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Cisplatin and PI3Kinase Inhibition decrease Invasion and Migration of Human Ovarian Carcinoma Cells and regulate Matrix-Metalloproteinase Expression

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Abstract

Targeting of the PI3K (phosphoinositide3-kinase)/Akt/mTOR pathway in human ovarian cancer cells is a promising novel therapeutic strategy. We investigated the effects of cisplatin and the PI3K inhibitor LY294002 on invasion, migration and the expression of essential matrix metalloproteinases (MMPs) in ovarian cancer cells. SKOV3, OVCAR5 and IGROV1 human ovarian cancer cell lines were treated with cisplatin, LY294002 and a combination of both drugs. Invasion and migration of treated cells was assessed using Matrigel and uncoated PET membrane assays. Expression levels of pro-MMP2, MMP2, TIMP1, TIMP2 and MT1-MMP were determined using Western Blotting. Gel zymography was used to quantitate the functional levels of active MMP2. All three cell lines showed significantly reduced invasion and migration after treatment with cisplatin, LY294002, and the combination of both drugs compared to untreated controls. In SKOV3 cells, cisplatin alone and in combination with LY294002 resulted in a 6.3 and 7.1-fold reduction in the total amount of activated MMP2. TIMP1 expression decreased by 5.0, 6.6 and 28.4-fold with cisplatin, LY294002 and the combination respectively ($p<0.05$). In contrast, only cisplatin and the combination of both drugs resulted in a significant, 3.7 and 5.1-fold reduction in the level of TIMP2. Expression levels of MT1-MMP remained unchanged. These observations were corroborated in IGROV1 cell lines that showed similar changes of activated MMP2 and TIMP2 expression, but no significant decrease in TIMP1 levels. Our data suggests that inhibition of ovarian cancer cell motility is mediated via down-regulation of activated MMP2, TIMP1 and TIMP2 expression under these treatment conditions.

Keywords

Matrix metalloproteinase 2; tissue inhibitor of matrix metalloproteinase; cisplatin; LY294002; PI3Kinase; ovarian cancer

Introduction

The oncogenic PI3K (phosphoinositide3-kinase)/Akt/mammalian target of rapamycin (mTOR) pathway plays an important role in various cancers, including human ovarian cancer (Crijns et al. 2006; Philp et al. 2001). Activation of PI3K by various growth factors like platelet derived growth factor (PDGF) or insulin growth factor (IGF) results in an increase of intracellular, membrane bound phosphatidylinositol-(3,4)-diphosphate (PIP2) and phosphatidylinositol-(3,4,5)-triphosphate (PIP3). The accumulation of PIP3 recruits the

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proto-oncogene Akt to the cell membrane, where it is phosphorylated by PIP3-dependent kinases (PDKs) at Thr308 and Ser473 for full activation. Phosphorylated Akt (pAkt) is released from the membrane and elicits downstream effects mainly by phosphorylating signal transduction proteins like BAD, Caspase 9 and mTOR (Bellacosa et al. 2005). Activation of these downstream signals leads to an increase in cell proliferation, invasiveness, drug resistance and neo-angiogenesis (Altomare and Testa 2005).

Activation of the PI3K/Akt/mTOR pathway in ovarian cancer has been demonstrated in several studies (Yuan et al. 2000). High levels of pAkt in human ovarian cancer tissue is found in about 50% of papillary serous ovarian cancers and in up to 70–80% of endometrioid and clear cell adenocarcinomas of the ovary (Campbell et al. 2004; Wang et al. 2005). Various genetic aberrations in the PI3K/Akt/mTOR pathway have been described in ovarian cancer, including increased copy numbers of the PIK3CA gene which encodes the p110α subunit of PI3K, and mutations within the PTEN gene (Levine et al. 2005).

Cis-Dichlorodiammine platinum (II) or cisplatin has emerged as a major chemotherapeutic agent in the treatment of ovarian cancer. The antineoplastic effects of cisplatin are mediated by the covalent formation of DNA intra-and inter-strand as well as DNA-protein crosslinks (Jamieson and Lippard 1999; Loehrer and Einhorn 1984). In addition, cisplatin has been shown to activate pro-apoptotic pathways through mitochondrial (Cullen et al. 2007) and Fas-associated mechanisms (Friesen et al. 1999), and exert anti-invasive mechanisms which are still incompletely understood (Ramer et al. 2007).

Matrix-metalloproteinases (MMPs) are zinc-dependent endopeptidases with the ability to degrade various extracellular matrix proteins. MMPs are involved in the cleavage of cell surface receptors, the release of apoptotic ligands such as the FAS ligand, and have emerged as key regulators of cancer cell invasion and metastasis (Curran and Murray 2000; Stamenkovic 2000). MMP2 (Gelatinase A) degrades Type IV collagen, an integral part of the basement membrane, and is assumed to play a key role in the metastatic process (Bjorklund and Koivunen 2005; Hornebeck et al. 2002). MMP2 is present in the cell as an inactive proenzyme (pro-MMP2) that is activated by membrane-type matrix-metalloproteinase 1 (MT1-MMP) (Strongin et al. 1995). This activation requires the presence of tissue inhibitor of matrix metalloproteinase-2 (TIMP2) (Butler et al. 1998; Jo et al. 2000).

TIMPs are the naturally occurring inhibitors of MMPs. Several studies have highlighted the dual functions of TIMP2 in regulating pro-MMP2 processing and inhibition of the active enzyme (Butler et al. 1998; English et al. 2006; Jo et al. 2000). In contrast, TIMP1 has emerged as a key MMP2 inhibitor with several studies demonstrating a correlation between elevated TIMP1 levels and diminished MMP2 activity and invasiveness (Khokha et al. 1992; Park et al. 2005; Ramer and Hinz 2008). In addition, TIMP1 and TIMP2 are able to modulate cell growth (Corcoran and Stetler-Stevenson 1995; Hayakawa et al. 1992). TIMP1 in particular can act as a potent serum mitogen that promotes cell survival and suppresses apoptosis in breast epithelial cells (Li et al. 1999). These functions are distinct from their MMP inhibiting activity (O'Shea et al. 1992).

