Cancer Therapy: Preclinical

The PDGFRβ–AKT Pathway Contributes to CDDP-Acquired Resistance in Testicular Germ Cell Tumors

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Abstract

Purpose: We examined whether PI3K–AKT or extracellular signal–regulated kinase (ERK) signaling pathways could play a role in the development of cisplatin (CDDP) resistance in testicular germ cell tumor (TGT) cells.

Experimental Design: We compared AKT and ERK activation levels in CDDP-sensitive testicular tumor cells and in their corresponding CDDP-resistant–derived cells. We also analyzed these pathways in orthotopic testicular tumors and human patient samples.

Results: Our results indicated that there was overactivation of AKT in CDDP-resistant cells compared with sensitive cells, but no effect on activated ERK levels. We observed an increase in mRNA and protein levels for platelet-derived growth factor (PDGF) receptor β and PDGF-B ligand. These were responsible for AKT overactivation in CDDP-resistant cells. When PDGFRβ levels were decreased by short hairpin RNA (shRNA) treatment or its activation was blocked by pazopanib, CDDP-resistant cells behaved like sensitive cells. Moreover, CDDP-resistant cells were more sensitive to incubation with PDGFRβ inhibitors such as pazopanib or sunitinib than sensitive cells, a finding consistent with these cells being dependent on this signaling pathway. We also found overexpression of PDGFRβ and pAKT in CDDP-resistant choriocarcinoma orthotopic tumor versus their CDDP-sensitive counterparts. Finally, we found high PDGFRβ levels in human testicular tumors, and overexpression in CDDP-resistant testicular choriocarcinomas compared with the CDDP-sensitive and nontreated tumors.

Conclusions: The PDGFRβ–AKT pathway plays a critical role in the development of CDDP resistance in testicular tumoral cells. *Clin Cancer Res; 20(3); 658–67. ©2013 AACR.*

Introduction

Cisplatin (CDDP) treatment is the first-line chemotherapy drug used in patients affected by various types of tumors, including metastatic testicular and ovarian tumors. Testicular germ cell tumors (TGT) are the main cause of cancer in men between 15 and 35 years of age (1). These tumors have excellent cure rates, with more than 90% of patients achieving a complete response to CDDP-based treatment, either alone or combined with surgery. Metastatic TGT has the highest cure rate, with a survival rate of 80%. However, a proportion of patients relapse or develop refractory diseases after CDDP treatment. For these patients, any new treatment would be considered an alternative treatment and, therefore, poor prognosis is often the result (2).

Resistance to chemotherapy is one of the major causes of death in patients with cancer. The cellular mechanisms for CDDP resistance involve a decrease in drug uptake or an increase in its expulsion from tumor cells, CDDP inactivation due to binding to sulfur-rich proteins, alterations in the capacity of DNA repair or a lack of detection of DNA damage, and a failure to enter cell death after DNA damage (3–5). The latter mechanism may result from different alterations in tumors, including induction of antiapoptotic factors or decrease in proapoptotic factors, but it may also be due to alterations in signal transduction pathways that normally regulate apoptosis, survival, and proliferation (5). Therefore, tumors that present wild-type p53, a key protein for inducing apoptosis after DNA damage, respond well to CDDP compared with those tumors that present p53-inactivating mutations. This is seen in TGTs that are particularly sensitive to CDDP because they are one of the few cancers

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Translational Relevance

Our results from testicular germ cell tumor (TGT) cells in orthotopic testicular tumors and human patient samples indicate an increase in platelet-derived growth factor receptor β (PDGFRβ)–AKT pathway activity as a new mechanism for developing cisplatin (CDDP)-acquired resistance in testicular cancer cells. These results reinforce the value of reagents such as pazopanib or sunitinib, which combine antiangiogenic and antitumoral effects, as resensitizing therapies for the subgroups of patients with poor-prognosis CDDP-resistant or refractory testicular cancer.

in which p53 is rarely inactivated (6). Activation of p38 MAPK, a kinase involved in apoptosis induction, is also altered in CDDP-resistant lung cells (7, 8). Prosurvival signals, such as PI3K–AKT or extracellular signal–regulated kinases (ERK), are overstimulated in some CDDP-resistant cells, such as lung or ovarian cell lines (3). However, Fung and colleagues described that blocking MAP–ERK kinase (MEK)/ERK led to cellular protection against CDDP-induced apoptosis in TGT cell lines (9). Our study examines the possible contribution of some of these signaling pathways to the acquisition of CDDP resistance in human testicular tumor cells.

Materials and Methods

Chemical compounds

Pazopanib (Votrient) and lapatinib (Tyverb) were kindly provided by GlaxoSmithKline. Sunitinib was kindly provided by Pfizer. Gefitinib (Iressa) was kindly provided by AstraZeneca, Ly2109761 was kindly provided by Eli Lilly, and Ly294002 and UO126 were obtained from Calbiochem. All the above compounds were dissolved in dimethyl sulfoxide (DMSO). CDDP was provided by Pfizer and was diluted in sterile serum. Platelet-derived growth factor (PDGF)-BB and fibroblast growth factor (FGF)-2 were provided by R&D, and epidermal growth factor (EGF) was provided by Sigma.

Cell culture

The human teratocarcinoma cell line SuSa, or SuSaS ("S" for sensitive to CDDP; ref. 10), and GCT27, or GCT27S cells (from embryonic carcinoma origin; ref. 11) were kindly provided by Dr. Yong-Jie Lu (Barts Cancer Institute, Queen Mary University of London, London, UK), as well as their respective CDDP-resistant ("R")–derived cell lines (SuSaR and GCT27R; refs. 12, 13). Both cell lines were authenticated performing the short tandem repeat profile in November 2012 by the Authentication Services of the Health Protection Agency Culture Collections, United Kingdom. SuSa cells were cultivated in RPMI media (Gibco) supplemented with 20% fetal calf serum (FCS), whereas GCT27 cells were cultivated in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FCS. Then, 50 U/mL of penicillin, 50 μg/mL of streptomycin sulfate, and 2 mmol/L of

glutamine were added to all cell culture media. All cells were grown at $37°C$ in a humidified atmosphere with 5% CO_2.

