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Enhancement of doxorubicin cytotoxicity of human cancer cells by tyrosine kinase inhibition of insulin receptor and type I IGF receptor

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Abstract

The type I insulin-like growth factor receptor (IGF1R) contributes to cancer cell biology. Disruption of IGF1R signaling alone or in combination with cytotoxic agents has emerged as a new therapeutic strategy. Our laboratory has shown that sequential treatment with doxorubicin (DOX) and anti-IGF1R antibodies significantly enhanced the response to chemotherapy. In this study, we examined whether inhibition of the tyrosine kinase activity of this receptor family would also enhance chemotherapy response. *Cis*-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP) inhibited IGF1R and insulin receptor (InsR) kinase activity and downstream activation of ERK1/2 and Akt in MCF-7 and LCC6 cancer cells. PQIP inhibited both monolayer growth and anchorage-independent growth in a dose-

dependent manner. PQIP did not induce apoptosis, but rather, PQIP treatment was associated with an increase in autophagy. We examined whether sequential or combination therapy of PQIP with DOX could enhance growth inhibition. PQIP treatment together with DOX or DOX followed by PQIP significantly inhibited anchorage-independent growth in MCF-7 and LCC6 cells compared to single agent alone. In contrast, pre-treatment with PQIP followed by DOX did not enhance the cytotoxicity of DOX in vitro. Furthermore, OSI-906, a PQIP derivative, inhibited IGF-I signaling in LCC6 xenograft tumors in vivo. When given once a week, simultaneous administration of OSI-906 and DOX significantly enhanced the anti-tumor effect of DOX. In summary, these results suggest that timing and duration of the IGF1R/InsR tyrosine kinase inhibitors with chemotherapeutic agents should be evaluated in clinical trials. Long-term disruption of IGF1R/InsR may not be necessary when combined with cytotoxic chemotherapy.

Keywords

Insulin; Insulin-like growth factors; Tyrosine kinase inhibitor; Doxorubicin; Autophagy

Introduction

Targeted therapies function by interfering with specific proteins (often kinases) involved in cancer growth and progression [1, 2]. One such promising target is the type I insulin-like growth factor receptor (IGF1R). IGF1R is a receptor tyrosine kinase highly expressed in most types of cancer [3, 4]. It belongs to the IGF/insulin signaling system. This complex system involves three ligands (IGF-I, IGF-II, and insulin), and three receptor tyrosine kinases (IGF1R, insulin receptor (InsR), and the IGF1R/InsR hybrid receptor). Both IGF1R and InsR are composed of two extracellular α subunits covalently linked to two β subunits that contain the tyrosine kinase domains [5]. IGF1R and InsR share high homology, especially within the kinase domain. In addition, InsR and IGF1R hetero-tetramerize to form hybrid receptors [6]. After ligand binding, the kinase function of these receptors is activated, leading to the engagement of multiple downstream signaling pathways, including the extracellular-signal-regulated kinases (ERK1/2) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway [7].

Because IGF1R plays critical roles in cancer cell proliferation and metastasis, many anti-IGF1R drugs, including monoclonal antibodies and tyrosine kinase inhibitors (TKIs), have been developed by pharmaceutical companies and research laboratories [8]. TKIs target directly to the catalytic domain and most interfere with the binding of ATP [9]. A tyrosine kinase inhibitor against IGF1R, *cis*-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP) was reported to inhibit IGF-I and IGF-II signaling and xenograft tumor growth of NIH 3T3 cells overexpressing human *igf1r* gene [10]. Its derivative, OSI-906, contains identical structural components as PQIP to bind to the kinase domain of IGF1R but has an alcohol group substitution at the C3 cyclobutyl group [11]. Both of these TKIs inhibit IGF1R and InsR activity, yet OSI-906 has a better pharmacokinetic profile and is being studied in clinical trials [11].