Recent evidence suggests that PI3K/Akt/mTOR signaling is involved in the regulation of invasion and metastasis in several tumor models including ovarian cancer (Park et al. 2001). Activation of PI3K has been shown to increase MMP2 activity and cell motility (Furuya et al. 2006; Vasko et al. 2004). Treatment of several ovarian cancer cell lines with the PI3K inhibitor LY294002 significantly decreased gonadotropin-induced MMP2/MMP9 secretion in one study (Choi et al. 2006). In vivo, the PI3K inhibitor LY294002 has been found to block metastatic spread and decrease cell motility of thyroid tumors in a mouse model of

thyroid cancer (Furuya et al. 2007). In addition, PI3K inhibition was shown to decrease TIMP1 mRNA abundance and protein content in immortalized human trophoblast cell lines (Qiu et al. 2004), human leukemic cell lines and erythroid progenitor cells (Kadri et al. 2000). In contrast, LY294002 failed to alter TIMP2 protein content in EGF (epidermal growth factor) stimulated trophoblast cells (Qiu et al. 2004).

Inhibition of PI3K pathway signaling using PI3K or mTOR inhibitors has been shown to sensitize ovarian cancer cell lines to the apoptosis-inducing effect of platinum compounds (Altomare et al. 2004; Winograd-Katz and Levitzki 2006, Westfall, 2005 #63). In addition, activation of the PI3K/Akt/mTOR pathway in ovarian cancer cell lines contributes to cisplatin resistance (Lee et al. 2005).

The present study investigates the mechanisms underlying the cisplatin and PI3K mediated inhibition on invasion and migration of human ovarian cancer cells. We demonstrate that the expression levels of MMP2, TIMP1 and TIMP2 are decreased by cisplatin and the PI3K/Akt/mTOR pathway.

Materials and Methods

Materials

The PI3K inhibitor LY294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride) and cis-Dichlorodiammine platinum (II) (cisplatin) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). The MMP2/MMP9 gelatin zymography standard was acquired from Chemicon (Temecula, CA) and activated for 1 hour at 37°C in a Tris-buffered solution of 1mM APMA (Sigma-Aldrich Corp. St. Louis, MO) prior to each experiment.

Cell culture and treatment

SKOV-3 ovarian cancer cells were purchased from the American Type Culture Collection (Manassas, VA). IGROV1 cells were obtained from the National Cancer Institute (Bethesda, Maryland). OVCAR5 cells were a kind gift from Timothy Lane (Department of Obstetrics and Gynecology, David Geffen School of Medicine at UCLA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Corp. St. Louis, MO) with 5mM L-Glutamine, 25 mM HEPES buffer, 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37°C. For monolayer cultures, the cell lines were maintained on tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ).

Cell Invasion and Migration Assays

Invasion and migration assays were performed using a dual chamber system. 2.5×10^5 cells were plated in serum free DMEM onto a BD Falcon ™ Cell Culture insert with transparent PET membranes (8.0 μm pores, Falcon, Becton Dickinson, Franklin Lakes, NJ). The cells were treated with 10 or 20 μg/ml cisplatin, 20μM LY294002, or a combination of 10 or 20 μg/ml cisplatin and 20μM LY294002 added to the upper chamber. To assess invasion, membranes were coated with 10 μg of BD Matrigel ™ (BD Biosciences, Franklin Lakes, NJ). The bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. For invasion assays, cells were incubated for 48 hours at 37°C, the non-invasive cells were removed from the upper chamber, and filters were fixed with 100% methanol and stained with a 0.05% Crystal Violet solution. The number of invading cells was manually counted as the sum of 3 randomly selected fields at a 40 X magnification. The same experimental design was used for migration experiments except that membranes were not coated with BD Matrigel and cells were incubated for 24 hours. All experiments were performed in triplicates.

Cell Proliferation and Viability Assays

SKOV3, IGROV1 and OVCAR cells were seeded in tissue culture treated 48-well micro titer plates (2.5×10^5 per well) and treated with cisplatin 24 hours later. Cell proliferation was assessed via cell counting on a Vi-CELL Cell Viability Analyzer (Beckman Coulter) at 24 and 48 hours. The percentage of viable cells (trypan blue exclusion) was divided by the number of total cells to yield viability ratios 24 and 48 hours after treatment.

Antibodies

MMP2 and TIMP1 antibodies were purchased from Calbiochem (San Diego, CA), TIMP2, and MT1-MMP antibodies were acquired from R&D Systems (Minneapolis, MN). Antibodies against phospho-4E-BP1 (T37/46), phospho-Akt (S473), Akt (total), PTEN, phospho-S6, S6, HRP-linked goat anti-mouse antibody, and HRP-linked goat anti-rabbit antibody were purchased from Cell Signaling (Beverly, MA). β-actin antibodies, HRP-linked donkey anti-mouse, and HRP-linked donkey anti-rabbit were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA).

Western Blotting

To investigate the regulation of MMP2, TIMP1, TIMP2, and MT1-MMP proteins, SKOV3 and IGROV1 cells were treated with 10 or 20 μg/ml cisplatin, 20μM LY294002, or a combination of 10 or 20 μg/ml cisplatin and 20μM LY294002. Following treatment for 24 hours, conditioned medium was collected and centrifuged at 4°C. The supernatant was used for immunoblotting and zymography. The cell pellet from the same experiment was lysed in ice-cold cell lysis buffer (Cell Signaling Technology, Inc. Beverly, MA) supplemented with 1 mM AEBSF. Total protein lysate was separated by electrophoresis under reducing conditions on 10% SDS-polyacrylamide gels. For analysis of conditioned media, ice-cold conditioned media was obtained and clarified by centrifugation to remove cells and debris. The clarified conditioned media was concentrated 10 to 20 fold using Centricon® Centrifugal Filters with Ultracel YM-10 membranes (Millipore Corporation Billerica, MA). 10 μg of protein was electrophoresed under reducing conditions on 12% SDS-polyacrylamide gels. After electrotransferring the proteins to PVDF membranes (Millipore Corporation Billerica, MA), the membranes were immunoblotted using specific primary antibodies at 4°C overnight. The signals were detected with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour and visualized using the enhanced chemiluminescence ECL Plus ™ system (GE Healthcare Barrington, IL) and Typhoon ™ 9400 Scanner (GE Healthcare Barrington, IL). The image analysis and quantification were performed using ImageQuantTM TL software (GE Healthcare Barrington, IL). The experiments were repeated up to three times.

