Therapeutic Discovery

Compensatory Insulin Receptor (IR) Activation on Inhibition of Insulin-Like Growth Factor-1 Receptor (IGF-1R): Rationale for Cotargeting IGF-1R and IR in Cancer

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Abstract

Insulin-like growth factor-1 receptor (IGF-1R) is a receptor tyrosine kinase (RTK) and critical activator of the phosphatidylinositol 3-kinase–AKT pathway. IGF-1R is required for oncogenic transformation and tumorigenesis. These observations have spurred anticancer drug discovery and development efforts for both biological and small-molecule IGF-1R inhibitors. The ability for one RTK to compensate for another to maintain tumor cell viability is emerging as a common resistance mechanism to antitumor agents targeting individual RTKs. As IGF-1R is structurally and functionally related to the insulin receptor (IR), we asked whether IR is tumorigenic and whether IR-AKT signaling contributes to resistance to IGF-1R inhibition. Both IGF-1R and IR(A) are tumorigenic in a mouse mammary tumor model. In human tumor cells coexpressing IGF-1R and IR, bidirectional cross talk was observed following either knockdown of IR expression or treatment with a selective anti–IGF-1R antibody, MAB391. MAB391 treatment resulted in a compensatory increase in phospho-IR, which was associated with resistance to inhibition of IRS1 and AKT. In contrast, treatment with OSI-906, a small-molecule dual inhibitor of IGF-1R/IR, resulted in enhanced reduction in phospho-IRS1/phospho-AKT relative to MAB391. Insulin or IGF-2 activated the IR-AKT pathway and decreased sensitivity to MAB391 but not to OSI-906. In tumor cells with an autocrine IGF-2 loop, both OSI-906 and an anti–IGF-2 antibody reduced phospho-IR/phospho-AKT, whereas MAB391 was ineffective. Finally, OSI-906 showed superior efficacy compared with MAB391 in human tumor xenograft models in which both IGF-1R and IR were phosphorylated. Collectively, these data indicate that cotargeting IGF-1R and IR may provide superior antitumor efficacy compared with targeting IGF-1R alone. *Mol Cancer Ther; 9(10); 2652–64. ©2010 AACR.*

Introduction

The role of insulin-like growth factor-1 receptor (IGF-1R) in tumor cell proliferation and survival is well established (1). IGF-1R is a receptor tyrosine kinase (RTK) with a di-dimeric $\alpha_2\beta_2$ structure and is activated on binding the growth factor ligands IGF-1 and IGF-2 (2). IGF-1R couples to the phosphatidylinositol 3-kinase (PI3K)–AKT signaling pathway via interactions with the adaptor protein insulin receptor substrate (IRS). IGF-1R is required for oncogenic transformation and tumorigenesis (3, 4), and disruption of IGF-1R activity by either genetic

(5, 6) or pharmacologic (7–9) approaches can reduce tumor cell proliferation and promote apoptosis. Increased expression of IGF-1R and its ligands is associated with etiology, progression, and prognosis for many human cancer types (10, 11). IGF-1R signaling is a key contributor of resistance to cytotoxic chemotherapeutics, ionizing radiation, and certain targeted agents, including inhibitors of epidermal growth factor receptor (EGFR), HER2, and mammalian target of rapamycin (12–15). IGF-1R has been intensely pursued as a cancer target, and both biological and small-molecule tyrosine kinase domain inhibitors (TKI) of IGF-1R are under investigation in oncology clinical trials (16–19). Given the important role for IGF-1R signaling as an adaptive survival mechanism against a diverse array of antitumor agents, combination therapies centered on IGF-1R inhibitors are being widely explored.

IGF-1R is closely related to the IR, sharing 70% amino acid identity overall and 84% identity within the catalytic domain (20, 21). IR can exist as either of two isoforms [IR(A) and IR(B)] due to alternative splicing of exon 11 (22). IR(A) (short form) is a fetally expressed isoform that lacks a region within exon 11. IGF-1R and IR $\alpha\beta$ monomers

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homodimerize or heterodimerize, and dimers are differentially activated by the ligands, insulin, IGF-1, and IGF-2. Insulin is the canonical ligand for IR and most potently activates IR homodimers. However, the ability of IGF-2 to activate IR is also well established (23–25). Interestingly, affinity of IGF-2 for IR(A) is 5-fold tighter than IR(B) homodimers (22, 26–28). In addition to the role of IR in metabolic signaling for tissues that regulate glucose homeostasis, IR can also promote cell proliferation and survival. Increased IGF-2–mediated IR signaling can rescue mouse embryonic development to prevent dwarfism in mice caused by knockout of the *IGF1R* gene (24). A growing body of data indicates that tumor cells can also exploit IR to promote proliferation and survival (25, 29, 30). Ectopic expression of IR oncogenically transforms NIH3T3 fibroblasts and 184B5 mammary epithelial cells (31, 32). Signaling through the IR(A) isoform specifically has been shown to mediate mitogenic signaling in tumor cells, which is especially important for tumor cells harboring an IR(A)–IGF-2 autocrine loop (22, 26–28). Ablation of pancreatic islet cells in rodent models reduces the growth rates of implanted xenograft tumors, suggesting that insulin-mediated IR signaling can promote tumor growth (33, 34). Epidemiologic studies have shown that elevated levels of insulin and C-peptide are associated with poor prognosis and accelerated tumor growth for several tumor types, including carcinomas of the breast, prostate, colon, endometrium, liver, and ovary (1, 35, 36). Furthermore, clinical studies of an inhaled form of insulin for the treatment of type I diabetes were recently halted due to an increased risk of developing lung cancer (37).

Compensatory RTK signaling is emerging as a major mode of resistance to antitumor agents that selectively target a single RTK in tumor cells. Resistance to inhibition of EGFR or HER2 can be mediated by an adaptive increase in MET or IGF-1R activity (38, 39). There are also data showing reciprocal cross talk between IGF-1R and IR. In mouse embryogenesis, compensatory IR signaling driven by IGF-2 can fully maintain normal embryonic growth in IGF-1R$^{-/-}$ mice, whereas double knockouts, IGF-1R$^{-/-}$ IR$^{-/-}$, are nonviable (24). In osteoblasts, where IGF-1R stimulates growth and differentiation, genetic ablation of *IGF1R* results in increased IR activation that is associated with enhanced insulin-driven AKT and extracellular signal-regulated kinase (ERK) signaling (40). On loss of IGF-1R function, osteoblasts shift from IGF- to insulin-mediated growth and differentiation. Therefore, upregulated IR signaling can compensate for loss of IGF-1R to maintain cellular function in several biological systems. More recent data have indicated that cross talk between IR and IGF-1R may also occur in tumor cells, as increased insulin signaling is observed on downregulation of IGF-1R (41).

Although mitogenic signaling by IR has been described in some tumor cell models, the potential tumorigenicity of this receptor has not been shown, nor have cellular codependence on IGF-1R and IR and cross talk between these receptors been extensively studied. We sought to determine whether IR(A) is tumorigenic and can mediate resistance to selective inhibition of IGF-1R and whether coinhibition of IR and IGF-1R could provide superior inhibition of AKT signaling as well as inhibition of tumor cell proliferation compared with selective inhibition of IGF-1R. We show that both IR(A) and IGF-1R can independently promote tumorigenesis and drive the IRS-PI3K-AKT pathway in a mouse mammary tumor cell model. IGF-1R and IR are coexpressed in a wide range of human tumor cell lines, and ablation of signaling through either receptor using short hairpin RNA (shRNA) toward IR or a neutralizing monoclonal antibody (mAb) specifically directed against IGF-1R (MAB391) resulted in increased phosphorylation of the reciprocal receptor. Furthermore, OSI-906, a selective dual inhibitor of IGF-1R and IR, more effectively inhibited the IRS1-AKT pathway compared with MAB391 in several human tumor cell lines. In xenograft tumors with readily detectable basal levels of phospho–IGF-1R and phospho-IR, dual-receptor inhibition by OSI-906 resulted in enhanced antitumor activity compared with MAB391, where treatment was associated with an increase in phospho-IR. Either insulin or IGF-2 was able to activate the IR-AKT pathway and decrease the sensitivity of tumor cells to selective inhibition of IGF-1R by the anti–IGF-1R mAb. In contrast, activation of the IR-AKT pathway by insulin or IGF-2 was fully blocked by OSI-906. Collectively, these data support the hypothesis that drugs cotargeting IGF-1R and IR, such as OSI-906, may provide superior efficacy compared with mAbs selective for IGF-1R by preventing IR/IGF-1R–mediated compensatory signaling.