IGF1R has been reported to play a vital role in the development of resistance to chemotherapy, which provides a rationale to combine the anti-IGF1R therapy with chemotherapy [12]. Recent studies from us and others have suggested that combination of targeted therapy with chemotherapy may be sequence dependent [13–15]. We have previously shown that the best anti-proliferative effect was obtained by doxorubicin (DOX) followed by anti-IGF1R antibodies, AVE-1642 and scFv-Fc. In contrast, giving anti-IGF1R antibodies first caused cell resistance to chemotherapy [15]. Given the long half-life of monoclonal antibodies, it may be difficult to study these sequencing effects in clinical trials. Given the short half-life of IGF1R TKIs, it might be easier to study sequencing effects using these drugs.

The study presented here describes the in vitro and in vivo activity of PQIP and its derivative OSI-906, alone or in combination with DOX. The primary goal of this study was to determine the optimal sequence of combining PQIP with DOX. Furthermore, we have discovered that PQIP triggers autophagy in cancer cells. Our results support the idea that sequencing of anti-IGF1R TKIs with chemotherapy can optimize the antitumor effect and have significant implications for the clinical development of this strategy.

Materials and methods

Reagents

All reagents and chemicals were purchased from Sigma-Aldrich, and cell culture reagents were from Invitrogen/Life Technologies unless otherwise noted. IGF-I was purchased from GroPep (Adelaide, Australia). ERK 1/2 antibody was from Cell Signaling. IGF1R α and β antibodies were from Santa Cruz Biotechnology. The microtubule-associated protein light chain 3 (LC3) antibody (5F10) was from Nanotool (Teningen, Germany). Anti-rabbit and anti-mouse secondary antibodies were from GE Healthcare Biosciences (Piscataway, NJ).

Cell lines and culture

MCF-7 and LCC6 cells were cultured according to the literature [16].

MTT assay

Cells were plated in triplicate in 24-well plates at a density of 20,000 cells per well in growth media. After 24 h, cells were switched to SFM for 18–24 h. Cells were then treated with various doses of PQIP for 72 h. Growth was estimated using the 3-[4,5-Dimethylthiazol 2-yl] 2,5-diphenyltetrazolium bromide (MTT) assay as described previously.

Immunoblotting

Serum-starved cells were pretreated with PQIP for 30 min and stimulated with 5 nM IGF-I or 10 nM insulin for 10 min at 37°C. Cells lysate were collected and separated by SDS–PAGE gels [15]. Proteins were transferred to nitrocellulose and immunoblotted with the various antibodies following manufacturers' instructions.

Immunoprecipitation

Pre-cleared total cellular lysates were incubated with IGF1R or InsR antibody overnight followed by incubation with protein A agarose for 4 h at 4°C. Samples were run on SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted for phosphotyrosine residues.

Anchorage-independent growth assay

Anchorage-independent growth assays were performed as previous described [15]. The bottom agar was overlaid with 800 µl of a 0.45% top agar mixture containing 10,000 LCC6 cells per well in the presence of DOX, PQIP or both, and incubated at 37°C for 24 h. The second treatment was given on the top of agar. After 9–10 days, colonies were counted using a light microscope with an ocular grid. Five random fields were counted per well, and only colonies exceeding two-thirds of a grid square were scored.

Cell cycle analysis

Confluent MCF-7 cells were plated at a density of 0.4×10^6 cells per 60 mm dish. After 24 h, cells were switched to SFM for 24 h. Cells were then treated with PQIP and with or without IGF stimulation. Cells were collected in PBS and stained with propidium iodide. Cell cycle analysis was performed using flow cytometry.

LC3 staining of autophagy

MCF-7 cells were seeded on cover slips 24 h prior to treatment. Cells were then treated with PQIP or AVE1642 (20 µg/ml) for 24 h. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton/PBS. After blocking in 1% FBS/PBS, cells were stained with LC3 antibody at 1:50 dilution overnight, followed by incubation with secondary Alexa 488 goat anti-mouse antibody (1:500) in blocking solution at RT for 30 min. Cells were mounted, and images were taken using an Olympus Fluo-view FV500 laser scanning confocal system.