Gel Zymography

SKOV3 and IGROV1 cells were treated with 10 or 20 μg/ml cisplatin, 20μM LY294002, or a combination of 10 or 20 μg/ml cisplatin and 20μM LY294002. The medium was harvested and clarified by centrifugation to remove cells and debris. The clarified conditioned media was concentrated 10 to 40 fold using Centricon® Centrifugal Filters with Ultracell YM-30 membranes (Millipore Corporation Billerica, MA) at 4°C and 4000 rpm. 10 μg of protein was electrophoresed under non-reducing conditions on 10% SDS-polyacrylamide gels containing 1 mg/ml of gelatin Type A from porcine skin (Sigma-Aldrich Corp. St. Louis, MO). The gels were washed twice in 2.5% (v/v) Triton X-100 at room temperature before incubation in 50mM Tris-HCl (pH 7.6), 10mM CaCl2, 50mM NaCl and 0.05% Brij35 overnight at 37°C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 for at least 1 hour and destained with methanol/acetic acid to reveal zones of gelatinase activity. The gels were analyzed using the Typhoon ™ 9400 Scanner (GE Healthcare Barrington,

IL). Image quantification was performed using ImageQuant ™ TL software (GE Healthcare Barrington, IL)..

Statistical Analysis

Differences in invasion, migration, protein and enzyme levels between groups were analyzed using 1 way ANOVA GraphPad Prism 5.00 (GraphPad Software, San Diego CA). Results were considered significant when $p<0.05$.

Results

Cisplatin Inhibits Invasion and Migration

To rule out the possibility that the decrease in invasion and migration was caused by considerable unspecific, cisplatin or LY294002 induced cell toxicity, we assessed cell proliferation and cell viability following treatment of cells under conditions used for migration and invasion assays. The viability decreased slightly with cisplatin treatment (−7.4%, −14.8%, −29.6% at 24 hours; −25.7, −17.8% and −19.0% at 48 hours in SKOV3, IGROV1 and OVCAR5 cells respectively). Similarly, PI3K inhibition alone using LY294002 did result in only minor changes in cell viability (+0.475%, −13.2%, −1.77% at 24 hours; −3.15%, −21.4%, −5.03% at 48 hours in SKOV3, IGROV1 and OVCAR5 cells respectively). The combination treatment with cisplatin and LY 294002 resulted in 24.1%, 28.9% and 28.0% decrease in cell viability at 24 hours, and a 30.8%, 17.5%, and 16.9% decrease in SKOV3, IGROV1 and OVCAR5 cells respectively. Overall, the changes in cell viability were considered modest and insufficient to account for the significant decrease in cell migration and invasion under these treatment conditions.

We assessed the effect of cisplatin, LY294002, or a combination of both agents on invasion and migration of SKOV3, OVCAR5, and IGROV1 human ovarian cancer cells, the results were normalized to the respective viability ratios for each cell line, therapy and exposure time. Figure 1A shows photomicrographs of a representative invasion experiment in SKOV3 cells. Treatment of SKOV3 cells with 20 μg/ml cisplatin or 20μM LY294002 resulted in a statistically significant 7.67 (95% CI 3.01 to 19.5, $p<0.001$) and 5.64 (95% CI, 2.85 to 11.2, $p<0.001$) fold reduction in the number of invading SKOV3 cells compared to untreated controls at 48 hours (Figure 1A,B). Combination treatment with cisplatin and LY294002 resulted in a 28.5 fold reduction in invasion (95% CI 10.4 to 78.0, $p<0.001$). Similar results were obtained in migration assays. Cisplatin, LY294002 and combination treatment resulted in a 2.00 (1.18 to 3.37, $p<0.01$), 1.72 (1.18 to 2.52, $p<0.01$), and 3.52 (1.91 to 6.51, $p<0.001$ fold) reduction in the number of migrating SKOV3 cells at 24 hours (Figure 1C). IGROV1 cells showed a 3.02 (2.01 to 4.53, $p<0.001$), 3.53 (2.35 to 5.29, $p<0.001$), and 15.4 (10.3 to 23.2, $p<0.001$) fold reduction in invasion, and a 2.43 (1.39 to 4.25, $p<0.01$), 1.07 (0.61 to 1.87, p=NS), and 2.41 (1.38 to 4.22, $p<0.01$) fold reduction in migration. Similarly, in OVCAR5 cells, invasion was reduced by 1.43 (0.94 to 2.20, p=NS), 1.84 (1.20 to 2.82, $p<0.01$), and 3.78 (2.47 to 5.79, $p<0.001$) fold, and migration decreased by 1.60 (1.13 to 2.27, $p<0.01$), 1.60 (1.21 to 2.13, $p<0.01$), and 2.69 (1.90 to 3.81, $p<0.001$) fold under the respective treatment conditions.

LY294002 Inhibits Akt Phosphorylation and Activation of Downstream Effectors

In order to confirm that LY294002 decreases PI3K/Akt/mTOR pathway signaling, SKOV3, IGROV1 and OVCAR5 cells were incubated with 20 μM LY294002. Protein lysates were subjected to immunoblotting for the PI3K/Akt/mTOR pathway related proteins pAkt, total Akt, p4EBP1, pS6 and total S6. LY294002 caused a marked decrease in the levels of pAkt in all three cell lines (Figure 2A and 2B). In addition, levels of pS6 and p4E-BP1 as two of the main downstream targets of mTOR were significantly reduced. Total levels of Akt and

S6 remained unchanged. This data suggests that LY294002 is an effective inhibitor of PI3K/Akt/mTOR pathway signaling in the cell lines examined.

We observed differences in relative pAkt and pS6 levels between cell lines that are at least partially attributed to different mutations in oncogenic pathway related genes. IGROV1 cells contain a heterozygous deletion mutation in the PTEN gene that results in constitutively increased pAkt levels due to the lack of PTEN phosphatase activity. SKOV3 cells harbor an activating mutation (H1047R) in the PIK3CA gene, which encodes for the catalytic p110α subunit of PI3K and results in continuous phosphorylation of Akt. OVCAR5 cells contain an activating mutation (G12V) in the K-ras gene (G12V) but lack known pAkt activating mutations in PI3K pathway related genes.

