Validation of the type 1 insulin-like growth factor receptor as a therapeutic target in renal cancer

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Abstract

Purpose: Expression of the type 1 insulin-like growth factor receptor (IGF1R) confers adverse prognosis in clear cell renal cell cancer (CC-RCC). We recently showed that IGF1R expression is inhibited by the von Hippel-Lindau (VHL) tumor suppressor, and the IGF1R is up-regulated in CC-RCC, in which VHL is frequently inactivated. We tested the hypothesis that IGF1R up-regulation mediates resistance to cancer therapeutics, evaluating the effects of IGF1R depletion on sensitivity to cytotoxic drugs, which are ineffective in RCC, and the mammalian target of rapamycin (mTOR) inhibitor rapamycin, analogues of which have clinical activity in this tumor.

Experimental Design: This study used CC-RCC cells harboring mutant VHL, and isogenic cells expressing functional VHL. Cells were transfected with nonsilencing control small interfering RNA (siRNA), or with one of two different IGF1R siRNAs. The more potent siRNA was modified by 2′-O-methyl derivatization for in vivo administration.

Results: CC-RCC cells expressing mutant VHL and higher IGF1R were more chemoresistant than cells expressing functional VHL. IGF1R depletion induced apoptosis, blocked cell survival, and sensitized to 5-fluorouracil and etoposide. These effects were significantly greater in CC-RCC cells expressing mutant VHL, supporting the hypothesis that IGF1R up-regulation makes a major contribution to the chemoresistance associated with VHL loss. IGF1R depletion also enhanced sensitivity to mTOR inhibition, at least in part due to suppression of rapamycin-induced Akt activation. Administration of stabilized IGF1R siRNA was shown to sensitize CC-RCC xenografts to rapamycin in vivo.

Conclusion: These data validate IGF1R as a therapeutic target in CC-RCC, and support the evaluation of IGF1R-inhibitory drugs in patients with renal cancer.

Introduction

The type 1 insulin-like growth factor receptor (IGF1R) plays a key role in the regulation of transformation, cell survival, and proliferation. These effects are achieved via recruitment to the IGF1R of docking proteins including insulin receptor substrate-1 (IRS-1), and activation of multiple downstream effectors, including the phosphatidylinositol-3-kinase (PI3K) phosphoinositide-depending kinase-1 AKT and mitogen-activated protein kinase pathways (1). Our recent investigations focused on the regulation of IGF1R expression in clear cell renal cell carcinoma (CC-RCC), a highly chemoresistant tumor characterized by frequent inactivating mutations of the von Hippel-Lindau (VHL) gene (2–4). Our studies revealed that the VHL protein is capable of suppressing Sp-1-mediated IGF1R promoter activation, and destabilizing IGF1R mRNA via interaction with the RNA-binding protein, HuR (5). This represents a new, non-HIF-dependent role for VHL. Consistent with the frequent loss of functional VHL in CC-RCC, we detected significant overexpression of IGF1R mRNA in CC-RCCs compared with paired samples of benign kidney. Although we could detect IGF1R transcripts in all tested samples of CC-RCC tumors (5), IGF1R protein is reportedly detectable by immunohistochemical staining in only ~60% of cases (6), indicating further regulation at the posttranscriptional level. IGF1R positivity on immunohistochemistry has been shown to correlate with higher tumor grade, and poor prognosis even in lower stage disease (6, 7). Moreover, IGF signaling is known to regulate survivin expression, identified as a mediator of poor prognosis in CC-RCC (8, 9). These findings suggest that IGF1R expression in CC-RCC is of biological significance.

