A newly synthesized platinum-based compound (PBC-II) increases chemosensitivity of HeLa ovarian cancer cells via inhibition of autophagy

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ABSTRACT

There are many mechanisms of resistance, chemoresistance of HeLa cells to anti-cancer agents seems to be autophagy-mediated. While using very effective anti-cancers such as Doxorubicin and cisplatin, cells overcome the cytotoxicity of these drugs through promotion of so-called cytoprotective autophagy. Here in this study, we sought to introduce a novel platinum-based compound PBC-II that possesses anti-cancer activity. Our data showed that PBC-II is able to induce apoptosis at relatively low concentrations, with no detectable reactive oxygen species (ROS). However, further experiments demonstrated that exposure of HeLa cells to PBC-II did not promote autophagy; rather, it resulted in accumulation of p62 and decrease in LC3-II levels. Autophagy was then promoted in HeLa cells pharmacologically by Doxorubicin and genetically by siRNA IL-10. In order to confirm promotion of autophagy in our model, we performed acridine orange staining to assess for autophagy under microscope as well as via flow cytometry. We then measured protein level of autophagy markers p62 and LC3 by western blot. Our data indicated that PBC-II interferes with therapy-induced autophagy. We also determined PI3K activity while co-incubation of PBC-II with autophagy inducers. It was clear that PI3K activation decreased when PBC-II was co-administered with autophagy inducers. Collectively, PBC-II exerts unique anti-proliferative effects associated with inhibition of autophagy, which indicates that PBC-II is potentially a promising agent to be used in resistant ovarian tumors.

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1. Introduction

Ovarian cancer is considered one of the major tumors that threaten women’s lives worldwide (Ferlay et al., 2010; Sankaranarayanan and Ferlay, 2006). Although incidence of ovarian cancer is relatively low, its mortality rate is highest compared to other gynecological tumors (Razi et al., 2016; Beral et al., 2008). At early stages, ovarian cancer shows no clinical signs, leading patients to be presented in clinic when cancer is metastasizing and invading the surrounding organs at late stages. The five-year survival rate of ovarian cancer does not exceed 43% with over 80% of patients will suffer from tumor relapse, making the disease prognosis very poor (Srivastava et al., 2017; Piccart et al., 2003). The current therapeutic approach for ovarian cancer is composed of surgical excision of tumor with six to eight courses of combination of paclitaxel and platinum-based compounds such as cisplatin (Piccart et al., 2003). Even though remission can reach 80%, relapse and resistance to ovarian cancer occur in 60% of women, causing the high mortality of disease (Lurvero et al., 2014; Mantia-Smaldone et al., 2011; Tomao et al., 2017). Therefore, ovarian cancer is still a serious problem to be managed.

Here in this work, we sought to find a newly synthesized platinum-based drug that effectively promotes cell killing mechanisms in ovarian carcinoma cells to overcome resistance that limits the use of cisplatin. PBC-II is novel platinum (II) was recrystallized.
from dichloromethane/acetonitrile. This compound has shown a potential anticancer activity with fewer side effects (data under publication). While exposing HeLa cells to PBC-II, our results showed that PBC-II induces apoptosis at micromolar range. Surprisingly, our data showed that PBC-II inhibits autophagy with no evidence of generation of reactive oxygen species. We then used genetic and pharmacological approaches to promote autophagy in combination with PBC-II. Our data suggest that PBC-II potently inhibits drug-induced autophagy in HeLa ovarian carcinoma. In conclusion, PBC-II exerts a very potent effect on human ovarian carcinoma cells with novel characteristics in inhibition of autophagy, which comprises a mode of resistance in this particular type of tumors.

2. Materials and methods

2.1. Cell culture

HeLa ovarian carcinoma cells were obtained from ATCC and kept in 10% DMSO (Sigma Chemical, St. Louis, MO) with Fetal Bovine Serum (FBS) (GIBCO Life Technologies, Gaithersburg, MD) and stored frozen in liquid nitrogen until day of use. Cells were thawed off and cultured in a T75 flask (Cellstar) in RPMI 1640 medium with 5% fetal bovine serum, 5% bovine calf serum, 2 mM L-glutamine, and penicillin/streptomycin 0.5 mL/100 mL medium (10,000 units/mL penicillin and 10 mg/mL streptomycin (GIBCO Life Technologies, Gaithersburg, MD) and incubated at 37 °C, 5% CO2, in a moisturized environment. Once cells reached 80% confluency, cells were washed with 1X PBS (GIBCO) and harvested by 0.25% trypsin-EDTA (GIBCO) (incubation for 5 min). Trypsin then was deactivated by addition of 5 mL of serum-containing RPMI 1640, cells were collected and centrifuged at 1,500 rpm for 5 min. Media and trypsin were removed and 5 mL of new sterile medium was added to the pellet; cells were resuspended and 500 mL of suspension was placed into 96 mm3 plate filled with 10 mL of RPMI 1640 medium. In every experiment, cells were cultured under identical conditions and incubated overnight to allow for adherence before treatment with continuous drug exposure. PBC-II agent was generously provided by our collaborator Dr. Isab, Department of Chemistry, College of Sciences, King Fahad University of Petroleum and Minerals.