Effect of Cisplatin and LY294002 on Expression of MMP2

We investigated the molecular mechanisms underlying the decrease in cell migration and invasion in SKOV3 and IGROV1 cells. Gel zymography of conditioned cell culture medium was performed to assess cisplatin and PI3K inhibition induced changes in MMP2 activation. Treatment of SKOV3 cells with cisplatin, or the combination of cisplatin and LY294002 was associated with a 6.3 (1.91 to 20.0, $p < 0.01$), and 7.1 (2.14 to 25.1, $p<0.01$) fold decrease in the level of the fully activated 62 kDa MMP2 respectively (Figure 3A,C). PI3K inhibition alone using LY294002 reduced levels of active MMP2, but this effect was not statistically significant (mean ratio of active MMP2 in LY294002 treated cell to control = 2.34, 0.69 to 7.76). These results were corroborated in IGROV1 cells (Figure 3A, B). Levels of pro-MMP2 did not change with any of the treatment conditions. The mean ratio of pro-MMP2 in the treated cells to control SKOV3 cells was 1.45 (0.82 to 2.57) for cisplatin, 1.08 (0.62 to 1.91 for LY294002, and 1.62 (0.83 to 2.88) for cisplatin and LY294002. Similarly, pro-MMP2 protein levels remained unchanged in IGROV1 cells under these treatment conditions (Figure 3A, C).

Cisplatin and LY294002 Decrease Levels of TIMP1 and TIMP2

We further investigated possible mechanisms of MMP2 down-regulation by cisplatin and PI3K inhibition. The classical model for cell surface activation of MMP2 involves the formation of a trimolecular complex comprised of MT1-MMP, TIMP2 and pro-MMP2 (Strongin et al. 1995). The transmembrane protein MT1-MMP interacts with TIMP2 in order to bind the 72 kDa pro-MMP2. Pro-MMP2 is initially cleaved to an intermediate form by a nearby free MT1-MMP molecule and subsequently converted into the fully active 62 kDa MMP2 (Atkinson et al. 1995). The binding of a second TIMP2 molecule or a TIMP1 molecule to MMP2 prevents its full activation.

We investigated whether cisplatin or LY294002 treatment of SKOV3 and IGROV1 cells induces changes in the expression of TIMP1 and TIMP2. Conditioned media from treated SKOV3 and IGROV1 cells was subjected to immunoblotting. Cisplatin, LY294002 and the combination of cisplatin and LY294002 induced a significant 4.9 (1.18 to 20.9, $p<0.05$), 6.5 (1.55 to 27.5, $p<0.05$), and 28.4 (6.75 to 115, $p<0.001$) fold decrease in the level of TIMP1 expression in SKOV3 respectively. Conversely, treatment of IGROV1 cells with cisplatin, and the combination of cisplatin and LY294002 did not result in a significant reduction of TIMP1 levels with 1.05 (0.72 to 1.95), and 1.22 (0.8 to 2.62) fold decrease respectively, treatment with LY294002 alone paradoxically resulted in a marginally significant 2.05 fold (1.09 to 19.2 fold $p<0.05$) decrease in the level of the protein (Figure 4A, B). Treatment of SKOV3 cells with cisplatin and the combination of cisplatin and LY294002 resulted in a 3.7 (1.66 to 8.43, $p<0.01$) and 5.1 (2.26 to 11.5, $p<0.001$) fold decrease in the level of TIMP2 protein respectively. In contrast, treatment with LY294002 alone did not result in a significant decrease in the level of TIMP2 (mean ratio of treated to control cells = 1.43 (0.64

to 3.23). IGROV1 cells showed a similar reduction in TIMP2 levels under treatment with cisplatin, LY294002 or the combination of both drugs with a 1.98 fold decrease (1.25 to 4.74 fold, $P<0.01$), 1.55 fold decrease (1.06 to 2.84 fold, $P<0.05$) and 1.57 fold decrease (1.08 to 2.92 fold, $P<0.05$) respectively (Figure 4A, C).

We further determined whether the observed decrease in pro-MMP2 activation induced by cisplatin and PI3K inhibition could potentially be explained by changes in the level of MT1-MMP expression. Protein lysates of control, cisplatin, LY294002 and combination treated SKOV3 and IGROV1 cells were subjected to immunoblotting for MT1-MMP. No significant changes in the levels of activated MT1-MMP were detected under any treatment condition. In SKOV3 cells, the mean ratio of activated MT1-MMP in treated cells to control was 0.86 (0.58 to 1.27, cisplatin), 1.05 (0.71 to 1.55, LY294002), and 1.04 (0.70 to 1.54, cisplatin and LY294002). IGROV1 cells showed slightly reduced MT1-MMP levels resulting in a 1.67 (1.03 to 4.35), 1.82, (1.09 to 5.56) and 1.79 fold decrease (1.08 to 5.26) for treatment with cisplatin, LY294002 or the combination of both drugs respectively (Figure 5).

Discussion

Our study demonstrates that cisplatin and inhibition of PI3K/Akt/mTOR signaling have a profound effect on invasion and migration of human ovarian cancer cells. In addition, we demonstrate significant changes in the expression levels of several matrix-metalloproteinases and their inhibitors induced by cisplatin and PI3K inhibition.

Previously published studies have demonstrated that cisplatin effects the motility of various cell lines, including developing granules cells (Pisu et al. 2005), phagocytes (Fumarulo et al. 1985; Reddy and Chatterjee 1999), and human osteosarcoma cells (Fukaya et al. 2005). Similarly, inhibition of PI3K using LY294002 has been found to block metastatic spread and decrease cell motility, for example, in a murine thyroid cancer model (Furuya et al. 2007). Our data is consistent with these observations. Cisplatin, LY294002 and the combination of both drugs inhibited invasion and migration of SKOV3, IGROV1 and OVCAR5 in our in vitro model system. The number of cells migrating was found to be consistently lower in IGROV1 cells compared to SKOV3 and OVCAR5 cells, an effect that is likely related to cell line specific differences in migratory potential. The concentrations of cisplatin utilized in our study are clinically relevant, since similar levels are achievable in patients receiving intravenous infusions of cisplatin (Holding et al. 1991).

The effect of PI3K inhibition alone is expected to be most pronounced in cell lines with activated PI3K/Akt/mTOR signaling. In our study, the motility of all three cell lines was decreased by LY294002 alone. This is an interesting observation since only SKOV3 and IGROV1 cells have constitutive active PI3K/Akt/mTOR pathway signaling due to activating mutations while OVCAR5 cells harbor a mutation in K-ras (K-ras G12V). The role of Akt in the control of invasion and migration of cancer cells has been subject to some controversy. Several studies have shown that Akt activation enhance tumor proliferation and suppress cell death (Vanhaesebroeck et al. 2001; Vivanco and Sawyers 2002). In contrast, others have demonstrated that pAkt is able to suppress tumor cell migration and/or metastasis by promoting the breakdown of pro-tumorigenic tuberous sclerosis complex 2 (TSC2) or inhibiting the transcriptional activity of NFAT (nuclear factor of activated T cells) (Liu et al. 2006; Wyszomierski and Yu 2005; Yoeli-Lerner et al. 2005).

