Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment

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Edited by Dennis A. Carson, University of California at San Diego, La Jolla, CA, and approved August 16, 2012 (received for review March 29, 2012)

Protein disulfide isomerase (PDI), an endoplasmic reticulum chaperone protein, catalyzes disulfide bond breakage, formation, and rearrangement. The effect of PDI inhibition on ovarian cancer progression is not yet clear, and there is a need for potent, selective, and safe small-molecule inhibitors of PDI. Here, we report a class of propynoic acid carbamoyl methyl amides (PACMAs) that are active against a panel of human ovarian cancer cell lines. Using fluorescent derivatives, 2D gel electrophoresis, and MS, we established that PACMA 31, one of the most active analogs, acts as an irreversible small-molecule inhibitor of PDI, forming a covalent bond with the active site cysteines of PDI. We also showed that PDI activity is essential for the survival and proliferation of human ovarian cancer cells. In vivo, PACMA 31 showed tumor targeting ability and significantly suppressed ovarian tumor growth without causing toxicity to normal tissues. These irreversible small-molecule PDI inhibitors represent an important approach for the development of targeted anticancer agents for ovarian cancer therapy, and they can also serve as useful probes for investigating the biology of PDI-implicated pathways.

oral bioavailability | drug resistance | BODIPY-conjugation

Ovarian cancer is one of the leading causes of death in women with gynecological cancers in the United States. About 70% of ovarian cancer cases are diagnosed at a late stage and therefore, poorly treatable (1). Although the current standard treatment for ovarian cancer involving the use of paclitaxel and carboplatin after aggressive surgical cytoreduction usually results in multiyear survival, prolonged use of platinum-based chemotherapy often induces drug resistance, which causes ovarian cancer relapse and eventually, death of patients (2). In this context, there is an urgent medical need for breakthrough drugs with effective therapeutic impact on ovarian cancer.

Protein disulfide isomerase (PDI) is a 57-kDa chaperone protein located in the endoplasmic reticulum (ER) (3). Acting as a thiol oxidoreductase, PDI catalyzes the formation, breakage, and rearrangement of disulfide bonds and therefore, regulates oxidative protein folding as well as cell viability (4, 5). Located in the two PDI active sites in the α- and α’-domains are two conserved cysteine residues within the CGHC motif, which are essential for the activity of PDI and the cycle between oxidized (disulfide) and reduced (dithiol) states (6). It has been reported that ER stress and unfolded protein response can activate PDI expression (7). Increased PDI levels have been documented in a variety of human cancers, including ovarian (8), prostate (9), and lung cancers (10, 11) as well as lymphoma (11), glioma (12, 13), acute myeloid leukemia (7), and melanoma (5, 14). Inhibition of PDI activity leads to apoptosis in cancer (5), suggesting that PDI is a promising druggable target. Moreover, small-molecule PDI inhibitors have been reported to inhibit HIV-1 entry into cells (15). Several small molecules were previously reported as selective irreversible PDI inhibitors that suppressed apoptosis caused by misfolded proteins in a model of Huntington disease (4). A peptide antibiotic, bacitracin, interacts with and inhibits PDI through disulfide bond formation with activity in the high micromolar range (16). Although bacitracin is widely used as a PDI inhibitor in research, its clinical use is hampered by its nephrotoxicity and low membrane permeability (17–19). Therefore, the development of safer and more effective small-molecule PDI inhibitors remains an attractive approach for cancer treatment.

Previously, we reported that a class of propynoic acid carbamoyl methyl amides (PACMAs) showed a broad spectrum of cytotoxicity in a panel of human cancer cell lines, with relatively selective potency in ovarian cancer cells resistant to doxorubicin and paclitaxel (20). Herein, we designed and synthesized a series of PACMA derivatives exhibiting significant cytotoxicity in human ovarian cancer. We also established that these small molecules act as potent irreversible PDI inhibitors. Among these molecules, PACMA 31 (bold numbers are used to indicate compounds) exhibited in vivo activity with oral bioavailability in a mouse xenograft model of human ovarian cancer. PACMA 31 is an orally active small-molecule PDI inhibitor with desirable pharmacological properties for cancer treatment. Most importantly, this study shows that PDI is a druggable target for cancer therapy, and it opens a promising area of research to develop treatments with a unique mechanism of action.