Materials and Methods

IGF-1R/IR inhibitors

OSI-906 was synthesized as previously described (9). MAB391, IGFBP3, and the IGF-2–neutralizing antibody were from R&D Systems.

Cell lines

Cell lines were obtained from the American Type Culture Collection or other sources, as indicated in Supplementary Materials and Methods, banked after receipt, and passaged for <6 months before use in experiments. Cell viability was assayed at 72 hours after drug treatment using CellTiter-Glo (Promega Corp.).

Preparation of protein lysates and Western blotting

Lysates for Western blotting were prepared as previously described (42). Antibodies included IGF-1R and IR (1:200 dilution; Santa Cruz Biotechnology); phospho-p42/p44, phospho-AKT(S473), phospho-AKT(T308), phospho-S6, and phospho-PRAS40 (1:1,000 dilution; Cell Signaling Technology); and phospho-IRS1^{Y612} (1:1,000 dilution; Biosource). Where indicated, IGF-1/IGF-2 (40 ng/mL) or insulin (5 or 50 μIU/mL) was added for 5 minutes before lysis. All other lysates were collected from cells growing under basal (10% FCS) growing conditions.

Analysis of RTK phosphorylation via a proteome array

RTK phosphorylation states were determined by Proteome Profiler arrays (R&D Systems) and processed according to the manufacturer's protocol. RTKs included on the array are described in Supplementary Materials and Methods.

Taqman assays

Gene expression assays for *IGF2, IGF1, IGF-1R*, and *IR* (Applied Biosystems) were conducted as described by the manufacturer using 50 ng template. Primer/probe sets used for IR(A) were specific for this receptor isoform as previously described (30). Data were normalized to the fourth quartile expression for a given gene within the 32–cell line panel.

Preparation of IGF-1R and IR(A) direct complementation tumor cell lines

Direct complementation (DC) tumor cell lines for IGF-1R–IGF-2 and IR(A)–IGF-2 were derived from inducible HER2-driven breast primary cultures (43). Using mechanical chopping and enzymatic treatment (collagenase), individual tumor cells were isolated, plated in culture dishes in RPMI 1640 + 10% fetal bovine serum, and propagated for up to six passages.

IR shRNA

GEO tumor cells grown to 20% confluence were treated with shRNA lentiviral particles (TRCN0000000379, Sigma) at a multiplicity of infection of 2. After 48 hours, cells were transferred to medium containing 10 μg/mL puromycin for selection.

In vivo antitumor efficacy studies

Female *nu/nu* CD-1 mice were used for xenograft studies. To assess antitumor efficacy, cells were implanted s.c. in the right flank and established to 200 ± 50 mm^3 before randomization into treatment groups. OSI-906 and MAB391 were administered as indicated according to daily or every third day schedules. Tumor volumes were determined twice weekly from caliper measurements by $V = (\text{length} \times \text{width}^2)/2$. Tumor growth inhibition (TGI) was determined by %TGI = $\{1 - [(T_t/T_0)/(C_t/C_0)]/1 - [C_0/C_t]\} \times 100$, where T_t = tumor volume of treated animal × at time t, T_0 = tumor volume of treated animal × at time 0, C_t = median tumor volume of control group at time t, and C_0 = median tumor volume of control at time 0. Median %TGI was calculated for the entire dosing period for each group. Significant antitumor activity is defined as median %TGI >50%. Rank ANOVA with Dunnett's comparison was used to compare groups, and P values of ≤ 0.05 were deemed statistically significant.

In vivo pharmacodynamic analysis

To assess the ability of OSI-906 or MAB391 to inhibit pharmacodynamic end points in tumor tissue, animals with established tumors of 300 ± 50 mm^3 size were dosed orally with OSI-906 dissolved in 25 mmol/L tartaric acid or i.p. with MAB391 diluted in PBS at indicated doses. Tumor samples were collected, snap frozen in liquid nitrogen, and homogenized in a Precellys-24 homogenizer (MO BioLaboratories) with tumor lysis buffer [1% Triton X-100, 10% glycerol, 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl$_2$, 1 mmol/L EDTA supplemented with protease and phosphatase inhibitor cocktails (Sigma), 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate]. Homogenates were clarified by centrifugation (14,000 × g for 5 minutes at 4°C), and supernatants were analyzed by Western blot or phospho-RTK array.

Results

Tumor cells with elevated expression of genes associated with the IGF-1R/IR signaling axis are sensitive to OSI-906

We sought to determine if gene expression or mutations within the IGF-1R/IR axis were predictive of sensitivity to OSI-906, a small-molecule, reversible, ATP-competitive, dual inhibitor of IGF-1R and IR (Fig. 1A). The synthetic route as well as the mechanism of action for OSI-906 have been described (44, 45). OSI-906 selectively inhibits both IGF-1R (IC$_{50}$ = 35 nmol/L) and IR (IC$_{50}$ = 75 nmol/L) and is far less potent (<50% inhibition at 1 μmol/L) against a broad panel (n = 116) of additional RTKs and other protein kinases (45). A panel of 32 tumor cell lines representing 10 tumor types was selected based on differential sensitivity to OSI-906 in cell proliferation assays. Cell lines were categorized as either sensitive (EC$_{50}$ < 1 μmol/L) or insensitive (EC$_{50}$ > 10 μmol/L) to OSI-906 (Fig. 1B). For sensitive tumor cell lines, growth inhibition by OSI-906 was dose dependent (Fig. 1C). Mutations in *KRAS* or *BRAF* did not preclude sensitivity to OSI-906. Ten of 19 OSI-906–sensitive tumor cells harbored mutations in either *KRAS* or *BRAF*, whereas these mutations were less frequent (3 of 13) in OSI-906–insensitive tumor cells (Fig. 1B). In contrast, mutations in *PIK3CA* were observed in nearly half (6 of 13) of the OSI-906–insensitive tumor cell lines but did not occur in any cell line that was sensitive to OSI-906. IGF-1R and IR couple strongly to the PI3K-AKT pathway, and therefore, mutations resulting in constitutive downstream signaling may mitigate the activity of IGF-1R/IR RTK inhibitors.