Multiple molecular mechanisms regulate invasion and migration in human ovarian cancer cells. MMP2 plays a key role in this process, and its activity is regulated by complex protein-protein interactions on the posttranslational level (Brown et al. 1990; Overall and

Sodek 1990; Overall et al. 1991). Our findings support this concept, since cisplatin, PI3K inhibition alone, and the combination of cisplatin and PI3K inhibition all resulted in a decrease in the amount of active MMP2, but no changes in the levels of MMP2 pro-enzyme. The parallel decrease in TIMP2 protein expression with cisplatin and combination treatment suggests an important role of TIMP2 in regulation of cisplatin and PI3K mediated regulation of MMP2 in ovarian cancer cells. This observation is consistent with previously published data demonstrating that TIMP2 overexpression protects neoplastic cells from cisplatin-induced apoptosis while enhancing MMP2 expression in ovarian cancer cell lines (Kim et al. 2006).

TIMP1 has been shown to regulate MMP2 activity. Several studies have demonstrated a correlation between elevated TIMP1 levels and diminished MMP2 activity and invasiveness (Khokha et al. 1992; Park et al. 2005; Ramer and Hinz 2008). Our findings show that cisplatin treatment in SKOV3 cells significantly decreases TIMP1 expression,. In IGROV1 cells, TIMP1 levels remained unchanged. In contrast, Ramer et al demonstrated that cisplatin increases the expression of TIMP1 in HeLa human cervical carcinoma cells (Ramer et al. 2007). Several mechanisms might explain these contrasting observations. First, the effects of cisplatin on TIMP1 expression might be cell line specific. Second, TIMP1 has anti-apoptotic properties and hence its decrease could contribute to the cytotoxic effects of cisplatin (Hayakawa et al. 1992; Li et al. 1999). TIMP1 also plays an important role in the activation as well as the inhibition of MMP9 (Kolkenbrock et al. 1995). However, we could not demonstrate any significant MMP9 activity in our ovarian cancer cell lines again suggesting a more important role for MMP2 in these models. In addition, MMP2 activation is highly dependent on TIMP2 as has been shown in a TIMP2 knock-out model (English et al. 2006). The variable effects of treatment on TIMP1 as opposed to a consistent decrease in TIMP2 levels underscores the greater importance of TIMP2 in the activation of MMP2 as opposed to the more limited role of TIMP1 in our ovarian cancer models.

Conclusions

Our results suggest that cisplatin and PI3K inhibition decrease human ovarian cancer cell invasion and migration by affecting MMP2, TIMP1 and TIMP2 expression. The combination of PI3K or mTOR inhibition with cytotoxic chemotherapy has been described as a novel therapeutic clinical strategy for ovarian cancer patients, but the mechanism of this treatment approach needs to be further elucidated.

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References

Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005; 24(50):7455–7464. [PubMed: 16288292]

Altomare DA, Wang HQ, Skele KL, De Rienzo A, Klein-Szanto AJ, Godwin AK, Testa JR. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. Oncogene. 2004; 23(34):5853–5857. [PubMed: 15208673]

Atkinson SJ, Crabbe T, Cowell S, Ward RV, Butler MJ, Sato H, Seiki M, Reynolds JJ, Murphy G. Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. J Biol Chem. 1995; 270(51):30479–30485. [PubMed: 8530478]

Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res. 2005; 94:29–86. [PubMed: 16095999]

Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta. 2005; 1755(1):37–69. [PubMed: 15907591]

Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG. Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res. 1990; 50(19):6184–6191. [PubMed: 2169338]

Butler GS, Butler MJ, Atkinson SJ, Will H, Tamura T, Schade van Westrum S, Crabbe T, Clements J, d'Ortho MP, Murphy G. The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. A kinetic study. J Biol Chem. 1998; 273(2):871–880. [PubMed: 9422744]

Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, Cristiano BE, Pearson RB, Phillips WA. Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res. 2004; 64(21):7678–7681. [PubMed: 15520168]

Choi JH, Choi KC, Auersperg N, Leung PC. Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. Cancer Res. 2006; 66(7):3912–3920. [PubMed: 16585220]

Corcoran ML, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism. J Biol Chem. 1995; 270(22):13453–13459. [PubMed: 7768948]

Crijns AP, Duiker EW, de Jong S, Willemse PH, van der Zee AG, de Vries EG. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer. 2006; 16 Suppl 1:152–165. [PubMed: 16515584]

Cullen KJ, Yang Z, Schumaker L, Guo Z. Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer. J Bioenerg Biomembr. 2007; 39(1):43–50. [PubMed: 17318397]

Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer. 2000; 36(13 Spec No):1621–1630. [PubMed: 10959048]

English JL, Kassiri Z, Koskivirta I, Atkinson SJ, Di Grappa M, Soloway PD, Nagase H, Vuorio E, Murphy G, Khokha R. Individual Timp Deficiencies Differentially Impact Pro-MMP-2 Activation. J. Biol. Chem. 2006; 281(15):10337–10346. [PubMed: 16469749]

Friesen C, Fulda S, Debatin KM. Cytotoxic drugs and the CD95 pathway. Leukemia. 1999; 13(11): 1854–1858. [PubMed: 10557062]

Fukaya Y, Ishiguro N, Senga T, Ichigotani Y, Sohara Y, Tsutsui M, Shioura T, Iwamoto T, Hamaguchi M. A role for PI3K-Akt signaling in pulmonary metastatic nodule formation of the osteosarcoma cell line, LM8. Oncol Rep. 2005; 14(4):847–852. [PubMed: 16142341]

Fumarulo R, Riccardi S, Pantaleo R, Coluccia M, Giordano D, Aresta M. In vitro cisplatin effects on phagocyte functions. Int J Cancer. 1985; 35(6):777–780. [PubMed: 4040125]

Furuya F, Hanover JA, Cheng SY. Activation of phosphatidylinositol 3-kinase signaling by a mutant thyroid hormone beta receptor. Proc Natl Acad Sci U S A. 2006; 103(6):1780–1785. [PubMed: 16446424]

Furuya F, Lu C, Willingham MC, Cheng SY. Inhibition of phosphatidylinositol 3-kinase delays tumor progression and blocks metastatic spread in a mouse model of thyroid cancer. Carcinogenesis. 2007; 28(12):2451–2458. [PubMed: 17660507]

Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K. Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. FEBS Lett. 1992; 298(1):29–32. [PubMed: 1544418]

