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Insulin-like growth factor-I receptor tyrosine kinase inhibitor cyclolignan picropodophyllin inhibits proliferation and induces apoptosis in multidrug resistant osteosarcoma cell lines

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Abstract

Insulin-like growth factor-I receptor (IGF-IR) is an important mediator of tumor cell survival and shows prognostic significance in sarcoma. To explore potential therapeutic strategies for interrupting signaling through this pathway, we assessed the ability of cyclolignan picropodophyllin (PPP), a member of the cyclolignan family, to selectively inhibit the receptor tyrosine kinase activity of IGF-IR in several sarcoma cell line model systems. Of the diverse sarcoma subtypes studied, osteosarcoma cell lines were found to be particularly sensitive to IGF-IR inhibition, including several multidrug resistant osteosarcoma cell lines with documented resistance to various conventional anticancer drugs. PPP shows relatively little toxicity in human osteoblast cell lines when compared with osteosarcoma cell lines. These studies show that PPP significantly inhibits IGF-IR expression and activation in both chemotherapy-sensitive and chemotherapy-resistant osteosarcoma cell lines. This inhibition of the IGF-IR pathway correlates with suppression of proliferation of osteosarcoma cell lines and with apoptosis induction as measured by monitoring of poly(ADP-ribose) polymerase and its cleavage product and by quantitative measurement of apoptosis-associated CK18Asp396. Importantly, PPP increases the cytotoxic effects of doxorubicin in doxorubicin-resistant osteosarcoma cell lines U-2OS$_{MR}$ and KHOS$_{MR}$. Furthermore, small interfering RNA downregulation of IGF-IR expression in drug-resistant cell lines also caused resensitization to doxorubicin. Our data suggest that inhibition of IGF-IR with PPP offers a novel and selective therapeutic strategy for ostosarcoma, and at the same time, PPP is effective at reversing the drug-resistant phenotype in osteosarcoma cell lines. [Mol Cancer Ther 2009;8(8):2122–30]

Introduction

Primary bone tumors account for 5% to 10% of all new pediatric cancer diagnoses in the United States (1). Osteosarcoma is the most common malignant bone tumor in children and adolescents and accounts for about 60% of malignant bone tumors diagnosed in the first two decades of life (2). Standard treatment for osteosarcoma is surgery and chemotherapy. Chemotherapy treatment has significantly improved survival rates from 11% with surgery alone to approximately 70% when combined with chemotherapy (3, 4). Unfortunately, for the 40% of patients with progression after frontline therapy, further therapy with additional chemotherapy is palliative and too often toxic. It is estimated that less than 30% of patients with recurrent metastasis will be cured. The development of chemoresistance is associated with many events, such as defective apoptotic signaling in response to chemotherapy, overexpression of antiapoptotic proteins, and overexpression of multidrug resistance (MDR) gene protein Pgp (5–7). Successful management of osteosarcoma would be greatly aided by novel agents that interfere with both the intrinsic and acquired mechanisms of drug resistance mechanisms.

The insulin-like growth factor (IGF) signaling pathway has been found to be critical for tumor cell proliferation, differentiation, and apoptosis (8–10). *In vitro* and *in vivo* studies have implicated IGF in the pathogenesis of breast, prostate, lung, and colon cancers (11–14). A member of the IGF family, the IGF-I receptor (IGF-IR), is a ubiquitously expressed type 1 transmembrane heterotetrameric receptor composed of two α-subunits (M_r 130,000) and two β-subunits (M_r 90,000 each), with intrinsic tyrosine kinase activity (10, 15). On binding specific ligands (IGF-I and IGF-II), IGF-IR undergoes autophosphorylation of tyrosine residues, leading to subsequent tyrosine phosphorylation of insulin receptor substrate 1, Src, and collagen homology proteins (Shc), resulting in the activation of the phosphatidylinositol 3-kinase/Akt, RAS/mitogen-activated protein

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kinase, and Janus-activated kinase/signal transducer and activator of transcription pathways (10, 15). These activated pathways regulate the function and expression of proteins involved in cell proliferation and survival (10, 16). IGF-IR thereby plays an important role in malignant transformation. Expression of functional IGF-IR is required for malignant cell transformation by known cellular and viral oncogenes (17). Studies on breast cancer have suggested that overexpression of IGF-IR is associated with local tumor recurrence, and circulating concentrations of IGF-I or its major binding protein, IGF binding protein 3, are associated with increased risk of common cancers (18, 19). IGF-IR activation protects cells from a variety of apoptosis-inducing agents including osmotic stress and anticancer drugs (20–22). Expression of IGF-IR in synovial sarcoma is associated with an aggressive phenotype and with a high incidence of lung metastases (23). *In vitro*, overexpression of IGF-IR reduces growth factor requirement for tumor cell growth and cellular susceptibility to apoptosis (24). Pediatric tumors such as Ewing's sarcoma, neuroblastoma, rhabdomyosarcoma, and Wilms' tumor express high levels of IGF-IR and, in the presence of IGF, increase proliferation and decrease apoptosis (10). Conversely, impairment of the IGF-IR function by antisense strategies, antibodies, or dominant-negative mutants causes large-scale apoptosis of tumor cells, abrogation of tumor growth, and metastasis (10, 15, 20). In particular, Ewing's sarcoma xenografts are sensitive to anti–IGF-IR treatment (25). Therefore, modulating the IGF-IR pathway is now an attractive anticancer treatment strategy.

IGF-I levels in humans are highest during the adolescent growth spurt, which coincides with the peak incidence of osteosarcoma, supporting the hypothesis that IGF-I may contribute to the pathogenesis of osteosarcoma (26). *In vitro* studies have shown that osteosarcoma cell lines express IGF-IR, depend on IGF-I ligand for proliferation and antiapoptosis, and are growth inhibited with IGF-IR blockade (27). Finally, a recent study observed in a human osteosarcoma cell line, HOS 58, that proliferative activity was associated with high mRNA levels of IGF-IR, and the rate of proliferation decreased with a decline in IGF-IR expression (28).