Expression of *IGF1, IGF2, IGF-1R*, and *IR* mRNAs was measured by quantitative reverse transcription-PCR. For each gene, expression was normalized to the fourth quartile of expression for that gene within the 32–cell line panel. We then ranked the cell lines according to collective expression of ligands and receptors. Cell lines exhibiting the highest expression of genes in the IGF axis were significantly more sensitive to OSI-906 (P = 0.0004, when comparing IGF axis gene expression in sensitive and insensitive cell lines by two-tailed t test; Fig. 1D). Among 19 OSI-906–sensitive cell lines, 14 exhibited expression of *IGF1* or *IGF2* mRNAs at levels that fell within the top
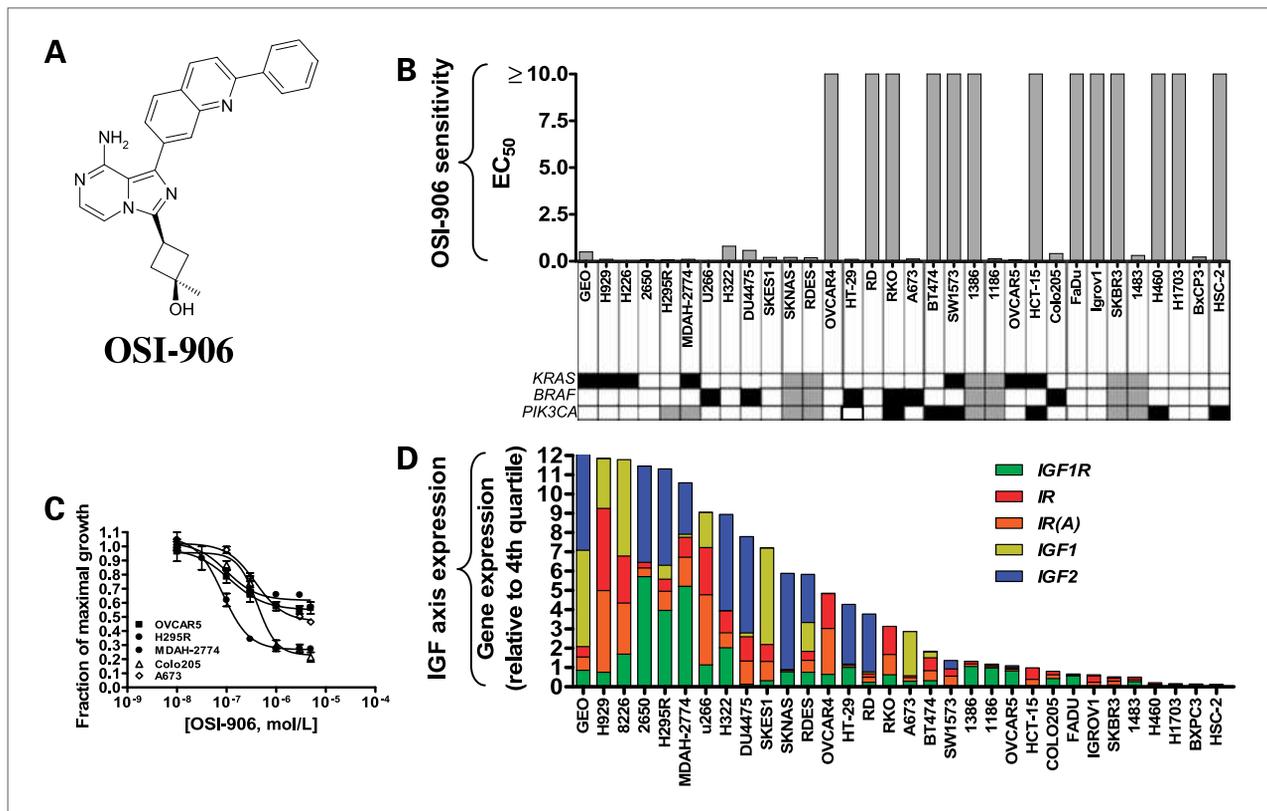
Figure 1. Elevated expression of IGF receptor-ligand pairs is observed among tumor cell lines sensitive to OSI-906. A, structure of OSI-906. B, sensitivity to OSI-906 for a panel of 32 tumor cell lines derived from 10 tumor types, expressed as EC_{50} values. Cell lines were categorized as either sensitive ($EC_{50} < 1$ μmol/L) or insensitive ($EC_{50} > 10$ μmol/L) to OSI-906. Mutational status for *KRAS, BRAF*, and *PIK3CA* is indicated (black = mutant, white = wild-type, gray = unreported), as indicated by Sanger Wellcome database. C, effect of varying concentrations of OSI-906 on cell growth for a panel of five sensitive tumor cell lines. D, expression of *IGF1, IGF2, IGF1R, IR*, and *IR(A)* mRNA by quantitative PCR. Primer/probe sets used for *IR(A)* were specific for this receptor isoform as previously described (1). Gene expression was normalized to the fourth quartile expression for a given gene within the 32–cell line panel.

quartile of expression across the entire 32–cell line panel. Interestingly, expression of *IGF1* and *IGF2* mRNAs was nearly mutually exclusive, with elevated autocrine *IGF1* mRNA expression (top quartile) frequent in tumor cells derived from hematologic malignancies (U266, H929, and 822) or sarcomatoid tumor types (A673, RDES, and SKES), and elevated *IGF2* mRNA expression (top quartile) frequent in tumor cells of epithelial derivation (GEO, HT-29, MDAH-2774, DU4475, and H322). Within the panel, only GEO tumor cells exhibited elevated (top quartile) expression of both *IGF1* and *IGF2*. In 9 of 19 OSI-906–sensitive cell lines, we observed high (top quartile) coexpression of mRNAs encoding ligand (either *IGF1* or *IGF2*) along with mRNAs encoding receptor (either *IGF1R* or *IR*). In contrast, elevated coexpression of ligand and receptor mRNAs was not observed for any of 13 OSI-906–insensitive cell lines evaluated. These data support a model in which elevated coexpression of receptor-ligand pairs in the IGF-1R/IR axis, consistent with tumor cell autocrine signaling, may be predictive for response to OSI-906.

Both IGF-1R and IR(A) are tumorigenic

Both IGF-1R and IR can provide cell growth cues during embryogenesis and can promote a transformed phenotype *in vitro* (23, 31). IGF-1R is also tumorigenic *in vivo*; however, the tumorigenicity for IR has not yet been firmly established. Because data suggest that IR(A) is more potently activated by IGF-2 than IR(B), and because we observed expression of IR(A) in tumor cell lines sensitive to OSI-906, we sought to assess the tumorigenic potential of IR(A) compared with IGF-1R. We used a DC mouse mammary tumor model driven by an inducible human HER2 oncogene under doxycycline-directed expression (46), where repression of *HER2* expression by doxycycline withdrawal was followed by introduction of genes encoding either human IGF-1R or human IR(A), in combination with human IGF-2 (43). Both IR(A) and IGF-1R, expressed in combination with IGF-2, were able to complement tumor growth (Fig. 2A, top). The IGF-1R and IR(A) DC tumors exhibited strong penetrance (>70%) and median tumor-free latency of 7 and 20 days, respectively. These data show that IR(A) is tumorigenic, albeit perhaps
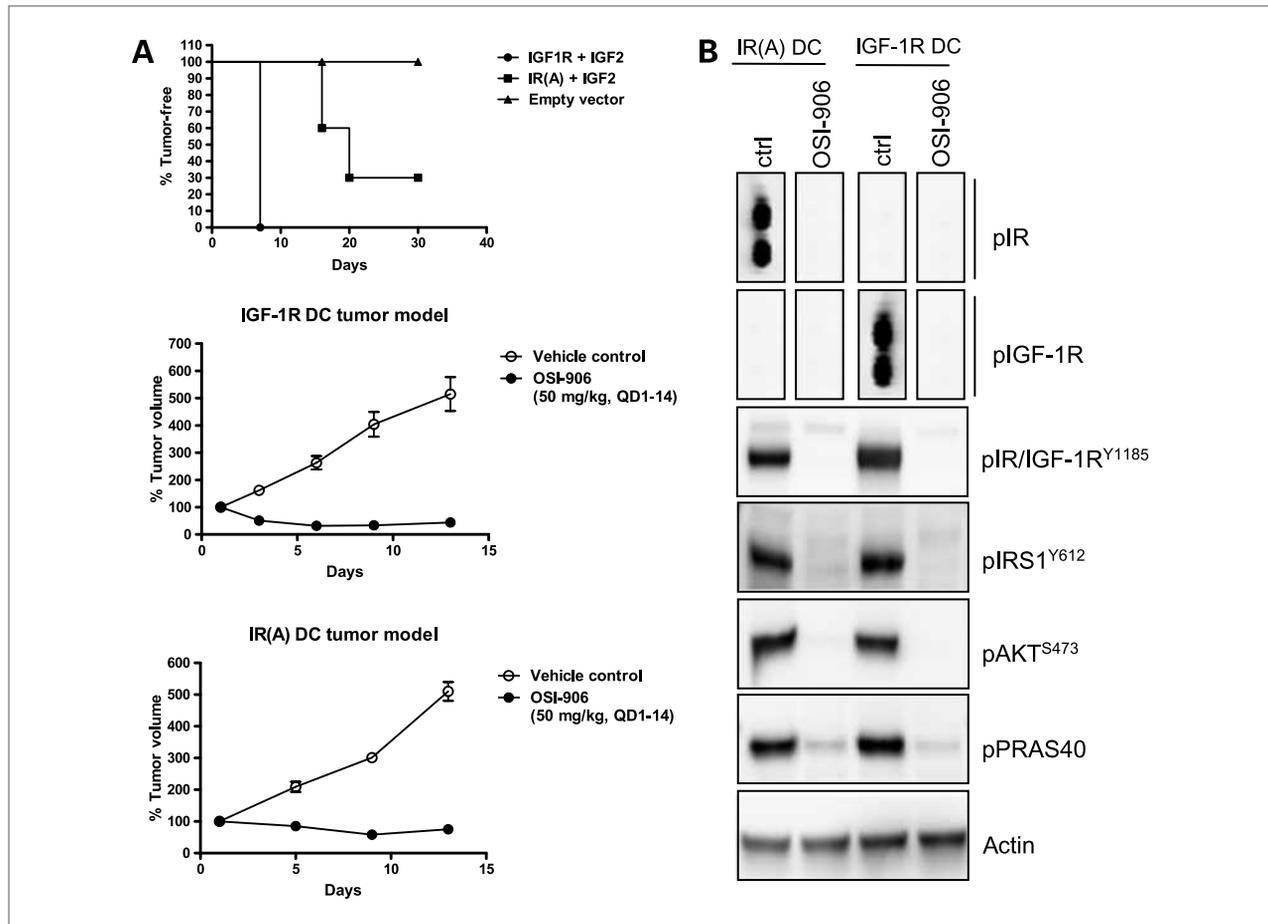
Figure 2. Both IGF-1R and IR(A) are tumorigenic and activate the IRS1-AKT pathway in a mouse mammary tumor model. A, top, effect of introduction of either IGF-1R or IR (+IGF2) on tumor growth, following doxycycline withdrawal, in a mouse mammary tumor model; middle and bottom, effect of OSI-906 treatment on the growth of IGF-1R or IR DC tumors. QD, daily. B, effect of OSI-906 (3 μmol/L) on the phosphorylation of IR or IGF-1R or downstream phosphorylation of IRS1^{Y612}, AKT^{S473}, and PRAS40 for IR and IGF-1R DC cell lines. Phosphorylation of IR and IGF-1R was determined by RTK capture array (top), as described in Materials and Methods, or by a dual phospho–IGF-1R/phospho-IR antibody (Epitomix).