Results

PACMAs Show Cytotoxicity in a Panel of Ovarian Cancer Cell Lines. To establish more informative structure–activity relationships and gain key insights on the likely protein targets of PACMAs in cancer, we designed and synthesized a series of PACMA derivatives (SI Materials and Methods, Scheme S1, and Fig. S1). The compounds were tested in human ovarian cancer cell lines OVCAR-8, NCI/ADR-RES, HEY, and OVCAR-3. Many of these compounds exhibited cytotoxicity with IC_{50} values below 10 μM (Table S1). It is important to note that the NCI/ADR-RES cell line shares a large number of karyotypic abnormalities with OVCAR-8 (21) but expresses high levels of MDRI (multidrug resistance protein 1) P-glycoprotein (22), resulting in resistance to multiple anticancer drugs in clinical use, including paclitaxel and doxorubicin. In addition, the human ovarian cancer cell line HEY is naturally resistant to cisplatin. Therefore, these results implicate our

Author contributions: S.X., N.A.P., and N.N. designed research; S.X., R.Y., Y.Z., B.D., R.D., and E.Z. performed research; A.N.B. contributed new reagents/analytic tools; S.X., Y.Z., B.D., N.A.P., and N.N. analyzed data; and S.X., N.A.P., and N.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1205226109/-/DCSupplemental.
PACMA compounds’ potential ability to overcome the current drug resistance issue in ovarian cancer therapy.

Active PACMA Analogs Covalently Bind to Their Cellular Target Protein in Human Ovarian Cancer Cells. Based on the electron-deficient nature of the propynoic acid amide moiety and confirmed by the structure–activity analysis (SI Results), we anticipated that the active PACMAs would be able to react irreversibly with certain nucleophilic groups, such as the thiol groups of cysteine side chains, to form covalent adducts. This property can be used to identify the protein target responsible for their activity and selectivity. To test this hypothesis, we first conjugated one of the most active analogs, 31, to the fluorescent dye BODIPY (boron-dipyrromethene), resulting in 57. We also synthesized 58, a close analog of 57 that lacks the propynoyl group and is expected to be inactive, as well as the BODIPY compound 59 with acylated linker that can serve as the control (Fig. 1A). Fluorolog was used to determine the fluorescent properties of 57 (ex = 490 nm, em = 537 nm) (Fig. S2). The ability of 31, 57, 58, and 59 to inhibit ovarian cancer cell growth was compared. PACMAs 31 and 57 exhibited similar potency (Fig. 1B), indicating that the conjugation of BODIPY to 31 did not affect the cytotoxic activity of 31. No considerable cytotoxicity was observed with 58 or 59, showing that the electrophile alkyne is essential for potency and that the BODIPY moiety does not contribute to cytotoxicity. In addition, 57 and 58 displayed comparable fluorescent activity and were slightly less fluorescent than 59 (Fig. 1C), suggesting that the conjugations quenched the fluorescence of BODIPY only to a small extent.

To examine whether the active analog 57 covalently binds to its target protein, we treated OVCAR-8 cells with 57, 58, 59, or equal amounts of DMSO. Cells were lysed after treatment and subjected to SDS/PAGE. A fluorescent band (∼57 kDa) was only observed in the lane with 57-treated samples (Fig. 1D). The interaction of 57 with its cellular protein target of ∼57 kDa is covalent, because it was preserved under the denaturing conditions of the SDS/PAGE.

Identification of PDI as the Target of PACMAs. To identify the 57-kDa protein, we performed 2D gel electrophoresis with whole-cell lysates from 57-treated OVCAR-8 cells (Fig. 2A). Using MS, PDI was identified as a protein target of compound 57 (Fig. S3).

To confirm PDI as the target, we treated OVCAR-8 cells with 57–59 or DMSO. Whole-cell lysates were subjected to immunoprecipitation with anti-PDI antibody. A strong fluorescent band of ∼57 kDa was detected only in the lane with 57-treated OVCAR-8 cells (Fig. 2B), indicating that 57 covalently bound to PDI. In addition, subcellular colocalization of PDI and 57 was determined in OVCAR-8 cells using confocal microscopy (Fig. 2C). To evaluate whether the parent PACMA 31 binds to the same site in PDI as its fluorescent analog 57, we performed a competition assay using purified recombinant PDI protein; 31 pretreatment blocked recombinant PDI protein from binding 57 (Fig. 2D), showing that the conjugation with BODIPY moiety does not change the target site of 31. Fig. 2E shows that 57 binds to PDI protein in a time-dependent manner. The presence of DTT considerably increased this interaction (comparing lane 8 with lane 2), indicating that 57 targets free cysteine residues. Additionally, the binding of 57 to PDI is temperature-dependent (Fig. S4). We also evaluated the selectivity of the active analogs for PDI. After incubating 57 with an equal amount of PDI, BSA, or GRP78 (78-kDa glucose-regulated protein) core domain (another important molecular chaperone within the ER), we showed that 57 selectively binds to PDI, whereas no detectable fluorescence was observed from 57-treated BSA or GRP78 core domain (Fig. 2F and Fig. S5). Together, these results indicate that the active PACMAs selectively target and covalently bind to PDI.