Tumor growth in athymic mice

Into the mammary fat pad of the mice (Foxn1nu strain from Harlan Sprague–Dawley, Indianapolis, IN, USA), 5×10^6 LCC6 cells in serum-free Iscove's modified essential medium were injected. Tumor growth was measured every 3 days, and tumor volume was estimated from bidirectional measurements using the formula $\text{length} \times \text{breadth}^2/2$. When the tumors reached palpable mass, the mice were randomized by tumor volume into six groups of five animals each. The mice were treated with PBS, DOX (3 mg/kg/week, ip), OSI-906 (OSI) (30 mg/mouse/week, orally), DOX and OSI simultaneously, DOX followed by OSI, or OSI followed by DOX, respectively. Treatment was given weekly for 4 weeks.

IGF-I signaling in OSI-906 treated xenograft tumors

LCC6 cells were injected into the mammary fat pad of female athymic mice. When tumors reached a volume of $\sim 500 \text{ mm}^3$, mice were gavaged with or without OSI-906 (30 mg/kg). Four hours later, mice were injected with 100 µg of IGF-I intraperitoneally for 40 min then killed. Tumors were snap-frozen, and lysates were prepared as previously described [15]. Tumor cell lysates were analyzed by Western blot.

Statistical analysis

One-way ANOVA analysis with Tukey's multiple comparison test was performed in colony growth assay. If not otherwise indicated, error bars in all experiments represent standard deviation error (SE). To analyze the data in mice tumor growth experiments, individual profile plots were first generated to explore tumor growth patterns. Transformation on tumor volume was conducted because of presence of non-linear patterns, and tumor volume in a square root scale showed a linear time trend individually. After transformation, general linear mixed model was used to analyze longitudinal measures of tumor volume in mice where tumor volume at day 7 was the baseline [17]. In the model, treatment, day, and treatment*day interaction were the fixed effects and random intercept and slopes were the random effects. Day was a continuous time variable, for which only linear term was considered in the model after square root transformation on tumor volume. In addition, the variance-covariance structure for two random effects was unstructured and that for random errors was first-order autoregression (AR (1)). These structures were determined based on likelihood ratio test or Akaike and Bayesian information criteria (AIC and BIC). Statistical analysis on tumor volume was conducted using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). A *P* value < 0.05 indicated statistical significance.

Results

PQIP inhibited both IGF-I and insulin signaling

The MCF-7 breast cancer cell line expresses both IGF1R and InsR. IGF-I and insulin has been reported to stimulate the growth of this cell line [18]. To assess whether PQIP inhibits both IGF1R and InsR, MCF-7 cells were treated with either IGF-I or insulin, and with increasing concentrations of PQIP. After immunoprecipitation with anti-IGF1R antibody, the phosphorylation of IGF1R upon IGF-I treatment was examined. As shown in Fig. 1a, IGF-I stimulated the phosphorylation of IGF1R, and PQIP inhibited the phosphorylation of IGF1R in a dose-dependent manner. Similarly, PQIP dose-dependently inhibited the phosphorylation of InsR upon insulin stimulation. However, PQIP was about three-fold more potent toward inhibiting the phosphorylation of IGF1R than that of InsR, consistent with previous reports that IGF1R is more sensitive to PQIP than InsR when overexpressed in NIH3T3 cells [10].