Tumor samples

We used two orthotopic TGTs models for our studies: a choriocarcinoma (TGT38) and its cisplatin-resistant counterpart (TGT38R), both of which have been described in Castillo-Avila and colleagues (14). All animal studies were approved by the local committee (IDIBELL, Barcelona, Spain) for animal care.

shPDGFRβ lentivirus transduction

Sigma MISSION pLKO.1 lentiviral vectors were used to permanently silence PDGFRβ expression in GCT27R cells. The negative vector without short hairpin RNA (shRNA) sequence (Sigma MISSION pLKO.1-pure empty vector) was used as negative control.

As these vectors express puromycin resistance, cell lines expressing lentiviral vectors were established in constant culture of puromycin-containing media (2 μg/mL). To confirm the correct PDGFRβ silencing by lentiviral vectors, protein samples from the cell lines were collected and processed in a Western blot analysis.

Cell viability assay

Cell viability was determined by measuring the metabolic activity using the MTT assay (Sigma Chemical). Cells were plated in 96-well plates, 1,000 cells per well, in quadruplicate, and allowed to grow for 24 hours. SuSaS or R and GCT27S or R cells were then treated with 0 to 10^{-2} mg/mL of CDDP for 4 days. DMSO was used as a negative control when a different drug was added to the CDDP dose curve in the GCT27S or R cells. When appropriate, a constant dose of pazopanib (0.5 μg/mL) or Ly294002 (4 μmol/L) was added. Subsequently, when treatment was finished, 10 μmol/L MTT was added to each well and incubated for an additional 4 hours. The blue MTT formazan precipitate was dissolved in DMSO and the optical density was measured (absorbance at 570 nm) on a multiwell plate reader. The pazopanib (0–10 μg/mL) curve was measured in the same manner.

Western blot analysis

Samples from cells or tumors were lysed using radio-immunoprecipitation assay lysis buffer. Protein lysates were processed as previously described (14). Antibodies used in this study are described in the Supplementary Materials and Methods.

ELISA

Human PDGF-BB protein levels were measured using the ELISA Kit from RayBio, following the manufacturer's instructions. When appropriate, PDGF-BB was quantified on cell-cultured media without FBS for 16 hours.

Quantitative real-time PCR

Total RNA from tumors or cells was extracted using the RNAeasy Plus Mini Kit (Qiagen). cDNA was obtained after a
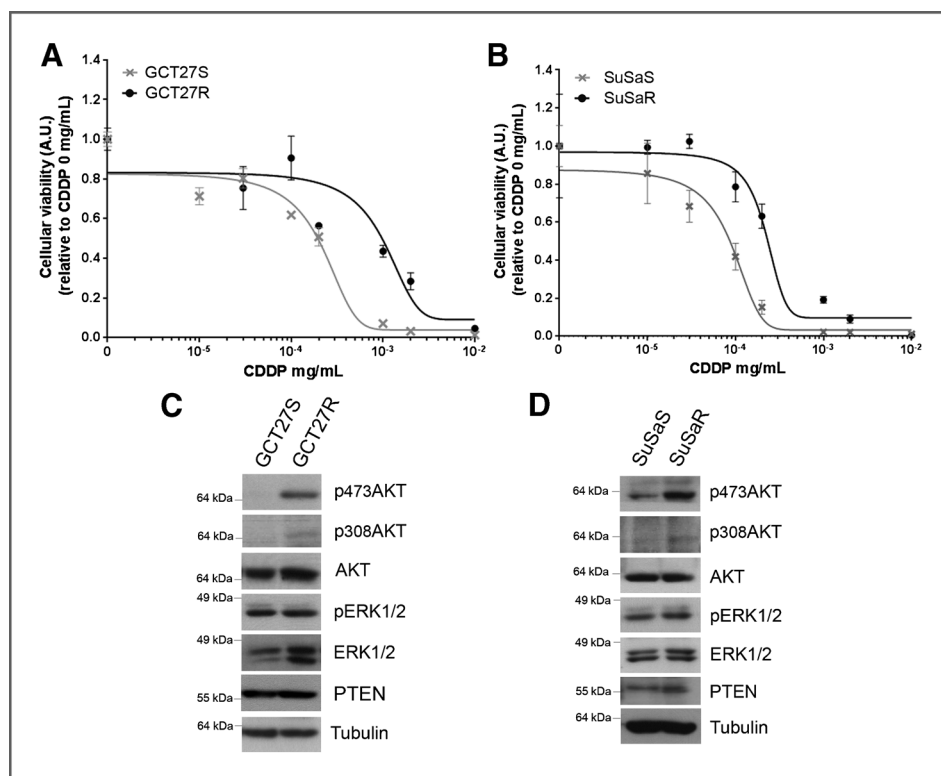
Figure 1. CDDP-resistant cells present high levels of phosphoAKT. A and B, parental sensitive, S, or resistant, R, GCT27 (A) or SuSa (B) testicular tumor cells incubated for 4 days in the absence or presence of a range of CDDP concentrations. Cell viability measured by MTT assay. Results are expressed relative to 0 mg/mL CDDP. Each data point represents the mean and SD of four independent determinations. C and D, expression of phosphorylated Ser473 AKT (p473AKT), phosphorylated Thr308 AKT (p308AKT), total AKT, phosphorylated ERK1/2 (pERK1/2), total ERK1/2, PTEN and tubulin in GCT27S and R cells (C) and in SuSaS and R cells (D) analyzed by Western blot analysis. A blot representative of five independent experiments is shown.

reverse transcription reaction (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Real-time PCR of cDNA obtained from tumors or cell lines was carried out as previously described (14). The human-specific primers used are described in the Supplementary Materials and Methods. Results are presented with the values $2^{(-\Delta\Delta C_t)}$ relative to the corresponding sensitive phenotype.

Immunohistochemistry in human samples and scoring

PDGFRβ expression was analyzed on samples representative of 71 patients diagnosed with nonseminomatous TGTs (NSTGT), 52 of whom were treated with CDDP at the Institut Català d'Oncologia (Barcelona, Spain) between 1989 and 2004. Eighteen patients were considered to be CDDP resistant, defined according to whether progression or relapse occurred, despite adequate first-line chemotherapy treatment. Patients with mature teratoma were not considered for analysis.