Picropodophyllin (PPP), a member of the cyclolignan family, is a new inhibitor of IGF-IR (29). The inhibitory effect of PPP on IGF-IR did not co-inhibit insulin receptor or compete with ATP in *in vitro* kinase assays, suggesting that it may inhibit IGF-IR autophosphorylation at the substrate level (30). PPP inhibits tyrosine phosphorylation of Y1136 in the activation loop of the IGF-IR kinase domain. This agent has been shown to induce tumor regression and inhibition of metastasis in several models of human cancer, and *in vitro* studies suggest development of only limited resistance in tumor cells after long-term PPP exposure (29–32). Recent studies showed that oral PPP is well tolerated *in vivo* and inhibits IGF-IR expression and growth of melanoma (33). To date, however, the effect of PPP on osteosarcoma and especially multidrug resistant osteosarcoma cells is undefined.

In this study, we determined whether the IGF-I signaling pathway is of functional importance in osteosarcoma. We further investigate the effect of PPP on constitutive expression of IGF-IR, and whether a combination of minimally or nontoxic doses of PPP induces apoptosis, overcomes drug resistance, or enhances drug sensitivity in drug-resistant osteosarcoma cell lines.

Materials and Methods

Cell Lines, Patient Tumor Samples, and Antibodies

Human osteoblast cell line HOB-c (hipbone derived) was purchased from PromoCell GmbH. The human osteosarcoma cell line U-2OS, KHOS, human uterine sarcoma cell line MES-SA, and its doxorubicin-selected drug-resistant cell line MES-SA/Dx5 were purchased from the American Type Tissue Collection. The multidrug resistant U-2OS$_{MR}$ was established as previously reported (6, 34). Briefly, the doxorubicin-resistant cell lines were selected over a period of 6 to 10 mo by continuous culture in media containing stepwise increasing concentrations of doxorubicin. Dr. Efstathios Gonos (Institute of Biological Research and Biotechnology, Athens, Greece) provided the multidrug (selected with doxorubicin) resistant KHOS R2 (referred in the text below as KHOS$_{MR}$) cell line (35). Dr. Katia Scotlandi (Institute Orthopedics Rizzoli, Italy) provided ET-743 resistant TC-ET 6 nM and TC-ET 12 nM cell lines (36). Eight cases of osteosarcoma samples (1–8) were analyzed. Samples 1 to 4 were tissues from patients without chemotherapy and samples 5 to 8 were tissues from patients with chemotherapy. The Pgp1 monoclonal antibody C219 was purchased from Signet. The goat anti-rabbit-horseradish peroxidase (HRP) and goat anti-mouse-HRP were purchased from Bio-Rad. SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce. The rabbit polyclonal antibodies to human IGF-IR, AKT, pAKT, and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technologies. The rabbit polyclonal antibody to human phosphor-IGF-IR (1158/1162/1163) was purchased from Invitrogen. The mouse monoclonal antibody to actin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich.

Drugs

The IGF-IR inhibitor PPP was purchased from Calbiochem. Stock solutions were prepared in DMSO and stored at −20°C; they were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration was <0.1%. Doxorubicin and paclitaxel were obtained through unused residual clinical material provided by the pharmacy at the Massachusetts General Hospital. The stock solution of drugs were prepared according to the drug specifications and stored at −20°C.

Cell Culture

Human osteoblast cells HOB-c were cultured in osteoblast growth medium (PromoCell) with 10% fetal bovine serum (FBS). All other cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 units/mL

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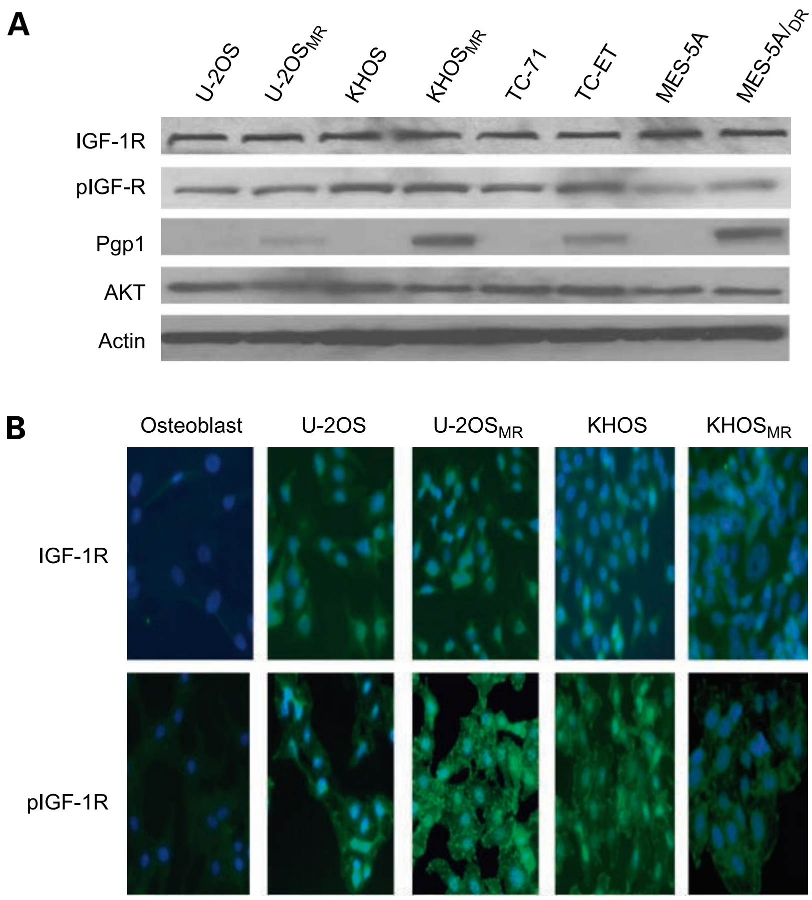

Figure 1. Expression of IGF-IR and pIGF-IR in the sarcoma cell lines as detected by Western blotting (**A**) and immunofluorescence (**B**). **A,** expression of IGF-IR and pIGF-IR in drug-sensitive and drug-resistant sarcoma cell line pairs, determined by Western blot. Total cellular protein isolated from the indicated cell lines and immunoblotted with specific antibodies as described in Materials and Methods. The blots were also probed with an anti-actin monoclonal antibody to assess the relative protein levels in the sample lanes. **B,** confirmation of expression of IGF-IR and pIGF-R in drug-sensitive and drug-resistant osteosarcoma cell lines by immunofluroescence. Stained cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope.

penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO_2-95% air atmosphere and passaged when near-confluent monolayers were achieved using trypsin-EDTA solution. Drug-resistant cell lines were periodically cultured in the respective drug to confirm their drug resistance characteristics. Cells were free from *Mycoplasma* contamination as tested using the MycoAlert Mycoplasma Detection Kit from Cambrex.