slightly less potent than IGF-1R. The growth of both IGF-1R and IR(A) DC tumors was inhibited by OSI-906, where OSI-906 achieved >100% TGI in each model accompanied by 42% (IR(A) DC) and 68% (IGF-1R DC) tumor regression (Fig. 2A, middle and bottom). Using tumor cells derived from the IR(A) and IGF-1R DC tumors, we found that IGF-1R or IR(A) could independently drive signaling through the IRS1-PI3K-AKT pathway and impart sensitivity to OSI-906 (Fig. 2B; Supplementary Fig. S1). Both the IGF-1R–DC and IR(A)-DC tumor cell lines had detectable levels of phospho-IRS1^{Y612} and phospho-AKT and were sensitive to OSI-906 in proliferation assays (Fig. 2B; Supplementary Fig. S1). Importantly, phospho-IRS1^{Y612} is a docking site for p85-PI3K, acting as a conduit to the AKT pathway. Accordingly, OSI-906 treatment reduced both phospho-IRS1^{Y612} and phospho-AKT in these cell lines (Fig. 2B), and effects were dose dependent (Supplementary Fig. S1). These results indicate that both IGF-1R and IR(A) have tumorigenic

potential and both receptors have the capacity to independently drive tumor cell growth through the IRS1-AKT pathway.

Inhibition of IGF-1R is associated with a compensatory increase in IR signaling

Phospho–IGF-1R and phospho-IR are often simultaneously detectable in human tumor cell lines (Fig. 3A, left). We determined whether coinhibition of IGF-1R and IR was required for maximal inhibition of downstream signaling through IRS1 and AKT. We compared OSI-906 to the selective anti–IGF-1R mAb MAB391 in tumor cell signaling assays measuring the phosphorylation of IGF-1R and IR as well as cytoplasmic signaling intermediates, including phospho-IRS1^{Y612}, phospho-AKT, and phospho-ERK. MAB391 exhibits pharmacologic properties similar to many anti–IGF-1R mAb drug candidates currently in clinical development by inhibiting signaling from both IGF-1R homodimers and IGF-1R/IR

heterodimers but not from IR/IR homodimers. OSI-906 decreased phospho–IGF-1R by >90% and phospho-IR by >50% in each cell line tested (Fig. 3A). MAB391 was similarly effective at decreasing phospho–IGF-1R but only moderately inhibited (50%) phospho-IR in one of nine tumor cell lines tested (Colo205). Interestingly, MAB391 treatment resulted in a substantial increase in phospho-IR in seven of nine cell lines evaluated, supporting a model of compensatory IGF-1R/IR signaling.

The ability of IGF-1R inhibitors to block downstream AKT and ERK signaling is associated with their ability to decrease tumor cell proliferation and survival. In SK-N-AS (neuroblastoma) tumor cells, phospho–IGF-1R but not phospho-IR was detectable and associated with