We hypothesized that the IGF1R makes a significant contribution to the resistance to clinical therapy that characterizes CC-RCC. We tested this hypothesis by evaluating the effects of IGF1R blockade on sensitivity to cytotoxic and biological therapies. Given the known homology between the IGF1R and the insulin receptor (10), and the ability of many IGF1R drug candidates to block insulin
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Translational Relevance

The data presented in this article indicate that the type 1 insulin-like growth factor receptor (IGF1R) is an important mediator of chemoresistance in renal cancer. IGF1R depletion was shown to enhance sensitivity to two cytotoxic drugs and also to rapamycin, analogues of which have clinical activity in patients with this tumor type. These results support the evaluation of drug candidates that target the IGF1R in patients with renal cancer, as a means of enhancing sensitivity to mammalian target of rapamycin inhibition, and potentially also to chemotherapy.

Materials and Methods

Cell Culture and siRNA Transfection

Human CC-RCC cell lines, 786-0/EV, 786-0/VHL, RCC4/EV, and RCC4/VHL, were obtained from Cancer Research UK Laboratories, Clare Hall, Hertfordshire, United Kingdom. The cell lines had been stably transfected with HA-VHL Laboratories, Clare Hall, Hertfordshire, United Kingdom. The cells were negative for Mycoplasma infection.

RCC cells were transfected at 30% to 50% confluence with HA-tagged wild-type VHL (786-0/VHL, RCC4/VHL) or empty vector (786-0/EV, RCC4/EV; ref. 15). The cells were cultured in DMEM supplemented with 10% FCS and 0.5 mg/mL of G418 (Invitrogen) in a humidified atmosphere of 5% CO2 and 95% air. All cell lines were negative for Mycoplasma infection.

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RCC cells were transfected at 30% to 50% confluence with nonsilencing control (NSC) siRNA (sense strand, 5′-UUCGAGGCUACGUGAGUGTT-3′; antisense strand, 5′-ACGUGACAGCUUCCAGGAAATT-3′) or one of two IGF1R siRNAs targeting different regions of the IGFR1 transcript. Duplex R1 was designed by conventional criteria (16) to target nucleotides 168 to 186 of human IGF1R mRNA (10), and has the sequence: sense strand, 5′-CGAUCAGCAUGCAGCUGAAGATT-3′; antisense strand, 5′-CUUCAGGUCGU-UAGUCUAGTT-3′. The duplex designated R4 targeted IGFR1 mRNA at nucleotides 639 to 657, identified in an array-based screen as a highly accessible region of the IGFR1 transcript (13). This siRNA has the sequence: sense strand, 5′-CAUAGAUCUCAACUCACCAGT-3′; antisense strand, 5′-GCGUAGUUCACUAGUUGTT-3′. All siRNAs were from Qiagen. Cells were transfected with 100 nmol/L of siRNA using OligofectAMINE (Invitrogen) as described previously (5), and were incubated at 37°C for 24 to 48 h before further analysis.

Immunoblotting

Cells were lysed for 30 min on ice in IGF1R lysis buffer containing 50 mmol/L of HEPES, 100 mmol/L of sodium chloride, 10 mmol/L of EDTA, 1% Triton X-100, 4 mmol/L of sodium pyrophosphate, 2 mmol/L of sodium orthovanadate, 10 mmol/L of sodium fluoride with 1.5 mmol/L of Pefabloc SC Plus (Roche Diagnostics), and EDTA-free protease inhibitor cocktail (Roche). Lysates were centrifuged at 13,000 rpm for 15 min to pellet insoluble material, and were analyzed by SDS-PAGE and immunoblotting. Primary antibodies were to IGF-1R (Cell Signaling Technology), HIF-1α (BD Biosciences), and HIF-2α (Abcam), HA tag (Covance), phosphorylated (S612) IRS-1 (clone L7B8; Cell Signaling), phosphorylated (S473) and total Akt (Cell Signaling), phosphorylated (T241/S242) p70 S6 kinase (Cell Signaling), phosphorylated (S240/244) and total S6 peptide (Cell Signaling), β-tubulin (clone TUB2.1; Sigma), and actin (Abcam). IGF1R levels were quantified using ImageJ software and were corrected for β-tubulin or actin loading. IGF1R levels in siRNA-transfected cultures were compared by paired t test or ANOVA using GraphPad Prism v.4.0 software (GraphPad), for paired and multiple samples, respectively.