Western Blotting

IGF-IR, phosphor-IGF-IR, AKT, and Pgp1 proteins were analyzed in total cell lysates. Total cell lysates were prepared, and Western blot analysis was done as previously described. Briefly, the cells were lysed in 1× radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology) and protein concentration was determined by the detergent-compatible protein assay (Bio-Rad). Twenty-five micrograms of total protein were resolved on NuPage 4% to 12% Bis-Tris gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween 20 with gentle agitation overnight at 4°C. HRP-conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween 20, at 1:2,000 dilution, for 1 h at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate. Densitometric analysis of Western blot results was done with Adobe Photoshop 7.0 as described in the User Guide.[4]

Immunofluorescence

For immunostainings of cultured cells, osteosarcoma cells were grow in eight-well chamber (Nalge Nunc International) for 48 h and fixed in 3.7% buffered paraformaldehyde. Immunostainings were done using antibodies against phosphorylated IGF-IR (Tyr1131) (1:200) for 1 h. After washing, the cells were incubated with Alexa Fluor secondary antibodies (Invitrogen). To counterstain the nuclei, the cells were incubated with PBS containing 1 μg/mL Hoechst 33342 (Invitrogen) for 1 min. Stained cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp.) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc.).

MTT Cell Proliferation Assay

Drug cytotoxicity was assessed *in vitro* using the MTT assay as previously described (37). Briefly, 2×10^3 cells per well were plated in 96-well plates in culture medium (RPMI

[4] http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html

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1640 supplemented with 10% FBS and penicillin/streptomycin) containing increasing concentrations of PPP, doxorubicin, or both. After 96 h of culture, 10 μL of MTT (5 mg/mL in PBS, obtained from Sigma) were added to each well and the plates were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a SPECTRAmax Microplate Spectrophotometer (Molecular Devices). The absorbance values were normalized by assigning the value of the control line in the medium without drug to 1.0 and the value of the no cell control to 0. Experiments were done in triplicate. Dose-response curves were fitted with use of GraphPad PRISM 4 software (GraphPad Software).

IGF-IR Small Interfering RNA Assay

The human *IGF-IR* On-Targetplus SMARTpool small interfering RNA (siRNA) was purchased from Dharmacon, Inc. and was used according to the manufacturer's instructions. For transfection, cells were either plated on 96-well plates for MTT assays or plated on dishes for Western blot protein detection. Transfections were done with siPORT NeoFX siRNA transfection reagents (Ambion, Inc.) as directed by the manufacturer. The Silencer *EGFP* siRNA (Ambion) and siRNA Control reagent (Dharmacon) were used as positive and negative controls in all experiments. For *IGF-IR* inhibition, the final concentration of siRNA was 100 nmol/L. Medium was replaced with RPMI 1640 supplemented with 10% FBS 24 h after transfection. Total protein was isolated after 72 h of *IGF-IR* siRNA transfection.

Apoptosis Assay

Whole-cell lysates were immunoblotted with specific antibodies to PARP (Cell Signaling Technologies) and its cleavage products. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate. Quantification of apoptosis was also evaluated using the M30-Apoptosense ELISA assay kit as per manufacturer's instructions (Peviva AB). U-2OS$_{MR}$ and KHOS$_{MR}$ cells were seeded at 8,000 per well in a 96-well plate for 24 h before the addition of doxorubicin and PPP. The cells were then treated with 0.5 μmol/L doxorubicin, 1 μmol/L doxorubicin, 0.01 μmol/L PPP alone, or a combination of doxorubicin and PPP for an additional 48 h. The cells were then lysed by adding 10 μL of 10% NP40 per well, and the manufacturer's instructions for the apoptosis assay were then followed.

Results

Expression of IGF-IR in Osteosarcoma

We observed the expression of IGF-IR in four pairs of multidrug resistant sarcoma cell lines (two osteosarcoma cell lines, a Ewing's sarcoma cell line, and a uterine sarcoma cell line). IGF-IR and phosphorylated IGF-IR (pIGF-IR) are expressed in these cell lines as measured by Western blot (Fig. 1A). For confirmation, we measured the prevalence and activation of IGF-IR in osteosarcoma cells by immunoflourescence, staining with antibodies against IGF-IR and pIGF-IR. We also observed that osteosarcoma