2.2. IL-10 and siRNA transfection

Transient transfection of siRNA was achieved using lipofectamine transfection 3000 reagent (Invitrogen, Carlsbad, CA), as described before (Alotaibi et al., 2018). In brief, ovarian carcinoma (HeLa) cells were plated in triplicates with cells density of 20,000 per well in 12-well plates for 16 h until cells reached approximately 70% confluence. One hour before transfection the cells were cultured in antibiotic-free medium. The cells were incubated with transfection mixes containing 20 nM of GAPDH- or IL-10-siRNA (Invitrogen, Carlsbad, CA), reverse transcriptase polymerase chain reaction (RT-PCR) assay and the real time RT-PCR assay were done to confirm silencing as previously described (Alotaibi et al., 2018).

2.3. Annexin V-PI staining for apoptosis

In cells undergoing early apoptosis, phosphatidyl serine (PS), which is originally located in the inner cell membrane, transfers to the outer cell membrane. Translocated phosphatidyl serine (PS) can be bound with V attached to a fluorescent chromophore (FITC) to identify early apoptosis. Propium iodide (PI) penetrates non-intact cellular membranes and stains cells, serving as a marker of late apoptosis or necrosis. In this experiment, cells were plated overnight to permit cells to adhere. On the following day, cells were treated with the drug. At several time points, adherent and non-adherent cells were harvested by 0.25% trypsin and centrifuged at 2,000 rpm for 5 min. Cells were then resuspended in 1 mL of 1X PBS, transferred to 2 mL tubes, and centrifuged at 2,000 rpm for 5 min. PBS was removed and 100 mL of 1X binding buffer (BD Biosciences) was added to pellets per tube. 5 mL of Annexin V-FITC (BD Biosciences) and 5 mL of PI at 10 μg/mL (BD Biosciences) were then added per tube. In the dark, cells were softly vortexed and incubated for 15 min at room temperature. Before analysis by flow cytometry, 400 mL of 1X binding buffer was added per tube and samples were analyzed by flow cytometry technique as soon as possible to measure the fluorescence at 530 nM.

2.4. Evaluation of autophagy by acridine orange staining

200,000 cells were seeded in 6-well plates and permitted to adhere overnight and drug-treated the next day. At various time points, drug was removed and cells washed once with 1X PBS. Acridine orange dye was diluted in PBS in a ratio of 1:10000 (prepared in the dark) and added to cells for staining, and incubated for 15 min. Dye then was aspirated and plates were washed with 1X PBS and fresh medium was added. Photographs were taken with an Olympus 1X 70 microscope and an Olympus SC 35 camera.

Flow analysis was utilized to count the cell population positively stained with acridine orange. Treated cells were harvested, collected, and centrifuged at speed of 1500 rpm. Supernatant was removed and pellets were resuspended in 1 mL of fresh medium. Cell suspension was filtered through standard flow cytometry 40 μm filter (BD falcon). A 10:1000 dilution of acridine orange in 1X PBS was prepared (in the dark) and protected from light until ready for use. At flow cytometry, 10 μL of acridine orange solution was added to per sample to make the dilution of 1:10000. When analyzed by flow cytometry using the FC500 Flow cytometer with CXP software (Beckman Coulter, USA), the acridine orange dye is excited at wavelength 525 nM for green fluorescence and 620 nM for red fluorescence.

2.5. Senescence by β-Galactosidase staining

Detection of senescence was by β-Galactosidase staining after exposure to the drug treatment. Cells were washed once with 1X PBS and fixed with 2% formaldehyde/ 0.2% glutaraldehyde for 5 min. Fixing solution was aspirated and cells were washed twice with PBS and fixed with 2% formaldehyde/ 0.2% glutaraldehyde for 4 h. Cells were washed twice with PBS and pictures were taken.

