The c-MET/PI3K Signaling Is Associated with Cancer Resistance to Doxorubicin and Photodynamic Therapy by Elevating BCRP/ABCG2 Expression

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ABSTRACT

Overexpression of BCRP/ABCG2, a xenobiotic efflux transporter, is associated with anticancer drug resistance in tumors. Proto-oncogene c-MET induces cancer cell proliferation, motility, and survival, and its aberrant activation was found to be a prognostic factor in advanced ovarian cancers. In the present study, we investigated the potential crossresistance of doxorubicin-resistant ovarian cancer cells to the photosensitizer pheophorbide a (Pba)-based photodynamic therapy (PDT), and suggest c-MET and BCRP/ABCG2 overexpression as an underlying molecular mechanism. The doxorubicin-resistant A2780 cell line (A2780DR), which was established by incubating A2780 with stepwise increasing concentrations of doxorubicin, showed low levels of cellular Pba accumulation and reactive oxygen species generation, and was more resistant to PDT cytotoxicity than A2780. In a microarray analysis, BCRP/ABCG2 was found to be the only drug transporter whose expression was upregulated in A2780DR; this increase was confirmed by Western blot and immunocytochemical analyses. As functional evidence, the treatment with a BCRP/ABCG2-specific inhibitor reversed A2780DR resistance to both doxorubicin and PDT. We identified that c-MET increase is related to BCRP/ABCG2 activation. The c-MET downstream phosphoinositide 3-kinase (PI3K)/AKT signaling was activated in A2780DR and the inhibition of PI3K/AKT or c-MET repressed resistance to doxorubicin and PDT. Finally, we showed that the pharmacological and genetic inhibition of c-MET diminished levels of BCRP/ABCG2 in A2780DR. Moreover, c-MET inhibition could repress BCRP/ABCG2 expression in breast carcinoma MDA-MB-231 and colon carcinoma HT29, resulting in sensitization to doxorubicin. Collectively, our results provide a novel link of c-MET overexpression to BCRP/ABCG2 activation, suggesting that this mechanism leads to crossresistance to both chemotherapy and PDT.

Introduction

The anthracycline antibiotic doxorubicin (Dox; adriamycin) is an effective drug for the treatment of a variety of solid tumors including ovarian cancer. Although its activity is not fully understood, it has been known that Dox induces apoptosis in cancer by DNA intercalation, inhibition of DNA synthesis, and reactive oxygen species (ROS) generation (Müller et al., 1998; Minotti et al., 2004). Due to a dose-limiting cardiotoxicity, the development of Dox resistance can finally cause treatment failure. Cellular defects, including p53 loss and death receptor impairment are related to poor therapeutic response to courses of Dox treatment (Müller et al., 1998). In addition, increased expression of drug efflux transporters, such as MDR1/ABCB1 and BCRP/ABCG2, has been observed in refractory tumors (Jamieson and Boddy, 2011).

Photodynamic therapy (PDT) has been approved worldwide as an alternative treatment modality for several types of cancer (Dougherty et al., 1998; Hopper, 2000; Dolmans et al., 2003). In PDT, a photosensitizer within tumor tissue is activated by light irradiation, generating singlet oxygen ($^{1}\text{O}_2$) by transferring energy to adjacent molecular oxygen. In addition, it has been reported that the activated photosensitizer can directly produce ROS by reacting with oxygen. Because singlet oxygen and ROS are damaging to cellular components, targeted cancer cells undergo apoptotic or necrotic cell death (Sharman et al., 2000; Buytaert et al., 2007). In a clinical setting, the use of PDT is largely limited to superficial tumors because the wavelengths of light used in PDT cannot penetrate the body. Pheophorbide a (Pba) is a chlorophyll derivative from silkworm excreta and the Chinese medicinal herb Scutellaria barbarta (Park et al., 1989; Chan et al., 2006). Unlike two Food and Drug Administration–approved photosensitizers Photofrin and Levulan (5-aminolevulinic acid), Pba can be excited by a longer

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ABBREVIATIONS: Dox, doxorubicin; HGF, hepatocyte growth factor; H342, Hoechst 33342, 2’-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2’-bi-1H-benzimidazole trihydrochloride trihydrate; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1’2’:1,6’]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylhydantoin ester hydrate; LY294, LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Pba, pheophorbide a; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDT, photodynamic therapy; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; RT, reverse-transcriptase; SU112, SU11274, (3Z)-N-(3-chlorophenyl)-3-(3,5-dimethyl-4-[[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrl-2-ylmethylene)-N-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide.
wavelength of light, enabling enhanced penetration into peritoneal cavity–located tumors such as ovarian cancer (Xodo et al., 2012). Pba was shown to cause mitochondria-dependent apoptosis in cultured cancer cells (Tang et al., 2009). In vivo animal models also demonstrated that Pba impedes tumor growth in colon carcinoma and hepatoma xenograft models (Hajri et al., 2002; Li et al., 2007). However, the development of PDT resistance has been displayed: colon cancer cells repeatedly exposed to PDT agents developed resistance to PDT (Singh et al., 2001). Although there are many discordant reports, cross-resistance between PDT and chemotherapy has been suggested. Fibrosarcoma cells resistant to PhotoFrin-PDT showed low levels of cisplatin cytotoxicity and cisplatin-DNA adduct formation (Moorehead et al., 1994).