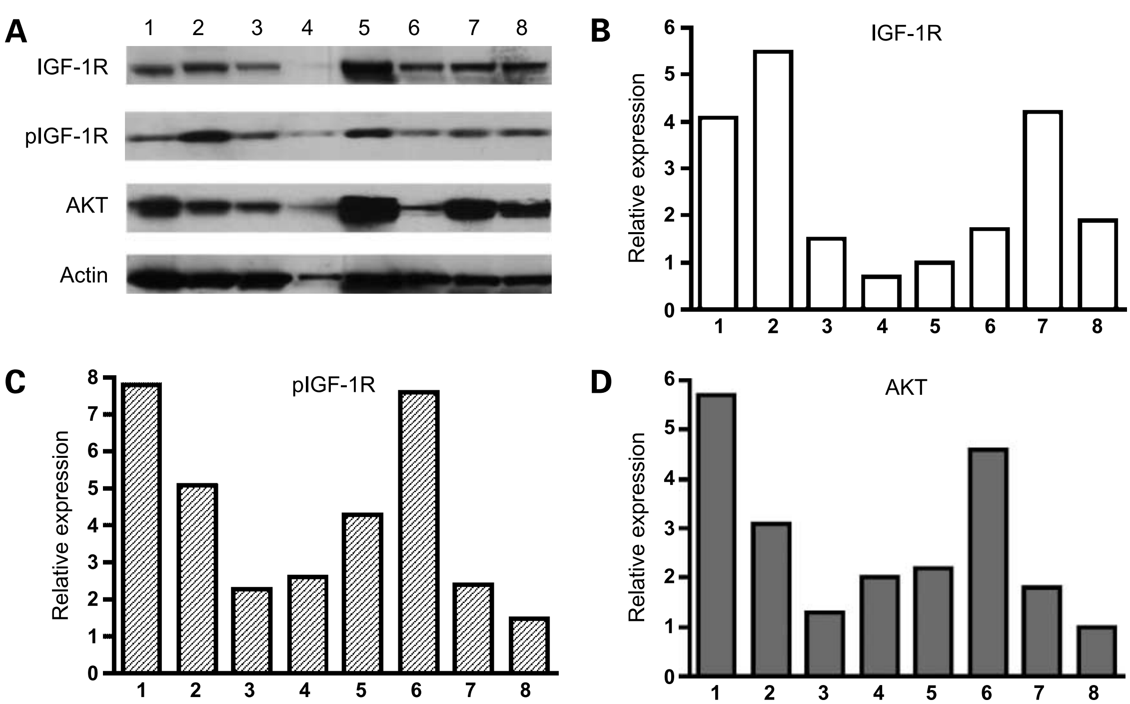

Figure 2. Expression of IGF-IR, pIGF-IR, and AKT in osteosarcoma tissues, determined by Western blot. Total cellular protein isolated from the osteosarcoma tissues and immunoblotted with specific antibodies as described in Materials and Methods. **A,** expression of IGF-IR, pIGF-IR, and AKT. Numbers 1 to 8 represent eight different tissue samples. **B** to **D,** Western blots from **A** were analyzed by densitometry, which was carried out in Photoshop and normalized to β-actin expression. Quantitative results for each protein were presented as relative expression. **B,** IGF-IR; **C,** pIGF-IR; **D,** AKT.

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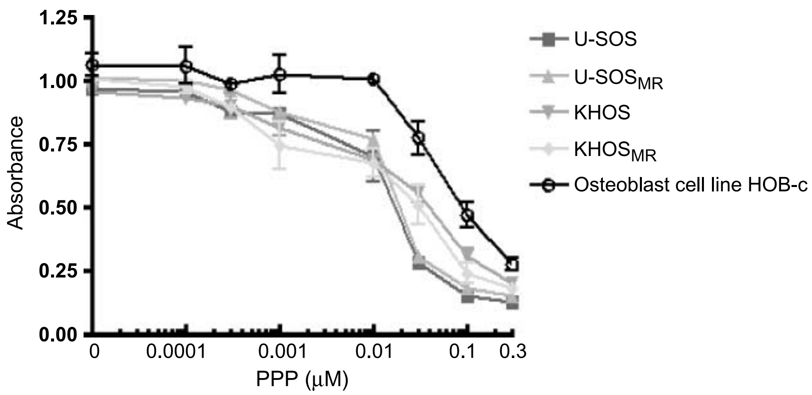

Figure 3. Effects of PPP on the proliferation of osteosarcoma and normal osteoblast cells. Cells were treated with PPP in RPMI 1640 complete medium at the indicated concentrations. The relative sensitivity of each line to PPP was determined by MTT analysis 96 h after treatment.

cell lines, including multidrug resistant cell lines, exhibited high levels of IGF-IR and pIGF-IR when compared with normal osteoblast cell lines (Fig. 1B). Furthermore, multidrug resistant cell lines KHOS$_{MR}$, TC-ET, and MES-5A/Dx5 showed high expression of Pgp1 (multidrug resistance protein 1). To preclude the possibility that IGF-IR expression is an artifact induced by *in vitro* propagation, we also examined eight freshly isolated primary osteosarcoma specimens, including samples from patients with resistance to conventional chemotherapy. Expression of IGF-IR and pIGF-IR was also universally observed in these primary patient samples (Fig. 2). Because the phosphatidylinositol

