RESEARCH ARTICLE

Alkylglyceronephosphate Synthase (AGPS) Alters Lipid Signaling Pathways and Supports Chemotherapy Resistance of Glioma and Hepatic Carcinoma Cell Lines

Yu Zhu[1&*], Xing-Jun Liu[2&], Ping Yang[1&], Meng Zhao[3&], Li-Xia Lv[1], Guo-Dong Zhang[1], Qin Wang[1], Ling Zhang[1]

Abstract

Chemotherapy continues to be a mainstay of cancer treatment, although drug resistance is a major obstacle. Lipid metabolism plays a critical role in cancer pathology, with elevated ether lipid levels. Recently, alkylglyceronephosphate synthase (AGPS), an enzyme that catalyzes the critical step in ether lipid synthesis, was shown to be up-regulated in multiple types of cancer cells and primary tumors. Here, we demonstrated that silencing of AGPS in chemotherapy resistance glioma U87MG/DDP and hepatic carcinoma HepG2/ADM cell lines resulted in reduced cell proliferation, increased drug sensitivity, cell cycle arrest and cell apoptosis through reducing the intracellular concentration of lysophosphatidic acid (LPA), lysophosphatidic acid-ether (LPAe) and prostaglandin E2 (PGE2), resulting in reduction of LPA receptor and EP receptors mediated PI3K/AKT signaling pathways and the expression of several multi-drug resistance genes, like MDR1, MRP1 and ABCG2. β-catenin, caspase-3/8, Bcl-2 and survivin were also found to be involved. In summary, our studies indicate that AGPS plays a role in cancer chemotherapy resistance by mediating signaling lipid metabolism in cancer cells.

Keywords: AGPS - Lipid metabolism - cancer chemotherapy resistance

Asian Pac J Cancer Prev, **15 (7)**, 3219-3226

Introduction

The hallmarks of cancer are keys for cancer treatment. The most fundamental hallmark of cancer cells is their ability to sustain proliferation (Marquardt et al., 2012). This trait is the target of chemotherapy agents, an old but still widely used class of cancer therapeutic medicine. These drugs could be divided into several types. One type is called alkylating agents, the typical drug is cisplatin (Srivastava et al., 2010). Another type is topoisomerase inhibitors, with doxorubicin is the typical one (Pommier et al., 2010). Cisplatin and doxorubicin are extensively used to treatment different types of cancer. However, resistance to these drugs is rather common.

Reprogramming of energy metabolism is an emerging hallmark of cancer in the last decade. Cancer cells reprogram the cellular energy metabolism in order to support continuous cell growth and proliferation (Morandi et al., 2014). One well known such alteration is the Warburg effect (Vander et al., 2009). Recent research suggests that alterations in metabolism could not be viewed as just a response to cell proliferation, but metabolites themselves can be oncogenic by altering cell signaling and blocking cellular differentiation (Weinstein et al., 2014). Targeting cancer cell metabolism is hence an interesting strategy. Here we focus on the abnormal lipids metabolism. Lipids are not just structural components of cell but can also have important roles in signaling. Deregulation of lipide metabolism is common in cancer and can affect numerous cellular processes, including cell growth and proliferation (Prabhu et al., 2013). Many lipid synthesis related genes have been considered involved in tumor procedure, for example that prostaglandin-endoperoxide synthase 2 (PTGS2) and phospholipase A2 group IIA (PLA2G2A) genes encode enzymes involved in arachidonic acid and prostaglandin biosynthesis, which is associated with carcinogenesis (Liu et al., 2014). Recently, alkylglyceronephosphate synthase (AGPS), an enzyme that catalyzes the critical step in ether lipids synthesis, is shown to up-regulate in multiple types of cancer cell and primary tumor. AGPS alters the balance of structural and signaling lipids to fuel cancer pathogenicity (Benjamin et al., 2013). Nonetheless, whether AGPS is involved in chemotherapy resistance has remained unclear.

*[1]Department of Clinical Laboratory, Tianjin Huan Hu Hospital, Tianjin Key Laboratory of Cerebral Vessels and Neural Degeneration, [2]Department of Neurosurgery, Tianjin Haihe Hospital, [3]Department of Immunology and Department of Biochemistry, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China [&]Equal contributors *For correspondence: zhuyutj@126.com*

In this study, we showed that AGPS elevated in glioma U87MG/DDP and hepatic carcinoma HepG2/ADM cell lines. Knockdown of AGPS restored the drug sensitivity in these cells. We also showed that silencing of AGPS led to reduced cellular level of lysophosphatidic acid (LPA), lysophosphatidic acid-ether (LPAe) and prostaglandin E2 (PGE2), which could fuel aggressive and progress of cancer (Hull et al., 2004; Brindley et al., 2013). Knockdown of AGPS also results in down-regulation of multi-drug resistance proteins and anti-apoptosis proteins.

Materials and Methods

Cell culture and establishment of drug-resistant cell clones

U87MG and HepG2 cell lines were obtained from Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was DMEM supplemented with 10% fetal bovine serum (FBS) for HepG2 and U87MG. Cisplatin-resistant U87MG/DDP cell line and Doxorubicin-resistant HepG2/ADM cell line were established by continuing exposure of the parental cells to gradient increasing concentration of respective drug.

Silencing of AGPS by shRNA

Lentivirus-shRNA mediated AGPS silencing was employed as previously described (Nomura et al., 2010). Lentiviral plasmids (pLKO.1) containing anti-AGPS shRNA (Open Biosystems) were transfected into cancer cells using Fugene HD transfection reagent (Promega). The transfected cells could be selected out over 3 days with 1 μg/mL puromycin. Control shRNA lentiviral particles were also purchased from Open Biosystems. The sequences of shRNA used to silence AGPS were as followes: shRNA #1: AAGGATTTCTTCTCTAGCAGC; shRNA #2: AATTCGCTCAAACATTCCTTC. Silencing of AGPS was confirmed by western blotting and realtime PCR assay.