ABC transporters such as BCRP/ABCG2 control the active efflux of intracellular xenobiotic chemicals through the plasma membrane (Jamieson and Boddy, 2011; Stacy et al., 2013). The active form of BCRP/ABCG2 is a homodimer (140 kDa) located in the plasma membrane. BCRP/ABCG2 was first reported as a xenobiotic transporter in a multidrug-resistant human breast cancer cell line (Doyle et al., 1998). After this, it was revealed that BCRP/ABCG2 overexpression is responsible for drug resistance to anticancer Dox, mitoxantrone, and topotecan (Litman et al., 2000). In addition, increased BCRP/ABCG2 expression was negatively correlated with accumulation of porphyrin-type photosensitizers such as Pba and protoporphyrin IX (Robey et al., 2005), which leads to poor responsiveness to PDT. The involvement of several response elements in the 5′-flanking region has been suggested as an underlying mechanism of BCRP/ABCG2 expression control. These regulating sequences include response elements for PPAR, hypoxia, and progesterone (An and Ongkeko, 2009). Overexpression of PPARγ was shown to elevate BCRP/ABCG2 expression in human dendritic cells (Szatmari et al., 2006). The incubation of human placental cells with progesterone and estradiol increased BCRP/ABCG2 expression via the progesterone receptor (Wang et al., 2006). Binding of the hypoxia-inducible factor 1α to the BCRP/ABCG2 promoter increased BCRP/ABCG2 gene transcription, implying the association of BCRP/ABCG2 overexpression within tumors (Krishnamurthy et al., 2004). The tert-butylhydroquinone-inducible BCRP/ABCG2 expression was mediated through NF-E2-related factor 2 (NRF2) in HepG2 cells (Adachi et al., 2007).

The receptor tyrosine kinase c-MET is primarily expressed in epithelial and endothelial cells. It transduces signaling from hepatocyte growth factor (HGF) primarily produced by mesenchymal and stromal cells (Ghiso and Giordano, 2013; Gravel et al., 2013). HGF/c-MET signaling has been associated with the oncogenesis of various cancers by enhancing cell proliferation, motility, and survival. The expression of c-MET is highly elevated in lung, prostate, and ovarian cancers; high expression levels are strongly associated with poor prognosis (Humphrey et al., 1995; Ichimura et al., 1996; Sawada et al., 1996). Upon stimulation, the autophosphorylation of the c-MET homodimer mediates downstream signaling via multiple molecular pathways, including the mitogen-activated protein kinases, phosphatidylinositol-4,5-bisphosphate 3-kinase [phosphoinositide 3-kinase (PI3K)]–AKT, and signal transducer and activator of transcription (Ghiso and Giordano, 2013; Gravel et al., 2013).

In this study, we investigated the development of potential crossresistance between chemotherapy and PDT using the ovarian carcinoma Dox-resistant A2780 (A2780DR) cell line, which has acquired adaptive resistance to Dox. A2780DR exhibits resistance to Pba-based PDT when compared with its parent cell line A2780. Cellular Pba accumulation, ROS generation, and cytotoxicity of Pba-based PDT are lowered in A2780DR. We showed that the elevated level of BCRP/ABCG2 is responsible for A2780DR resistance to Dox and PDT. Further analyses with microarrays and pharmacological inhibitors revealed that c-MET overexpression and the resultant activation of the P13K/AKT signaling cascade is involved in increased expression of BCRP/ABCG2 and crossresistance to Dox and PDT.

**Materials and Methods**

Pba was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dox, Ko143 ([3S,6S,12aS]-1,2,3,4,6,7,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxoiazepin(o1,2,3,4,6)-pyrido[3,4,6]indole-3-propanoic acid 1,1-dimethyl ester; hydrate), LY294002 (LY294; 2-morpholin-4-yl-8-phenylchromen-4-one), SU11274 (SU1112; (3Z)-N-(3-chlorophenyl)-3(3,5-dimethyl-4-(4-methylpiperazin-1-yl)carbonyl)-1H-pyrrol-2-ylmethylene)-N-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide), propidium iodide, 3,4-(5-dimetithythiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT), and puromycin were purchased from Sigma-Aldrich (Saint Louis, MO). Hoechst 33342 (H342; 2′(4-ethoxyphenyl)-5-[4-(methyl-1-piperazinyl)-2,5-bis-1H-benzimidazole tris hydrochloride trihydrate), trans-1,2′-methoxyvinylpyrene, carboxy-2′, 7′-dichlorofluorescein diacetate, and Alexa Fluor 488 goat anti-rabbit IgG (H + L) were purchased from Life Technologies (Carlsbad, CA). The SYBR Premix Ex Taq system was obtained from Takara (Otsu, Japan). Antibodies recognizing c-MET, P13K (P13K regulatory subunit-1α), AKT, p-AKT, and BCRP/ABCG2 were obtained from Cell Signalling Technology (Danvers, MA). β-Tubulin antibody was purchased from Santa Cruz Biotechnology. Cisplatin and 5-fluorouracil were purchased from Sigma-Aldrich. Paclitaxel was obtained from MedKoo Biosciences (Chapel Hill, NC).