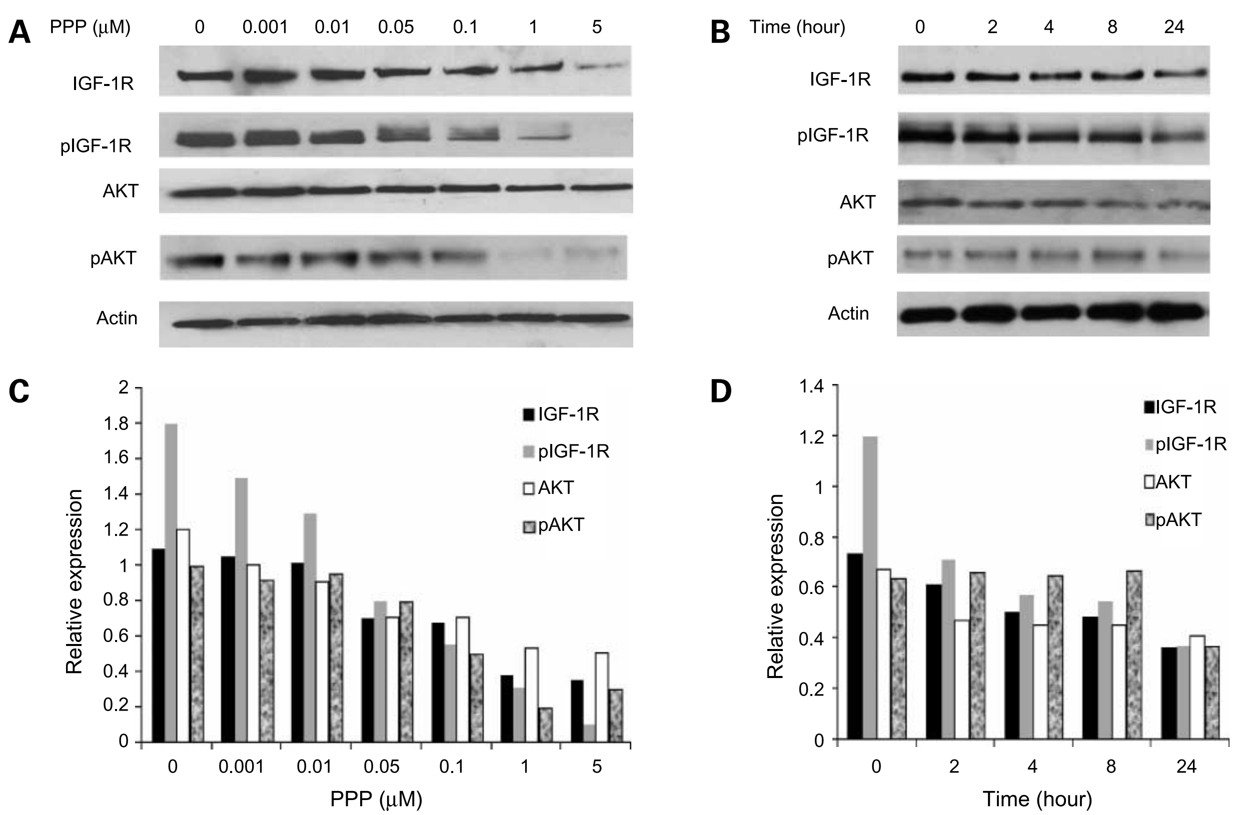

Figure 4. Dose- and time-dependent of PPP down-regulated IGF-IR proteins in drug-resistant osteosarcoma cells. U-2OS$_{MR}$ was treated with PPP in dose-dependent (**A**) or time-dependent (**B**) manner in fresh complete medium. Total cellular proteins were subjected to immunoblotting with specific antibodies to IGF-IR, pIGF-IR, AKT, and β-actin as described in Materials and Methods. **A,** PPP reduces IGF-IR proteins in U-2OS$_{MR}$ cells in a dose-dependent manner. U-2OS$_{MR}$ cells were cultured for 24 h in the presence of various concentrations of PPP as indicated. **B,** U-2OS$_{MR}$ cells were cultured for the time indicated (in hours) in the presence of 1 μmol/L of PPP. **C** and **D,** Western blots from **A** and **B** were analyzed by densitometry as described in Materials and Methods.

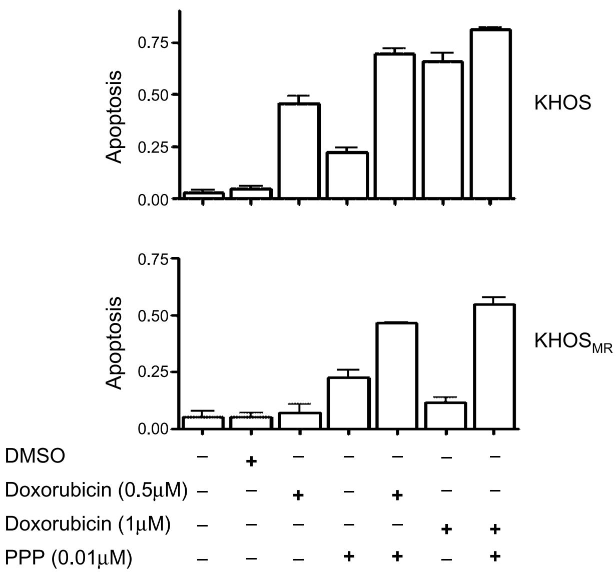

Figure 5. PPP enhances apoptosis induced by doxorubicin in drug-resistant osteosarcoma cells. KHOS or KHOS$_{MR}$ cells were seeded at a density of 8,000 per well in a 96-well plate for 24 h. Cells were then treated with different drugs for an additional 48 h. The cells were lysed with 10% NP40, and the M30-Apoptosense ELISA assay was done as described in Materials and Methods.

3-kinase-AKT pathway is known to be involved in the transmission of IGF-IR signals, we assayed for expression of AKT and observed that all osteosarcoma cell lines and primary tumor samples that express IGF-IR also express AKT (Figs. 1A and 2A and D).

Effects of PPP on Cellular Proliferation in Osteosarcoma Cell Lines

We examined the effect of PPP on the proliferation of osteosarcoma cell lines. After exposing the cell lines to PPP in complete cell culture medium for 96 hours, the relative numbers of viable cells were determined by MTT assay. The osteosarcoma cell lines were growth inhibited by PPP (Fig. 3). Incubation of both drug-sensitive and drug-resistant osteosarcoma cell lines with increasing concentrations of PPP was found to decrease the amount of MTT absorbance in a dose-dependent manner. U-2OS and U-2OS$_{MR}$ were more sensitive to PPP compared with normal osteoblast cells. A slightly higher resistance to PPP was observed in normal osteoblast cell lines. In addition, osteosarcoma cell lines and its drug-resistant subclones showed similar responses to PPP.

Effect of PPP on IGF-IR Expression

Because IGF-IR has been shown to be expressed to a high degree in drug-sensitive and drug-resistant osteosarcoma cell lines as compared with normal osteoblast cell lines (Fig. 1), the effects of PPP on the expression of IGF-IR, pIGF-R, and AKT in osteosarcoma cell line were analyzed by Western blot. The osteosarcoma cell lines were incubated either with a range of concentrations of PPP for 24 hours or with 1 μmol/L PPP for 0, 2, 4, 8, and 24 hours. Figure 4 shows the representative results from the cell line U-2OS$_{MR}$, which has shown high expression of IGF-IR (Fig. 1). Quantitatively analysis of Western blot data showed that PPP reduced IGF-IR, pIGF-IR, AKT, and pAKT expression in a dose- and time-dependent manner (Fig. 4). PPP-induced inhibition could be observed as early 2 h (1 μmol/L) and at a concentration as low as 0.01 μmol/L. These results are consistent with previous studies of PPP in breast cancer and melanoma cells (29, 38). Similar results were found in osteosarcoma cell lines U-2OS, KHOS, and KHOS$_{MR}$ (data not shown).