2.6. Detection of reactive oxygen species

Cells were plated and treated with indicated concentrations for 24 h. On experiment day, cells were collected and centrifuged at 1500 rpm and mixed with 1X assay buffer (Muse Oxidative Stress Kit MCH100111, milliMol). Then, oxidative stress reagent (Muse Oxidative Stress Kit MCH100111, milliPore) was diluted in 1X buffer (1:100) to make intermediate solution. After that, intermediate solution was diluted again in 1X buffer (1:80) to make working
Cells were then mixed with working solution and incubated for 30 min at 37 °C. Samples were read using Muse Cell Analyzer (0500–3115), millipore.

### 2.7. Determination of PI3K activation

Cells were plated and treated with indicated concentrations for 24 h. On experiment day, cells were collected and centrifuged at 1500 rpm, and washed with 1X PBS. Cells were then fixed with fixation buffer (Muse PI3K Activation Dual Detection Kit, MCH200103) followed by washing step. Cells were then permeabilized by permeabilization buffer for 5 min on ice. Mixture of antibody cocktail with 1X assay buffer was made and incubated at room temperature for 3 min in dark. Cells were then resuspended in the mixture and run through Muse Cell Analyzer (0500–3115), millipore.

### 2.8. Western blotting

In order to determine whether some proteins were induced, degraded, or downregulated, we conducted a specific assay to analyze protein levels. After the indicated time points, we collect viable and non-viable cells in a pellet. These collected cells were mixed with 100 to 200 µL of 1X Tris lysis buffer (1 M Tris (pH 6.8), 10% SDS, and dH2O) containing protease inhibitors and boiled for 5 min. Protein concentration was measured using the Bradford protein assay (BioRad) and 40 µg of total cell lysate was separated in a 12% gel using SDS-PAGE. Proteins were transferred onto nitrocellulose membrane for 1.5 h and washed 3 times with PBS containing 0.01% Tween for 5 min in each time. Membrane was blocked for one hour with TBS-Tween 20 buffer containing 5% nonfat dry milk. The proteins were then blocked with the primary antibody and left overnight in 4°C. In the following day, the primary antibody was washed out and the immunofluorescent secondary antibody was added for an hour at room temperature. Membrane was washed three times and bands were detected using enhanced chemiluminescence detection reagents from Pierce (Rockford, IL).

### 2.9. Statistical analysis

Statistics were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, United States) using ANOVA followed by post-hoc analysis. The significance of group values was determined based on a p-value of p < 0.05.

## 3. Results

### 3.1. PBC-II induces apoptosis in HeLa ovarian carcinoma cells

In order to investigate whether PBC-II promotes cell death in our model of interest, we exposed HeLa cells to different concentrations of the indicated drug to 24 h. First, we screened for apoptosis as one of the most desired effects in treatment of cancer in an intent to kill tumor cells. Our data shows that PBC-II significantly enhances apoptosis in concentrations as low as 1 µM. Percentage of apoptotic cells increased in a dose-dependent manner (Fig. 1A). To understand whether apoptosis was accompanied with elevated levels of reactive oxygen species, we tested this certain question based on dihydroethidium (DHE) reagent. The results showed that PBC-II does not generate reactive oxygen species (ROS), indicating that apoptosis was induced independently of oxidative stress (Fig. 1B).

### 3.2. Lack of autophagy and senescence promotion by PBC-II

Autophagy (Macro-autophagy) is a self-induced degradative process of eukaryotic cells that leads to breakdown of unnecessary intracellular material within lysosomes or macro-autophagosomes (Galluzzi et al., 2017). Several studies reported that autophagy is triggered and upregulated in cancer cells during exposure to anticancer drugs (Gewirtz, 2014). Here in this study, we investigated whether autophagy is promoted in HeLa cells in response to PBC-II. During induction of autophagy, autophagosomes fuse with lysosomes to form acidic vacuoles, which are stainable by acridine orange dye (François et al., 1976). While exposure of HeLa cells to PBC-II, acridine orange staining did not present evidence of accumulation of acidic vacuoles, even at high concentrations of PBC-II (Fig. 2A). In addition, we also monitored other markers of autophagy such as LC3-II protein and p62 protein. LC3-I is a cytoplasmic protein that converts to LC3-II when lipidated and binds to intracellular material within lysosomes or macro-autophagosomes (Scherz-Shouval et al., 2007). While treating HeLa cells with PBC-II, we found that LC3-II protein level decreases as drug concentration increases. Furthermore, autophagic flux was measured via p62 protein degradation. Consistent with LC3-I, p62 protein clearly increases in response to PBC-II at dose-dependent manner, suggesting that autophagy was not promoted in response to PBC-II (Fig. 2B).
were treated with 0, 1, 3, 10, 30 μM of PBC-II for 24 h. At indicated point, acridine orange (1:10000) diluted in medium was added to cells. Images were taken under a fluorescent microscope at a magnification power of 20X. B. Conversion of LC3-I to LC3-II and completion of autophagy process was confirmed via measurement the LC3-I expression and the degradation of p62 by western blotting. HeLa ovarian cancer cells were treated with 0, 1, 3, 10, 30 μM of PBC-II for 24 h and levels of LC3-I and p62 protein was measured. There was no clear conversion of LC3-I neither degradation of p62 in response to indicated concentrations of PBC-II.