**Cell Culture.** Human ovarian cancer cell line A2780 was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). A2780DR was established in our previous study (Shim et al., 2009). These cells were maintained in RPMI 1640 (HyClone, Logan, UT) with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (WelGene Inc., Daegu, South Korea). The human breast cancer cell line MDA-MB-231 and colon cancer cell line HT29 were obtained from American Type Culture Collection (Rockville, MD). A2780 and A2780DR were plated at a density of 5 × 10^4 cells/well in 96-well plates and after 24 hours, PDT was performed as described in a previous study by Choi et al. (2014). Briefly, the cells were treated with Pba (0.025–2.5 μg/ml) for 6 hours, and then the Pha-containing medium was removed. The 670 nm LED Hybrid Lamp system (Quantum Spectra Life, Barneveld, WI) was used to irradiate the cells with a 1.2 J/cm² laser. Then, the cells were recovered in a complete medium for 18 hours for further analyses.

**MTT Analysis.** Cells were plated at a density of 3 × 10^4 cells/well in 96-well plates. After the treatment with PDT or anticancer agents, the MTT solution (2 mg/ml) was incubated for 4 hours. The MTT solution was removed and 100 μl of dimethylsulfoxide was added to each well. The absorbance was measured at 570 nm using a SPECTROstar Nano microplate reader (BMG Laboratory Technologies, Offenburg, Germany).

**Measurement of ROS.** Cells were plated at a density of 7 × 10^4 cells/well on a cover glass slide (SPL Life Sciences, Gyeonggi-do, Korea). After PDT, the cells were washed with phosphate-buffered saline (PBS) and incubated with trans-1,2′-methoxyvinylpyrene (50 μM) or carboxy-2′,7′-dichlorofluorescein diacetate (30 μM) at 37°C as
Results

A2780DR was More Resistant to Dox Accumulation and PDT Cytotoxicity Than A2780. The A2780DR cell line was established by maintaining human ovarian carcinoma A2780 cells in Dox-containing medium (Shim et al., 2009). When A2780 and A2780DR cells were incubated with Dox (0.2–1.6 μM) for 72 hours, the A2780DR cells showed much higher viability than the parental A2780 cells (Fig. 1A). The anthracycline chromophore group in Dox is also a fluorophore; therefore, cellular accumulation of Dox can be visualized and quantified using a fluorescent cell imager. Incubation with Dox increased the level of cellular fluorescence in a concentration-dependent manner (1–4 μM) in both cell lines; however, the magnitude of increase is significantly smaller in A2780DR cells (Fig. 1B). The cellular Dox level increased up to 11-fold following 4 μM Dox incubation for 6 hours in A2780, whereas only a 5.5-fold increase was observed in A2780DR. The A2780DR sensitivity to other anticancer drugs such as cisplatin, paclitaxel, and 5-fluorouracil was not significantly different from A2780, suggesting that Dox resistance of A2780DR had developed through a specific mechanism (Supplemental Fig. 1).

PDT has been approved as an effective treatment option for various solid tumors. The identification of molecular determinants of the responsiveness of PDT is necessary to optimize the therapeutic effect of PDT. In this aspect, we hypothesized that Dox-resistant A2780DR cells may have differential susceptibility to PDT and we investigated the responsiveness of A2780DR to Pa-based PDT. MTT analysis showed that incubation with Pba (24 hours) up to 2.5 μg/ml did not affect cell viability in both A2780 and A2780DR (Fig. 1C). When Pba-incubated A2780 was irradiated with laser light for 60 seconds, the viable cell number was decreased in a concentration-dependent manner; only 25 and 14% of cells survived following PDT with 0.125 and 0.25 μg/ml Pba, respectively (Fig. 1D). A2780DR was clearly resistant to PDT cytotoxicity: 83% cells were viable following 0.25 μg/ml Pba-PDT. These results indicate that A2780DR is crossresistant to Dox and Pba-PDT.

PDT-Induced ROS Increase and Cellular Pba Accumulation Were Mitigated in A2780DR. The levels of cytotoxic oxygen species were significantly lower following PDT in A2780DR than in A2780. The incubation of cells with singlet oxygen-specific dye showed an increase in green fluorescence right after laser irradiation in A2780. Fluorescence intensity

**Immunocytochemical Analysis.** Cells were plated at a density of 7 × 10^3 cells/well on cover glass slides and drug treatment was initiated. The cells were washed with cold PBS three times and were fixed in cold methanol for 10 minutes. Then, anti-BCRP/ABCG2 (1:200) antibody was incubated in fixed cells at 4°C overnight. The next day, the cells were washed with PBS and the Alexa Fluor 488 goat anti-rabbit antibody (1:500) incubation was performed at room temperature for 1 hour. For nuclear staining, propidium iodide (1:250) incubation was executed for 10 minutes. Fluorescence images were obtained using the LSM 710 confocal microscope (Carl Zeiss).

**c-MET siRNA Transfection.** Predesigned c-MET siRNA (#51, 5'-GUGAAGALUCGUGCAUGCUA-3' and 5'-UGACACAUUGAGGUCCUC-3') and a scrambled control siRNA were obtained from Bioneer. The cells were seeded in 60 mm plates at a density of 5 × 10^3 cells and grown overnight. The next day, cells were transfected with c-MET siRNA using a Lipofectamine 2000 reagent (Life Technologies). RNAs and proteins were extracted after recovery for 24 hours in complete medium.