Apotosis Assay

Forty-eight hours after siRNA transfection, CC-RCC cells were re-seeded at 40,000 cells per well into 96-well plates coated with poly-HEMA as previously described (17) to prevent adherence. Caspase 3/7 activation assays (Apo-1; Promega) were done according to the instructions of the manufacturer, by adding assay substrate, incubating at 37°C for up to 12 h, and analysis on a FluorScan Universal Microplate Spectrophotometer. Apoptosis was expressed as relative fluorescence light units.

MTS and Clonogenic Assays

5-Fluorouracil (5-FU; Sigma) and etoposide (Sigma) were prepared as stock solutions of 42.5 mmol/L, cisplatin (Sigma) was prepared as a 20 mmol/L stock solution, and rapamycin (Sigma), an inhibitor of mTOR (18), was prepared as a 1 mmol/L stock, all in sterile DMSO. These compounds were aliquoted and stored at −20°C, and further diluted in culture medium (DMEM) to the correct final concentration.

Twenty-four hours after siRNA transfection, CC-RCC cells were re-seeded in 96-well plates at 3,000 cells per well, and the following day, were treated with compounds as above or with solvent (DMSO). After 3 days, MTS assays (Promega) were done according to the instructions of the manufacturer, and the absorbance at 490 nm was quantified on a plate reader (Becton Dickinson Labware). The results were expressed as a percentage of OD590 in control cells treated with DMSO without added drug.

Clonogenic assays were done as previously described (14, 17). In brief, 48 h after siRNA transfection, cells were re-seeded into 10 cm dishes at 1,000 to 1,500 cells per dish, depending on the cell line. The remaining cells were...
pelted by centrifugation at 13,000 rpm at 4°C, washed with ice-cold PBS, and lysed for immunoblotting to quantify IGFR1 gene silencing. Clonogenic assay dishes were incubated at 37°C in 5% CO₂ for 10 to 14 days until discrete colonies were visible. For the investigation of drug sensitivity, RCC cells were transfected with siRNAs, reseeded as above, and the following day, were treated with DMSO or drug in DMEM with 10% FCS. After 24 h, the medium was removed and fresh medium without drug was added. Following incubation for 10 to 14 days, visible colonies were fixed in methanol/acetic acid (3:1), stained with crystal violet (400 μg/mL; Sigma), and counted on an automated colony counter (ColCount, Oxford Optonix). Data from MTS and clonogenic assays were analyzed using nonlinear regression to fit the data to a curve, from which IC₅₀ values (dose required to kill 50% of the cells) were calculated. In cells transfected with the R1 and R4 siRNAs, respectively, using Prism v.4.0 software. Changes in drug sensitivity were expressed as fold sensitization, calculated as the ratio of the IC₅₀ value for the control transfectants to the IC₅₀ value for the IGFR1 siRNA transfectants.

Serum Stability Assay

To assess siRNA stability, 200 ng of unmodified or 2′-O-methyl (2′-OMe)–modified siRNAs were added to nine volumes of FCS and incubated at 37°C for 0 to 48 h. Samples were mixed with RNase-free loading buffer and analyzed by electrophoresis on 2% Seakem GTG agarose (NuSieve) gels in parallel with 25 bp DNA size ladder (New England Biolabs). Gels were photographed and digitized using a Fluorchem camera and AlphaEase image analysis software (version 2.0, Alpha Innotech Corporation).