Holding JD, Lindup WE, Bowdler DA, Siodlak MZ, Stell PM. Disposition and tumour concentrations of platinum in hypoalbuminaemic patients after treatment with cisplatin for cancer of the head and neck. Br J Clin Pharmacol. 1991; 32(2):173–179. [PubMed: 1931468]

Hornebeck W, Emonard H, Monboisse JC, Bellon G. Matrix-directed regulation of pericellular proteolysis and tumor progression. Semin Cancer Biol. 2002; 12(3):231–241. [PubMed: 12083853]

Jamieson ER, Lippard SJ. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. Chem Rev. 1999; 99(9):2467–2498. [PubMed: 11749487]

Jo Y, Yeon J, Kim HJ, Lee ST. Analysis of tissue inhibitor of metalloproteinases-2 effect on pro-matrix metalloproteinase-2 activation by membrane-type 1 matrix metalloproteinase using baculovirus/insect-cell expression system. Biochem J. 2000; 345 Pt 3:511–519. [PubMed: 10642509]

Kadri Z, Petitfrere E, Boudot C, Freyssinier JM, Fichelson S, Mayeux P, Emonard H, Hornebeck W, Haye B, Billat C. Erythropoietin induction of tissue inhibitors of metalloproteinase-1 expression and secretion is mediated by mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. Cell Growth Differ. 2000; 11(11):573–580. [PubMed: 11095246]

Khokha R, Zimmer MJ, Graham CH, Lala PK, Waterhouse P. Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16-F10 melanoma cells. J Natl Cancer Inst. 1992; 84(13):1017–1022. [PubMed: 1318976]

Kim T-J, Rho SB, Choi Y-L, Choi CH, Lee J-W, Bae D-S, Ahn G, Lee J-H, Kim B-G. High expression of tissue inhibitor of metalloproteinase-2 in serous ovarian carcinomas and the role of this expression in ovarian tumorigenesis. Human Pathology. 2006; 37(7):906–913. [PubMed: 16784992]

Kolkenbrock H, Orgel D, Hecker-Kia A, Zimmermann J, Ulbrich N. Generation and activity of the ternary gelatinase B/TIMP-1/LMW-stromelysin-1 complex. Biol Chem Hoppe Seyler. 1995; 376(8):495–500. [PubMed: 7576248]

Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol. 2005; 97(1):26–34. [PubMed: 15790433]

Levine DA, Bogomolniy F, Yee CJ, Lash A, Barakat RR, Borgen PI, Boyd J. Frequent mutation of the PIK3CA gene in ovarian and breast cancers. Clin Cancer Res. 2005; 11(8):2875–2878. [PubMed: 15837735]

Li G, Fridman R, Kim H-RC. Tissue Inhibitor of Metalloproteinase-1 Inhibits Apoptosis of Human Breast Epithelial Cells. Cancer Res. 1999; 59(24):6267–6275. [PubMed: 10626822]

Liu H, Radisky DC, Nelson CM, Zhang H, Fata JE, Roth RA, Bissell MJ. Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. Proc Natl Acad Sci U S A. 2006; 103(11):4134–4139. [PubMed: 16537497]

Loehrer PJ, Einhorn LH. Drugs five years later. Cisplatin. Ann Intern Med. 1984; 100(5):704–713.

O'Shea M, Willenbrock F, Williamson RA, Cockett MI, Freedman RB, Reynolds JJ, Docherty AJ, Murphy G. Site-directed mutations that alter the inhibitory activity of the tissue inhibitor of metalloproteinases-1: importance of the N-terminal region between cysteine 3 and cysteine 13. Biochemistry. 1992; 31(42):10146–10152. [PubMed: 1420137]

Overall CM, Sodek J. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. J Biol Chem. 1990; 265(34):21141–21151. [PubMed: 2174435]

Overall CM, Wrana JL, Sodek J. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. J Biol Chem. 1991; 266(21):14064–14071. [PubMed: 1649834]

Park BK, Zeng X, Glazer RI. Akt1 induces extracellular matrix invasion and matrix metalloproteinase-2 activity in mouse mammary epithelial cells. Cancer Res. 2001; 61(20):7647–7653. [PubMed: 11606407]

Park MJ, Lee JY, Kwak HJ, Park CM, Lee HC, Woo SH, Jin HO, Han CJ, An S, Lee SH, et al. Arsenic trioxide (As2O3) inhibits invasion of HT1080 human fibrosarcoma cells: role of nuclear factor-kappaB and reactive oxygen species. J Cell Biochem. 2005; 95(5):955–969. [PubMed: 15962302]

Philp AJ, Campbell IG, Leet C, Vincan E, Rockman SP, Whitehead RH, Thomas RJ, Phillips WA. The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors. Cancer Res. 2001; 61(20):7426–7429. [PubMed: 11606375]

Pisu MB, Roda E, Guioli S, Avella D, Bottone MG, Bernocchi G. Proliferation and migration of granule cells in the developing rat cerebellum: cisplatin effects. Anat Rec A Discov Mol Cell Evol Biol. 2005; 287(2):1226–1235. [PubMed: 16247801]

Qiu Q, Yang M, Tsang BK, Gruslin A. EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. Reproduction. 2004; 128(3): 355–363. [PubMed: 15333786]

Ramer R, Eichele K, Hinz B. Upregulation of tissue inhibitor of matrix metalloproteinases-1 confers the anti-invasive action of cisplatin on human cancer cells. Oncogene. 2007; 26(39):5822–5827. [PubMed: 17369856]

Ramer R, Hinz B. Inhibition of Cancer Cell Invasion by Cannabinoids via Increased Expression of Tissue Inhibitor of Matrix Metalloproteinases-1. J. Natl. Cancer Inst. 2008; 100(1):59–69. [PubMed: 18159069]

Reddy TB, Chatterjee S. Cisplatin inhibits folic acid chemotaxis and phagocytotic functions in Dictyostelium discoideum. Cell Biol Int. 1999; 23(3):227–233. [PubMed: 10562444]

Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol. 2000; 10(6):415–433. [PubMed: 11170864]

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem. 1995; 270(10):5331–5338. [PubMed: 7890645]

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem. 2001; 70:535–602. [PubMed: 11395417]

Vasko V, Saji M, Hardy E, Kruhlak M, Larin A, Savchenko V, Miyakawa M, Isozaki O, Murakami H, Tsushima T, et al. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. J Med Genet. 2004; 41(3):161–170. [PubMed: 14985374]

Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002; 2(7):489–501. [PubMed: 12094235]

Wang Y, Helland A, Holm R, Kristensen GB, Borresen-Dale AL. PIK3CA mutations in advanced ovarian carcinomas. Hum Mutat. 2005; 25(3):322. [PubMed: 15712344]

Winograd-Katz SE, Levitzki A. Cisplatin induces PKB/Akt activation and p38(MAPK) phosphorylation of the EGF receptor. Oncogene. 2006; 25(56):7381–7390. [PubMed: 16785992]

Wyszomierski SL, Yu D. A knotty turnabout?: Akt1 as a metastasis suppressor. Cancer Cell. 2005; 8(6):437–439. [PubMed: 16338656]

Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell. 2005; 20(4):539–550. [PubMed: 16307918]

Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, Coppola D, Nicosia SV, Cheng JQ. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene. 2000; 19(19):2324–2330. [PubMed: 10822383]

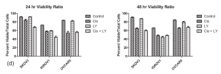

Figure 1.

