt, K. Bosse, H. Köppler, A. N. Tutt, R. K. Schmutzler, *Clin. Oncol.* **2009**, *21*, 448.
[46] S. K. Pal, J. Mortimer, *Maturitas* **2009**, *63*, 269.
[47] T. Byrski, M. Foszczynska-Kloda, T. Huzarski, R. Dent, J. Gronwald, C. Cybulski, T. Debniak, R. Gorski, J. Lubinski, S. Narod, *J. Clin. Oncol.* **2009**, *27 (Suppl)*, Abstr. 1099.
[48] P. S. Brzovic, J. E. Meza, M.-C. King, R. E. Klevit, *J. Biol. Chem.* **2001**, *276*, 41399.
[49] P. S. Brzovic, J. R. Keeffe, H. Nishikawa, K. Miyamoto, D. Fox III, M. Fukuda, T. Ohta, R. Klevit, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5646.
[50] W. Wu, A. Koike, T. Takeshita, T. Ohta, *Cell Div.* **2008**, *3*, 1.
[51] S. J. Berners-Price, T. A. Frenkiel, U. Frey, J. D. Ranford, P. J. Sadler, *J. Chem. Soc., Chem. Commun.* **1992**, 789.
[52] R. Ishida, Y. Takaoka, S. Yamamoto, T. Miyazaki, M. Otaka, S. Watanabe, A. Komatsuda, H. Wakui, K. Sawada, H. Kubota, H. Itoh, *FEBS Lett.* **2008**, *582*, 3879.
[53] I. Cass, R. L. Baldwin, T. Varkey, R. Moslehi, S. A. Narod, B. Y. Karlun, *Cancer* **2003**, *97*, 2187.
[54] A. D. Frankel, J. M. Berg, C. O. Pabo, *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4841.
[55] T. Zhao, F. L. King, *J. Inorg. Biochem.* **2010**, *104*, 186.
[56] M. El-Khateeb, T. G. Appleton, L. R. Gahan, B. G. Charles, S. J. Berners-Price, A. M. Bolton, *J. Inorg. Biochem.* **1999**, *77*, 13.
[57] P. C. Roehm, J. M. Berg, *Biochemistry* **1997**, *36*, 10240.
[58] B. A. Krizek, D. L. Merkle, J. M. Berg, *Inorg. Chem.* **1993**, *32*, 937.
[59] J. S. Magyar, H. A. Godwin, *Anal. Biochem.* **2003**, *320*, 39.
[60] J. M. Matthews, K. Kowalski, C. K. Liew, B. K. Sharpe, A. H. Fox, M. Crossley, J. P. Mackay, *Eur. J. Biochem.* **2000**, *267*, 1030.
[61] M. P. Byrne, W. E. Stites, *Protein Sci.* **1995**, *4*, 2545.
[62] R. Miao, O. Yang, Y. Miao, Y. Mei, J. Hong, C. Zhao, L. Zhu, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1031.

[63] D. Petrović, B. Stojimirović, B. Petrović, Z. M. Bugarčić, Ž. D. Bugarčić, *Bioorg. Med. Chem.* **2007**, *15*, 4203.
[64] M. Ebadi, P. L. Iversen, *Gen. Pharmacol.* **1994**, *25*, 1297.
[65] B. L. Zhang, W. Y. Sun, W. X. Tang, *J. Inorg. Biochem.* **1997**, *65*, 295.
[66] Y. Saga, H. Hashimoto, S. Yachiku, T. Iwata, M. Tokumitsu, *Int. J. Urol.* **2004**, *11*, 407.
[67] J. R. Eckardt, D. L. Bentsion, O. N. Lipatov, I. S. Polyakov, F. R. MacKintosh, D. A. Karlin, G. S. Baker, H. B. Breitz, *J. Clin. Oncol.* **2009**, *27*, 2046.
[68] J. Holford, F. Raynaud, B. A. Murrer, K. Grimaldi, J. A. Hartley, M. Abrams, L. R. Kelland, *Anti-Cancer Drug Des.* **1998**, *13*, 1.
[69] S. W. Provencher, J. Glöckner, *Biochemistry* **1981**, *20*, 33.
[70] G. Engel, *Anal. Biochem.* **1974**, *61*, 184.

Received October 2, 2009