MTS assay to determine the drug sensitivity of the cells

0.1 ml of cells suspension (HepG2/ADM: 1×10^5/ml, U87MG/DDP: 3×10^4/ml) were seeded into each well of the 96-well microplate, and incubated overnight to get the cells to adherence. Then different concentration of Cisplatin or Doxorubicin was added to each well and the cells were cultured for another 72hrs. CellTiter 96 Aqueous One Solution Reagent (Promega) was used according to the manufacturer's instructions. 4hrs later the cell viability was determined by measuring the absorbance at 490nm using a microplate reader.

Flow cytometry to determine intracellular Rhodamine-123, cell cycle distribution, apoptosis rate and fluorescence-based assay to determine activity of caspase-3/8

About 1×10^6 of the cells were collected and resuspended in 0.1 ml culture medium. The cells were stained with 10μM rhodamine-123 (Sigma-Aldrich) for 1 hr and the intracellular concentration was determined by flow cytometry (BD, FACSCalibur). For cell cycle analysis, PI (BD Biosciences) was used to stain DNA. For apoptosis analysis, 20 μM cisplatin for U87MG/DDP or 2 μM doxorubicin for HepG2/ADM was added for 24h, then Annexin V-FITC/PI dual stain (BD Biosciences) was employed. For caspase-3/8 activation analysis, 2μM cisplatin for U87MG/DDP or 0.1 μM doxorubicin for HepG2/ADM was added for 24h, then activity of caspase-3/8 was measured by Caspase 3 and Caspase 8 activity assay kits (Promega) .

Western blotting

The whole proteins were obtained by RAPI lysis buffer (Millipore) extraction and centrifugation at 12,000 g for 10 min. Total protein concentrations of the supernatants were measured by the BCA kit (Sigma Aldrich). 20 μg proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes. Target proteins were detected by incubation overnight at 4°C with rabbit anti-AGPS (1:800), MDR1 (1:800), MRP1 (1:800), ABCG2 (1:800), Bcl-2 (1:800), survivin (1:800), β-catenin (1:800), Akt (1:800), *p*-Akt (1:400), β-actin (1:5000) antibody (Santa Cruz). All primary antibodies were diluted according to the instruction manual. Membranes were washed and incubated for 1 hr with peroxidase-labelled anti-rabbit IgG (Santa Cruz, diluted at 1:2000). Finally, membranes were washed three times in TNT and exposed to the ImmobilonTM Western chemiluminescent HRP substrate (Millipore) for 1 min, and then exposed autoradiography film for 2~3 min in the dark.

Real-time PCR analysis

About 3×10^6 of cells were harvested for RT-PCR analysis. The total mRNA was extracted from the cells by the Dynabeads mRNA Direct Kit (Invitrogen) according to the manual instruction. Total mRNA was then reverse transcribed for 1hr at 42°C in incubation buffer containing 250 μM of each deoxynucleotide triphosphate, 5 μM oligo (dT)20, 25 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics). The transcription level of target proteins was detected by semiquantitative real-time PCR using the icycler iQ detection system (Bio-Rad). The PCR condition was as following: decontamination at 50°C for 2min, denaturation at 95°C for 2min, followed by n cycles at 95°C for 20 sec. and at hybridization T°C for 40 sec. The full details were shown in Table 1.

Table 1. Oligonucleotide Sequences Used for Real-Time PCR

Gene	Primer Sequences (5'-3')	Hybridization T°C	Cycles
AGPS	Forward: ACCAGATTCCCTGGAGTTCA Reverse: GAACCACCAGGTCCTCGATA	65	40
MDR1	Forward: AAAAAGATCAACTCGTACCACTC Reverse: GCACAAAATACACCAACAA	65	37
MRP1	Forward: CTGGGAACATGATTAGGAAGC Reverse: GAGGATTTCCCAGAGCCGAC	65	42
ABCG2	Forward: GGGTTCTCTTCTTCCTGACGACC Reverse: CAGACAACCAGTTAGAGTGTTGGT	65	38
BCL-2	Forward:ACGGGGTGAACTGGGGGAGGA Reverse:TGTTTGGGGCAGGCATGTTGACTT	65	40
Survivin	Forward: GCATGGGTGCCCCGACGT TG Reverse: GCTCCGGCCAGAGGCCTCAA	60	38
β-catenin	Forward: GGATTCTGGAATCCATTCTGG Reverse: TCTGAGCCCTAGTCATTGCAT	60	38
β-actin	Forward: TGAGCGCGGCTACAGCTT Reverse: TCCTTAATGTCACGCACGATTT	60	40