PPP Enhances Apoptosis and Reduces Resistance in Drug-Resistant Human Osteosarcoma Cell Lines

Inhibition of IGF-IR signaling in KHOS and KHOS$_{MR}$ may increase the levels of apoptosis induced by chemotherapy drugs. The addition of PPP to cells exposed to doxorubicin resulted in greater levels of apoptosis in both multidrug resistant osteosarcoma cell lines KHOS and KHOS$_{MR}$ (Fig. 5). Additionally, MTT assay, which measures a combination of cellular proliferation and cytotoxicity, also showed that PPP has an additive effect on paclitaxel-induced cell death in U-2OS and U-2OS$_{MR}$ (Supplementary Fig. S1). These effects were also seen in KHOS and KHOS$_{MR}$ cells exposed to sublethal doses of doxorubicin and PPP (0.005 μmol/L; data not shown). From these results, it is evident that PPP increased doxorubicin- or paclitaxel-induced cell death and partially overcame drug resistance.

PPP Induced Apoptosis in Multidrug Resistant Osteosarcoma Cell Lines

The effect of PPP on the induction of apoptosis was further investigated by immunoblotting for PARP cleavage. PARP cleavage was detected after the incubation of U-2OS$_{MR}$ or KHOS$_{MR}$ cells with PPP. A dose-response analysis revealed the appearance of PARP cleavage products starting at a PPP concentration of 0.005 μmol/L when cells were allowed to incubate for 24 hours (Supplementary Fig. S2).

Effects of Inhibition of IGF-IR Expression by siRNA on Drug Sensitivities

The evaluation of the contribution of *IGF-IR* mRNA expression levels to multidrug resistance took two steps. First, the *IGF-IR* gene in U-2OS$_{MR}$ cells was down-regulated with siRNA. Western blot showed that IGF-IR expression was significantly decreased after the cells were treated with siRNA (Fig. 6A). Then, relative drug sensitivities were evaluated by comparing the IC_{50} values determined by MTT in siRNA-treated and control multidrug resistant cell lines. Cytotoxicity was measured 96 hours after treatment with *IGF-IR* siRNA (see Materials and Methods). The results showed that IGF-IR down-regulation by siRNA partially recovered sensitivity to doxorubicin (Fig. 6B). Treatment of IGF-IR siRNA knockdown cells with PPP showed a modest higher resistance to PPP as compared with cells without IGF-IR knockdown (Fig. 6C). These data are consistent with the results showing that PPP inhibits osteosarcoma cell growth and induces apoptosis (Fig. 3). Moreover, whereas *IGF-IR* siRNA could significantly inhibit expression of

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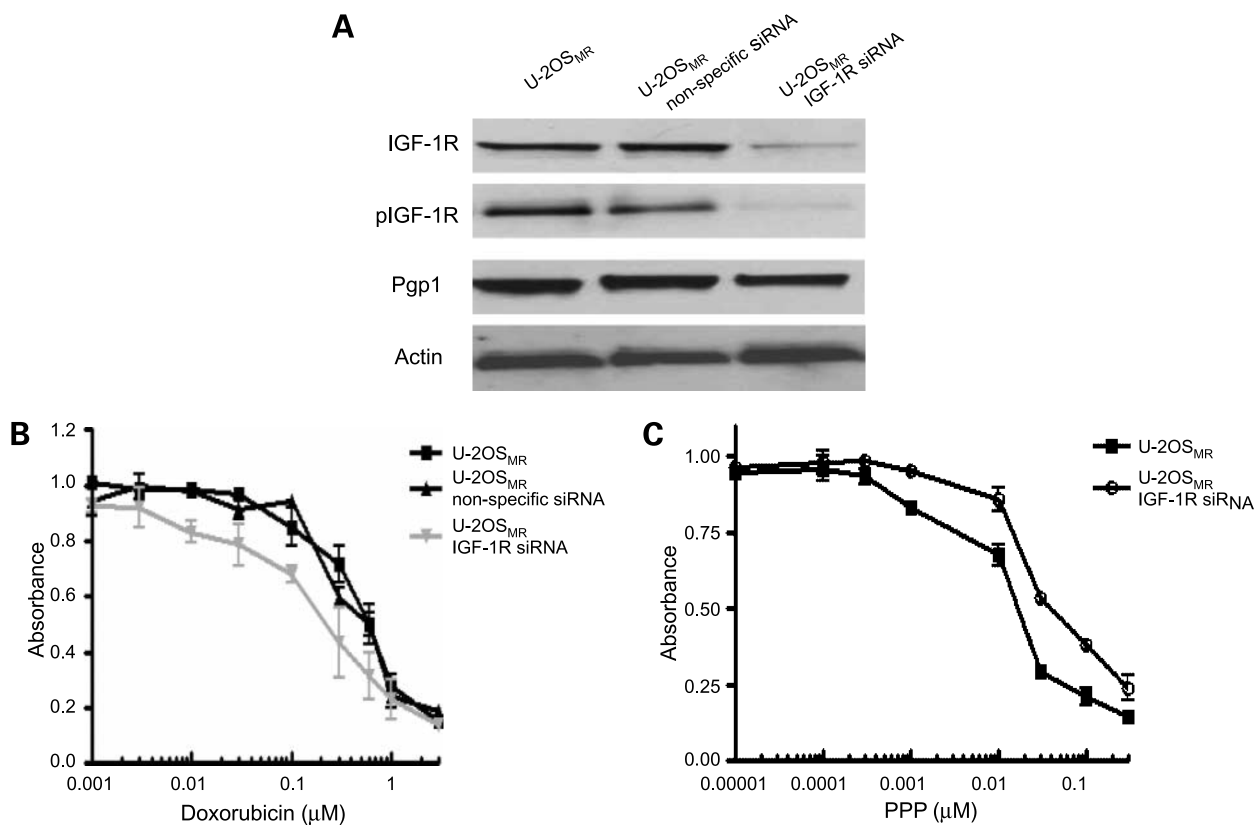