**Statistical Analysis.** Statistical significance was determined by a Student’s paired t test followed by one-way analysis of variance and the Student’s Newman-Keuls test for multiple comparisons using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).
was elevated by 1.3- and 1.6-fold in the 0.25 and 2.5 μg/ml group, respectively (Fig. 2A), whereas the presence of singlet oxygen was significantly lower in A2780DR. Similarly, PDT-treated A2780 exhibited a high level of cellular ROS not observed in A2780DR (Fig. 2B). These findings support reduced cytotoxic responsiveness of A2780DR to PDT. In particular, it was noted that the generated singlet oxygen level is low in A2780DR, which implies that the cellular level of photosensitizer Pba might be differential. Following Pba incubation for 6 hours, cellular red fluorescence intensities derived from Pba were elevated by 28-fold in A2780 with 2.5 μg/ml Pba. On the other hand, A2780DR cells accumulated less Pba: the fluorescent intensity increase was 10.5-fold in 2.5 μg/ml Pba (Fig. 2C). When the cell lysates from the lower concentrations of Pba-incubated cells (0.25, 0.5, and 1 μg/ml), and were measured with fluorometry, similar differences were observed (Supplemental Fig. 2). These results indicate that PDT susceptibility is low in Dox-resistant ovarian cancer cells, and this phenomenon is associated with a diminished cellular level of Pba.

**BCRP/ABCG2 Activation-Induced Dox Resistance in A2780DR.** The efflux of cellular Dox is mediated by ABC transporters such as MDR1/ABCB1, BCRP/ABCG2, and MRP1/ABCC1 (Jamieson and Boddy, 2011). In an attempt to elucidate the causative reason of lower cellular levels of Dox and Pba, the transporter expression pattern of A2780DR was compared with that of A2780. It is especially notable that BCRP/ABCG2 was the only gene among the 50 sampled ABC transporters in which expression is elevated more than 50% in A2780DR. The transcript level of BCRP/ABCG2 was 45-fold higher in A2780DR compared with A2780 (Table 1).

When transcript levels for BCRP/ABCG2, MDR1/ABCB1, and MRP1/ABCC1 were determined using real-time RT-PCR analysis, the BCRP/ABCG2 mRNA level was 71 times higher in A2780DR, implicating its potential role in differential Pba accumulation (Fig. 3A). Similarly, the Western blot analysis showed that the protein level of BCRP/ABCG2 was substantially higher in A2780DR compared with A2780 (Fig. 3B). In addition, immunocytochemical analysis showed that elevated BCRP/ABCG2 expression is largely located in the plasma membrane in A2780DR (Fig. 3C). Functional implications of increased expression of BCRP/ABCG2 can be confirmed by cellular staining with H342, a substrate of BCRP/ABCG2. A2780 showed nuclear staining following H342 dye incubation for 10 minutes, whereas A2780DR displayed extracellular accumulation of H342, resulting in low cellular level (Fig. 3D). Differential distribution of H342 was abolished by incubation with the BCRP/ABCG2-specific inhibitor Ko143, confirming the predominant role of BCRP/ABCG2 in H342 transport (data not shown), whereas cellular levels of 5-chloromethylfluorescein diacetate, a substrate of MRP/ABCC, were similar in both cell lines (Fig. 3E).

Finally, BCRP/ABCG2 expression was strongly associated with differential Dox accumulation: the reduced cellular levels of Dox in A2780DR were completely restored to that of A2780 by incubation with BCRP/ABCG2 inhibitor Ko143 (Fig. 3F). Consistently, pretreatment of Ko143 diminished viable cell numbers in A2780DR following Dox treatment (data not shown). Collectively, these results show that BCRP/ABCG2 expression is distinctly elevated during the acquisition of Dox resistance in A2780, and this mechanism, in turn, leads to the attenuation of Dox accumulation in resistant cells.

**BCRP/ABCG2 Activation Was Responsible for Reduced Pba Responsiveness in A2780DR.** To clarify whether elevated BCRP/ABCG2 expression is responsible for PDT resistance, the BCRP/ABCG2 mRNA level was 71 times higher in A2780DR, implying its potential role in differential Pba accumulation (Fig. 3A). Similarly, the Western blot analysis showed that the protein level of BCRP/ABCG2 was substantially higher in A2780DR compared with A2780 (Fig. 3B). In addition, immunocytochemical analysis showed that elevated BCRP/ABCG2 expression is largely located in the plasma membrane in A2780DR (Fig. 3C). Functional implications of increased expression of BCRP/ABCG2 can be confirmed by cellular staining with H342, a substrate of BCRP/ABCG2. A2780 showed nuclear staining following H342 dye incubation for 10 minutes, whereas A2780DR displayed extracellular accumulation of H342, resulting in low cellular level (Fig. 3D). Differential distribution of H342 was abolished by incubation with the BCRP/ABCG2-specific inhibitor Ko143, confirming the predominant role of BCRP/ABCG2 in H342 transport (data not shown), whereas cellular levels of 5-chloromethylfluorescein diacetate, a substrate of MRP/ABCC, were similar in both cell lines (Fig. 3E).

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resistance of A2780DR, we first monitored cellular Pba levels following BCRP/ABCG2 inhibitor treatment. Lower levels of Pba (0.25 and 2.5 µg/ml) in A2780DR were completely recovered to those of A2780 following Ko143 preincubation (Fig. 4A). The pretreatment of A2780DR with MK-571, a specific inhibitor of MRPs, did not alter Pba accumulation; however, this confirmed the predominant role of BCRP/ABCG2 in Pba transport (data not shown). Ko143 pretreatment was shown to elevate PDT-stimulated singlet oxygen levels in A2780DR (1.38- to 2.35-fold), but not in A2780 (Fig. 4B). The increase in PDT cell viability in A2780DR was concordantly blocked by Ko143 treatment (Fig. 4C). These results indicate that increased BCRP/ABCG2 function in A2780DR leads to enhanced extracellular export of Pba, and thus consequent protection against PDT cytotoxicity.