To identify the precise cysteine residues in PDI that form covalent bonds with 31, online LC-Orbitrap CID (collision induced dissociation) and ETD (electron transfer dissociation) MS/MS were used (technical details are described in SI Materials and Methods and workflow for sample preparation and data analysis is shown in Fig. S6A). The addition of 31 led to a defined mass change of one peptide derived from the digestion of recombinant PDI protein that was directly measured by high-resolution MS with high confidence (Fig. S6B). This peptide fragment contains PDI’s active site cysteines: C(397)GHC(400). The mass shift suggests that recombinant PDI was modified by 31 at either Cys397 or Cys400. Interestingly, Cys397 and Cys400 were not modified simultaneously, presumably because of steric hindrance caused by the binding of a PACMA 31 molecule. CID and ETD fragmentation MS/MS were also used to localize the modification site with single amino acid resolution. Detection of precursor ions at high resolution and a nearly complete series of fragmentation ions from both CID (Fig. S6C) and ETD (Fig. S6D) allowed the accurate sequencing and assignment of the modification site to Cys397/Cys400. Integrating all of the CID and ETD results, Protein Discoverer 1.3 automatically assigned potential modification sites at either Cys397 or Cys400 with high confidence (Fig. S6 E and F).

When 31 was docked on Cys397 of PDI using Protein Data Bank structure ID code 3UEM, we obtained a fitness score of 40.73, forming a covalent bond between the terminal carbon atom of the propynoic moiety of 31 and the sulfur atom of Cys397. We also observed two additional π–π interactions: one between 31’s phenyl ring in R1 and PDI’s Trp396, and the other between 31’s thienyl ring (R2) and PDI’s Phe304 (Fig. 3A). When 31 was docked on Cys400 of PDI, we obtained a fitness score of 33.53. PACMA 31 bound PDI through a covalent bond with Cys400 on the opposite side of the Cys397 site (Fig. 3B). In addition, 31’s amide (–NH) formed a hydrogen bond with an oxygen within Pro395. We also
observed an extra π-cation interaction between 31’s phenyl ring in R₁ and a side chain nitrogen of Lys401.

Active PACMAs Affect PDI Secondary Structure and Inhibit PDI Activity.

Circular dichroism spectroscopy was performed to examine whether covalent binding of the active PACMAs to PDI would affect its secondary structure (23). Based on the circular dichroism data analysis using the K2D2 web server (www.ogic.ca/projects/k2d2) (24), PACMAs 57 and 31 affected the secondary structure of PDI, whereas no substantial difference was observed between the spectra of recombinant PDI treated with vehicle control DMSO and the inactive analog 56 (Table 1 and Fig. S7A, representative curves). No substantial changes were observed in the secondary structure of the control protein BSA treated with 31, 56, or 57 (Table 1 and Fig. S7B, representative curves), indicating that covalent binding of active PACMAs to PDI affects its secondary structure.

Changes in protein structure are usually associated with variations in protein activity. PDI in the ER of mammalian cells is in its reduced state, allowing PDI to reduce and isomerize non-native disulfide bonds of target proteins (25). We, therefore, examined the reductase activity of PDI with or without PACMA treatment in the insulin aggregation assay, a well-established assay for evaluating the activity of PDI (15); 31 significantly inhibited the activity of PDI in a dose- and time-dependent manner, producing complete inhibition at 100 μM (Fig. 4A).

Direct comparison of 31 and phenylarsine oxide (PAO; a previously reported small-molecule PDI inhibitor) (26) (Fig. 4B) showed that 31 (IC₅₀ of 10 μM) is a more potent PDI inhibitor than PAO (IC₅₀ of 85 μM). However, 56 was inactive as expected (Fig. 4C). These results show that covalent binding of the active PACMAs to PDI inhibits its enzymatic activity.