the ability of either OSI-906 or MAB391 to decrease phospho-AKT levels (Fig. 3B). However, in three of four cell lines with detectable basal phospho-IR and phospho–IGF-1R (H322, H295R, and A673), OSI-906 decreased phospho-AKT or phospho-ERK levels by >80%. In contrast, MAB391 had a minimal effect on phospho-ERK or phospho-AKT levels (<10%). This was especially striking in the H295R advanced colorectal cancer cell line. Despite its ability to promote a 70% to 90% decrease in IGF-1R expression (presumably by internalization and degradation), MAB391 was still unable to maximally decrease phospho-AKT. These data support a role for IR in maintaining downstream signaling when IGF-1R is selectively inhibited.
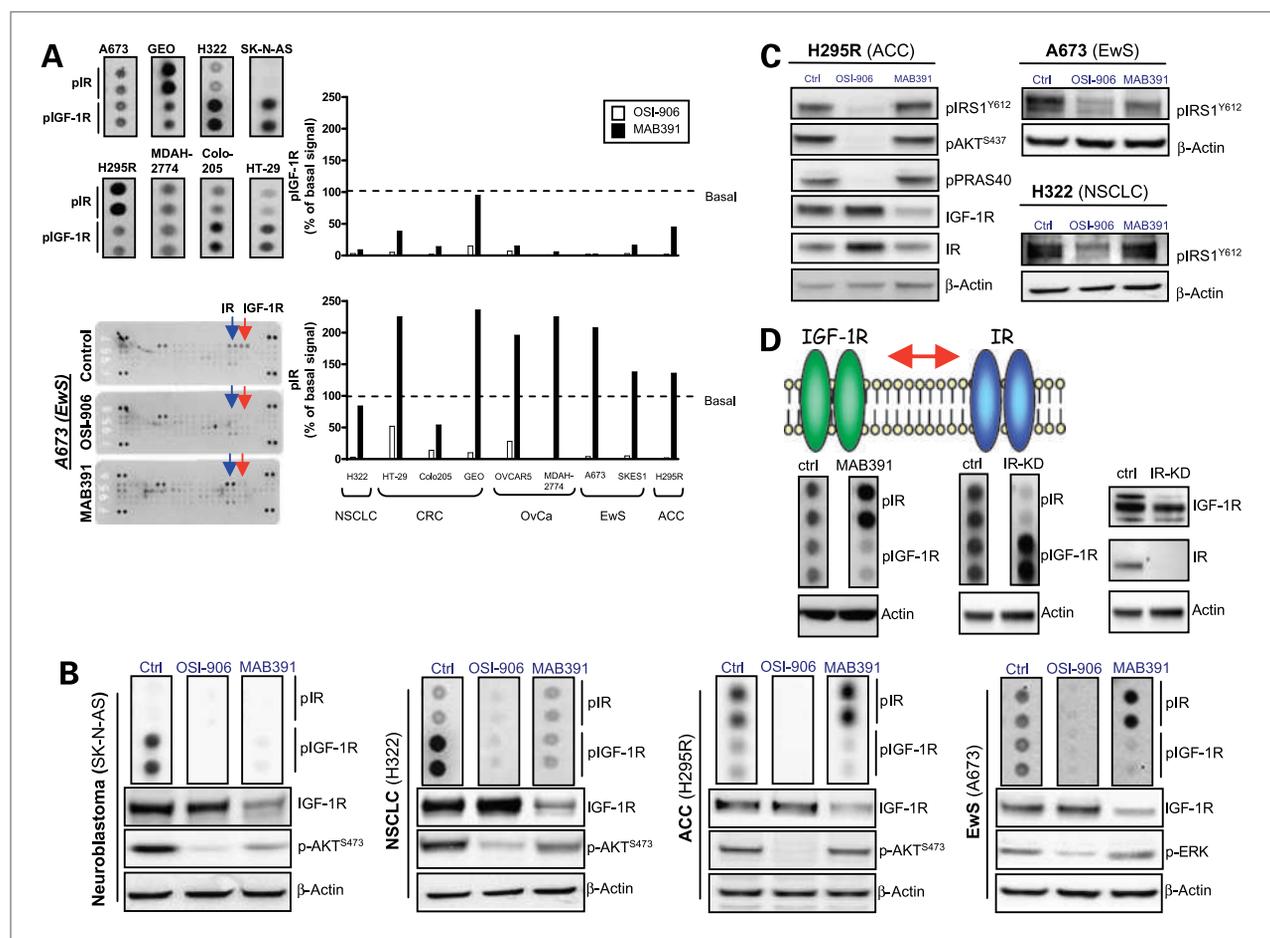
Figure 3. The IGF-1R–neutralizing antibody MAB391 confers a compensatory increase in IR phosphorylation, and cotargeting IGF-1R and IR achieves enhanced inhibition of the IRS1-AKT pathway for select tumor cells. A, top left, phosphorylation of IR and IGF-1R for a group of eight human tumor cell lines under basal growing conditions (10% FCS); right, effect of OSI-906 (3 μmol/L) or MAB391 (3 μg/mL) on phosphorylation of IR and IGF-1R for a panel of nine OSI-906–sensitive tumor cell lines. Data are captured 16 h after dosing and expressed as percentage of basal phosphorylation. Data were realized by RTK capture array as described in Materials and Methods. A set of representative array images is shown for A673 Ewing's sarcoma tumor cells (EwS). NSCLC, non–small cell lung cancer; CRC, colorectal cancer; ACC, advanced colorectal cancer. B, effect of 16-h treatment with OSI-906 (3 μmol/L) or MAB391 (3 μg/mL) on phosphorylation of IR or IGF-1R, total IGF-1R expression, and phospho-AKT^{S437} or phospho-ERK for a panel of four tumor cell lines (H322, SK-N-AS, H295R, and A673). C, effect of OSI-906 (3 μmol/L) or MAB391 (3 μg/mL) on phospho-IRS1^{Y612} for H295R, A673, and H322 cells. Also shown are phospho-AKT^{S473}, phospho-PRAS40, and total IGF-1R and IR levels under basal conditions or on treatment with OSI-906 or MAB391 for H295R cells. Results shown are typical of three or more independent experiments. D, effect of either treatment with MAB391 or generation of stable IR-KD on the phosphorylation of IGF-1R and IR in GEO tumor cells. The control for IR-KD was derived from cells treated with a nonspecific shRNA.

Inhibition of phospho-IRS1^{Y612}, the p85-PI3K docking site, is associated with activity of IGF-1R inhibitors (12), and OSI-906 inhibited phospho-IRS1^{Y612} in the DC models. In A673, H322, and H295R tumor cell lines, OSI-906, but not MAB391, strongly inhibited phospho-IRS1^{Y612} (98%; Fig. 3C). In H295R cells, MAB391 promoted a 70% decrease in total IGF-1R expression levels, consistent with its ability to promote internalization and degradation of this receptor; however, MAB391 had no effect on total levels of IR and resulted in increased phospho-IR (Fig. 3C). For H295R cells, inhibition of phospho-IRS1 and phospho-AKT by OSI-906 (94%), but not MAB391 (6%), was associated with decreased phospho-PRAS40 (98%), a direct substrate of AKT. Interestingly, the induced activation of phospho-IR by MAB391 was not accompanied by further activation of phospho-AKT, suggesting that both IGF-1R and IR likely contribute to activation of downstream AKT signals in tumor cells at the level of IRS1.

To determine if IR was important for cellular growth and if the cross talk between IGF-1R and IR was bidirectional, we generated GEO cells with stable IR knockdown (KD) using shRNA that would allow KD of either IR(A) or IR(B). IR-KD was accompanied by a decrease in proliferation rate of ~50% (data not shown), and although treatment of parental GEO cells with MAB391 promoted an increase in IR phosphorylation, ablation of IR signaling in GEO IR-KD cells was accompanied by an increase in phospho–IGF-1R (Fig. 3D). Collectively, these data indicate that bidirectional cross talk can occur in tumor cells that express both IGF-1R and IR, and targeting both receptors results in greater inhibition of the IRS1-AKT pathway than specifically targeting IGF-1R alone.

Dual inhibition of IR and IGF-1R is associated with enhanced antitumor activity *in vivo*

Dual inhibition of IR and IGF-1R was investigated *in vivo* in two xenograft tumor models: GEO and SK-N-AS. Both express *IGF2* mRNA and similar levels of *IGF1R* mRNA. However, GEO cells, but not SK-N-AS cells, also express *IR(A)* mRNA (Fig. 4A, left). SK-N-AS cells have readily detectable levels of basal phospho–IGF-1R, but not phospho-IR, whereas GEO cells contain high levels of both phospho–IGF-1R and phospho-IR (Figs. 3B and 4A). In SK-N-AS tumors, treatment with OSI-906 resulted in significant median TGI of 71% over the dosing period ($P < 0.001$). MAB391 was also efficacious in this model (median %TGI of 67% over the dosing period; $P < 0.009$). Treatment with a single dose of either OSI-906 or MAB391 resulted in decreased phospho-AKT (Fig. 4A; Supplementary Fig. S2). Similar effects on phospho-PRAS40, a substrate of AKT, were also observed (data not shown). In GEO tumors, treatment with OSI-906 resulted in significant TGI (median %TGI of 80% over the dosing period; $P < 0.004$), whereas MAB391 was inactive, corresponding to a median %TGI of only 8% over the dosing period (Fig. 4A). Both drugs were well tolerated, with minimal (<10%) body

weight loss. The efficacy of OSI-906 in GEO tumors was reflected by decreased phospho-AKT, whereas treatment with MAB391 did not result in decreased phospho-AKT (Fig. 4A; data not shown). For GEO tumors, differential effects of OSI-906 and MAB391 on phospho-AKT correlated with their effects on phospho-IR (Fig. 4B). Although treatment with either OSI-906 or MAB391 resulted in decreased phospho–IGF-1R (>50%), only treatment with OSI-906 resulted in a significant decrease in phospho-IR (>50% for at least 16 hours). In contrast, treatment with MAB391 had no significant effect on phospho-IR for the first 48 hours after dosing, and by 72 hours after dosing, phospho-IR levels increased by >2-fold compared with control tumors (Fig. 4B). These data are consistent with our *in vitro* observations, where treatment with MAB391 resulted in a compensatory increase in phospho-IR. Therefore, in GEO tumors, cotargeting of IGF-1R and IR resulted in enhanced inhibition of phospho-AKT, corresponding with improved TGI. Taken together, the pharmacodynamic and efficacy studies in the GEO and SK-N-AS tumors indicate that inhibition of both IGF-1R and IR may be required for optimal efficacy in cancers where both receptors are present and activated.