Migration and invasion of cisplatin (Cis), LY294002 (LY) and combination treated SKOV3, IGROV1 and OVCAR5 cells. (a) Photomicrograph of SKOV3 cells invading through Matrigel-coated membranes stained with Crystal violet. (b) Mean number of invaded cells normalized to their respective viability ratio ± SEM under different treatment conditions. (c) Mean number of migrated cells normalized to their respective viability ratio ± SEM under different treatment conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by 1 way ANOVA. (d) Ratio of viable over total tumor cells as determined using a Vi-CELL Cell Viability Analyzer at 24 and 48 hours of incubation under treatment conditions.

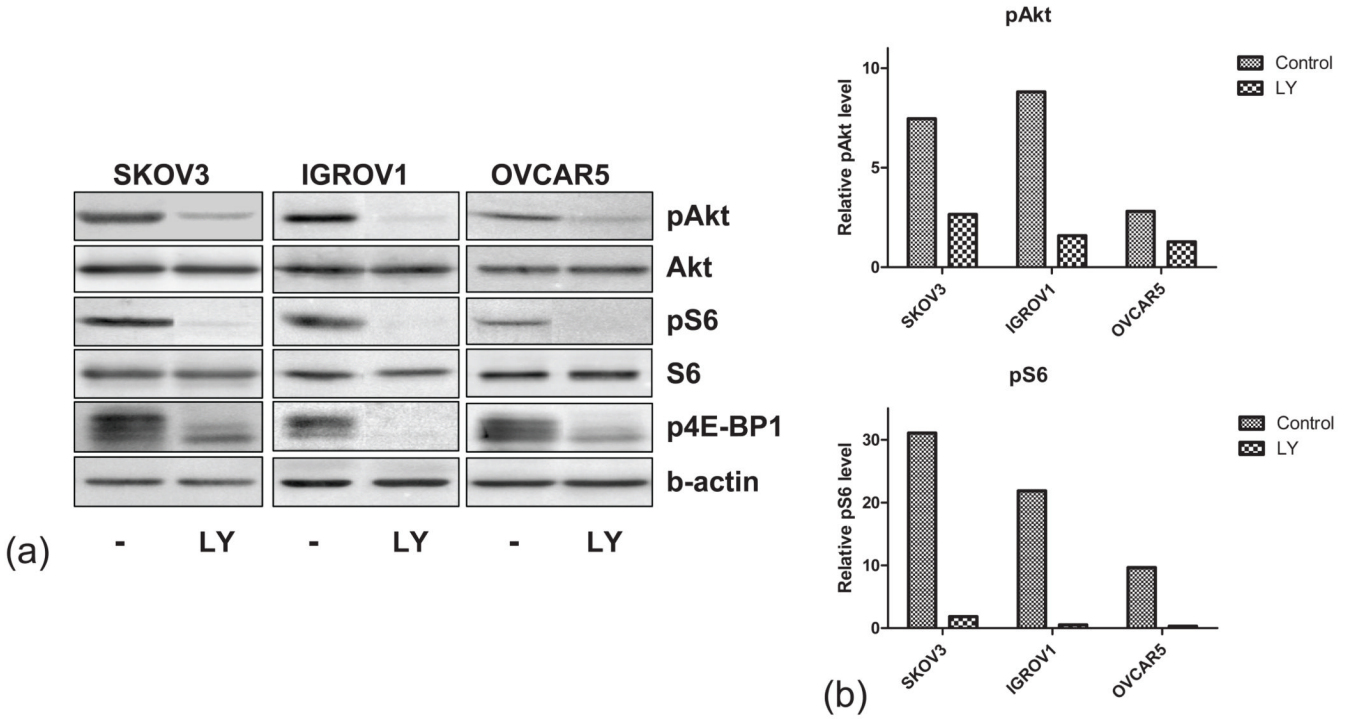

Figure 2.

Inhibition of pAkt signaling by LY294002 in SKOV3, IGROV1 and OVCAR5 cells. (a) Western blot analysis of control and LY294002 treated SKOV3, IGROV1 and OVCAR5 cell lysates using antibodies against pAkt, Akt and its downstream effectors pS6, S6 and p4E-BP1. β-actin was used to control for equal protein loading. (b) The relative amounts of activated pAkt and pS6 protein were determined by densitometric analysis of their respective band intensities in each cell line.