Although PDI has been reported to play an important role in cancer progression (5, 12, 23, 27), it may be cancer- and cell type-specific. Therefore, we evaluated the viability of human ovarian cancer cells by silencing PDI. PDI siRNA substantially down-regulated PDI expression in OVCAR-8 cells between 24 and 96 h (Fig. 5A). This finding was consistent with the significant inhibition of OVCAR-8 cell growth in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 5B).
Additionally, silencing of PDI significantly inhibited colony formation by OVCAR-8 cells (Fig. 5C). Similarly, 31 significantly inhibited colony formation in OVCAR-8 cells in a dose-dependent manner (Fig. 5D). These results indicate that silencing of PDI is sufficient to cause considerable cytotoxicity in ovarian cancer cells.

**PACMA 31 Suppresses Tumor Growth in Human Ovarian Cancer Mouse Xenografts.** To evaluate the tumor targeting ability of active PACMAs in vivo, we tested 37–59 in a mouse xenograft model of human OVCAR-8 ovarian cancer. After 3 d of continuous i.p. administration, tumor, liver, and brain tissues were collected and prepared for frozen sections that were analyzed using fluorescent microscopy. PACMA 57-treated tumor sections exhibited strong fluorescence intensity, whereas no fluorescence was detected in 58- or 59-treated tumor sections (Fig. 6A). Consistent with our in vivo data, one fluorescent band at ~57 kDa was observed only in the 57-treated tumor sample (Fig. 6A), confirming that PDI is covalently modified by 57 in the tumors. Because the liver is the major organ for drug metabolism, we also examined the fluorescence intensity in the liver of 57–59-treated animals by fluorescence microscopy using the same settings and conditions used to examine the tumor sections. Compared with 57-treated tumor sections, the liver sections of 57-treated animals exhibited substantially lower fluorescence intensity (Fig. 6A). In comparison, liver sections from 58-treated animals exhibited higher fluorescence intensity than liver sections from 57-treated animals. No fluorescence emission was detected in 59-treated liver sections as expected. In addition, no fluorescence emission was detected in brain sections with treatments of active or inactive compounds. Taken together, these data show that the active PACMAs selectively target and accumulate in ovarian tumors in vivo, and they do not cross the blood–brain barrier.

To determine the in vivo efficacy of 31, we tested its effect on established tumors from OVCAR-8 cells through i.p. or per os administration; 31 was given i.p. at 20 mg/kg per day for the first 3 wk, with 5-d on and 2-d off treatment cycles. The dose was escalated to 40 mg/kg per day for the next 7 d. After 30-d i.p. treatment, the mouse xenografts were left untreated for an additional 32 d (Fig. 8A). A second xenograft study was conducted to evaluate per os administration of 31. Treatment was initiated with a dose of 20 mg/kg per day, and it was gradually increased by 20 mg/kg per day with each dose for 3 d before it was orally dosed at 200 mg/kg per day for an additional 32 d. The total duration of the oral treatment was 62 d (Fig. S8). Compared with the control group, i.p. or per os administration of 31 significantly inhibited tumor growth by 85% (from 796.6 to 117.0 mm³, \( P = 0.009 \)) and 65% (from 796.6 to 280.1 mm³, \( P = 0.015 \)) at day 62, respectively (Fig. 6B). Thus, 31 not only suppresses tumor growth in vivo but also is orally bioavailable. One mouse was found dead on day 30 in the i.p. administration group, but no obvious abnormalities were observed in other 31-treated mice. The tumors in the i.p. treatment group did not aggressively grow after treatment was stopped on day 30. This finding may be at least partially because of the prolonged duration of drug action of 31, a common feature of irreversible inhibitors. Compared with mice in the control group, no substantial body weight loss was detected in i.p. or per os groups during the study (Fig. S9A), indicating that 31 did not exert severe adverse effects on the mice at its effective anticancer dose. All mice were dissected at the end of the study (day 62). H&E staining of tumor sections showed extensive areas of necrosis in both 31 treatment groups (i.p. and per os) compared with the control group (Fig. 6C). In addition, no detectable abnormalities were observed in the organs examined, including liver, kidney, spleen, heart, lung, and pancreas (Fig. S9B), further showing the safety of PACMA 31.