OSI-906 inhibits insulin-driven AKT signaling

Elevated insulin is associated with poor prognosis in several tumor types (1, 35, 36). We confirmed that insulin at 50 µIU/mL, a level corresponding to mild fasting hyperinsulinemia, increased both phospho-IR and phospho-AKT, but not phospho–IGF-1R, in HT-29 colorectal cancer cells (Fig. 5A and B). Only OSI-906 fully inhibited phospho–IGF-1R, phospho-IR, and phospho-AKT in HT-29 cells treated with either 5 or 50 µIU/mL insulin, corresponding to normal fasting insulin levels and mild hyperinsulemic levels, respectively. In contrast, MAB391 significantly reduced phospho–IGF-1R in HT-29 but had minimal effects on phospho-IR and phospho-AKT under all conditions tested (Fig. 5A and B). Treatment with IGFBP3, which can neutralize IGF-1 or IGF-2 ligands, but not insulin, resulted in effects on phospho-AKT similar to those observed for MAB391 and far less significant than those caused by OSI-906 (Fig. 5B). These data indicate that even mild increases in insulin levels may provide survival signals to tumor cells, which may mitigate the activity of IGF-1R–selective therapies.

IGF-2 can drive IR-AKT signaling

Increased expression of IGF-2 has been observed in several tumor types, caused in some instances by loss of imprinting (47–53). Because IGF-2 can activate IR, we asked whether it also signals through AKT in an autocrine loop independently of IGF-1R. MDAH-2774 OvCa tumor cells use an IGF-2 autocrine loop and are sensitive to OSI-906 *in vitro*. We treated MDAH-2774 cells with OSI-906 or MAB391 alone or in the presence of insulin, IGF-1, or IGF-2. Insulin (50 µIU/mL) activated IR, but not IGF-1R, as reflected by increased receptor phosphorylation (Fig. 6A). Treatment with 40 ng/mL IGF-1 or
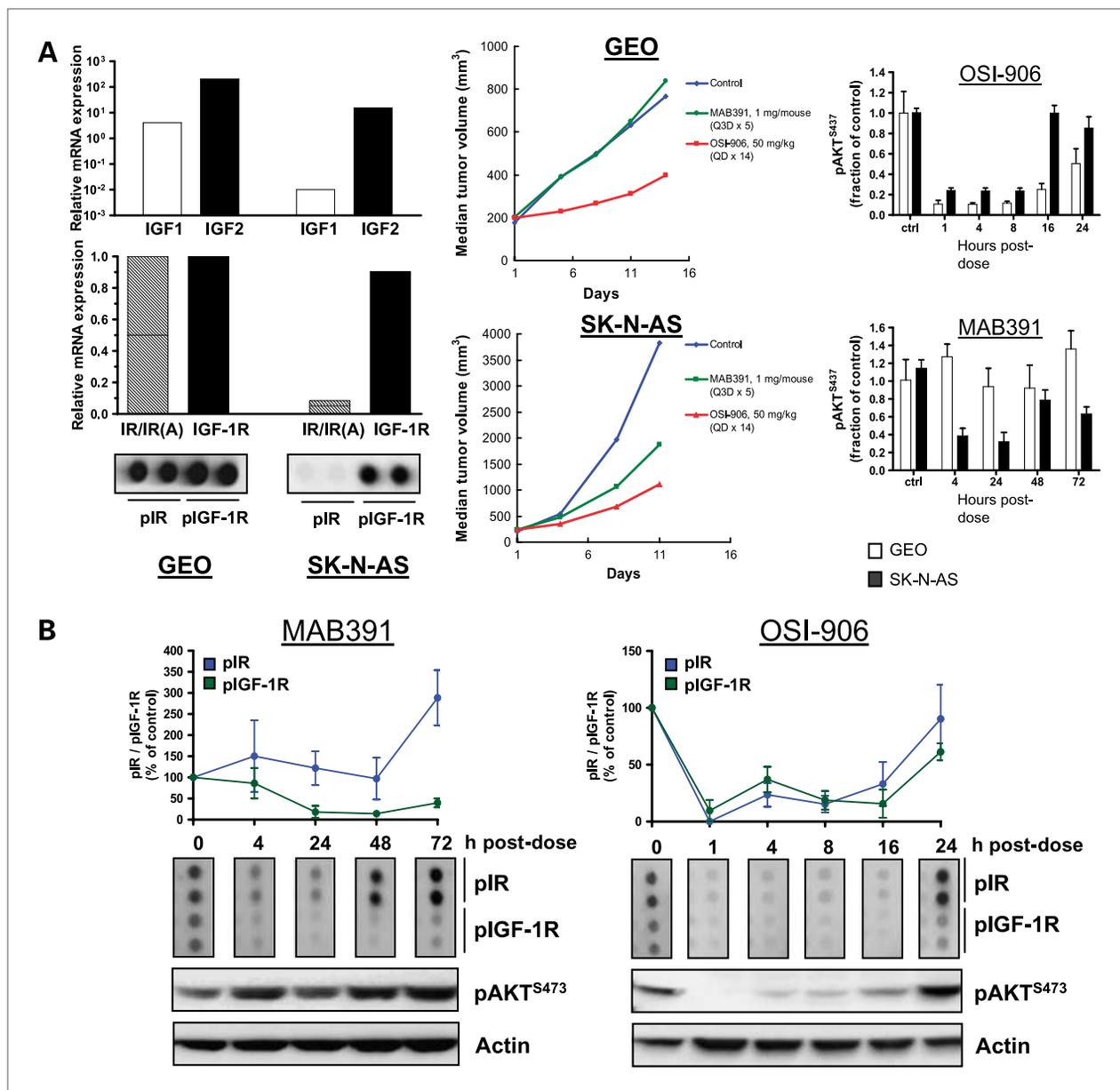
Figure 4. Xenograft tumors coexpressing phospho–IGF-1R and phospho-IR are sensitive to OSI-906 but not MAB391, whereas tumors expressing phospho–IGF-1R and not phospho-IR are sensitive to both OSI-906 and MAB391. A, left, expression of IGF1, IGF2, IGF-1R, and IR(A) as determined by quantitative PCR and expression of phospho-IR and phospho–IGF-1R as determined by capture array. Quantitative PCR for IR was conducted using primer/probe sets that could detect either IR(A) or IR(B), whereas PCR for IR(A) was conducted using primer/probe sets that would specifically detect only IR(A), as previously described (1). Middle, mice bearing SK-N-AS or GEO tumors were treated with either OSI-906 (50 mg/kg daily, orally) or MAB391 (1 mg/mouse every third day, i.p.), as indicated, and tumor growth was determined over a 14-d period. Right, time course pharmacodynamic effect of single-dose OSI-906 or MAB391 on phosphorylation of AKT for GEO and SK-N-AS tumors. B, top, time course pharmacodynamic effect of OSI-906 or MAB391 on the phosphorylation states for IR and IGF-1R *in vivo* for GEO tumors over the dosing period (i.e. 24 h for OSI-906 or 72 h for MAB391). Representative images from the RTK array are shown. Bottom, effects of MAB391 or OSI-906 on tumor phospho-AKT^{S473} over the dosing period.