To elucidate how PQIP affected downstream signaling, we investigated the Akt and ERK1/2 pathways upon IGF-I or insulin treatment in cancer cells. As shown in Fig. 1b, MCF-7 cells have relatively low basal phosphorylation of ERK1/2 and Akt. After IGF-I treatment, ERK1/2 and Akt phosphorylation was dramatically increased. Insulin stimulated the phosphorylation of ERK1/2 and Akt to a lesser extent. Pre-treatment of cells with PQIP dose-dependently inhibited phosphorylation of IRS-1 and IRS-2, Akt and ERK1/2. Because insulin signaling was weaker than IGF-I induced signaling, 0.1 μ M of PQIP was able to efficiently block downstream effects of insulin. LCC6 is a derivative of the MDA-MB-435 cell line. While there has been some debate regarding the origin of these cells [19, 20], IGF1R has been shown to play a critical role in the proliferation and metastasis [16, 21]. Recently, we have also shown that downregulation of InsR in these cells inhibited cancer cell proliferation and metastasis [22]. Therefore, LCC6 cells provide a good model to assess

the anti-tumor effects of PQIP. ERK1/2 is constitutively active in these cells thus was not regulated by ligands or PQIP (Fig. 1b, lower panel). In contrast, the phosphorylation of Akt was activated by IGF-I and to a lesser extent, by insulin. PQIP dose-dependently inhibited the phosphorylation of Akt in LCC6 cells. Our results suggest that PQIP is a potent inhibitor against both IGF-I and insulin signaling in MCF-7 and LCC6 cells.

PQIP inhibited MCF-7 proliferation and progression into S phase

To assess the activity of PQIP on cell proliferation, MCF-7 cells were treated with or without IGF-I, and increasing concentration of PQIP. The IC₅₀ concentration for PQIP was determined using MTT assays. As shown in Fig. 2a, MCF-7 cells treated with PQIP exhibited a dose-dependent decrease in monolayer growth compared with untreated cells. The IC₅₀ of PQIP was in the submicromolar range in the presence of IGF-I, consistent with the IC₅₀ measured in NIH 3T3 fibroblasts and GEO human colorectal cancer cells [10].

We further evaluated the effect of PQIP on anchorage-independent growth. MCF-7 cells were grown in soft agar with 1% FBS plus IGF-I, in the absence or presence of increasing concentration of PQIP. As shown in Fig. 2b, PQIP dose-dependently inhibited anchorage-independent growth with or without IGF-I stimulation. IGF-I is a well-known mitogen that stimulates G1 to S phase progression [3]. To investigate the ability of PQIP to interfere with IGF-I mediated cell cycle progression, we analyzed MCF-7 cell cycle distribution by flow cytometry. Serum-starved MCF-7 cells were treated with PQIP (0.3 μ M) in the absence or presence of IGF-I for 24 h, and cell cycle progression was analyzed. As shown in Fig. 2c, about 20% of serum-deprived cells were found in the S phase and IGF-I induced a significant increase of S phase cell population; PQIP completely inhibited the IGF-I stimulated S phase progression. Together these results show that PQIP is a potent inhibitor of IGF-I dependent cell proliferation in vitro.

PQIP induced autophagy, but not apoptosis

To investigate potential mechanisms by which PQIP inhibits cell proliferation, we studied whether PQIP induced cell apoptosis in the presence of IGF-I. We have previously shown that monoclonal antibodies directed against IGF1R induced apoptosis [23]. MCF-7 and LCC6 cells were incubated in serum-free media or 1% FBS in the absence or presence of PQIP for 24 h. After treatment, both adherent and non-adherent cells were collected and subjected to Western blotting analysis to detect the cleaved fragment of poly (ADP) ribose polymerase (PARP), a caspase substrate. As shown in Fig. 3a, increased concentration of PQIP failed to induce PARP cleavage in MCF-7 and LCC6 cells while the monoclonal antibody AVE1642 did (data not shown and [23]). In addition, annexin V did not increase when MCF-7 and LCC6 cells were treated with PQIP (data not shown). These results suggest that PQIP does not induce cell apoptosis.