**c-MET Overexpression in A2780DR and Its Association with Dox Resistance.** Based on the obtained results showing the overexpression of BCRP/ABCG2 and consequent resistance to PDT and Dox treatment in A2780DR, we next attempted to identify the underlying molecular mechanisms of BCRP/ABCG2 increase. It is particularly notable that the expression of proto-oncogene c-MET is relatively high in A2780DR when compared with that of its parental A2780 cells (Supplemental Table 1). c-MET transduces a signal to the PI3K/AKT pathway for enhanced tumor growth, migration, and survival (Graveel et al., 2013), and A2780 has been reported to express very low levels of c-MET (Sawada et al., 2007). The transcript level of c-MET in A2780DR was found to be 3.6-fold higher in microarray analysis (Supplemental Table 1), and a similar increase was confirmed in real-time RT-PCR analysis (Fig. 5A). Nonetheless, there were no significant differences in transcript levels for HGF, AKT, catalytic subunits of PI3K, and phosphatase and tensin homolog between A2780DR and A2780. Only PI3K regulatory subunit-1 was increased by 1.92-fold (Supplemental Table 1). In western blot analysis, A2780DR cells showed increased levels of c-MET (140 kDa) in A2780DR (Fig. 5B). With a similar pattern observed in microarray analysis, the level PI3K regulatory subunit-1 of PI3K was marginally elevated in A2780DR. Moreover, the protein level of phosphorylated AKT, downstream from activated PI3K, was substantially high in A2780DR cells, confirming the activation of c-MET/PI3K/AKT signaling in these cells.

Since there are previous reports showing that the activation of PI3K/AKT signaling can upregulate BCRP/ABCG2 activity (Mogi et al., 2003; An and Ongkeko, 2009), elevated c-MET expression may be involved in increased expression of BCRP/ABCG2 in A2780DR. This potential relationship can be supported by the effects of pharmacological inhibitors of PI3K and c-MET. After preincubation of A2780DR with PI3K-specific inhibitor LY294 (5 or 10 µM) for 3 hours, cellular levels of Dox increased to the levels of A2780 (Fig. 5C). Similarly, when A2780DR was pretreated with c-MET–specific tyrosine kinase inhibitor SU112 (1 or 2 µM) for 3 hour, the intracellular level of Dox increased by 50% (Fig. 5D). In addition, A2780DR resistance to Dox was significantly attenuated by SU112 (Fig. 5E). These results suggest...
that elevated c-MET/Pi3K signaling may be responsible for suppressed Dox accumulation in A2780DR.

c-MET/Pi3K/AKT Signaling Was Related to PDT Resistance of A2780DR. To investigate the role of c-MET/Pi3K signaling in A2780DR PDT resistance, we examined the effects of pharmacological inhibitors on PDT cytotoxicity, singlet oxygen levels, and Pba accumulation. When cells were preincubated in LY294 (5 μM), the level of accumulated Pba was enhanced in A2780DR but not in A2780 (Fig. 6A). In addition, the level of singlet oxygen in A2780DR was increased to the level of A2780 (Fig. 6B). Finally, PDT cell viability was affected by Pi3K inhibition in A2780DR; the percentage of viable cells decreased from 76% to 31% by LY294 incubation (Fig. 6C).

Next, the PDT response of cells pretreated with the c-MET-specific inhibitor SU112 (1 μM) was assessed. The c-MET inhibitor incubation restored intracellular accumulation of Pba (Fig. 6D) and elevated singlet oxygen level only in A2780DR (Fig. 6E). Accordingly, the cell viability of A2780DR decreased from 62% to 33% following PDT (Fig. 6F). These results indicate that increased c-MET expression and subsequent Pi3K signaling is strongly associated with PDT resistance in A2780DR.

c-MET Increase in A2780DR Was Linked to BCRP/ABC2 Overexpression. The involvement of c-MET/Pi3K signaling in BCRP/ABC2 expression was assessed by monitoring levels of c-MET, Pi3K, AKT, and BCRP/ABC2 following treatment with pharmacological inhibitors. First, when cells were incubated with the Pi3K inhibitor LY294 (5 μM) for 3, 6, and 24 hours, the levels of p-AKT decreased in A2780DR, confirming the effect of the inhibitor (Fig. 7A). Notably, the elevated levels of BCRP/ABC2 monomer in A2780DR were diminished by LY294 in a time-dependent manner. An immunocytochemical analysis also confirmed a decrease of BCRP/ABC2 expression in A2780DR; this was suppressed by LY294. These results show that Pi3K signaling is associated with BCRP/ABC2 overexpression in resistant cells. Next, we examined the effect of c-MET inhibitor on BCRP/ABC2 expression. The treatment of cells with SU112 for 3–24 hours decreased p-AKT levels in A2780DR, and BCRP monomer level was also repressed (Fig. 7C). This finding was also confirmed by immunocytochemical analysis: c-MET inhibitor treatment led to the reduction in plasma membrane BCRP/ABC2 levels (Fig. 7D). To confirm the linkage between c-MET and BCRP/ABC2, we transiently silenced c-MET expression in A2780DR using c-MET-specific siRNAs (#51 and #52) and examined BCRP expression. The results indicate that the genetic inhibition of c-MET effectively reduced BCRP protein level (Fig. 7E).