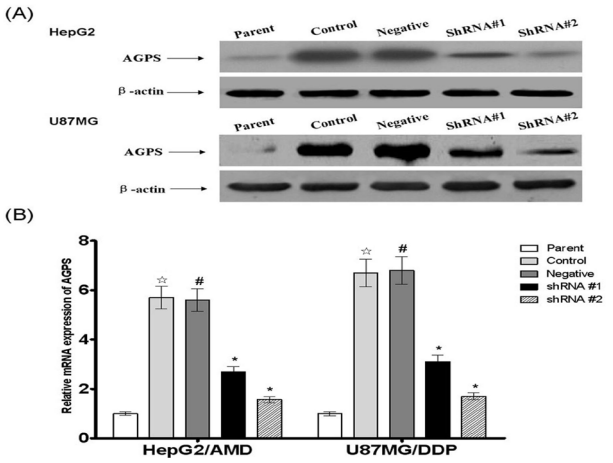

Figure 1. The Expression of AGPS in Human Glioma and Hepatic Carcinoma Cell Lines. A) Western blotting results showed that there was an increased protein expression of AGPS in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP cell lines compared with HepG2 and U87MG cell lines, meanwhile, there was a decreased protein expression of AGPS in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. β-actin was as an internal control. **B)** Realtime PCR results showed that there was an increased mRNA expression of AGPS in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP cell lines compared with HepG2 and U87MG cell lines, meanwhile, there was a decreased mRNA expression of AGPS in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. β-actin was as an internal control. Bars mean±SD. n=5. ☆p<0.05, compared to the parent group; #*p*>0.05, compared to the control group; **p*<0.05, compared to the control group

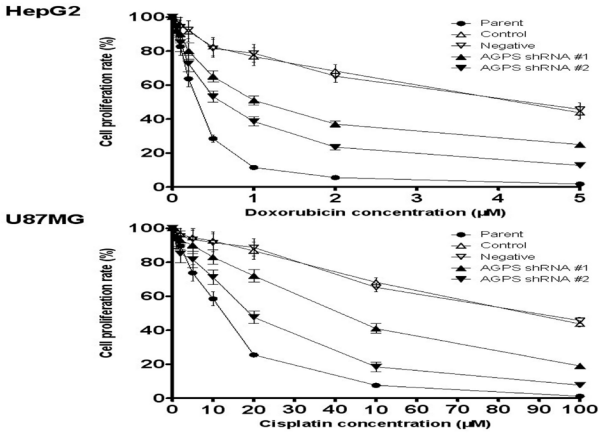

Figure 2. The Effect of AGPS Silencing on Drug Sensitivity in Human Glioma and Hepatic Carcinoma Cell Lines. The MTS results showed that there was an increased drug sensitivity in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=10

Extraction and quantization of LPA and LPAe

Cancer cells were starved in serum-free media for 4 h to minimize the contribution of serum-derived metabolites. About 1×10^6 cells were washed twice by PBS and harvested by scraping and centrifugation. Cell pellets were flash frozen at -80°C. Nonpolar lipid metabolites were extracted and analyzed by LC-MS/MS as described previously. Briefly, cell lysis was extracted by 4 mL of mixture of 2 (chloroform): 1 (methanol): 1 PBS with C12:0 dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol) as internal standards. Organic and aqueous layers were separated by centrifugation. The aqueous layer was acidified by adding 0.1% formic acid and reextracted by chloroform. The organic layer was dried by N2 and dissolved in 120 uL chloroform and a 10 μl aliquot was analyzed by Agilent 6430 LC-MS/MS.

Analysis of PGE2 by enzyme immunoassay

0.1 ml of cancer cells (HepG2/ADM: 1×10^6/ml, U87MG/DDP: 4×10^5/ml) were plated in 96-well plates and cultured overnight. The next day, cells were treated with 0.1ml arachidonic acid (final concentration 15 μM) for 1 h before collecting the culture medium. PGE2 levels in the medium were determined by PGE2 enzyme immunoassay kits (Cayman Chemical).

Measurement of PLD activity

Cancer cells were seeded into 24-well plate and allowed to reach to near 90% fusion. Cells were washed three times with PBS. Then 100 μl of an ice-cold Tris 50 mM (pH 8.0) was add to each well and the cells were broken by three freeze and thaw cycles. Samples were collected, mixed with an equal volume of the Amplex Red reaction buffer (Amplex Red Phospholipase D assay kit, Life Technologies), and the PLD activity was estimated after 1 h incubation at 37°C with a Bio-Rad fluorometer. A standard curve was performed with purified PLD from Streptomyces chromofuscus (Sigma-Aldrich). Data are given as the mean of six determinations performed on three different cell preparations.

Stimulation of EP receptors by PEG2 and respective agonists

Cancer cells were seeded into 96-well plate as MTS assay mentioned above. On the second day, PEG2 (5 μM) or respective agonists (19(R)-hydroxy PGE1, 1μM, EP1 and EP3 receptor agonist; CAY10399, 1μM, EP2 receptor agonist; L-902,688, 100nM, EP4 receptor agonist; Cayman Chemical) were added to each well 1h before adding different concentration of drugs to stimulate EP receptors. 72hrs later, CellTiter 96 Aqueous One Solution Reagent (Promega) was employed to determine the cell viability.

Statistical analyses

Data were expressed as means±SD., statistical analysis was carried out using One Way ANOVA and $p<0.05$ indicates statistical significance.

Results

The HepG2/ADM and U87MG/DDP cells were resistant to chemotherapy drug and over-expressed AGPS

Clones of drug resistant cancer cells were established by continuing exposure to chemotherapy agents. Significant over-expression of AGPS was found in these drug resistant cancer cells compared to the drug sensitive HepG2 and U87MG cells (parent group).