IGF-2 increased IR and IGF-1R phosphorylation. IGF-1 presumably increased phospho-IR within the context of IGF-1R/IR heterodimers, whereas IGF-2 presumably increased phospho-IR within the context of either IGF-1R/IR heterodimers or IR/IR homodimers. OSI-906 fully inhibited IGF-1R and IR phosphorylation in all cases. Although MAB391 also inhibited phospho–IGF-1R under all conditions, it had varied effects on phospho-IR, which were dependent on the stimulating ligand. Under basal conditions, MAB391 activated phospho-IR by ~2-fold. Insulin (50 µIU/mL) promoted a 7-fold increase in phospho-IR, and this was potentiated to >12-fold when
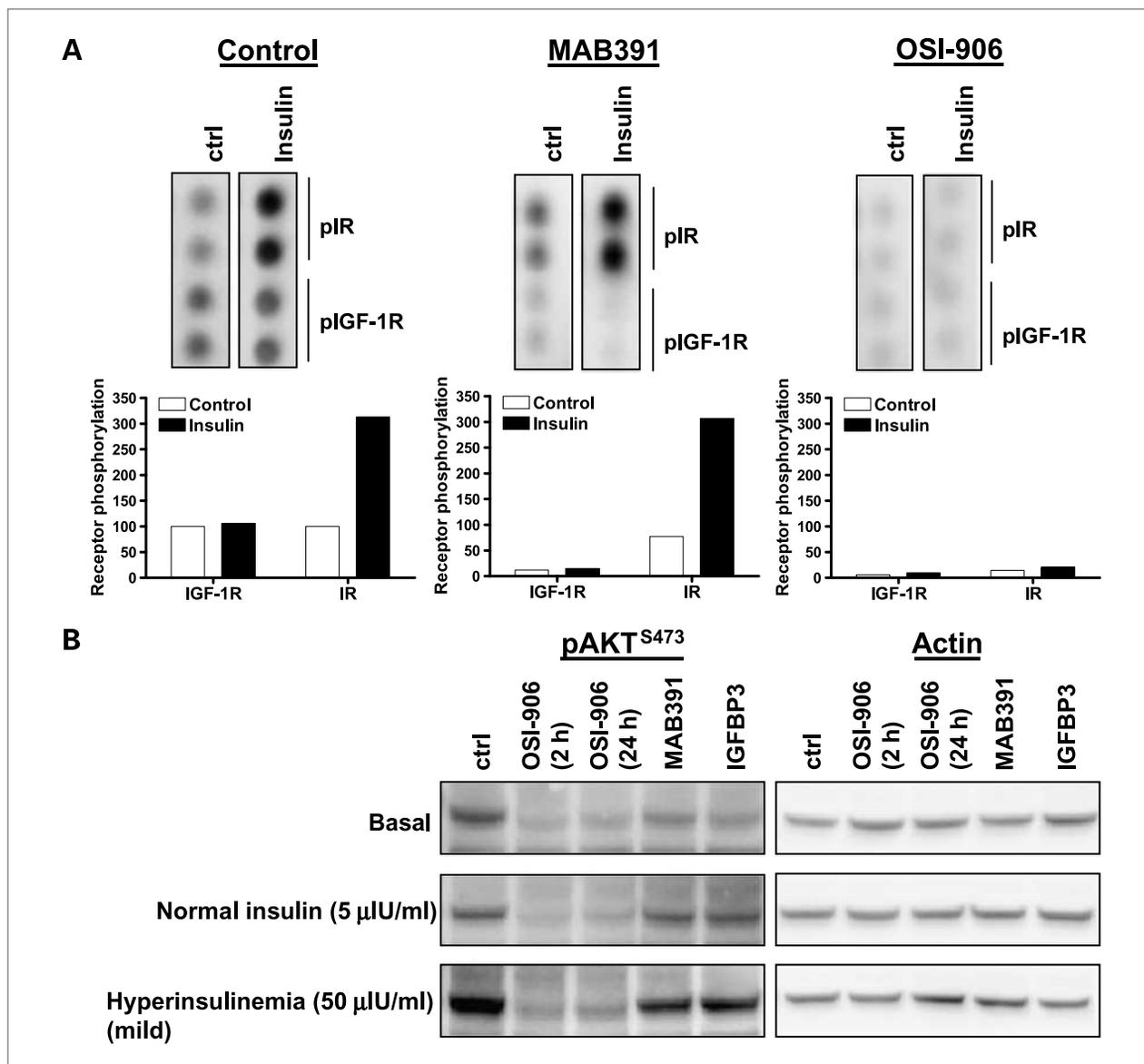
Figure 5. Insulin activation of tumor cell IR-AKT signaling is inhibited by OSI-906 but not MAB391. A, effects on phosphorylation of IGF-1R and IR for HT-29 tumor cells treated with either OSI-906 (3 μmol/L) or MAB391 (3 μg/mL) for 16 h followed by stimulation with 50 μIU/mL (300 pmol/L) insulin for 5 min before cell lysis. Receptor phosphorylation is expressed as percentage of untreated control cells. B, effect of OSI-906, MAB391, or IGFBP3 on phospho-AKT^{S473} for HT-29 cells under basal conditions or following stimulation with 5 μIU/mL (30 pmol/L) or 50 μIU/mL (300 pmol/L) insulin. Results shown are typical of three or more independent experiments, and a representative experiment along with quantitation is shown.

cells were cotreated with MAB391. Both IGF-1 and IGF-2 promoted increased phospho-IR; however, although MAB391 completely inhibited phospho-IR driven by IGF-1, it did not fully inhibit phospho-IR driven by IGF-2. Both ligands promoted downstream AKT signaling. MAB391 fully inhibited IGF-1 stimulation of phospho-AKT (Fig. 6B). However, in cells pretreated with MAB391, IGF-2 could partially rescue AKT phosphorylation. These data indicate that the potential for differential efficacy for agents that specifically inhibit IGF-1R, compared with

those that coinhibit IGF-1R and IR, may be affected by the levels of various ligands available within the intratumoral compartment. Elevated intratumoral concentrations of IGF-2 and/or insulin may indicate that cotargeting of IGF-1R and IR is required for maximal efficacy because both of these ligands can activate IR homodimers.

To further validate IGF-2–driven IR-AKT signaling, we evaluated the ability of an IGF-2–neutralizing antibody to decrease phospho-IR and phospho-AKT. Under basal conditions, MAB391 activated IR in a compensatory manner.

However, neutralization of IGF-2 achieved near-complete inhibition of IGF-1R and IR phosphorylation (Fig. 6C). Furthermore, greater inhibition of phospho-PRAS40 was caused by the IGF-2–neutralizing antibody compared with MAB391. These data indicate that the enhanced activity for OSI-906 against the IR-AKT pathway is specific, and indicate that IGF-2, in addition to insulin, can activate IR signaling in tumor cells to maintain survival signaling.

Discussion

The observation that a range of RTKs can function to drive tumorigenesis has revolutionized anticancer drug discovery and development efforts in recent decades. However, tumor cells exhibit a high degree of signaling plasticity, which can contribute to adaptive survival in the presence of RTK inhibitors, and identifying the mechanisms of acquired resistance for these agents is
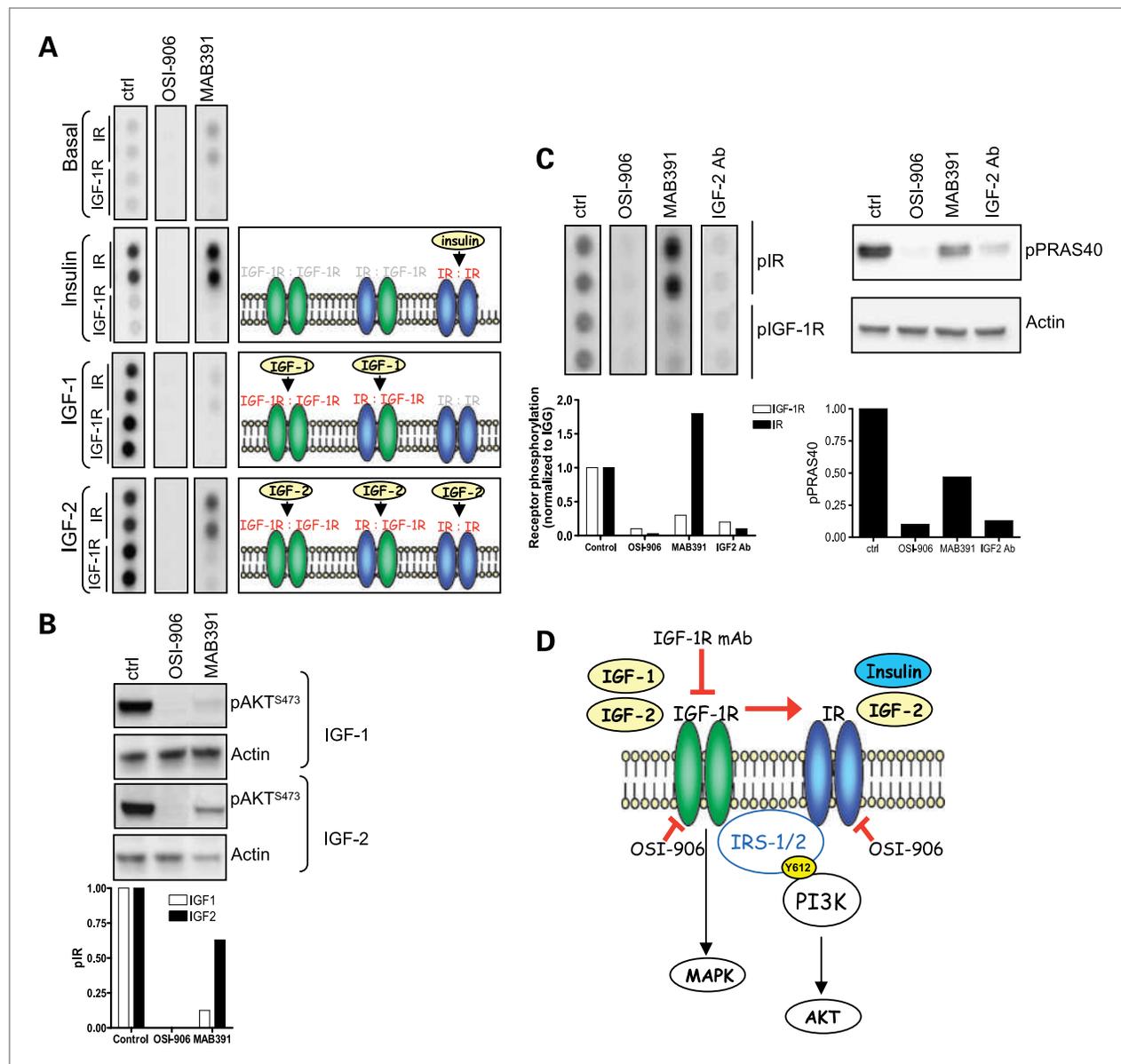
Figure 6. MAB391 inhibits IGF-1–mediated, but not IGF-2– or insulin-mediated, stimulation of phospho-IR and phospho–IGF-1R. A, effect of OSI-906 (3 μmol/L) or MAB391 (3 μg/mL) on phospho-IR and phospho–IGF-1R for control cells or cells treated with insulin (50 μIU/mL, 300 pmol/L), IGF-1 (40 ng/mL), or IGF-2 (40 ng/mL) for 5 min before lysis. B, effect of OSI-906 or MAB391 on phospho-AKT^{S473} in the presence of IGF-1 or IGF-2. C, effect of OSI-906 (3 μmol/L), MAB391 (3 μg/mL), or an IGF-2–neutralizing antibody (10 μg/mL) on phosphorylation of IGF-1R or IR (left) or phospho-PRAS40 (right) for MDAH-2774 cells following 16-h treatment. Results shown are typical of three or more independent experiments. D, cartoon illustrating compensatory signaling through IR that can occur on specific inhibition of IGF-1R.