c-MET-BCRP/ABC2 Signaling Was Confirmed in Additional Cancer Cell Lines. Our results indicate that activated c-MET signaling led to BCRP/ABC2 overexpression and refractoriness to Dox and PDT in A2780DR. Next, we questioned whether this phenomenon can be observed in additional cell lines. For this, we selected breast carcinoma MDA-MB-231 and colon carcinoma HT29 as the c-MET-overexpression cancer cell line systems, and investigated the effect of c-MET inhibition on BCRP/ABC2 expression and Dox response. First, Western blot analysis showed that c-MET protein is highly expressed in these two cell lines (Fig. 8A). Second, when c-MET-specific siRNA (#51) was transfected, BCRP/ABC2 protein levels were reduced in both cell lines (Fig. 8B). Accordingly, the c-MET siRNA-transfected MDA-MB-231 cells showed enhanced cell death following Dox treatment (Fig. 8C). Similarly, the SU112-treated HT29 exhibited increased Dox sensitivity compared with the control cells (Fig. 8D). These results confirm that increased c-MET signaling is associated with BCRP/ABC2 overexpression in additional cancer cells.

Discussion

In the present study, we have shown that the Dox-resistant ovarian carcinoma subcell line A2780DR overexpresses c-MET, and demonstrated that c-MET elevation led to an increased expression of drug efflux transporter BCRP/ABC2 via Pi3K/AKT signaling. Using pharmacological inhibitors and siRNA-mediated genetic inhibition, we provided evidence that elevated values of the c-MET/Pi3K/BCRP axis are associated with Dox
resistance in A2780DR. In addition, our results indicate that c-MET–mediated chemoresistance is linked to the reduced PDT response of this cell line. Intracellular PDT accumulation, PDT-induced ROS generation, and cytotoxicity were significantly lowered in A2780DR compared with its parental A2780 cells, whereas the inhibition of BCRP/ABCG2, PI3K/AKT, or PI3K/AKT/Bcl-2 pathways reversed this effect.

**Fig. 3.** Elevated BCRP/ABCG2 expression in A2780DR is associated with Dox resistance. (A) Transcript levels for BCRP/ABCG2, MDR1/ABCB1, and MRP1-2/ABCC1-2 were determined in A2780 and A2780DR using a relative quantification of RT-PCR analysis. The data represent ratios with respect to A2780 of each gene and are reported as the mean ± S.E. of 3–4 experiments. (B) Protein levels of BCRP/ABCG2 were determined using Western blot analysis. Similar blots were obtained from 3–4 independent experiments. (C) An immunocytochemical detection of BCRP/ABCG2 was performed using confocal microscopic observation. Nuclei were stained with propidium iodide (PI) (1000× magnification). (D) A2780 and A2780DR were incubated with BCRP/ABCG2 substrate H342 for 10 minutes and the cellular levels of H342 fluorescence intensities were quantified. The data represent ratios with respect to A2780 and are reported as the mean ± S.E. of 3–4 experiments. (E) Cells were incubated with MRP substrate 5-chloromethylfluorescein diacetate (CMF-DA; 1 and 2 μM) for 10 minutes, PBS washing followed, and cellular fluorescent intensities were quantified. The data represent ratios with respect to vehicle control of each cell line and are reported as the mean ± S.E. of 3–4 experiments. (F) A2780 and A2780DR were preincubated with BCRP/ABCG2 specific inhibitor Ko143 (0.5 and 1 μM) for 1 hour and then vehicle or Dox (2 μM) was added for a further 6-hour incubation. Intracellular levels of Dox were quantified using a Cell Insight system (Thermo Fisher Scientific). The data are ratios with respect to the A2780 vehicle control and are reported as the mean ± S.E. of 8–10 wells. *P < 0.05 compared with the A2780 Dox alone group. **P < 0.05 compared with A2780DR Dox alone group.

**Fig. 4.** A2780DR shows a lower level of cellular Pba accumulation via elevated BCRP/ABCG2 expression. (A) A2780 and A2780DR were preincubated with Ko143 (1 μM) for 1 hour and then Pba (0.25 and 2.5 μg/ml) was added for a further 6-hour incubation. The cellular level of Pba was visualized using confocal microscopic observation. Pba-derived red fluorescent intensities were quantified using a Cell Insight system (Thermo Fisher Scientific) (bar graph). The data are ratios with respect to the A2780 vehicle control and are reported as the mean ± S.E. of 3–4 experiments. (B) Levels of singlet oxygen were assessed in cells treated with Pba-PDT in the presence or absence of Ko143 (1 μM). Green fluorescence intensities from singlet oxygen reacted dye were quantified using a Cell Insight system (Thermo Fisher Scientific). The data are average fluorescence intensities and are reported as the mean ± S.E. of 3–4 experiments. (C) Viable cell numbers were monitored in PDT cells with or without Ko143 incubation (1 μM). Cells were incubated with Pba (0.25 μg/ml) for 6 hours and MTT analysis was performed at 24 hours after PDT. The data are percentages with respect to the no PDT group of each cell line and are reported as the mean ± S.E. of 8–10 wells. *P < 0.05 compared with A2780 vehicle group. **P < 0.05 compared with A2780DR vehicle group.
c-MET restored sensitivity to Dox and PDT by repressing BCRP/ABCG2 expression. Moreover, we showed that c-MET inhibition can enhance Dox sensitivity by repressing BCRP/ABCG2 expression in additional cell lines MDA-MB-231 and HT29. Overall, these data suggest a novel link between proto-oncogene c-MET and BCRP/ABCG2 activation followed by the subsequent poor response of cancer cells to Dox and PDT.