The drug resistant clones were transfected with

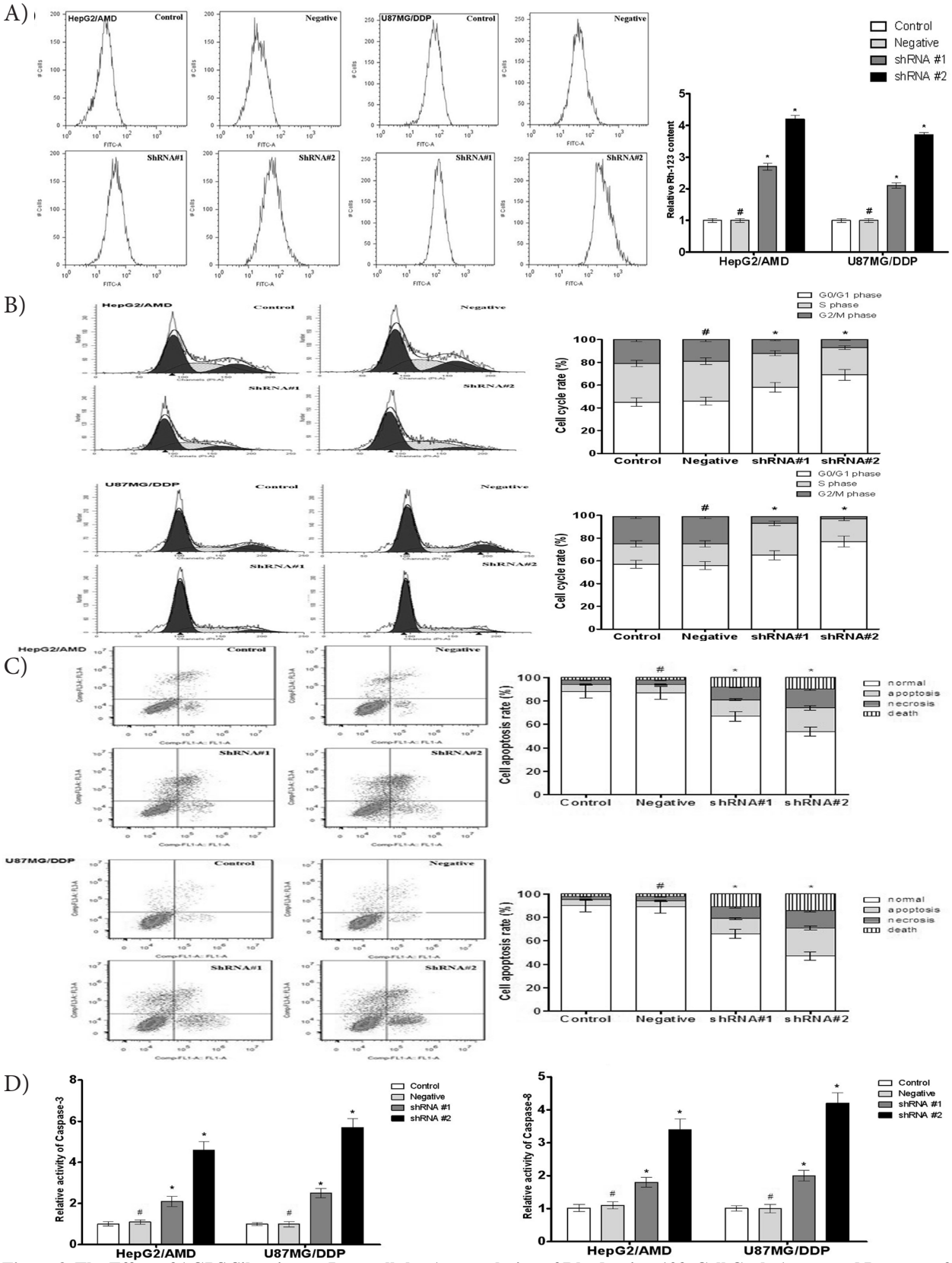

Figure 3. The Effect of AGPS Silencing on Intracellular Accumulation of Rhodamine-123, Cell Cycle Arrest and Increase of Apoptosis Rate and Caspase-3/8 Activities in Human Glioma and Hepatic Carcinoma Cell Lines. A) The FCM results showed that there was an increased intracellular accumulation of Rhodamine-123 in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=3. #p>0.05, compared to the control group; *p<0.05, compared to the control group. **B)** The FCM results showed that there was an increased G0/G1 phase in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=3. #p>0.05, compared to the control group; *p<0.05, compared to the control group. **C)** The FCM results showed that there was an increased apoptosis rate in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=3. #p>0.05, compared to the control group; *p<0.05, compared to the control group. **D)** The fluorescein results showed that there was an increased caspase-3/8 activity in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=5. #p>0.05, compared to the control group; *p<0.05, compared to the control group.

different sequences of AGPS-shRNA (shRNA #1 and shRNA #2) in order to produce two groups of cells with different AGPS silencing level. Both HepG2/ADM and U87MG/DDP cells transfected with AGPS-shRNA resulted in down-regulation of AGPS mRNA expression in shRNA #1 and shRNA #2 groups compared to the HepG2/ADM and U87MG/DDP cells (control group) ($p<0.05$). The blank vector HepG2/ADM and U87MG/DDP cells (negative group) had no effect on AGPS mRNA expression when compared to the HepG2/ADM and U87MG/DDP control groups ($p>0.05$), meanwhile, the AGPS protein expression was showed the similar tendency (Figure 1).

AGPS silencing restored the drug sensitivity

After knockdown of the AGPS, we examined the drug sensitivity of each cell clone by MTS assay. As shown in Figure 2, we could see the cell proliferation rate of the AGPS silencing cells were lower than that of the control group. The negatively transfected cells were nearly unaffected. The IC_{50} of shRNA#1 and shRNA#2 groups were 0.97μM and 0.53μM in HepG2/AMD cell lines for doxorubicin respectively, which was significant sensitive compared with the control group (IC_{50} was 4.4μM) ($p<0.05$). The reversal factor was 4.5 and 8.3, respectively. The IC_{50} of shRNA#1 and shRNA#2 groups were 31.3μM and 18.7μM in U87MG/DDP cell lines for cisplatin respectively, which was significant sensitive compared with the control group (IC_{50} was 94.2μM) ($p<0.05$). The reversal factor was 3.0 and 5.0, respectively. These results indicated that AGPS play a positive role in