Figure 6. Effect of IGF-IR inhibition on drug sensitivity in drug-resistant cells. Multidrug resistant cell line U-2OS$_{MR}$ was transfected with On-Targetplus SMARTpool IGF-IR siRNA as well as nonspecific siRNA. **A,** confirmation of IGF-IR protein knockdown by Western blot. Total protein was isolated 72 h after transfection, and IGF-IR, pIGF-IR, and Pgp1 expression was analyzed by Western blotting. **B,** the relative sensitivity of each treatment to doxorubicin was determined by MTT analysis 96 h after transfection. **C,** effects of IGF-IR knockdown by siRNA on PPP sensitivity in U-2OS$_{MR}$ cells.

IGF-IR, no significant changes in Pgp1 expression were observed in this IGF-IR knockdown cell line (Fig. 6A).

Discussion

Identification of both novel therapeutic strategies and potent drugs that are effective against sarcomas is a high-priority goal for sarcoma oncologists. The IGF-IR signaling pathway plays a significant role in the growth and proliferation of tumor cells. Several lines of evidence have shown that IGF-I production and IGF-IR expression may prevent cell death and lead to drug resistance through up-regulation of survival proteins in human cancers (10, 20, 21, 39). Therefore, IGF-IR proteins emerged as important targets for cancer therapy. Previous studies with IGF-IR inhibitors such as NVP-AEW541, R1507, and SCH-717454 have shown that these compounds have the capabilities of inhibiting tumor cell growth and inducing apoptosis in several human cancers (10, 15, 20). Cells with a high level of IGF-IR expression are sensitive to these inhibitors. In this study, we show that IGF-IR is highly expressed in chemotherapy-resistant osteosarcoma cell lines as well as in primary osteosarcoma tissues. Our findings further showed that PPP inhibits IGF-IR expression in osteosarcoma cell lines. Moreover, we found that the osteosarcoma cell lines were particularly sensitive to IGF-IR inhibition when compared with normal osteoblast cells or with cells that were genetically suppressed for IGF-IR expression by siRNA knockdown. The inhibition of the IGF-IR pathway in osteosarcoma cell lines correlated with suppression of proliferation and with induction of apoptosis.

The molecular mechanism of PPP action on different tumor cell is still unknown. PPP seems to block IGF-IR phosphorylation and promote IGF-IR degradation, whereas the homologous insulin receptor is not affected (29, 31). IGF-IR–null fibroblasts have been shown to be resistant to PPP, whereas PPP reduces the viability of cancer cell lines and causes tumor regression in mouse xenografts of multiple myeloma and uveal melanoma (32, 38). We also showed that osteosarcoma cell lines that were induced to no longer express IGF-IR by treatment with siRNA become insensitive to PPP (Fig. 6). PPP has been shown to associate with inhibition of the phosphatidylinositol 3-kinase/Akt and vascular endothelial growth factor pathways and activation of the extracellular signal–regulated kinase pathways (30, 31, 40). More recently, treatment of animals bearing constitutively active oncogenic K-ras (mutant K-ras^{G12D}) tumors with PPP resulted in a dramatic decrease in tumor mass of the main forms of basal-like breast cancer. PPP also was effective against xenografts of the human basal-like breast cancer cell line MDA-MB-231, which carries a *K-ras*^{G13D} mutation (41).