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in downgraded alcohols and distilled water. Antigen retrieval was carried out under high-pressure conditions for 2 minutes in citrate buffer, pH 6. Samples were then blocked with 1 of 50 horse serum for 30 minutes and incubated with 1 of 20 anti-PDGFRβ antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C. Sections were incubated with the specific secondary rabbit antibody EnVisionFLex (Dako), followed by the EnVisionFLex DAB developing system (Dako). Samples were counterstained with hematoxylin and visualized by light microscopy.

The intensity of PDGFRβ stain was scored using a grading scale, defined as follows: no detectable signal (0 points), low-intensity signal (1 point), moderate-intensity signal (2 points), or high-intensity signal (3 points). Labeling frequency was scored as the percentage of positive tumoral cells. The multiplicative index of intensity and labeling frequency was used in our analysis, as previously described (15).

Statistical analyses

Statistical analysis was carried out using SPSS (SPSS for Windows 13.0, SPSS, Inc.). Statistical significance of differences between groups was determined using the Mann–Whitney U test, statistical significance being concluded for values of $P < 0.05$ (*) or $P < 0.01$ (**) relative to the GCT27S value in all experiments.

Dose–response curves and IC_{50} statistics were generated using GraphPad Prism 6 (GraphPad Software, Inc.).

Results

To explore the mechanisms involved in CDDP resistance in depth, we used already existing CDDP-resistant–derived testicular cancer cells. Two cells lines were used: GCT27 (named GCT27S when referring to those with increased sensitivity to CDDP; ref. 11) and SuSa (SuSaS, for the sensitive line; ref. 10), and their resistant CDDP-derived cell lines GCT27R (12) and SuSaR (13). We confirmed resistance by measuring the cell viability of these cell lines over a range of CDDP concentrations (Fig. 1A and B). As observed, resistant-derived cells presented IC_{50}s in CDDP of
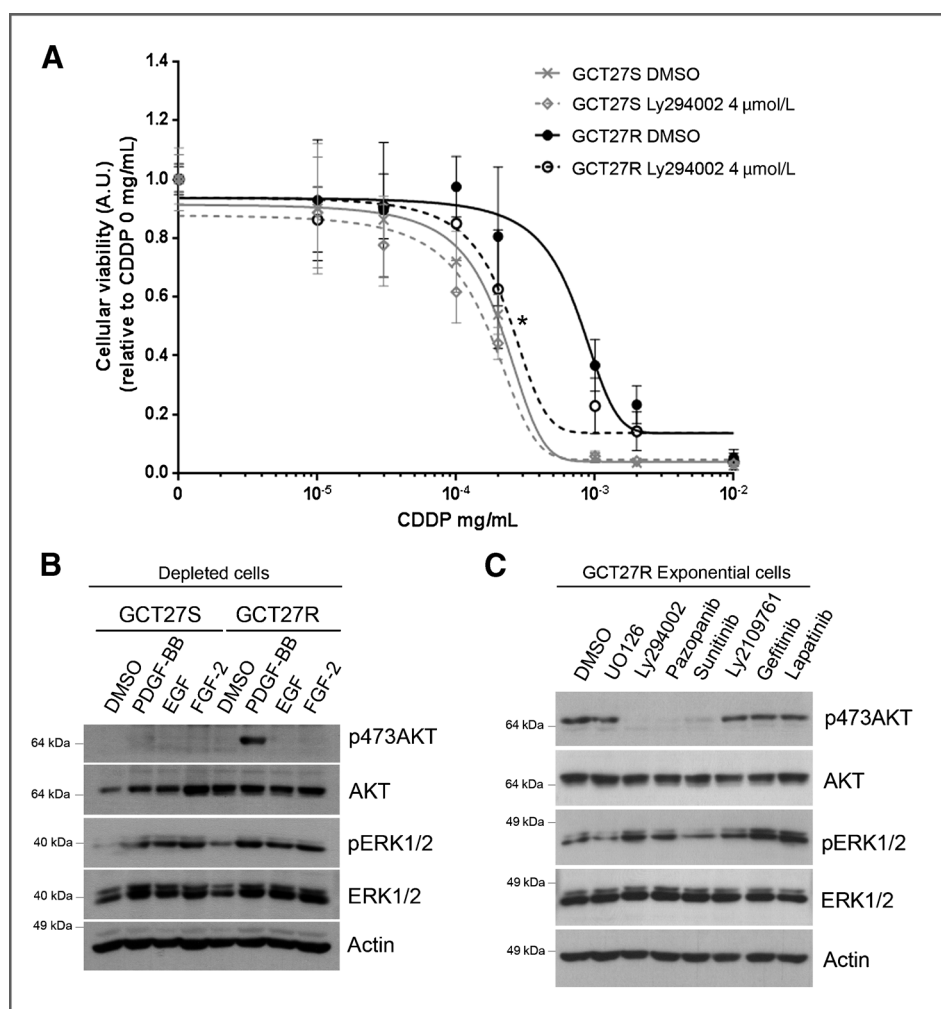
Figure 2. Blocking PI3K activity restores CDDP sensitivity and phosphoAKT levels depend on PDGF receptors. A, GCT27S and R cells incubated for 4 days in the presence of the indicated concentrations of CDDP and in the absence (DMSO), or presence of 4 μmol/L Ly294002 PI3K inhibitor. Cell viability was measured by MTT assay. Results are expressed as relative to CDDP 0 mg/mL dose condition. Each data point represents the mean and SD of four independent determinations. Differences between GCT27R and GCT27R + Ly294002 were considered statistically significant when $P < 0.05$ (*; Mann–Whitney U test). B, growth factor–depleted GCT27S or GCT27R cells stimulated for 15 minutes in the absence (DMSO) or presence of 20 ng/mL PDGF-BB, 20 ng/mL EGF, or 20 ng/mL FGF-2. Cells were lysed, and p473AKT, total AKT, pERK1/2, total ERK1/2, and actin expression analyzed by Western blot analysis. A blot representative of two independent experiments is shown. C, exponential GCT27R cells incubated for 3 hours in the absence (DMSO) or presence of 10 μmol/L U0126 (MEK inhibitor), 15 μmol/L Ly294002 (PI3K inhibitor), 5 μg/mL pazopanib (PDGFRs and c-KIT inhibitor), 10 μmol/L sunitinib (PDGFRs and c-KIT inhibitor), 2 μmol/L Ly2109761 (TGFβRII inhibitor), 10 μmol/L gefitinib (ErbB1 inhibitor), or 10 μmol/L lapatinib (ErbB1 and ErbB2 inhibitor). Cells lysed and p473AKT, total AKT, pERK1/2, total ERK1/2, and actin expression analyzed by Western blot analysis. A blot representative of two independent experiments is shown.