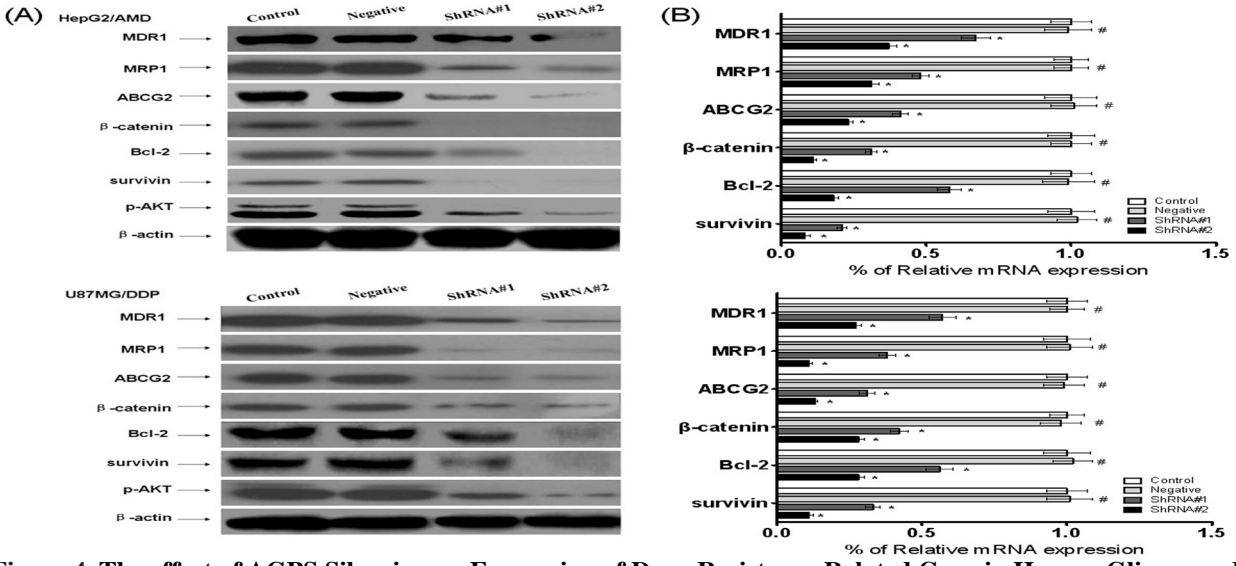

Figure 4. The effect of AGPS Silencing on Expression of Drug Resistance Related Gene in Human Glioma and hepatic Carcinoma Cell lines. A) Western blotting results showed that there was a decreased protein expression of MDR1, MRP1 and ABCG2, β-catenin, Bcl-2, survivin and *p*-AKT in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. β-actin was as an internal control. **B)** Realtime results showed that there was a decreased mRNA expression of MDR1, MRP1 and ABCG2, β-catenin, Bcl-2, survivin and *p*-AKT in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. β-actin was as an internal control. Bars mean±SD. n=5. *$p<0.05$, compared to the control group

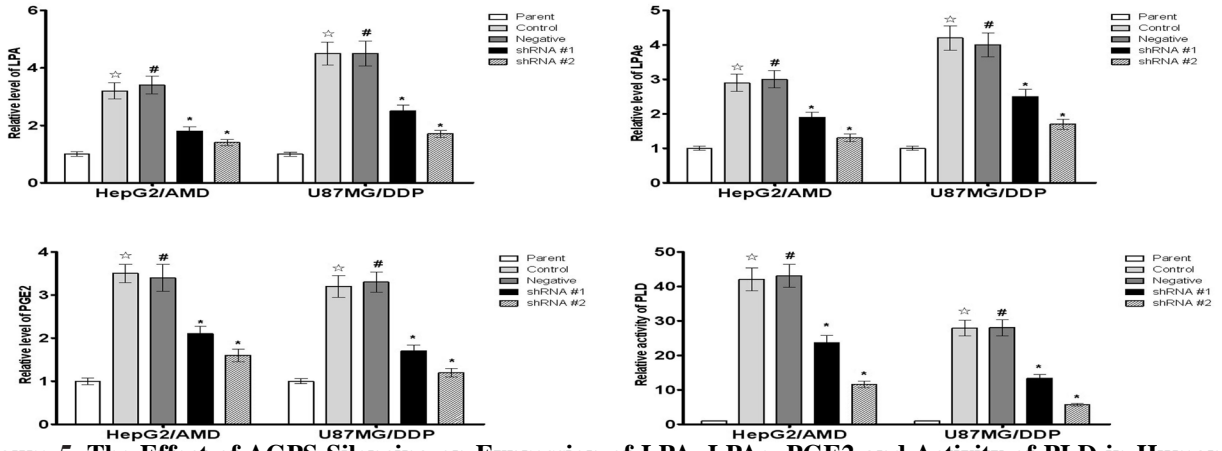

Figure 5. The Effect of AGPS Silencing on Expression of LPA, LPAe, PGE2 and Activity of PLD in Human Glioma and Hepatic Carcinoma Cell Lines. The results showed that there was an increased expression of LPA, LPAe, PGE2 and activity of PLD in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP cell lines compared with HepG2 and U87MG cell lines, meanwhile, there was a decreased expression of LPA, LPAe, PGE2 and activity of PLD in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Bars mean±SD. n=10. ☆$p<0.05$, compared to the parent group; #$p>0.05$, compared to the control group; *$p<0.05$, compared to the control group

the chemotherapy resistance of the cancer cells.