a major goal toward individualizing their use in the clinic. Multiple RTKs can be activated simultaneously within a single cell, and cross talk can exist between them. Cross talk between EGFR and either IGF-1R or MET can provide adaptive survival for tumor cells when EGFR is targeted individually (38, 39). Preclinical data highlighting reciprocity for these receptor pairs have spurred the evaluation of combinatorial RTK targeting in the clinic for EGFR inhibitors.

There is growing support for IR as a mitogenic driver for tumor cells, and there are several examples in which IGF-1R or IR can compensate for inhibition of the other in nontransformed cells. Indeed, the activity of IGF-2 on IR was first discovered in studying mouse development, where it was found that IR, activated by IGF-2, can compensate for IGF-1R disruption to rescue embryonic growth (24). Other studies have described enhanced signaling by insulin when IGF-1R is disrupted in tumor cells (41). Of particular significance is the observation that IGF-2 activation of the IR(A) fetal isoform can stimulate mitogenic signaling in tumor cells (22, 26–28). We showed here that either IGF-1R or IR(A), expressed along with IGF-2 ligand, can promote tumor growth in a mouse mammary tumor model. Mechanistically, both receptors can activate the IRS1-AKT pathway. Elevated phosphorylation of both IGF-1R and IR has been observed in many human tumor cell lines, and we have shown that IGF-1R/IR cross talk is another means exploited by tumor cells to maintain activation of cell survival pathways when IGF-1R is specifically targeted (Fig. 6D). Of particular relevance is our observation that treatment of tumor cell lines with a selective anti–IGF-1R mAb, MAB391, promoted a compensatory increase in phospho-IR in select cell lines. MAB391 functions similarly to IGF-1R–specific antibodies that are currently in clinical development because it promotes the downregulation of either IGF-1R homodimers or heterodimers with IR; however, it does not promote the downregulation of IR homodimers (7, 54, 55). Using shRNA directed toward IR, we further showed that compensatory signaling between IGF-1R and IR is bidirectional, as ablation of IR was accompanied by an increase in the phosphorylation state of IGF-1R.

In contrast to observations with the IGF-1R mAb, we showed that cotargeting IGF-1R and IR with OSI-906 resulted in enhanced inhibition of the IRS1-AKT signaling pathway. Finally, whereas both OSI-906 and MAB391 achieved efficacy in a human tumor xenograft model expressing only detectable phospho–IGF-1R, only OSI-906 was efficacious in a human tumor xenograft model in which both phospho-IR and phospho–IGF-1R were detectable. We speculate that in such a setting, both IGF-1R and IR are required in tumor cells to mediate growth and/or survival signals, and a dual IGF-1R/IR inhibitor such as OSI-906 may have enhanced efficacy against select tumors compared with an inhibitor that targets only IGF-1R. Although hyperglycemia is evident in mice treated with OSI-906, at maximum tolerated dose, OSI-906 is able to chronically suppress both IGF-1R and IR phosphoryla-

tion in tumor tissue, translating to effects on AKT signaling and TGI (44, 45). In the clinic, hyperinsulinemia has been observed in select patients treated with either IGF-1R–specific antibodies or small-molecule IGF-1R/IR TKIs including OSI-906 (56–58). However, in phase I clinical studies, hyperglycemia was transient and reversible, and OSI-906 was well tolerated with an acceptable safety profile and plasma pharmacokinetic profile that correlates with inhibition of IGF-1R and IR targets in blood.

Hyperinsulinemia has been implicated as an increased risk and poor-prognosis factor for certain cancers, and one hypothesis is that insulin is driving tumor cell survival through IR-AKT signaling. We have determined that treatment with either insulin or IGF-2 could maintain activation of the AKT pathway when IGF-1R was selectively targeted. Insulin concentrations corresponding to mild hyperinsulinemia promoted an increase in phosphorylation of IR and AKT, independent of IGF-1R, and insulin treatment promoted resistance toward inhibition of phospho-AKT by MAB391. Under basal conditions, MAB391 promoted a compensatory increase in phospho-IR in tumor cells by ~2-fold, which was increased further to 12-fold by addition of insulin. IGF-1R–selective drug candidates in clinical development have been shown to provoke an increase in systemic insulin levels (59); therefore, the compensatory increase in phospho-IR in response to an anti–IGF-1R antibody in tumor cells may be further enhanced by increased supplies of endocrine insulin.

IR, in addition to IGF-1R, can also be activated by IGF-2. MAB391 inhibited IGF-1– or IGF-2–stimulated phospho–IGF-1R. However, although MAB391 inhibited IR when activated by IGF-1, presumably mediated by transphosphorylation by IGF-1R within the context of IGF-1R/IR heterodimers, it had little effect on IGF-2–activated IR signaling. Furthermore, for tumor cells pretreated with MAB391, IGF-2, but not IGF-1, could partially rescue AKT signaling. These data indicate that IGF-2–mediated activation of IR homodimers may compensate for activation of the AKT pathway when IGF-1R is individually targeted. Finally, tumor cell lines with IGF-2 autocrine loops were especially sensitive to OSI-906 compared to MAB391.

Collectively, these data support the approach of cotargeting IGF-1R and IR to deliver enhanced and sustained antitumor activity for cancers that rely on signaling through both of these receptors. Moreover, because resistance to IGF-1R–specific antibodies may emerge via increased IR signaling, dual targeting of IGF-1R and IR by TKIs may be efficacious following failure of an anti–IGF-1R antibody. Identifying biomarkers associated with the activation of IGF-1R and IR will be important for optimally leveraging emerging therapeutic agents like OSI-906.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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Compensatory Insulin Receptor (IR) Activation on Inhibition of Insulin-Like Growth Factor-1 Receptor (IGF-1R): Rationale for Cotargeting IGF-1R and IR in Cancer

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