2.1×10^{-4} mg/mL (SD, 5.7×10^{-6}) in SuSaR, compared with 0.8×10^{-4} mg/mL (SD, 4.6×10^{-6}) in SuSaS normal cells, and 8.5×10^{-4} mg/mL (SD, 1.4×10^{-4}) in GCT27R, compared with 1.9×10^{-4} mg/mL (SD, 1.3×10^{-5}) in GCT27S normal cells. In both cases, the difference was found to be significant using the Mann–Whitney U test.

Next, different signal transduction pathways involved in cell survival and CDDP resistance, such as PI3K–AKT or ERKs (5), were analyzed. No differences in ERK activation levels between normal and CDDP-resistant cell lines (Fig. 1C and D) were detected. In contrast, phosphoAKT levels (phosphorylated in serine 473 or in threonine 308) were clearly higher in both CDDP-resistant cells than in normal

cells. We detected no differences in total AKT protein levels between normal and CDDP-resistant cell lines (Fig. 1C and D).

To assess the importance of overstimulation of PI3K–AKT to CDDP sensitivity, we incubated GCT27S or R cells over a range of CDDP concentrations and in the presence or absence of the pan-PI3K inhibitor, Ly294002 (4 μmol/L; Fig. 2A). GCT27R cells recovered their sensitivity to CDDP when PI3K activity was inhibited by 4 μmol/L Ly294002 incubation (7.2×10^{-4} mg/mL SD, 1.2×10^{-4} for GCT27R, 2.2×10^{-4} mg/mL SD, 6.0×10^{-5} for GCT27R with Ly294002, 2.0×10^{-4} mg/mL SD, 2.0×10^{-5} for GCT27S, and 1.5×10^{-4} mg/mL SD, 1.8×10^{-5} for GCT27S with

Ly294002). These results suggest PI3K dependence on CDDP resistance in GCT27R cells.

Overstimulation of PI3K/AKT activity could arise from intrinsic activating mutations in the PI3K protein, altered function of the antagonist phosphatase of PI3K, PTEN, or upstream overstimulation due to one of the multiple receptors that signal through the PI3K/AKT signaling pathway. To identify the mechanisms involved in overstimulating the PI3K/AKT pathway in CDDP-resistant cell lines, we first measured the levels of PTEN in GCT27 and SuSa cell lines. Levels of this phosphatase were comparable in the CDDP-sensitive and CDDP-resistant cells (Fig. 1C and D), ruling out the possibility of a decrease in PTEN levels as being the molecular target of CDDP resistance. Moreover, levels of phosphoAKT decreased after the depletion of growth factors (Fig. 2B, DMSO lane in GCT27R cells), indicating that the PI3K pathway was not overstimulated by an activating mutation. We then proceeded to treat exponential CDDP-resistant GCT27 cells with the following inhibitors of different growth factor receptors: sunitinib and pazopanib against PDGF receptors, VEGF receptors and the stem cell factor receptor, c-KIT; gefitinib against ErbB1 receptor; lapatinib against ErbB1 and ErbB2 receptors; Ly2109761 against TGFβRII; and the U0126 inhibitor of MEK-1 and Ly294002 inhibitor of PI3K activity. The results indicated not only that phosphoAKT levels were reduced by Ly294002 inhibitor, as expected, but also that PDGFRs, VEGFR, and c-KIT inhibitors (sunitinib and pazopanib) blocked AKT activity at a similar level (Fig. 2C). In contrast, no effect of ErbBs or TGF-β inhibitors was observed in these cells. We also observed a slight decrease in ERK1/2 activity with both the MEK-1 inhibitor and sunitinib. To confirm these results, depleted parental or CDDP-resistant GCT27 cells were incubated with each of the growth factors FGF-2, EGF, and PDGF-BB, which are known activators of PI3K. As illustrated in Fig. 2B, all these growth factors stimulated ERK1/2 at similar levels in normal and CDDP-resistant GCT27 cells. In contrast, AKT was activated only by PDGF-BB and, more importantly in GCT27 CDDP-resistant cells. This AKT stimulation by PDGF-BB was impeded by pazopanib treatment (data not shown).

Our results suggest a different capacity of stimulation by PDGF receptors between CDDP-resistant and normal (parental) cells. To confirm whether this was the case, we measured mRNA levels of PDGFRα and PDGFRβ in GCT27S and GCT27R cells. As shown in Fig. 3A, there was a 3-fold decrease in PDGFRα levels in resistant cells compared with CDDP-sensitive cells, rather than an increase. In contrast, mRNA PDGFRβ levels were 2.5-fold as high in GCT27R cells compared with normal cells. The results were confirmed by measuring PDGFR protein levels in these cell lines. In CDDP-resistant GCT27 cells, we found a 4-fold increase in total levels (Fig. 3B), and an increase in the amount of PDGFRβ in the plasmatic membrane (Supplementary Fig. S1). However, no differences were observed in PDGFRα protein levels (Fig. 3B). We also measured mRNA and protein levels for the PDGFRs ligands PDGF-A and PDGF-B. As shown in Fig.

3C, mRNA levels for this latter growth factor were 6.5-fold those in resistant cells, whereas no significant differences were detected for the PDGF-A growth factor. Protein PDGF-BB levels measured by ELISA were also higher in cell lysates (2.9-fold) and cell-culture media (6.9-fold) for resistant compared with cisplatin-sensitive cells (Fig. 3D). Similar results were obtained in SuSa cells (Supplementary Fig. S2). Together, these results indicate that CDDP-resistance in testicular tumor cells was associated with an increase in the activation of the PDGF-B/PDGFRβ/PI3K–AKT pathway.