Restore of drug sensitivity was accompanied with an intracellular accumulation of Rhodamine-123, cell cycle arrest and increase of apoptosis rate and caspase-3/8 activities

The FCM analysis showed that AGPS silenced cells had a greater intracellular fluorescent activity, which meant that more Rh-123 was retained in the cancer cells. Meanwhile, cell cycle analysis showed that in the AGPS attenuated cancer cells, more cells were distributed in G0/G1 phase, and the percentages of S phase and G2/M phase cells were reduced. After treatment with cisplatin or doxorubicin, the apoptosis rates of AGPS silenced cells were increased significantly when compared to the control cells. Further, the activiy of caspase-3/8 was increased significantly in AGPS silenced groups compared to the control group as shown in Figure 3.

AGPS modulated the expression of MDR1, MRP1, ABCG2, β-catenin, Bcl-2, survivin and p-AKT

We investigated whether expression of transporter proteins from ABC family, such as MDR1, MRP1 and ABCG2, which have the potency to transport both Rh-123 and chemotherapy agents, were modulated. Meanwhile, β-catenin could stimulate cell growth and proliferation via its interaction with TCF-4 in the nucleus (Castellone et al., 2005). Bcl-2 and survivin have been implicated in anti-apoptosis, and is also thought to be involved in resistance to conventional cancer treatment (Mita et al., 2008; Aust et al., 2013). As shown in Figure 4, the expression of MDR1, MRP1 and ABCG2, β-catenin, Bcl-2 and survivin was down regulated in AGPS silencing groups compared with control group. The RT-PCR results were consistent to the results from western blotting (Figure 4).

AGPS modulated drug resistance through LPA, LPAe and PGE2 mediated lipid signaling pathways

LPA, LPAe and PGE2 are several lipids that have been found to be involved in AGPS mediated cancer pathogenicity. Here we demonstrated that in all the three drug resistant cancer cells, LPA, LPAe and PGE2 levels increased when compared to the non-resistant cancer cells. Silencing of AGPS resulted in reduction of LPA, LPAe and PGE2. Since both LPA, LPAe and PLD binds to LPA receptors to drive multiple aspect of cancer (Blackburn et al., 2012; Madan et al., 2013), we tested the downstream signaling activities of LPA receptor. We found that both level of LPA, LPAe and PLD in control group of HepG2/ADM and U87MG/DDP was increased compared to the HepG2 and U87MG cells (parent group) ($p<0.05$), however, there was not significant modification between control and negative group of HepG2/ADM and U87MG/DDP ($p>0.05$). Further, both level of LPA, LPAe and PLD in shRNA#1 and shRNA#2 group of HepG2/ADM and U87MG/DDP was decreased to compared to the control group of HepG2/ADM and U87MG/DDP ($p>0.05$) (Figure 5).

Furthermore, the phosphorylaion of AKT was down-regulated as well, which meat the activity of PI3K/AKT pathway was attenuated. PI3K/AKT is a key signaling pathway downstream LPA receptor. To confirm the role of PEG2 in AGPS mediated drug resistance, we supplemented the AGPS silenced cells with PEG2 and EP receptor agonists and found that the drug sensitivity established by AGPS shRNA#1 and shRNA#2 was reversed (Figure 6).