PQIP inhibited IGF-I and insulin-induced Akt signaling. The mammalian target of rapamycin (mTOR) is a substrate for Akt and a key regulator of autophagy [24], so we examined whether PQIP led to the activation of autophagy. The LC3 protein is widely used to detect autophagy. We therefore examined the presence of cleaved LC3 (LC3-II) and the localization of LC3 in PQIP treated cells. The amount of LC3-II fragmentation is correlated

with the number of autophagosomes [25]. As shown in Fig. 3b, 0.3 μ M PQIP time-dependently increased the levels of LC3-II fragments. To support the specific induction of autophagy by inhibition of IGF1R activation, we also observed an increase in autophagy with the anti-IGF1R antibody, AVE-1642. While both drugs induced autophagy, PQIP resulted in a more rapid appearance of LC3-II fragments.

To confirm PQIP and AVE-1642 induced autophagy, we performed immunofluorescent staining for LC3 to detect the formation of autophagosomes. As shown in Fig. 3c, PQIP and AVE-1642 induced the formation of auto-phagosomes, indicating that PQIP treatment was associated with increased autophagy.

PQIP enhanced the cytotoxicity of DOX in vitro

Previous results from our laboratory have shown that anti-IGF1R antibodies enhanced the cytotoxicity of DOX in a sequence-dependent manner. Only DOX followed by antibody significantly enhanced DOX's effects in vitro and in vivo [15]. Herein, we examined whether PQIP could enhance the cytotoxicity of DOX using an anchorage-independent growth assay. We also studied whether there was sequence dependence. MCF-7 and LCC6 cells were treated with PQIP in combination with DOX as the following schedules: (1) IGF-I; (2) 25 ng/ml (MCF-7), 100 ng/ml (LCC6) DOX; (3) 0.1 μ M PQIP; (4) simultaneous DOX and PQIP treatment; (5) DOX for 24 h followed by PQIP; (6) PQIP for 24 h followed by DOX. While DOX alone inhibited colony formation in MCF-7 cells, PQIP significantly enhanced the cytotoxicity of DOX under two sequence conditions: (4) simultaneous DOX and PQIP and (5) DOX followed by PQIP (Fig. 4a). In contrast, PQIP followed by DOX had little effect on colony formation. To confirm the effects of PQIP on chemotherapy in other cancer cells, LCC6 cells were also examined. Unlike MCF-7 cells, PQIP or DOX alone did not significantly inhibit LCC6 colony formation. However, LCC6 colonies were similarly affected by simultaneous treatment of PQIP with DOX or DOX followed by PQIP as seen in MCF-7 cells (Fig. 4b). Therefore, sequencing of PQIP with DOX can significantly enhance the effectiveness over chemotherapy alone in different cancer cells.

A PQIP derivative compound, OSI-906, enhanced the effect of DOX in vivo

OSI-906 is a derivative compound of PQIP that has similar inhibitory effects on IGF1R and InsR activity, yet has better drug metabolism and pharmacokinetics in vivo [11]. We therefore used OSI-906, not PQIP, to examine sequencing with DOX and IGF-I signaling in vivo. LCC6 cells were injected into the mammary fat pad of athymic mice. When tumors were palpable, mice were pre-treated with or without OSI-906 for 4 h and then treated with IGF-I for 40 min. As shown in Fig. 5a, IGF-I significantly stimulated the phosphorylation of Akt, and pre-treatment of OSI-906 abolished the IGF-I induced Akt phosphorylation. The total levels of IGF1R and InsR remained unaffected. These results suggest that OSI-906 can effectively block IGF-I signaling in vivo.