To confirm that AKT activation by PDGFRβ caused CDDP resistance by modifying the potential viability of these cells, we evaluated the effect of the PDGFRs inhibitor pazopanib (0.5 μg/mL) on the CDDP dose–response curve. Pazopanib treatment in normal GCT27 cells did not significantly affect the CDDP IC_{50} (Fig. 4A). In contrast, when pazopanib was added to CDDP-resistant cells, they recovered sensitivity to CDDP, and an IC_{50} value similar to sensitive cells was noted (7.2×10^{-4} mg/mL SD, 3.4×10^{-4} for GCT27R, 2.81×10^{-4} mg/mL SD, 1.4×10^{-4} for GCT27S, and 2.56×10^{-4} mg/mL SD, 1.2×10^{-4} for GCT27R with pazopanib). IC_{50} values for GCT27R cells were significantly different in the presence or absence of pazopanib, but not between GCT27R pazopanib and GCT27S, with or without the inhibitor. These results indicated that blocking PDGFRs by pazopanib treatment reverted GCT27R cells to CDDP sensitivity.

Next, to assess whether PDGFRβ was sufficient to explain CDDP resistance, we inhibited its expression in GCT27R cells. By transducing lentiviral vectors expressing either PDGFRβ–shRNAs or a negative control using an empty vector (EV), GCT27R-EV cells as controls or GCT27R-shPβ cells were generated. We used four independent shRNA vectors but only one of them (shPβ4) partially reduced PDGFRβ expression protein without having effects on PDGFRα (Fig. 4B). This partial blocking of PDGFRβ expression also partially blocked phosphoAKT levels (Fig. 4B). As shown in Fig. 4C, decreased PDGFRβ levels in GCT27R–shPβ4 cells caused a partial recovery in CDDP sensitivity, indicating that inhibition of this receptor affected CDDP resistance. Although the difference in IC_{50} values between GCT27R and GCT27R–shPβ4 was not statistically significant, we observed a significant decrease in the sensitivity of GCT27R–shPβ4 compared with the resistant cell line when 10^{-4} mg/mL CDDP was used. Moreover, at this CDDP concentration, the sensitivity of these three cell lines to the drug was linearly proportional to the phosphoAKT levels detected by Western blot analysis (Fig. 4B and Supplementary Fig. S3).

We also analyzed PDGF dependence in CDDP-resistant cells compared with normal cells. To this end, we treated GCT27S or R cells with a range of concentrations of PDGFRs inhibitors (pazopanib, Fig. 4D, or sunitinib, data not shown), then studied cell viability. We observed that CDDP-resistant cells were more sensitive to these inhibitors, with levels of cell viability inhibition around 90%, compared with 70% in normal cells (Fig. 4D). These results indicated that overexpression of PDGF-B and PDGFRβ in

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Figure 3. PDGFRβ and its ligand PDGF-BB are overexpressed in GCT27R cells. A, mRNA levels of human PDGFRα and β analyzed by quantitative real-time PCR in GCT27S and R cells. Results are expressed as the mean and SD of mRNA expression in five GCT27R samples relative to mRNA expression levels in 4 GCT27S samples. Differences were considered statistically significant when *, $P < 0.05$ and **, $P < 0.01$ in a Mann–Whitney U test. B, PDGFRα, PDGFRβ, and actin protein levels analyzed by Western blot analysis in GCT27S and R cells. A blot representative of four independent experiments is shown. Densitometric quantification of PDGFRβ and PDGFRα from Western blots shown as the mean and SD of four independent samples, represented as arbitrary units relative to GCT27S cell group mean. The difference was considered statistically significant when $P < 0.05$ (*; Mann–Whitney U test). C, mRNA levels in human PDGF-A and -B analyzed by quantitative real-time PCR in GCT27S and R cells. Results are expressed as the mean and SD of mRNA expression in five GCT27R samples relative to mRNA expression levels in four GCT27S samples. D, human PDGF-BB protein levels measured by ELISA in cell lysates (pg of PDGF-BB/g of protein) and in cell culture media (pg of PDGF-BB/10^6 cells). Results are expressed as the mean and SD of three independent samples for each cell line.
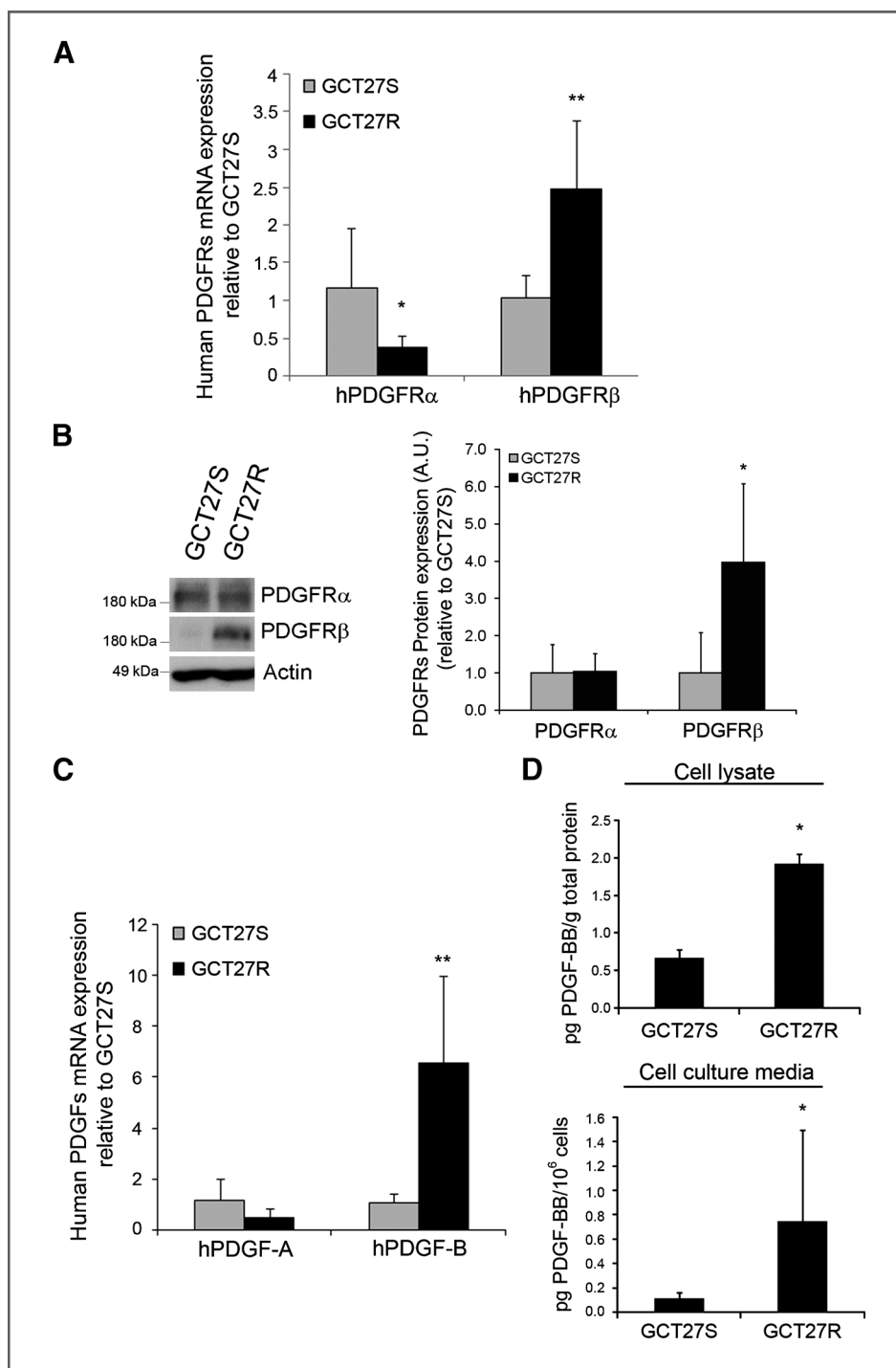
CDDP-resistant cells increases the degree of addiction of these cells to follow this pathway.