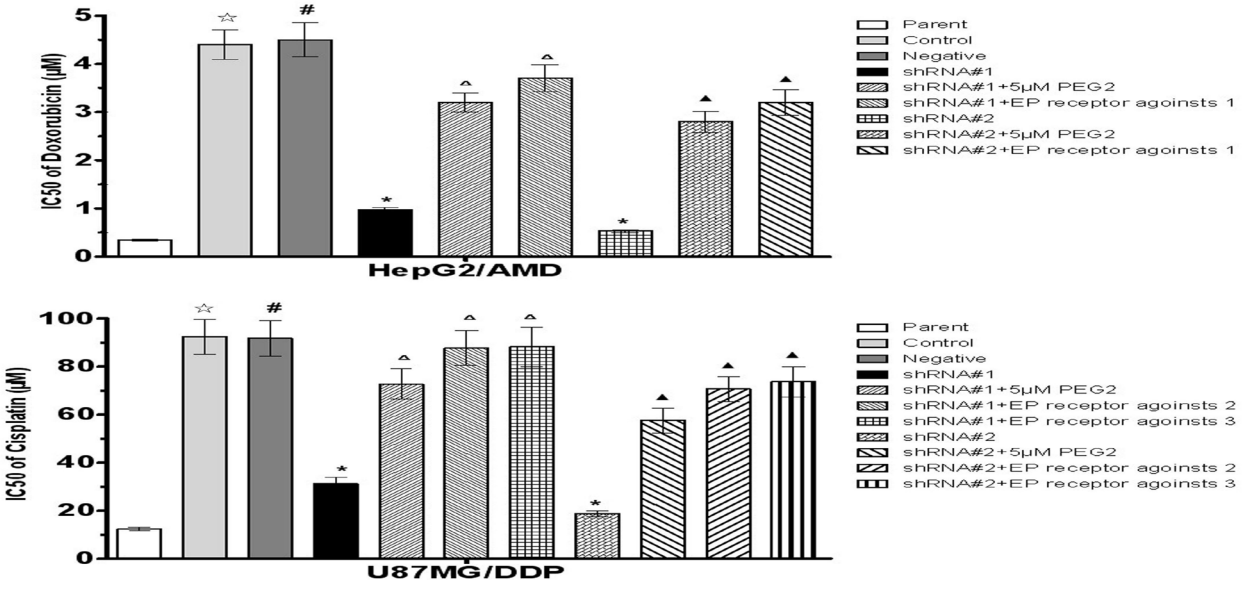

Figure 6. The Effect of AGPS Silencing on Drug Sensitivity Mediated by PEG2 and EP receptor in Human Glioma and Hepatic Carcinoma Cell Lines. The results showed that there was a decreased drug sensitivity in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP cell lines compared with HepG2 and U87MG cell lines, meanwhile, there was an increased drug sensitivity in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP AGPS silencing groups compared with HepG2/ADM and U87MG/DDP cell lines. Further, there was a decreased drug sensitivity in human hepatic carcinoma HepG2/ADM and glioma U87MG/DDP silencing cell lines by PEG2 and EP receptor agonists treatment. Bars mean±SD. n=10. ☆$p<0.05$, compared to the parent group; #$p>0.05$, compared to the control group; *: $p<0.05$, compared to the control group; *$p<0.05$, compared to the AGPS-shRNA#1 group; ▲$p<0.05$, compared to the AGPS-shRNA#2 group. 1. 19(R)-hydroxy PGE1, 1μM, EP1 and EP3 receptor agonist; 2. CAY10399, 1μM, EP2 receptor agonist; 3. L-902,688, 100nM, EP4 receptor agonist

Discussion

In order to investigate the role of AGPS in chemotherapy resistance, we used lentivirus to knock-down AGPS in several drug-resistant cancer cell lines in this study. In consistent with previous study that indicate AGPS play a role in the cancer pathogenesis, our MTS assay showed that drug sensitivity of the resistant cancer cells could be restored after AGPS silencing. Further investigations showed that after AGPS silencing, intracellular accumulation of Rh-123 increased, more cells were distributed to G0/G1 phase, and apoptosis rate increased after chemotherapy agent treatment. Therefore, we following these clues to explore the possible mechanism of AGPS mediated resistance.

One major mechanism that involved in drugs resistance is increased expression of multi-drug resistant proteins which efflux drug out of cancer cells, for example MDR1, MRP1 and so on (Louisa et al., 2014). The increase of intracellular concentration of Rh-123 means that drug efflux by the cancer cells is inhibited after AGPS silencing. Western blotting and RT-PCR showed that the expression of ABC family transporters MDR1, MRP1 and ABCG2 were down-regulated by AGPS silencing. Other mechanisms involve cell cycle promotion and anti-apoptosis effects. Cell cycle analysis showed that AGPS silencing resulted in more cancer cells distributing to G0/G1 phase. We found that a key cell cycle modulator protein, β-catenin, was regulated by AGPS. Furthermore, western blotting and RT-PCR showed that the expression of Bcl-2 and survivin, which are involved in cell apoptosis, were modulated by AGPS as well. The mechanisms that AGPS mediated the expression of these proteins could be through the PEG2 pathway as described below.

AGPS is an enzyme that catalyzes the synthesis of lipids. It has been reported that AGPS affects the level of LPA and LPAe, which could promote the cancer. Here we showed that after AGPS silencing, level of LPA and LPAe were all down-regulated in HepG2/ADM and U87MG/DDP. To confirm that LPA and LPAe play a role in the drug resistance of our cancer cells, we determined the downstream pathway. LPA and LPAe could activate the LPA receptor and further activate the PI3K/AKT pathway. Our western blotting assay showed that phosphorylation of AKT was downregulated with AGPS silencing. Furthermore, the activity of PLD, a phospholipase that transduces the activity of LPA receptor, was down-regulated as well. It has been reported that PLD plays a role in the drug resistance of cancer. LPA receptor stimulates PLD activation and then accumulates intracellular PA (phosphatidate). Elevation of PA could activate several pathways such as SK-1/S1P, Raf/ERK, mTOR and PKC-ζ to promote cancer cell migration and survival.

Another lipid-related substrate that is affected by AGPS is PEG2. Our EIA assay showed that intracellular PEG2 was down-regulated after AGPS silencing. Moreover, when supplementing the AGPS silenced cancer cells with PEG2, the drug resistance reemerged, which further valid the role of PGE2 in AGPS mediated drug resistance. In cancer cells, PEG2 could bind to respective receptors to activate the downstream signaling pathway. There are 4 subtype of receptors, named EP1~4 (Greenhough et al., 2009). It has been reported that in human hepatocellular carcinoma cells EP1 receptor transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness (Han et al., 2006; Gillibert-Duplantier et al., 2007). U87MG has been reported to express both EP2 and EP4 (Kambe et al., 2008). To confirm the role of EP receptors in respective cancer cells, we used selective agonist to stimulate the respective receptor. Selective EP1 and EP3 agonist 19(R)-hydroxy PGE1 could restore drug resistance of HepG2/ADM, while selective EP2 agonist CAY10399 and selective EP4 agonist L-902,688 had similar effect on U87MG. PGE2 is the key in COX-2 regulating the express of multidrug resistance proteins in cancer cells (Woodward et al., 1993; Tani et al., 2001; Takaoka et al., 2008; Henderson et al., 2011; Hu et al., 2013; Kashiwagi et al., 2013). COX-2/PGE2 pathway could enhance the activation of the APC/β-catenin pathway to promote cancer cell cycle. Furthermore, PGE2 could regulate the expression of Bcl-2 and survivin to prevent cancer cells from apoptosis (Krysan et al., 2004). These literature reports are consistent with our observation mentioned above. PI3K/AKT is key pathway downstream EP receptors as well. Hence, the downregulation of Akt phosphorylation mentioned above might be the joint action of modulation to LPA receptor and EP receptor.

In summary, we found that AGPS overexpression could be a causation of chemotherapy agent resistance of cancer cells. AGPS silencing could lead to drug uptake and cell cycle arrest and apoptotic cell death. Our study suggested that lipid signaling pathways was critical for AGPS mediated drug resistance.