Since PQIP enhanced the cytotoxicity of DOX in vitro, we sought to determine whether OSI-906 sequencing was relevant in vivo using xenograft models. When LCC6 xenografts reached a palpable size, mice were treated on the following schedules: (1) vehicle control; (2) DOX; (3) OSI-906; (4) DOX and OSI-906 simultaneously; (5) DOX for 24 h followed

by OSI-906; (6) OSI-906 treatment followed by DOX 24 h later. All treatments were given once weekly. As shown in Fig. 5b, c, both DOX and OSI-906 alone significantly inhibited tumor growth compared to control. Simultaneous OSI-906 and DOX treatment further enhanced the anti-tumor effect of DOX (DOX vs. DOX + OSI: $P = 0.0016$). DOX followed by OSI-906 also enhanced the effect of DOX, but to a lesser extent (DOX vs. DOX followed by OSI: $P = 0.067$). In contrast, OSI-906 followed by DOX was not statistically different than DOX alone as measured by tumor volumes (Fig. 5c). Therefore, our result indicated that OSI-906 enhanced the cytotoxicity of DOX when given once a week. Our data suggest that OSI-906 given 24 h prior to DOX was an inferior sequence. Simultaneous OSI-906 and DOX treatment appeared to be the optimal schedule to inhibit tumor growth. Moreover, a single dose of OSI-906 could enhance the effects of DOX.

Discussion

The importance of the IGF1R pathways in breast cancer is already well recognized based on numerous reports. Recently, several lines of evidence support the important function of InsR in cancer cell proliferation and metastasis [22, 26–28]. Furthermore, IGF1R and InsR play compensatory roles in regulating cancer cells [29, 30]. Therefore, TKIs, such as PQIP/OSI-906, may have better anti-tumor effects than monoclonal antibodies targeting IGF1R alone. The effect of combining anti-IGF1R/InsR TKIs with chemotherapy drugs has not been characterized in the past. Our study have shown that co-administration of TKIs with DOX has the best anti-tumor effects, providing significant implications for the clinical development of this strategy.

Our study also showed that PQIP can induce autophagy, but not apoptosis. Autophagy is activated to maintain cellular homeostasis under conditions of metabolic stress or nutrient deprivation [31]. Cells that undergo autophagy stop proliferation and in some instances may undergo senescence [32]. Whether cell death is induced by or is associated with autophagy is unclear since cancer cells can use autophagy as a survival mechanism [33]. Several recent studies have shown that antibodies and TKIs against other tyrosine kinases induced autophagy in cancer cells, such as cetuximab (an antibody against epidermal growth factor receptor), dasatinib (a TKI against Src/Abl), and multiple tyrosine kinase inhibitor sorafenib [34–36]. A common characteristic of these drugs is their ability to initiate autophagy through inhibition of PI3K/Akt/mTOR pathway, but not the ERK1/2 pathway. Consistent with this observation, constitutive activation of the PI3K/Akt pathway was able to reverse drug-induced autophagy [34, 35]. Therefore, PQIP and AVE-1624 likely induce autophagy due to their ability to inhibit the PI3K/Akt/mTOR pathway.

In our previous report, we studied the optimal sequencing treatment regimen to combine anti-IGF1R antibodies with DOX [15]. The anti-IGF1R antibody, AVE-1642, followed by DOX was the least effective treatment sequence and did not enhance the cytotoxicity of DOX. Similar results were obtained with PQIP and OSI-906. DOX intercalates with DNA and affects topoisomerase II activity [37]. Pre-treatment of anti-IGF1R antibodies followed by DOX decreased topoisomerase II activity [15], suggesting that pre-blockade of IGF-I signaling negatively regulates topoisomerase II activity. This could account for the negative

interaction of IGF1R inhibition, by either monoclonal antibodies or TKIs, prior to DOX exposure.

Because antibodies and TKIs have distinct mechanisms of inhibiting the IGF1R signaling and different in vivo pharmacodynamic and pharmacokinetic profiles, it could not be assumed that the findings we made with monoclonal antibodies would apply to the TKIs. Our studies suggest that in some aspects, the two strategies are similar. Both modalities induce autophagy and IGF1R inhibition after chemotherapy further enhances the effect of chemotherapy. However, the TKIs did not induced apoptosis, and this finding is also reported in a recent study where PQIP failed to induce apoptosis in colorectal cancer cell lines [38]. These results are in consistent with the suggestion that downregulation of cell surface IGF1R is necessary to induce cancer cell apoptosis [39].