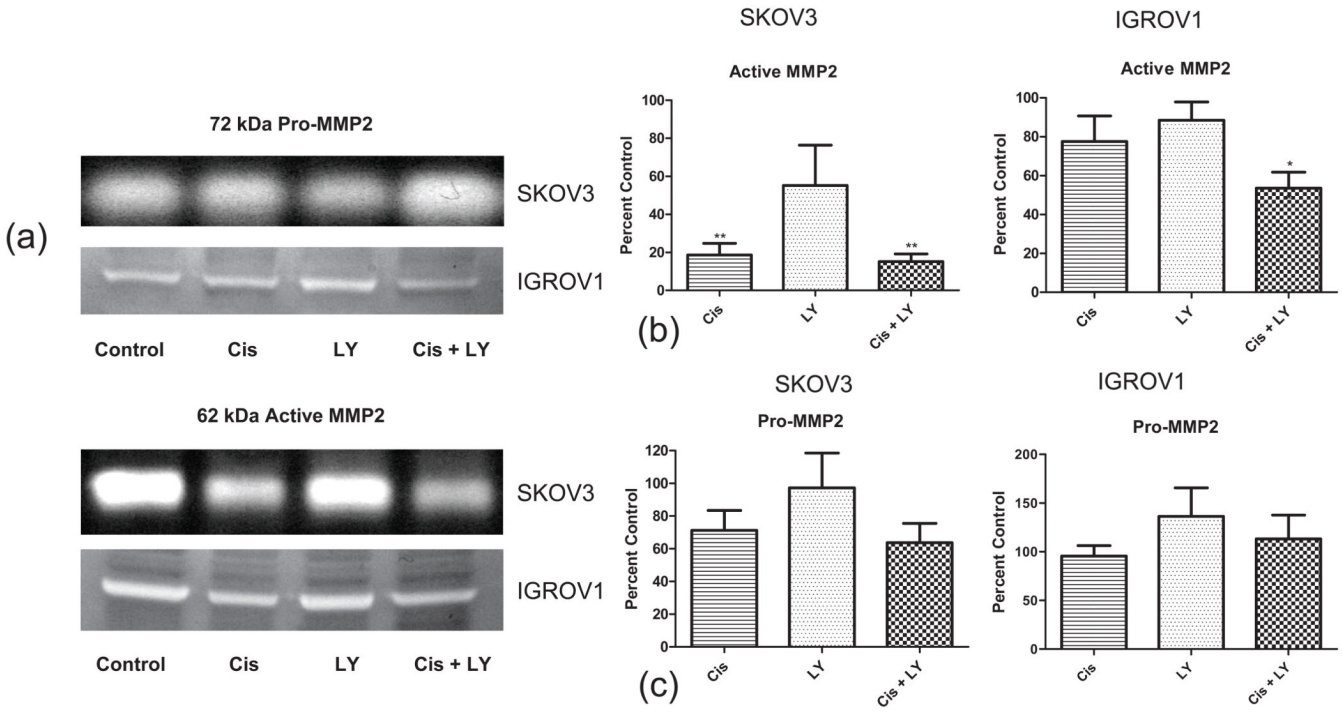

Figure 3.

MMP2 activity by gelatin zymography of conditioned medium from treated SKOV3 and IGROV1 cells. (a) Gelatin zymography for MMP2. The upper band corresponds to the 72 kDa proform MMP2 gelatinase, the lower band represents the 62 kDa activated form. (b), (c) Densitometric analysis of pro-MMP2 and MMP2 activity. The value of MMP2 and pro-MMP2 activity is shown as percent of untreated control cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by 1 way ANOVA.

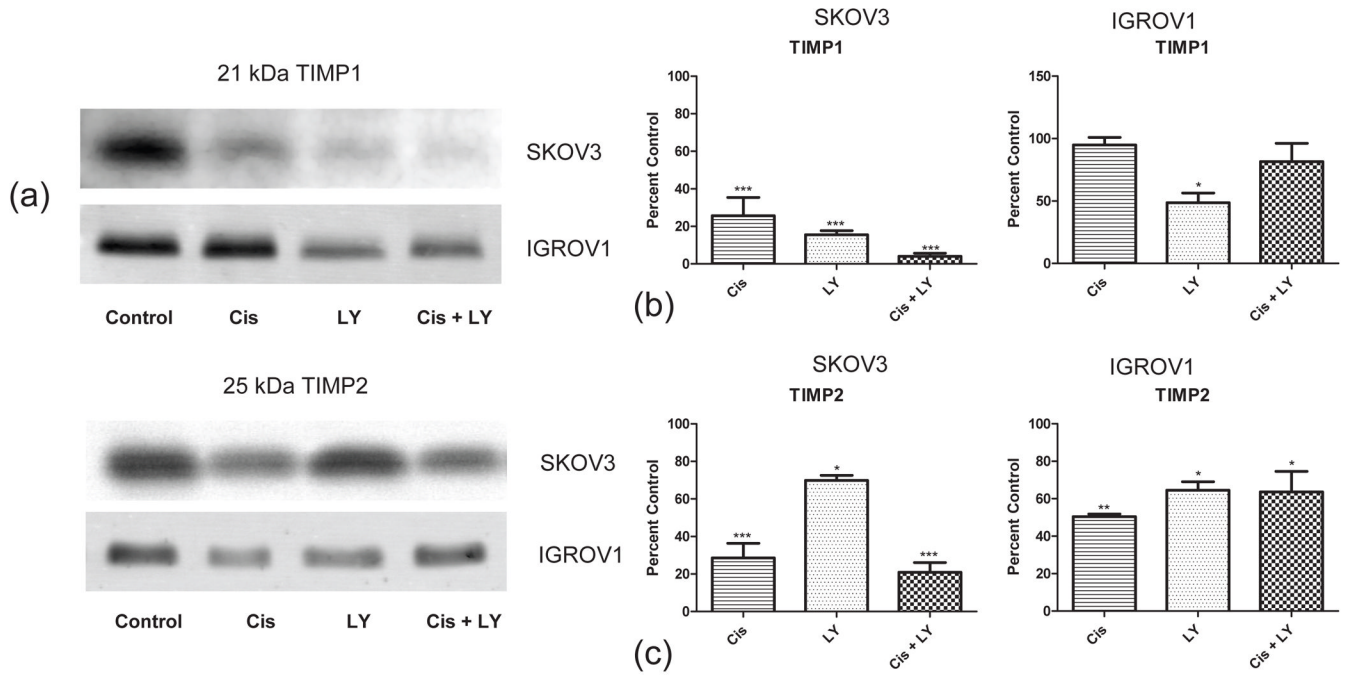

Figure 4.

Expression of TIMP1 and TIMP2 in SKOV3 and IGROV1 cells treated with cisplatin (Cis), LY294002 (LY) or combination. (a) Western blot analysis of conditioned media from control and treated SKOV3 and IGROV1. (b), (c) Densitometric analysis of TIMP1 and TIMP2 band intensities. The value of TIMP1 and TIMP2 protein levels is shown as percent of control cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by 1 way ANOVA.

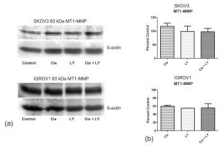

Figure 5.
MT1-MMP levels in SKOV3 and IGROV1 cells treated with cisplatin (Cis), LY294002 (LY) or the combination express similar levels of MT1-MMP. (a) Western blot of cell lysates from treated SKOV3 and IGROV1 cells using antibodies against MT1-MMP. (b) Densitometric analysis of MT1-MMP band intensities. The value of MT1-MMP protein levels in treated SKOV3 and IGROV1 cells is shown as percent of the control value.