**Results and Discussion**

**IGFR1 Depletion Inhibits CC-RCC Cell Survival and Expression of HIF-1α**

Assessment of IGFR1 levels in each pair of isogenic cell lines confirmed that IGFR1 levels were significantly higher in EV-transfected cells that lacked functional VHL (Fig. 1A), as we previously reported (5). In order to investigate the effects of IGFR1 depletion, human CC-RCC cells were transfected with IGFR1 siRNAs, using two previously validated siRNAs targeting different regions of IGFR1 mRNA (13, 14). The siRNA transfection resulted in inhibition of IGFR1 expression to 27% and 17% of control levels in cells transfected with the R1 and R4 siRNAs, respectively (P < 0.001 for each comparison with NSC transfectants; Fig. 1A). Within each pair of isogenic cell lines, there was a trend to increased resistance to apoptosis and increased clonogenic survival in empty vector (EV)–transfected cells lacking functional VHL, compared with isogenic cells expressing wild-type VHL, but these differences were not statistically significant (Fig. 1B and C). In all four sublines, IGFR1 depletion induced apoptosis upon loss of anchorage, and also inhibited clonogenic survival (Fig. 1B and C). Furthermore, cell survival was inhibited to a greater extent by IGFR1 knockdown in the absence of functional VHL: compared with results in NSC transfectants, cell survival was suppressed by the R4 IGFR1 siRNA to ~25% in 786-0/EV cells and ~50% in isogenic 786-0/VHL cells (P < 0.05). Similarly, IGFR1 depletion had a more profound inhibitory effect on survival in RCC4/EV cells than RCC4/VHL (Fig. 1C). These data suggest that the survival of CC-RCC cells expressing mutant VHL was more heavily dependent on the IGFR1 compared with the survival of cells expressing functional VHL.

RCC4 cells express both HIF-1α and HIF-2α, and it was noted that IGFR1 depletion led to HIF-1α down-regulation both basally, in RCC4/EV lacking functional VHL, and in response to hypoxia in RCC/VHL cells in which a functional response to normoxia was restored by re-expression of wild-type VHL. This effect on HIF-1α is consistent with the ability of IGFs to protect against hypoxia and to activate PI3K-AKT and mitogen-activated protein kinase signaling, known to induce HIF-1α translation and stabilization (19–21). The 786-0 cell line does not express HIF-1α, but both RCC4 and 786-0 express HIF-2α, the levels of which were not influenced by IGFR1 gene silencing (Fig. 1D). This finding is consistent with reports that expression of HIF-2α by breast cancer cells was unaffected by PI3K inhibition (21), although in other cell types, HIF-2α was reported to be IGF-inducible (22, 23).

**IGFR1 Depletion Enhances the Chemosensitivity of CC-RCC**

The ability of IGFR1 gene silencing to induce apoptosis and to block CC-RCC cell survival raises the question of whether IGFR1 targeting can enhance the sensitivity of
CC-RCC cells to cancer therapeutics. In other tumor types, chemosensitivity can be enhanced by blocking IGF signaling (24, 25). Work from our laboratory has shown that IGF1R depletion enhances the chemosensitivity and radiosensitivity of prostate cancer and melanoma (14, 17, 26). However, these tumors are routinely treated with chemotherapy, whereas RCC is intrinsically radioresistant and chemoresistant, and cytotoxic drugs currently play no part in routine treatment (27). The sole chemotherapeutic agent with reported activity in RCC is 5-FU, although recent data...
suggest that the addition of 5-FU to IFNα and interleukin 2 confers no benefit over IFNα alone (28).