A major difference between the two modalities relate to their half-life. Monoclonal antibodies have prolonged half-life while the TKIs are very short [40]. Our studies suggest that only brief inhibition of IGF1R/InsR could be effective when combined with DOX. Thus, it is possible that intermittent scheduling of an anti-IGF1R/InsR TKI could be more effective than continuous dosing. This could have the advantage of having only brief inhibition of host InsR. Recent data have suggested that use of an IGF1R/InsR TKI is effective in other models of breast cancer. Use of a TKI inhibited tumor growth but also resulted in hyperinsulinemia and hyperglycemia [41]. While this strategy may be effective in disrupting tumor growth by blocking both receptors, long-term hyperinsulinemia and hyperglycemia would likely have detrimental effects on normal tissue. An intermittent TKI dosing schedule, in combination with a cytotoxic agent, might be superior and deserves evaluation in clinical trials.

This study, in conjunction with our previous study [15], has important implications for the administration of anti-IGF1R therapy in combination with cytotoxic drugs. In lung cancer, an initial positive trial using chemotherapy in combination with an anti-IGF1R antibody was reported [42], but these promising initial results could not be reproduced in a larger phase III clinical trial (Antonio Gualberto, personal communication). There are potentially several reasons for this failure of this antibody trial. First, IGF1R antibody therapy results in increased serum levels of insulin, most likely due to the increased growth hormone and accompanying insulin resistance [43]. If InsR plays an important role in tumor biology, then upregulation of the ligand while leaving the InsR uninhibited could overcome any positive effects of IGF1R inhibition. Indeed, a recent study by Buck et al. has revealed that anti-IGF1R antibody treatment resulted in a compensatory increase in phosphorylation of InsR, which was associated with resistance to inhibition of IRS1 and AKT. While OSI-906 treatment resulted in an enhanced reduction of downstream signaling pathways [29]. Second, host toxicity, particularly hyperglycemia, due to the metabolic effects of the monoclonal antibody could affect response to cytotoxic chemotherapy. Given the long half-life of antibodies, it would be difficult to reduce this toxic effect with this class of drugs. Finally, as we have outlined, sequencing of the cytotoxic insult with anti-IGF1R inhibition may be important. Our data suggest that the cytotoxic agent should precede IGF1R/InsR inhibition. Again, the long half-life of monoclonal antibodies makes it difficult to achieve this sequencing. In this respect, the TKIs could be more effective in combination with

chemotherapy because of their pharmacokinetic properties. Further clinical development of these agents should focus on appropriate sequencing and scheduling to determine the benefit of these drug combinations.

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Abbreviations

IGF1R	The type I insulin-like growth factor receptor
PQIP	<i>Cis</i> -3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]-1-(2-phenylquinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine
InsR	Insulin receptor
DOX	Doxorubicin
ERK	Extracellular-signal-regulated kinases
PI3K	The phosphatidylinositol 3-kinase
TKIs	Tyrosine kinase inhibitors
PARP	Poly (ADP) ribose polymerase
mTOR	Mammalian target of rapamycin
LC3	Microtubule-associated protein 1 light chain 3

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Fig. 1.

PQIP inhibited both IGF I and insulin signaling. **a** Serum-starved MCF-7 cells were treated with or without 1 μ M PQIP, or with increasing concentration of PQIP (0.03, 0.1, 0.3, and 1 μ M) in the presence of 5 nM IGF-I or 10 nM insulin. Cells were lysed, and the IGF1R or InsR was immunoprecipitated and blotted with phosphotyrosine antibody. **b** Serum-starved MCF-7 or LCC6 cells were treated with or without 1 μ M PQIP, or with increasing concentration of PQIP (0.01, 0.03, 0.1, 0.3, and 1 μ M) in the presence of 5 nM IGF-I or 10 nM insulin. Cellular lysates were immunoblotted for IGF1R and InsR, and total and phospho ERK1/2, Akt

Fig. 2.