Acknowledgements

This study was supported by The Fund of the Health Bureau of Tianjin (2013KY17) and Tianjin Research Program of Application Foundation and Advanced Technology (14JCQNJC12000).

References

Aust S, Pils S, Polterauer S, et al (2013). Expression of Bcl-2 and the antiapoptotic BAG family proteins in ovarian cancer. *Appl Immunohistochem Mol Morphol*, **21**, 518-24.

Benjamin DI, Cozzo A, Ji X, et al (2013). Ether lipid generating enzyme AGPS alters the balance of structural and signaling lipids to fuel cancer pathogenicity. *Proc Natl Acad Sci USA*, **110**, 14912-7.

Blackburn J, Mansell JP (2012). The emerging role of lysophosphatidic acid (LPA) in skeletal biology. *Bone*, **50**, 756-62.

Brindley DN, Lin FT, Tigyi GJ (2013). Role of the autotaxin-lysophosphatidate axis in cancer resistance to chemotherapy and radiotherapy. *Biochim Biophys Acta*, **1831**, 74-85.

Castellone MD, Teramoto H, Williams BO, et al (2005). Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. *Science*, **310**, 1504-10.

Gillibert-Duplantier J, Neaud V, Blanc JF, et al (2007). Thrombin inhibits migration of human hepatic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol*, **293**, 128-36.

Greenhough A, Smartt HJ, Moore AE, et al (2009). The COX-

2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis*, **30**, 377-86.

Han C, Michalopoulos GK, Wu T (2006). Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J Cell Physiol*, **207**, 261-70.

Henderson MJ, Haber M, Porro A, et al (2011). ABCC multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux. *J Natl Cancer Inst*, **103**, 1236-51.

Hu S, Sun W, Wei W, et al (2013). Involvement of the prostaglandin E receptor EP2 in paeoniflorin-induced human hepatoma cell apoptosis. *Anticancer Drugs*, **24**, 140-9.

Hull MA, Ko SC, Hawcroft G (2004). Prostaglandin EP receptors: targets for treatment and prevention of colorectal cancer? *Mol Cancer Ther*, **3**, 1031-9.

Kambe A, Iguchi G, Moon Y, et al (2008). Regulation of EP4 expression via the Sp-1 transcription factor: inhibition of expression by anti-cancer agents. *Biochim Biophys Acta*, **1783**, 1211-9.

Kashiwagi E, Shiota M, Yokomizo A, et al (2013). Prostaglandin receptor EP3 mediates growth inhibitory effect of aspirin through androgen receptor and contributes to castration resistance in prostate cancer cells. *Endocr Relat Cancer*, **20**, 431-41.

Krysan K, Merchant FH, Zhu L, et al (2004). COX-2-dependent stabilization of survivin in non-small cell lung cancer. *FASEB J*, **18**, 206-8.

Liu F1, Wei WQ, Cormier RT, et al (2014). Association of single nucleotide polymorphisms in the prostaglandinendoperoxide synthase 2 (PTGS2) and phospholipase A2 Group IIA (PLA2G2A) genes with susceptibility to esophageal squamous cell carcinoma. *Asian Pac J Cancer Prev*, **15**, 1797-802.

Louisa M, Soediro TM, Suyatna FD (2014). *In vitro* modulation of P-glycoprotein, MRP-1 and BCRP Expression by Mangiferin in Doxorubicin-Treated MCF-7 Cells. *Asian Pac J Cancer Prev*, **15**, 1639-42.

Madan D, Ferguson CG, Lee WY, et al (2013). Non-invasive imaging of tumors by monitoring autotaxin activity using an enzyme-activated near-infrared fluorogenic substrate. *PLoS One*, **8**, 79065.

Marquardt JU, Andersen JB (2012). Next-generation sequencing: application in liver cancer-past, present and future? *Biology*, **1**, 383-94.

Mita AC, Mita MM, Nawrocki ST, et al (2008). Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res*, **14**, 5000-5.

Morandi A, Chiarugi P (2014). Metabolic implication of tumor:stroma crosstalk in breast cancer. *J Mol Med*, **92**, 117-26.

Nomura DK, Long JZ, Niessen S, et al (2010). Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. *Cell*, **140**, 49-61.

Pommier Y, Leo E, Zhang H, et al (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol*, **17**, 421-33.

Prabhu AV, Krycer JR, Brown AJ (2013). Overexpression of a key regulator of lipid homeostasis, Scap, promotes respiration in prostate cancer cells. *FEBS Lett*, **587**, 983-8.

Srivastava AN, Gupta A, Srivastava S, et al (2010). Cisplatin combination chemotherapy induces oxidative stress in advance non small cell lung cancer patients. *Asian Pac J Cancer Prev*, **11**, 465-71.

Takaoka K, Kishimoto H, Segawa E, et al (2008). In vitro susceptibility to anticancer agents of the human KB carcinoma cell line transfected with COX-2 cDNA. *Oncol Rep*, **20**, 645-9.

Tani K, Naganawa A, Ishida A, et al (2001). Design and synthesis of a highly selective EP2-receptor agonist. *Bioorg Med Chem Lett*, **11**, 2025-8.

Vander Heiden MG, Cantley LC, Thompson CB (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, **324**, 1029-33.

Weinstein D, Sarfstein R, Laron Z, et al (2014). Insulin receptor compensates for IGF1R inhibition and directly induces mitogenic activity in prostate cancer cells. *Endocr Connect*, **3**, 24-35.

Woodward DF, Protzman CE, Krauss AH, et al (1993). Identification of 19 (R)-OH prostaglandin E2 as a selective prostanoid EP2-receptor agonist. *Prostaglandins*, **46**, 371-83.