To determine whether these *in vitro* associations between acquired cisplatin resistance and activation of the PDGFR pathway were also present in tumors, we analyzed PDGFR expression in an orthotopic model of testicular germ cell choriocarcinoma tumor (TGT38) and its CDDP-resistant counterpart (TGT38R). These resistant tumors were gener-

ated in our laboratory using a mouse model bearing a TGT38 tumor subjected to prolonged CDDP treatment (14). We did not detect differences in mRNA or protein levels for the PDGFRs ligand PDGF-BB in this model (Supplementary Fig. S4). mRNA levels for PDGFRα were found to be equal in TGT38 and TGT38R tumors (Fig. 5A). In contrast, a 2.2-fold increase in PDGFRβ mRNA levels was found in resistant tumors relative to the CDDP-sensitive
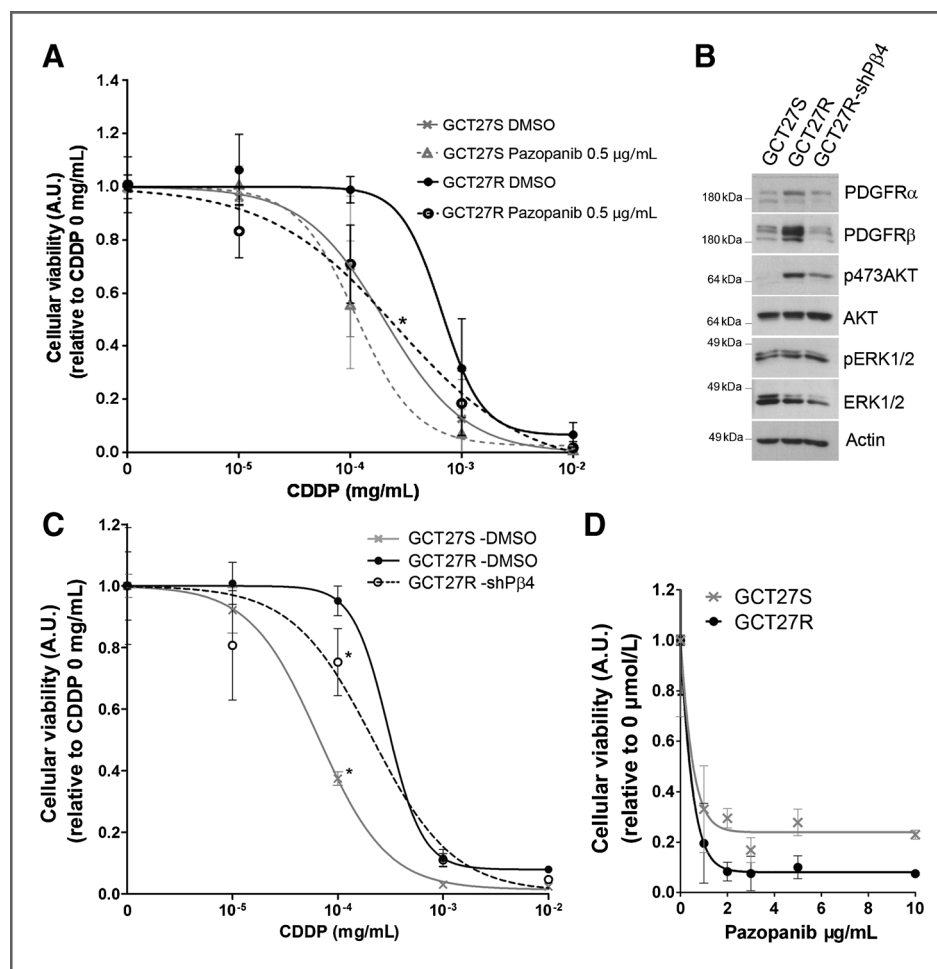
Figure 4. Blocking of PDGFRβ activity reverts CDDP resistance. A, GCT27S and GCT27R testicular tumor cells incubated for 4 days in a range of concentrations of CDDP in the absence or presence of 0.5 µg/mL pazopanib. Cell viability measured by MTT assay. Results are expressed relative to those for 0 mg/mL of CDDP condition. Each data point represents the mean and SD of six determinations measured in duplicate. Differences between GCT27R and GCT27R + pazopanib were considered statistically significant when $P < 0.05$ (*; Mann–Whitney U test). B, PDGFRα, PDGFRβ, p473AKT, total AKT, pERK1/2, total ERK1/2, and actin protein levels analyzed by Western blot analysis in GCT27S, GCT27R, and GCT27R-shPβ4 cell lysates. C, GCT27S, GCT27R, and GCT27R–shPβ4 cells incubated for 4 days, in the absence or presence of a range of CDDP concentrations. Cell viability was measured by MTT assay. Results are expressed relative to CDDP, 0 mg/mL dose condition. Each data point represents the mean and SD of four independent determinations. D, GCT27S or R testicular tumor cells incubated for 4 days in the absence or presence of a range of concentrations of pazopanib. Cell viability measured by MTT assay. Results are expressed relative to CDDP, 0 mg/mL dose condition. Each data point represents the mean and SD of three determinations.

tumors. This result was confirmed by Western blot analysis of PDGFRβ protein levels. In addition, a 3-fold increase in PDGFRβ expression levels was observed in the CDDP-resistant tumor along with a 2-fold increase in pAKT levels (Fig. 5B), confirming the relevance of the activation of the PDGFRβ–pAKT pathway in conferring cisplatin resistance.