over, it has been already shown that induction of autophagy is closely accompanied by promotion of senescence in cancer cells (Alotaibi et al., 2016). We have stained HeLa cells 24 h post-treatment with β-galactosidase staining, a remarkable marker of senescence. Consistent with our observation to autophagy markers, we failed to determine positively stained cells upon exposure to PBC-II (data not shown). Taken together, autophagy and senescence seem to be not responsible for the cytotoxicity of PBC-II, on the contrary, these astonishing data have added up another aspect of PBC-II action as a novel inhibitor of autophagy in addition to being a potent apoptosis inducer.

3.3. PBC-II inhibits genetically- and pharmacologically-induced autophagy in HeLa ovarian carcinoma cells

To determine how potent PBC-II inhibits autophagy, we sought to induce autophagy using pharmacological and genetic approaches. In our work, we utilized siRNA IL-10 and doxorubicin as autophagy inducers in HeLa ovarian carcinoma cells. IL-10 is an immunomodulatory cytokine that maintains immune homeostasis (Iyer and Cheng, 2012). Although the relationship between IL and autophagy seems to be controversial in literature (Wu et al., 2016), there is quite significant evidence that IL-10 may inhibit autophagy (Park et al., 2011; Wang et al., 2014; Santarelli et al., 2014). Therefore, silencing IL-10 might become an acceptable approach to induce autophagy in HeLa ovarian carcinoma cells. Also, the topoisomerase II inhibitor, doxorubicin, is a well-known drug that highly promote autophagy in many cancer cell lines including HeLa cells (Goethe et al., 2012; Di et al., 2009; Fang et al., 2017). Therefore, we applied both approaches as strategies in order to ensure whether promotion of autophagy in HeLa cells by different means is inhibitable by PBC-II. We exposed HeLa cells to 1 μM doxorubicin and siRNA for 24 h and stained them by acridine orange. Upon treatment, a significant fraction of the treated cell populations apparently demonstrated stained vacuoles per cell in treated groups compared to control (Fig. 3A). In addition, cells exposed to either siRNA IL-10 or doxorubicin became enlarged in comparison with control cells, demonstrating a morphological sign of autophagy. An additional technique was performed using flow cytometry to evaluate autophagy in cells based on the intensity of acridine orange staining. In this experiment, we found that cells treated with either siRNA IL-10 or doxorubicin showed a significant increase in the percentage of the cell population falling into the area of red-fluorescing cells, indicating these cells undergo autophagy when treated either with siRNA IL-10 or doxorubicin (Fig. 3B). Then, we investigated whether PBC-II is able to interfere with therapy-induced autophagy in HeLa ovarian carcinoma cells. We treated cells with autophagy inducers along with 30 μM PBC-II and assessed autophagy via LC3-I and p62 proteins. Our western blot data demonstrated that both autophagic flux and LC3-I conversion were drastically inhibited by PBC-II, indicating that inhibition of autophagy occurs at early steps. Therefore, we measured the activity of PI3 kinase as an early player of process of autophagy. While exposure of HeLa ovarian carcinoma cells to autophagy inducers, activated PI3 kinase levels became significantly elevated. Interestingly, co-treatment with PBC-II restored the activated PI3 kinase levels as low as control level (Fig. 3C). Collectively, PBC-II obviously targets therapy-induced autophagy at early steps.