**Discussion**

In this study, we designed and synthesized a series of PACMAs that showed in vitro and in vivo anticancer activity in human ovarian cancer by targeting PDI. As a prototype of the ER protein disulfide isomerase family, PDI catalyzes the formation, cleavage, and rearrangement of disulphide bonds and facilitates oxidative protein folding by acting as a molecular chaperone (28). Therefore, PDI inhibition causes accumulation of unfolded or misfolded proteins that leads to ER stress and the unfolded protein response (UPR) that results in cell death (5). Compared with normal tissues, PDI is overexpressed in ovarian tumors (8). In addition, estrogen has been reported to increase PDI expression (<\( P < 0.05 \)) and PAO. (B) Comparison of the inhibitory activity of 31 and PAO. (C) PACMA 56 did not exhibit significant effects on the enzymatic activity of PDI. Experiments were performed in triplicate.

**Fig. 4.** Active PACMAs inhibit PDI activity. (A) PACMA 31 significantly inhibited the activity of PDI in a dose- and time-dependent manner in the insulin aggregation assay. Curves were generated from mean values (BAR, SEM). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). (B) Comparison of the inhibitory activity of 31 and PAO. (C) PACMA 56 did not exhibit significant effects on the enzymatic activity of PDI. Experiments were performed in triplicate.

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**Table 1.** Active PMCMAs affected PDI secondary structure

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agents, fenretinide and velcade, in human melanoma cells (5). In addition, both bacitracin and a PDI monoclonal antibody can inhibit migration and invasion of glioblastoma cells (12). However, the role of PDI in apoptosis might be cell type- and tissue-specific. It was documented that PDI knockdown by PDI siRNA has significant effects on the viability of human cervical cancer HeLa cells (32). Therefore, PDI could be exploited as an important target for developing drugs against different cancers.

Irreversible drugs have proved to be successful therapies for various indications (33). To date, a large number of irreversible inhibitors have been reported to exhibit anticancer activities, such as α-difluoromethylornithine and covalent EGF receptor inhibitors (HKI-375, HKI-272, EKBS569, BIBW2992, and PF299804) (34). Similar to the binding of irreversible inhibitors, post-translational modifications of PDI also suppress its enzymatic activity. Nitrosative stress-induced S-glutathionylation of PDI inhibits its enzymatic activity, leading to the activation of UPR and cell death in human ovarian cancer SKOV3 cells (23). Additionally, NO-induced S-nitrosylation of PDI down-regulates its enzymatic activity, resulting in the accumulation of polyubiquitinated proteins and the activation of UPR in neurodegenerative disorders (35).

The chemical structure of 31 suggests that it may be a prodrug, because it is possible that the ester bond can be hydrolyzed into an acid in vivo, converting 31 into 32 (Fig. S1). Because the activity of 31 is mostly dependent on the terminal acetamido-acetic acid moiety, this potential change does not cause loss of activity. This possibility is also supported by the activity of 32, although it is less active (Table S1), probably because of the lower permeability of 32 caused by the negatively charged acidic group in solution.

PDI possesses two independent active sites: C(53)GHC(56) and both are located in the active C(397)GHC(400) site within the α-domain (36). Disruption of cysteines in either active site abolishes 50% of the catalytic activity of PDI, whereas disruption of cysteines in both active sites completely abolishes its catalytic activity (37). Using a liquid chromatography MS/MS method, we identified the PACMA 31 modification sites in the purified recombinant PDI protein as either Cys397 or Cys400, and both are located in the active C(397)GHC(400) site within the α-domain (36). Disruption of cysteines in either active site abolishes 50% of the catalytic activity of PDI, whereas disruption of cysteines in both active sites completely abolishes its catalytic activity (37). Using a liquid chromatography MS/MS method, we identified the PACMA 31 modification sites in the purified recombinant PDI protein as either Cys397 or Cys400, and both are located in the active C(397)GHC(400) site within

**Fig. 5.** Silencing of PDI inhibits cell growth of OVCAR-8 cells. (A) Representative Western blot of 24–96 h PDI silencing in OVCAR-8 cells. (B) PDI siRNA showed significant cytotoxicity as measured by MTT assay. The histogram shows the mean values of growth inhibition (%). (C) PDI knockdown significantly inhibited colony formation in OVCAR-8 cells. The histogram shows the mean number of colonies. (D) The 24-h treatment of 31 significantly inhibited the formation of OVCAR-8 colonies at indicated doses. The histogram shows the mean number of colonies (BAR, SEM). **P < 0.01; ***P < 0.001.