The development of multidrug resistance has posed major obstacles to the efficacy of chemotherapy for cancer treatment (2, 5, 6). We therefore investigated the effects of PPP in combination with conventional therapeutic agents that are currently used in the treatment of osteosarcoma. We observed that the MDR1 (Pgp1)-positive osteosarcoma cells are sensitive to PPP-induced cell death. At the same time, the synergistic activity of PPP with doxorubicin suggests that PPP could induce apoptosis in doxorubicin-resistant cells through mechanisms independent of inhibition of Pgp1. We found that PPP could increase the sensitivity of drug-resistant osteosarcoma cells to various cytotoxic chemotherapeutic agents (doxorubicin, paclitaxel). These studies provided a proof of principle that PPP is active against osteosarcoma cell lines with known resistance to conventional (doxorubicin, paclitaxel, vincrinstine) anticancer agents, as well as primary tumor cells from osteosarcoma patients. These preclinical studies provide the framework for clinical evaluation of PPP, either as a monotherapy or in combination with doxorubicin, to treat osteosarcoma and overcome drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Chou AJ, Geller DS, Gorlick R. Therapy for osteosarcoma: where do we go from here? Paediatr Drugs 2008;10:315–27.
3. Trent JC. Rapid evolution of the biology and treatment of sarcoma. Curr Opin Oncol 2008;20:393–4.
4. Siegel HJ, Pressey JG. Current concepts on the surgical and medical management of osteosarcoma. Expert Rev Anticancer Ther 2008;8:1257–69.
5. Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther 2006;6:1075–85.
6. Yang C, Yang S, Wood KB, et al. Multidrug resistant osteosarcoma cell lines exhibit deficiency of GADD45α expression. Apoptosis 2009;14:124–33.
7. Pasello M, Michelacci F, Scionti I, et al. Overcoming glutathione *S*-transferase P1-related cisplatin resistance in osteosarcoma. Cancer Res 2008;68:6661–8.
8. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915–28.
9. Ullrich A, Gray A, Tam AW, et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J 1986;5:2503–12.
10. Kim SY, Toretsky JA, Scher D, Helman LJ. The role of IGF-1R in pediatric malignancies. Oncologist 2009;14:83–91.
11. Lann D, LeRoith D. The role of endocrine insulin-like growth factor-I and insulin in breast cancer. J Mammary Gland Biol Neoplasia 2008;13:371–9.
12. Rowlands MA, Gunnell D, Harris R, Vatten LJ, Holly JM, Martin RM. Circulating insulin-like growth factor peptides and prostate cancer risk: a systematic review and meta-analysis. Int J Cancer 2009;124:2416–29.
13. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127–37.
14. Sachdev D, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. Mol Cancer Ther 2007;6:1–12.
15. Chitnis MM, Yuen JS, Protheroe AS, Pollak M, Macaulay VM. The type 1 insulin-like growth factor receptor pathway. Clin Cancer Res 2008;14:6364–70.
16. Surmacz E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. Oncogene 2003;22:6589–97.
17. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res 1995;55:249–52.
18. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004;363:1346–53.
19. Cohen P. Clinical implications of the IGF-cancer connection. Growth Horm IGF Res 2001;11:336–8.
20. Casa AJ, Dearth RK, Litzenburger BC, Lee AV, Cui X. The type I insulin-like growth factor receptor pathway: a key player in cancer therapeutic resistance. Front Biosci 2008;13:3273–87.
21. Oh SH, Jin Q, Kim ES, Khuri FR, Lee HY. Insulin-like growth factor-I receptor signaling pathway induces resistance to the apoptotic activities of SCH66336 (lonafarnib) through Akt/mammalian target of rapamycin-mediated increases in survivin expression. Clin Cancer Res 2008;14:1581–9.
22. Peretz S, Kim C, Rockwell S, Baserga R, Glazer PM. IGF1 receptor expression protects against microenvironmental stress found in the solid tumor. Radiat Res 2002;158:174–80.
23. Xie Y, Skytting B, Nilsson G, Brodin B, Larsson O. Expression of insulin-like growth factor-1 receptor in synovial sarcoma: association with an aggressive phenotype. Cancer Res 1999;59:3588–91.
24. Kurmasheva RT, Houghton PJ. IGF-I mediated survival pathways in normal and malignant cells. Biochim Biophys Acta 2006;1766:1–22.
25. Manara MC, Landuzzi L, Nanni P, et al. Preclinical *in vivo* study of new insulin-like growth factor-I receptor-specific inhibitor in Ewing's sarcoma. Clin Cancer Res 2007;13:1322–30.
26. Henderson BE, Ross RK, Pike MC, Casagrande JT. Endogenous hormones as a major factor in human cancer. Cancer Res 1982;42:3232–9.
27. Kappel CC, Velez-Yanguas MC, Hirschfeld S, Helman LJ. Human osteosarcoma cell lines are dependent on insulin-like growth factor I for *in vitro* growth. Cancer Res 1994;54:2803–7.
28. Viereck V, Siggelkow H, Pannem R, Braulke T, Scharf JG, Kubler B. Alteration of the insulin-like growth factor axis during *in vitro* differentiation of the human osteosarcoma cell line HOS 58. J Cell Biochem 2007;102:28–40.
29. Girnita A, Girnita L, del Prete F, Bartolazzi A, Larsson O, Axelson M. Cyclolignans as inhibitors of the insulin-like growth factor-1 receptor and malignant cell growth. Cancer Res 2004;64:236–42.
30. Vasilcanu D, Girnita A, Girnita L, Vasilcanu R, Axelson M, Larsson O. The cyclolignan PPP induces activation loop-specific inhibition of tyrosine phosphorylation of the insulin-like growth factor-1 receptor. Link to the phosphatidyl inositol-3 kinase/Akt apoptotic pathway. Oncogene 2004;23:7854–62.
31. Vasilcanu R, Vasilcanu D, Rosengren L, et al. Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor: potential mechanistic involvement of Mdm2 and β-arrestin1. Oncogene 2008;27:1629–38.
32. Menu E, Jernberg-Wiklund H, De Raeve H, et al. Targeting the IGF-1R using picropodophyllin in the therapeutical 5T2MM mouse model of multiple myeloma: beneficial effects on tumor growth, angiogenesis, bone disease and survival. Int J Cancer 2007;121:1857–61.
33. Economou MA, Andersson S, Vasilcanu D, et al. Oral picropodophyllin (PPP) is well tolerated *in vivo* and inhibits IGF-1R expression and growth of uveal melanoma. Invest Ophthalmol Vis Sci 2008;49:2337–42.
34. Yang C, Hornicek FJ, Wood KB, Schwab JH, Mankin H, Duan Z. RAIDD expression is impaired in multidrug resistant osteosarcoma cell lines. Cancer Chemother Pharmacol 2009;64:607–14.

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35. Lourda M, Trougakos IP, Gonos ES. Development of resistance to chemotherapeutic drugs in human osteosarcoma cell lines largely depends on up-regulation of Clusterin/Apolipoprotein J. Int J Cancer 2007;120:611–22.

36. Manara MC, Perdichizzi S, Serra M, et al. The molecular mechanisms responsible for resistance to ET-743 (Trabectidin; Yondelis) in the Ewing's sarcoma cell line, TC-71. Int J Oncol 2005;27:1605–16.

37. Duan Z, Foster R, Bell DA, et al. Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer. Clin Cancer Res 2006;12:5055–63.

38. Girnita A, All-Ericsson C, Economou MA, et al. The insulin-like growth factor-I receptor inhibitor picropodophyllin causes tumor regression and attenuates mechanisms involved in invasion of uveal melanoma cells. Clin Cancer Res 2006;12:1383–91.

39. Jones HE, Gee JM, Barrow D, Tonge D, Holloway B, Nicholson RI. Inhibition of insulin receptor isoform-A signalling restores sensitivity to gefitinib in previously *de novo* resistant colon cancer cells. Br J Cancer 2006;95:172–80.

40. Economou MA, Wu J, Vasilcanu D, et al. Inhibition of VEGF secretion and experimental choroidal neovascularization by picropodophyllin (PPP), an inhibitor of the insulin-like growth factor-1 receptor. Invest Ophthalmol Vis Sci 2008;49:2620–6.

41. Klinakis A, Szabolcs M, Chen G, Xuan S, Hibshoosh H, Efstratiadis A. Igf1r as a therapeutic target in a mouse model of basal-like breast cancer. Proc Natl Acad Sci U S A 2009;106:2359–64.

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