Initial assays investigated the effects of IGF1R depletion on sensitivity to 5-FU in 786-0/EV cells that express higher levels of IGF1R than isogenic 786-0/VHL cells (Fig. 1A). Clonogenic assays showed evidence of dose-dependent killing of 5-FU–treated 786-0/EV cells, with greater killing of IGF1R-siRNA–transfected cells than control transfectants at each drug concentration tested (Fig. 2A). Clonogenic and MTS assays gave similar results, with mean IC₅₀ values in control and IGF1R siRNA transfecteds of 26 ± 3 and 13 ± 2 μmol/L by clonogenic assay (2-fold sensitization; \( P < 0.01 \) by paired t test), and 23 ± 6 and 9 ± 3 μmol/L by MTS assay (2.5-fold sensitization; \( P < 0.05 \)). The minor reduction in 5-FU sensitization measured by the clonogenic assay may relate to the fact that clonogenic survival was measured over 10 days, beyond the duration of IGF1R depletion (Fig. 2B), whereas MTS assays were completed within 5 days. Next, we tested...
the effects of IGF1R depletion on sensitivity to two additional cytotoxic drugs, etoposide and cisplatin, which have no clinical activity in RCC. The 786-0/EV cells were highly resistant to the topoisomerase II inhibitor etoposide, with IC₅₀ values in excess of 50 μmol/L, decreasing to 21 ± 4 μmol/L following transfection with R4 IGF1R siRNA (Fig. 2C). There was little change in sensitivity to cisplatin following IGF1R depletion, with reduction in IC₅₀ values by a factor of 1.2 in clonogenic assay and by a factor of 1.7 in MTS assay, effects that were statistically significant (P < 0.05) but modest. Thus, we found evidence that IGF1R depletion induced significant sensitization to 5-FU and etoposide but only a minor reduction in IC₅₀ for cisplatin. This is a different pattern from that observed previously in prostate cancer, in which we found sensitization to DNA-damaging cytotoxic drugs but not to 5-FU or paclitaxel (14). The reasons for this difference are unclear, but could include differences in the spectrum of intrinsic chemosensitivity in these tumor types, and the observation that the IGF axis can influence the repair of DNA damage in some cells (26, 29).

Effect of IGF1R Depletion on Chemosensitivity is Greater in CC-RCC Cells Expressing Mutant Inactive VHL than Isogenic Cells Expressing Functional VHL

As a further test of the hypothesis that IGF1R overexpression contributes to CC-RCC chemoresistance, effects of IGF1R depletion were compared in 786-0/EV and 786-0/VHL cells that express relatively high and low IGF1R levels, respectively. These experiments used 5-FU and etoposide because these were the agents to which IGF1R depletion had sensitized 786-0/EV cells (Fig. 2). It was clear that control-transfected 786-0/EV cells were more resistant to 5-FU than 786-0/VHL (Fig. 3A; IC₅₀ 34 ± 2 versus 8 ± 1 μmol/L; P < 0.001), consistent with the known effects of VHL loss (4). IGF1R depletion induced 3.4-fold sensitization in 786-0/EV cells (IC₅₀ values of 34 ± 2 in NSC transfec-100ants, 10 ± 2 μmol/L in IGF1R siRNA transfectants; P < 0.001), whereas isogenic 786-0/VHL cells showed no significant change in IC₅₀ upon IGF1R depletion (8 ± 1 versus 5 ± 1 μmol/L, not significant). Next, we evaluated sensitiv-

![Figure 3](mct.aacrjournals.org)
etoposide than had been used previously (Fig. 2C), in an attempt to identify the IC₅₀ value in control transfectants. However, 786-0/EV cells showed striking resistance to this drug, with an IC₅₀ of >200 μmol/L, and as in assays of 5-FU sensitivity (Fig. 3A), the isogenic 786-0/VHL cells were more sensitive (IC₅₀ 34 ± 14 μmol/L). Etoposide sensitivity was significantly enhanced following IGF1R depletion, with IC₅₀ values of 31 ± 11 and 19 ± 3 μmol/L in cells transfected with the R1 and R4 siRNAs, respectively, representing at least 6.5-fold and 10.5-fold sensitization. IGF1R depletion also sensitized 786-0/VHL cells to etoposide, with IC₅₀ values in control transfectants of 34 μmol/L, decreasing to 9 μmol/L (P < 0.05) and 5 μmol/L (P < 0.05) using R1 and R4 siRNAs. This represents 3.8-fold and 6.8-fold sensitization, respectively, in 786-0/VHL cells, a significant effect, but of lesser magnitude than that induced in the 786-0/EV cells that lack functional VHL (Fig. 3B). In the clinical setting, maximal plasma concentrations of 20 to 60 μmol/L of etoposide can be attained after short infusions of 50 to 175 mg/m² etoposide, whereas constant infusion schedules achieve steady state levels of ∼5 μmol/L (30, 31). Thus, following IGF1R depletion, IC₅₀ values for etoposide fell into the range that can be achieved clinically.