Finally, we analyzed PDGFRβ expression in samples from TGTs patients gathered together in the tissue microarray (TMA). To achieve this, immunohistochemistry (IHC) for this receptor was performed on samples from patients affected by different non seminomatous TGTs (NSTGTs), which have different histologic components and responses to the CDDP treatment. The intensity of PDGFRβ staining was characterized as undetectable, low, moderate, or high, as illustrated in Fig. 5C. The analysis of these samples indicated

that 75% of the patients with NSTGT expressed moderate or high levels of PDGFRβ, although there was no difference between the CDDP-sensitive and CDDP-resistant phenotypes. However, when we focused our analysis on the patients who presented the choriocarcinoma histologic component, as a pure or mixed testicular tumor, we observed that 80% of the CDDP-sensitive patients expressed moderate or high levels of PDGFRβ. In contrast, in the CDDP-resistant patient group, 100% of patients expressed moderate or high levels of this receptor. The multiplicative index considering intensity and the percentage of positive cells revealed no differences between CDDP-treated and -untreated patients or between sensitive and resistant patients. In contrast, patients with resistant choriocarcinomas had a higher index than sensitive choriocarcinomas, and a significantly higher index than those untreated patients (Fig. 5D).
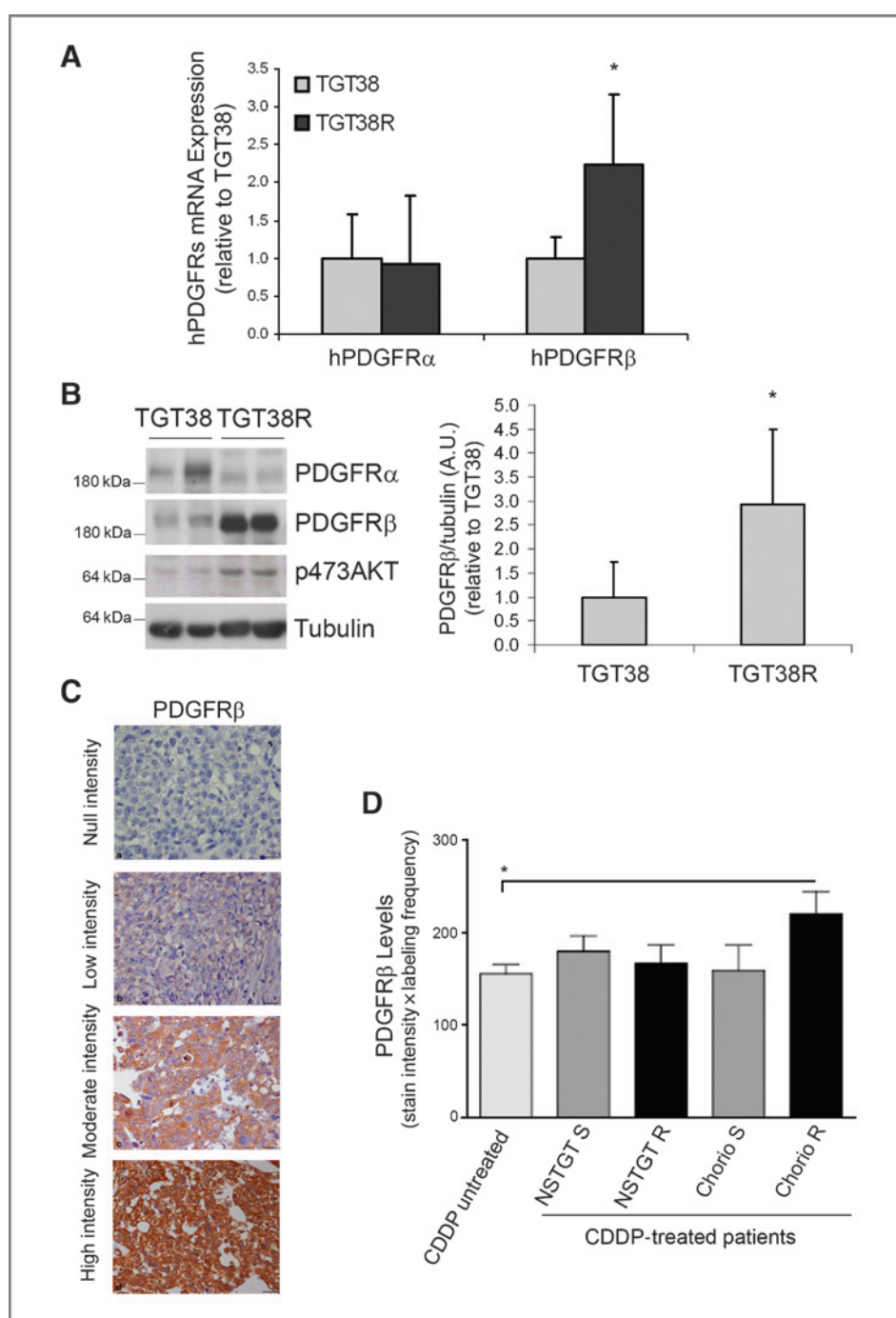
Figure 5. PDGFRβ overexpressed in CDDP-resistant orthotopic TGTs and in human choriocarcinoma tumors. A, mRNA levels of human PDGFRα and β analyzed by quantitative real-time PCR from samples of orthotopic human choriocarcinoma tumors CDDP-sensitive (TGT38) or its CDDP-resistant version (TGT38R). Results are expressed as the mean and SD of mRNA expression in four independent TGT38R tumors relative to mRNA expression levels in four independent TGT38 tumors. Differences were considered statistically significant when $P < 0.05$ in a Mann–Whitney U test. B, expression of human protein PDGFRα and β receptors, pAKT (p473AKT), and tubulin analyzed by Western blot analysis in two samples from TGT38 choriocarcinoma orthotopic TGTs (lanes 1 and 2) and two from CDDP-resistant TGT38R tumors (lanes 3 and 4). Densitometric quantification of PDGFRβ from Western blots shown as the mean and SD of five independent TGT38 tumors and four independent TGT38R tumors, represented as arbitrary units relative to the TGT38 group mean. Differences were considered statistically significant when $P < 0.05$ in a Mann–Whitney U test. C, examples representative of no staining (a), and low (b), moderate (c), and high (d) levels of positive PDGFRβ immunostaining in NSTGT patients samples. D, quantification of PDGFRβ levels (using the multiplicative index of the intensity of the stain and the labeling frequency) in tumor tissue sections from CDDP-untreated patients, patients with CDDP-sensitive or CDDP-resistant general NSTGT, and patients with CDDP-sensitive or CDDP-resistant choriocarcinoma tumor. Data analyzed from 19 patients with good-prognosis nonseminomatous germ cell tumor, 34 patients with CDDP-sensitive and 18 patients with CDDP-resistant nonseminomatous germ cell tumor, and 10 patients with CDDP-sensitive and 6 patients with CDDP-resistant choriocarcinoma tumor. Results are expressed as the mean and SD. The difference between non-CDDP-treated patients and CDDP-resistant choriocarcinoma patients was considered statistically significant when $P < 0.05$ (*; Mann–Whitney U test).