PQIP inhibited MCF-7 cell proliferation and progression into S phase in cell cycle. **a** Monolayer growth of MCF-7 cells treated with different concentrations of PQIP in the presence or absence of 5 nM IGF-I. **b** Anchorage independent growth of MCF-7 cells treated with different concentration of PQIP in the presence or absence of 5 nM IGF-I in 1% FBS. **c** Sub-confluent MCF-7 cells were exposed to either SFM, 5 nM IGF-I, 0.3 μ M PQIP or 5 nM IGF-I plus 0.3 μ M PQIP for 24 h. Cells were harvested, and cell cycle analysis was performed by flow cytometry. * $P < 0.05$

Fig. 3.

PQIP induced autophagy instead of apoptosis. **a** Cells were serum starved for 24 h and treated with or without 1 μ M PQIP, or with increasing concentration of PQIP (0.1, 0.3, 1, 3, and 10 μ M) in the presence of IGF-I (MCF-7 cells) or 1% FBS (LCC6 cells). Twenty four hours later, cells were lysed and PARP cleavage was assessed by immunoblotting. **b** MCF-7 cells were treated with 0.3 μ M PQIP or 20 μ g/ml AVE1642. Cells were collected at indicated hours and analyzed by immunoblotting using an LC3 antibody. *PC* positive control consisted of serum starved neuro 2A cellular lysates. **c** Cells were treated with 0.1 or 1 μ M PQIP, or 20 μ g/ml AVE-1642 for 24 h with 5% FBS. Cells were fixed, and fluorescent immunohistochemistry was performed to detect endogenous LC3. Representative confocal microscopy images were shown

Fig. 4.

PQIP and DOX inhibited anchorage independent growth in a sequence dependent manner MCF-7. **a** and LCC6 (**b**) cells with or without PQIP (*a*, 0.3 μ M; *b*, 1 μ M), DOX (*DOX*) or PQIP plus DOX were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plate for 24 h, and then, second treatment were added in the top of agar and continue incubating for 9–10 days. Colonies formed were counted on a portion of the well. Each treatment was done in triplicate, and the results are shown as the average number of colonies $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. The experiment was repeated three times with similar results, and a representative experiment is shown

Fig. 5.

OSI-906 enhanced the cytotoxicity of DOX in a sequence dependent manner in vivo. **a** LCC6 cells were injected into the mammary fat pad of female athymic mice to form xenograft tumors. Treatment was performed when mice tumors reached a volume of ~500 mm³. Three mice were pre treated with OSI 30 mg/kg for 4 h and then treated with 100 µg of IGF-I for 40 min. Two mice were treated with 100 µg of IGF-I for 40 min. One mouse was control. Then, mice were killed, and tumor samples were frozen in liquid nitrogen and homogenized in TNESV buffer. A total of 100 µg of tumor extract were immunoblotted for IGF1R, InsR, phospho Akt, and Akt. **b** Female athymic mice were injected with LCC6 cells in the mammary fat pads to form xenograft tumors. At day 7, mice were treated with vehicle control, DOX (3 mg/kg/week each, i.p.), OSI-906 (*OSI*) (50 mg/mouse/week, p.o.), DOX + OSI, DOX 24 h followed by OSI or OSI 24 h followed by DOX four times at a 7 day interval. The arrows indicate the days of drug administration. Tumors were measured every 3 days. **c** Tumor volumes of mice from different treatment groups on the day of killing. Tumor growth curves were analyzed as described in the Materials and methods section. Specifically, control versus DOX: $P = 0.073$; control versus OSI: $P = 0.022$; DOX versus DOX + OSI: $P = 0.0016$; DOX versus DOX followed by OSI: $P = 0.067$; DOX versus OSI followed by DOX: $P = 0.473$