PDT uses porphyrin derivatives to produce singlet oxygen and ROS through visible light irradiation, leading to apoptotic and necrotic tumor cell death (Hajri et al., 1999; Hibasami et al., 2000; Oleinick et al., 2002; Tang et al., 2006). Pba mediates mitochondrial membrane perturbation and consequent cytochrome c release, resulting in apoptotic cell death (Tang et al., 2009). In addition, PDT destroys tumor microvasculature and causes oxygen depletion within tumors (Dougherty et al., 1998). PDT also triggers inflammation as a secondary event: the PDT-induced alteration of the plasma membrane activates acute inflammatory signaling, in turn influencing immune cell attraction to tumor tissues (Dougherty et al., 1998; Castano et al., 2006). Due to its multilateral action, PDT has been successfully applied in the clinic for the treatment of cancer of the gastrointestinal tract, lung, head, and neck in several countries, including the United States, Japan, and Europe (Dougherty et al., 1998; Dolmans et al., 2003). In the case of ovarian cancer, multiple clinical trials have been conducted showing promising results with Photofrin-based PDT (Sindelar et al., 1991; Hendren et al., 2001; Hahn et al., 2006).

The development of chemoresistance has been the major hurdle in ovarian cancer treatment; the potential efficacy of PDT in chemoresistant tumors has therefore been explored. According to Casas et al. (2011), about two-thirds of the current reports demonstrate the absence of crossresistance of chemoresistant cancer cells to PDT. An ex vivo study using patient-derived ovarian cancer cells described that cisplatin-resistant cells can be sensitized to chemotherapy by PDT (Duska et al., 1999). In contrast, the remaining reports show crossresistance between chemotherapy and PDT: Dox-resistant uterine sarcoma cells displayed a reduced response to PDT (Olsen et al., 2013). Fibrosarcoma cells that are resistant to Photofrin-PDT showed low levels of cisplatin cytotoxicity and cisplatin-DNA adduct formation (Moorehead et al., 1994). These conflicting results suggest that the cellular response to PDT may be determined by multiple factors. Therefore, the potential cross-resistance of chemoresistant ovarian cancer to PDT and its underlying molecular mechanism should be identified. In our study, ovarian cancer cells with acquired Dox resistance were highly refractory to PDT cytotoxicity, supporting the development of crossresistance to PDT.
The involvement of BCRP/ABCG2 in PDT resistance has been demonstrated in several studies. The link between photosensitizers and BCRP/ABCG2 originally emerged from a study using bcrp-knockout mice. Mice that are deficient in bcrp were more sensitive to Pba-induced skin phototoxicity (Jonker et al., 2002). It was later shown that Pba is selectively transported by BCRP/ABCG2 and treatment with BCRP/ABCG2-specific inhibitors completely blocks Pba transport (Robey et al., 2005). In addition to Pba, Photofrin and Photochlor, clinically approved photosensitizers, are known to be substrates of BCRP/ABCG2 (Liu et al., 2007). The aforementioned reports support the results of our study, clearly indicating that BCRP/ABCG2 expression is an important factor in developing crossresistance of ovarian cancer cells to chemotherapy and PDT. In particular, it is notable that NRF2 signaling, which is a master regulator of antioxidant gene expression, is activated in A2780DR (Shim et al., 2009); therefore, the low ROS level in PDT-treated A2780DR might be caused by enhanced antioxidant proteins such as glutathione synthesis enzymes. Nevertheless, the contribution of elevated BCRP/ABCG2 to PDT resistance appears to be primary. The pharmacological inhibition of BCRP/ABCG2 activity in A2780DR largely reversed Pba accumulation, ROS generation, and cell viability to the levels of A2780 (Fig. 4).