4. Discussion

Progression of autophagy after exposure to chemotherapy in cancer treatment is a debatable topic. Many reports have indicated that autophagy play a significant role in proliferation, survival, and resistance (Liu et al., 2016; Peng et al., 2016; Lee et al., 2015). On the other hand, different studies in several laboratories demonstrated that autophagy can be also defined as type II-programmed cell death (Sharma et al., 2014; Shimizu et al., 2014; Biggers et al., 2013). Presumably, autophagy function differs between treatment models due to variations in cell types, used drugs, and duration of therapy. Since cisplatin is the drug of choice in ovarian cancers, cisplatin-induced autophagy seems to be...
cytoprotective in ovarian carcinoma according to many reports (Ma et al., 2019; Long et al., 2018; Wang and Wu, 2014). Hence, utilizing drugs that induce autophagy such as cisplatin in ovarian carcinoma is not a wise decision. Therefore, we need to introduce innovative autophagy inhibitors that possess anticancer properties. We examined PBC-II effect on HeLa ovarian carcinoma. Expectedly, PBC-II alone induced significant apoptotic populations at micromolar range, with no clear markers of autophagy and senescence. Thus, it may be beneficial to implement this drug in treatment of ovarian carcinoma, which is addicted to autophagy as a chemoresistance mode.

Resistance of ovarian cancer to cisplatin, the first-line treatment of ovarian cancer, is an obstacle and probably results in treatment failure (Damia and Broggini, 2019). Several reports have indicated that inhibition of autophagy resulted in increased sensitivity of ovarian cancer cells to cisplatin as well as other chemotherapeutic agents (Liu et al., 2018; Qiu et al., 2017; He et al., 2015; Liang et al., 2016). In addition, studies have revealed that NF-E2-related factor 2 (Nrf2) enhanced cisplatin resistance via activating autophagy in ovarian carcinoma (Bao et al., 2014). Furthermore, another study showed that the oncoprotein, YAP, enhanced resistance of ovarian carcinoma through increasing the levels of autophagy (Xiao et al., 2019).

Fig. 3. PBC-II inhibits therapy-induced autophagy in HeLa ovarian carcinoma cells. A. HeLa cells were treated with siRNA IL-10 and 1 µM doxorubicin for 1 day. Cells were then harvested and centrifuged at 1500 rpm. Acridine orange was diluted in PBS (1:10000) and was then added to the cells for staining. The extent of autophagy was counted based on the number of cell population in quadrants Q2 and Q4 from our raw data. This experiment was performed three times (*p < 0.05 compared to control). B. Cells were plated and treated with siRNA IL-10 and 1 µM doxorubicin. A day post-treatment, acridine orange (1:10000) diluted in medium was added to cells. During autophagy, acidic vacuoles are stained with acridine orange dye. Images were taken under a fluorescent microscope at amagnification power of 20X. Three different experiments were performed to confirm the result. C. Conversion of LC-3-I to LC3-II and completion of autophagy process in combination was assessed via measurement the LC3-I expression and the degradation of p62 by western blotting. HeLa ovarian cancer cells were treated with 1 µM doxorubicin, siRNA IL-10, 10 µM PBC-II, and combination for 24 h, and levels of LC3-I and p62 protein was measured. A clear inhibition of therapy-induced autophagy via PBC-II was observed in HeLa cells. D. Activation of PI3K was evaluated by flow cytometry. HeLa ovarian cancer cells were treated with 1 µM doxorubicin, siRNA IL-10, 10 µM PBC-II, and combination for 24 h, and levels of LC3-I and active PI3K level was measured. Data demonstrate that activation of PI3K during autophagy was clearly inhibited by PBC-II in HeLa cells (*p < 0.05 compared to control).
These findings build upon our previous work that supports the actions by reducing the activity of PI3 kinase that is necessary for ovarian carcinoma cells, as well as powerful effect in inhibition whether PBC-II could be used in addition to the promising PI3K inhibitor. Therefore, it would be worth studying treatment. However, the ability of PBC-II to reduce PI3K activation levels were decreased when PBC-II was added to the treatment. Moreover, the ability of PBC-II to reduce PI3K activation might also explain why apoptosis was significantly induced at relatively low concentrations. Therefore, it would be worth studying whether PBC-II could be used in addition to the promising PI3K inhibitors as synergistic regimen.

In summary, PBC-II was shown to induce apoptosis in HeLa ovarian carcinoma cells, as well as powerful effect in inhibition of autophagy. We have also confirmed that this compound may work as a chemo-sensitizing agent when used along with other treatment modalities by interference with autophagy. Interestingly, we found that this molecule can contribute to antitumor actions by reducing the activity of PI3 kinase that is necessary for chemoresistance, autophagy progression, and tumor proliferation. These findings build upon our previous work that supports the potential utility of developing PBC-II as a promising agent for cancer therapy.

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