**Fig. 6.** PACMA 31 suppresses tumor growth in a mouse xenograft model of human OVCAR-8 ovarian cancer. (A) Mice were treated with 57, 58, and 59 at 10 mg/kg for 3 d with two injections per day; 6 h after the last injection, tumor, liver, and brain tissues were prepared for frozen sections and fluorescent microscopy analysis. (Left) Representative fluorescence images of indicated tissue sections captured using fluorescence and Coomassie blue stain. (Right) Gel fluorescence and Coomassie blue analysis of homogenized tumor samples from the 57- and 58-treated mice. An ∼57-kDa fluorescent band in the 57-treated tumor is indicated by a red arrow. (B) Growth curves of s.c. tumors in mice treated with 31 through i.p. (red; n = 4) or per os administration (yellow; n = 4) or treated with vehicle (blue; n = 5). Treatment schedules are described in Fig. S8. Results are presented as mean tumor volume (BAR, SEM). (Inset) Comparison of tumor volumes between control and 31 i.p. (**P < 0.01) or 31 per os treatment group (*P < 0.05) on day 62 (BAR, SEM). (C) PACMA 31 treatment induced extensive areas of necrosis in OVCAR-8 tumors. Representative images of H&E-stained tumor sections from control (Left) and 31-treated (Center, i.p.; Right, per os) mice are shown. Arrows indicate areas of necrosis.
the α-domain (Fig. S6 B–F). The location of this modification sites is consistent with the ability of 31 to inhibit the enzymatic activity of PDI. Additional modifications at Cys53 and Cys56 may occur in vivo because of the different redox environment in the ER, where these four cysteines should be in a reduced state that is required for full catalytic activity. In the recombinant PDI protein produced in Escherichia coli, a disulfide bond is probably formed between Cys53 and Cys56, which was shown in a PDI structure (Protein Data Bank ID code 1MEK) solved from a recombinant protein also produced in E. coli (38). Such a disulfide bond can protect Cys53/56 from PACMA modification in vitro.

In summary, we identified PDI as a cellular protein target for PACMAs. We also showed that PDI knockdown in human ovarian cancer cells was cytotoxic and that our irreversibly PDI inhibitors exhibited both in vitro and in vivo anticancer activity in human ovarian cancer models with tumor targeting ability and no substantial toxicity to normal tissues. Moreover, PACMAs were effective on human ovarian cancer cell lines resistant to conventional chemotherapy. Resistance to first-line therapy occurs in all ovarian cancer patients and is a major cause of mortality. Therefore, development of effective and safe PDI inhibitors as anticancer agents may overcome the current treatment failure in ovarian cancer therapy.

Materials and Methods

Measurement of PDI Activity. PDI activity was assayed by measuring the PDI-catalyzed reduction of insulin in the presence of DTT, thus measuring the aggregation of reduced insulin B chains at 620 nm as described previously (15). Briefly, recombinant PDI protein (0.4 μM) was incubated with indicated compounds at 37 °C for 1 h in sodium phosphate buffer (100 mM sodium phosphate, 2 mM EDTA, 8 μM DTT, pH 7.0). After this incubation, the modified recombinant PDI protein was added to the reaction mixture containing 100 μM cysteine and 200 μM DTT (500 μM dihydroxybenzyl disulfide (130 μM Sigma). The reduction reaction was catalyzed by PDI at room temperature, and the resulting aggregation of reduced insulin B chains was measured at 620 nm.

In Vivo Tumor Xenograft Studies. OVCA-R8 cells in logarithmic growth phase from cell culture were implanted in athymic mice (C57BL/6×129SvEvTac; both sexes) and grown subcutaneously for 5 days to a size of 5 mm diameter. Treatment schedules are described in Fig. S8. Treatment of each animal was the longest and shortest diameters, respectively. For i.p. administration, tumors were allowed to grow to an average volume of 50 mm³. Mice were then randomly assigned into three groups: vehicle control (n = 5), i.p. treatment with 31 (n = 4; 20 mg/kg per day for the first 3 wk with 5-d on and 2-d off treatment cycles, and dose was escalated to 40 mg/kg per day for the next 7 d), and per os treatment of 31 (n = 4; the initial dose of 20 mg/kg per day was gradually increased by 20 mg/kg per day with each dose for 3 d before it was orally dosed at 200 mg/kg per day for an additional 3 d, increasing the dose from 20 to 200 mg/kg). Treatment schedules are described in Fig. S8. Treatment of each animal was based on individual body weight. The body weights and tumor volumes in each group were measured twice per week. The percentage of tumor growth inhibition was calculated as TC50 = 100 × (mean TW of treated group)/mean (control TW) of all ovarian cancer patients and is a major cause of mortality. Therefore, development of effective and safe PDI inhibitors as anticancer agents may overcome the current treatment failure in ovarian cancer therapy.

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