Discussion

This study has shown that an increase in activity of the PDGFRβ–AKT pathway is a hitherto unidentified mechanism of CDDP resistance in testicular cancer cells. Activation of PI3K as a mechanism of CDDP resistance has been previously described. For example, the increased activation of pAKT in human lung tumor tissues is inversely correlated with CDDP sensitivity in their primary derived culture counterpart (16). Moreover, a high level of PI3K activity in patients with CDDP-resistant non–small cell lung cancer through overexpression of ErbB2 receptor (17), or through EGFR/Her3 in glioma and ovarian cancer cells has also been described previously (18). Another mechanism involved in CDDP resistance is the downregulation of PTEN by induction of microRNAs (miRNA), such as miR-214 (19) and miR-93 (20) in ovarian cells, or miR-221 in osteosarcoma cells (21). In cisplatin-resistant testicular cancer cells, AKT phosphorylates p21 and induces its cytoplasmic accumulation, protecting cells from cisplatin-induced apoptosis (22). For PDGF factors, an autocrine loop involving PDGF-BB induction in lung cancer stem cells resistant to CDDP (23), in glioma CDDP-resistant cell lines (24), and in tumoral hepatic progenitor cells resistant to CDDP under hypoxia (25) has been described previously. These last two studies also describe PDGF-BB–induced AKT overactivation in resistant cells and its importance to the resistant phenotype. Moreover, stimulation of PI3K by PDGF renders human ovarian carcinoma cells resistant to paclitaxel (26). However, to our knowledge, this is one of the first times that not only an autocrine PDGF loop, but also regulation of PDGFR expression have been implicated in CDDP resistance. The molecular mechanisms contributing to this response require further investigation. We did not detect any differences in the methylation of the PDGFRβ promoter (data not shown), a classic mechanism known to induce or repress gene expression. Neither were there any differences in the regulation of PDGF-BB–PDGFRβ–pAKT activation by the TGF-β pathway (Fig. 2), as described in glioma models (27). Nevertheless, other mechanisms could be involved, such as miRNA regulation or transcription factor activity. All of these mechanisms are regulated by CDDP in various tumor cell types (15, 28).

Our results indicate that sensitivity to CDDP depends on the phosphoAKT levels in the cells. In fact, in testicular tumor cells, we observed a perfect correlation between phosphoAKT levels and cell viability upon CDDP treatment (Supplementary Fig. S3). Moreover, resistant cells recovered their sensitivity to CDDP when levels of phosphoAKT were reduced by Ly294002. Thus, phosphoAKT seems to be a key factor for CDDP resistance in testicular tumor cells, the signaling pathway being regulated by PDGFRβ. The IHC results from our tissue microarray (TMA) assay revealed no correlation between PDGFRβ expression and resistant or refractory TGTs. Only in tumors with the choriocarcinoma component, the least common but most aggressive NSTGT component, the resistance to CDDP was correlated with

higher PDGFRβ expression. This is the same histologic component as in the orthotopic tumors in which we found PDGFRβ overexpression in the CDDP-resistant phenotype. However, several signaling pathways can induce stimulation of phosphoAKT levels. The PDGFRβ pathway was identified in testicular tumor cells and choriocarcinoma tumors, but other signaling pathways (such as PDGFRα, c-KIT, and ErbBs) could contribute to AKT activation in other testicular tumors subtypes and may explain the lack of a close correlation between PDGFRβ expression and CDDP resistance in our TMA results. Moreover, despite our results about phosphoAKT, it is certain that patients with CDDP-resistant tumors have more than one mechanism of resistance. This adds to the complexity of interpreting the analytical results from patient samples (5).

We detected high levels of PDGFRβ in tumor cells from different types of testicular tumors. These results indicate that compounds such as sunitinib or pazopanib, in addition to their antiangiogenic response, also directly affect testicular tumor cells by blocking these PDGFRs. Sunitinib as a single agent was tested in three clinical trials of refractory TGT (29–31), giving modest results, with only a few cases of short-duration disease stabilization followed by rapid progressive disease in two studies (29, 31), but with three temporary partial responses (9%) and 41% of cases of stable disease in the other (30). Moreover, there was a decrease in the frequency of tumor markers following sunitinib treatment, suggesting that the targets of sunitinib may still be important in TGT biology (29, 31). Our results also indicate that CDDP-resistant testicular tumor cells are more sensitive to pazopanib or sunitinib than CDDP-sensitive cells. These findings indicate that the cells become addicted to the PDGF/PDGFR pathway and can explain the previous results of our group about sunitinib response in CDDP-resistant tumors compared with CDDP-sensitive tumors (14). Similar results have been reported in glioma cells that overexpress PDGF-BB and subsequently become more sensitive to PDGFR inhibitors (24). Thus, our findings reinforce the value of these antiangiogenic reagents as resensitizing therapies for subgroups of CDDP-resistant or refractory patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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