The BCRP/ABCG2 increase in cancer cells appears to be related to deviant signaling changes. In particular, there are several lines of evidence that AKT is implicated in BCRP/ABCG2 expression. The number of H342-refractory cells is decreased in akt null mice, and the reintroduction of Akt in these mutant cells increased H342-negative cells, indicating that BCRP/ABCG2 activity is influenced by Akt (Mogi et al., 2003). Another group later found that AKT inhibitors reduced plasma membrane BCRP/ABCG2 expression in hepatoma cells (Hu et al., 2008). Furthermore, the activation of EGFR, an upstream regulator of PI3K/AKT, decreased BCRP overexpression cells in head and neck squamous cell carcinoma (Chen et al., 2006). These all together indicate that translocation of BCRP/ABCG2 to the plasma membrane is a critical step for BCRP activation, and this process is stimulated by PI3K/AKT signaling, although specific molecular mechanisms are unveiled. As in these reports, the association of PI3K/AKT...
with BCRP/ABCG2 activity was verified in our A2780 system. The levels of phosphorylated AKT were higher in A2780DR than in A2780, and the inhibition of PI3K/AKT reversed BCRP/ABCG2 expression and Dox/PDT resistance. Further investigation showed that c-MET overexpression was related to activated PI3K/AKT signaling in A2780DR: the c-MET–specific inhibitor SU112 diminished BCRP/ABCG2 expression and Dox/PDT resistance (Fig. 7). One major difference in our study is that the c-MET/PI3K/AKT axis appears to activate transcription of BCRP/ABCG2. A2780DR showed substantial increases in BCRP/ABCG2 mRNA and monomer BCRP/ABCG2 level (72 kDa), as well as its plasma membrane level (Figs. 3 and 7). In addition, treatment with an inhibitor of c-MET or PI3K/AKT reduced the level of BCRP monomer present (Fig. 7, A and C). To our knowledge, there is no previous study describing the involvement of c-MET in BCRP/ABCG2 expression; therefore, the molecular mechanism of c-MET–mediated BCRP/ABCG2 upregulation has yet to be elucidated. One of the possible mechanisms would be the upregulation of BCRP/ABCG2 by cancer stem cell markers. There has been a report that stemness transcription factor OCT4 positively regulates BCRP/ABCG2 expression in chronic myeloid leukemia (Marques et al., 2010). Furthermore, lines of evidence have shown that c-MET is involved in the maintenance of cancer stem cell phenotypes (Boccaccio and Comoglio, 2013). These studies suggest that the increase in c-MET–mediated stemness markers may be associated with BCRP overexpression. In addition, since we previously observed that NRF2 signaling is enhanced in A2780DR (Shim et al., 2009), there is a possibility that elevated BCRP/ABCG2 expression is partly attributable to NRF2 activation. However, given that NRF2 regulates the expression of multiple transporters, including MRP/ABCC and MDR1/ABCB1 (Hayashi et al., 2003; Maher et al., 2005; Adachi et al., 2007), the exclusive increase of BCRP/ABCG2 in this case is still mysterious.

Aberrant activation of c-MET is strongly linked to increased tumorigenicity and metastasis potential in various types of cancer. In advanced ovarian carcinoma, high levels of c-MET expression were correlated with poor survival rates (Wong et al., 2001; Ayhan et al., 2005). Similarly, c-MET activation was related to increased expression of migration-associated genes such as MMP-2 and MMP-9 in ovarian cancer (Sawada et al., 2007). Therefore, blockade of c-MET signaling using pharmacological inhibitors may suppress cell growth, motility, and invasion in ovarian carcinoma cell lines (Koon et al., 2008). In addition to these phenotypic characteristics, c-MET was implicated in the chemoresistance of cancer cells. The incubation of gastric carcinoma cells with the c-MET inhibitor SU112 sensitized cells to irinotecan (Yashiro et al., 2013). c-MET was overexpressed in a chemoresistant multiple myeloma cell line; SU112 treatment countered this cell line’s resistance to melphalan, bortezomib, and Dox (Moschetta et al., 2013). Ovarian cancer patients with higher levels of relapse after chemotherapy
showed elevated expression of c-MET and HGF (Mariani et al., 2014). Our study supports the aforementioned reports in that it provides evidence that Dox resistance is attributed to an increase of c-MET levels in ovarian carcinoma cells. In addition, we present upregulation of BCRP/ABCG2 as a novel mechanism of c-MET chemoresistance in ovarian, breast, and colon carcinoma cells, although the genetic variations in these cell lines are divergent. Several factors have been suggested to be involved in the overexpression of c-MET. Particularly, the c-MET promoter analysis revealed that ETS1 directly activates c-MET transcription (Gambarotta et al., 1996). The ETS1 increase elevated c-MET expression and consequently enhanced expression of genes involved in malignant phenotypes (Furlan et al., 2008), whereas in A2780DR, ETS1 is not likely to induce an increase in c-MET expression: ETS-1 levels were not different from those in A2780 in our microarray analysis (data not shown). On the other hand, since wild-type p53 was reported to repress c-MET expression through changes in miR-34a (Hwang et al., 2011), a reasonable hypothesis is that continuous Dox exposure evokes mutation of p53, resulting in c-MET overexpression. In conclusion, our present study shows that c-MET overexpression is accompanied by the acquisition of adaptive resistance to Dox in A2780, suggesting that the consequent activation of c-MET/PI3K/AKT signaling elicits elevation of BCRP/ABCG2 levels, leading to resistance to both chemotherapy and PDT in cancer cells.

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Authorship Contributions

Participated in research design: Kwak.
Conducted experiments: Jung, Choi.
Contributed new reagents or analytic tools: Jung, Choi, Kwak.
Performed data analysis: Jung, Choi, Kwak.
Wrote or contributed to the writing of the manuscript: Kwak, Jung, Choi.

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Fig. 8. c-MET inhibition represses BCRP level in breast and colon carcinoma cell lines. (A) c-MET protein levels were determined in MDA-MB-231 and HT29 using Western blot analysis. (B) MDA-MB-231 and HT29 were transfected with c-MET siRNA, and then mRNA levels for c-MET and protein levels for BCRP were assessed. (C) MDA-MB-231 was transfected with c-MET siRNA, and Dox cytotoxicity was monitored. Dox was incubated for 48 hours, and MTT analysis was performed. (D) HT29 was preincubated with SU112, and Dox cytotoxicity was examined using MTT analysis. The data represent ratios with respect to the vehicle group and are reported as the mean ± S.E. of 8 wells. *P < 0.05 compared with the scrambled or Dox alone group. GADPH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; sc, si-control; si-c-MET, c-MET siRNA; veh, vehicle.

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