These findings indicate that CC-RCC cells harboring mutant VHL and relatively high IGF1R are more chemoresistant than cells expressing wild-type VHL, and can be significantly chemosensitized by IGF1R depletion. This supports the hypothesis that IGF1R up-regulation makes a major contribution to the chemoresistance associated with loss of functional VHL. There are several factors that could contribute to this effect. HIF1-α is known to mediate resistance to cytotoxic drugs, at least in part due to a reduction in drug-induced senescence (32, 33). However, the effects of IGF1R depletion documented here must presumably have been HIF-independent, because 786-0 cells lack HIF1-α, and express only HIF2-α, which is not IGF-regulated in these cells (Fig. 1D). Alternatively, the relatively high IGF1R level in 786-0/EV cells could itself render this cell line more sensitive to IGF1R targeting, given that high IGF1R expression has been linked to sensitivity to IGF1R blockade in other tumor models (34, 35).

### Rapamycin-Induced AKT Activation is Abrogated by IGF1R Depletion

We next wished to investigate whether there was any interaction between IGF1R blockade and one of the new generation of kinase inhibitors recently developed for the treatment of RCC (36). Activation of mTOR is a frequent event in CC-RCC (37). Rapamycin analogues have been shown to have objective activity in poor prognosis RCC, inducing significant delay in time to disease progression, although with a low incidence of objective regressions (38, 39). The IGF1R is a potent activator of mTOR via recruitment of IRS-1 and activation of PI3K and phosphoinositide-dependant kinase-1, which phosphorylates Akt on threonine 308 (40). The mTOR kinase exists in two multiprotein complexes, mTOR complexes 1 and 2, in which mTOR is complexed with raptor and rictor, respectively (41). The mTOR complex 2 complex induces S473 phosphorylation and activation of Akt, leading in turn to mTOR complex 1 activation to promote the translation of proteins mediating cellular growth and survival. This is achieved by mTOR complex 1–induced phosphorylation and inactivation of the translational inhibitor 4E-BP1, and by activation of p70 S6 kinase (41). S6 kinase phosphorylates translational targets including the ribosomal S6 peptide (S6), and also serine phosphorylates IRS-1, causing IRS-1 to dissociate from the IGF1R and to undergo proteasomal degradation (42, 43). These molecular events have consequences for the clinical use of mTOR inhibitors, because loss of this negative feedback loop may amplify receptor tyrosine kinase signaling to Akt, potentially mediating resistance to mTOR inhibition.

The effect of rapamycin on mTOR activity was monitored in 786-0/EV cells by measuring phosphorylation of mTOR effectors, revealing the inhibition of S6 kinase and its substrate S6 peptide. This effect was associated with dose-dependent inhibition of IRS-1 serine phosphorylation and increase in S473 Akt phosphorylation (Fig. 4A), suggesting that rapamycin had released mTOR-induced feedback inhibition on IGF1R signaling. Similar enhancement of Akt phosphorylation has been observed following rapamycin treatment in myeloblasts, myeloma, prostate and breast cancer cells in vitro, and in RCC biopsies following clinical mTOR inhibitor treatment (44–46), suggesting that this phenomenon is clinically relevant.

To assess whether IGF1R knockdown could influence signaling induced by rapamycin, control and IGF1R siRNA-transfected 786-0/EV cells were treated with rapamycin, and analyzed by immunoblotting for Akt phosphorylation. Control transfectants showed an increase in Akt phosphorylation in response to rapamycin, compatible with the effect in untransfected cells (Fig. 4A), and this effect was suppressed by IGF1R gene silencing (Fig. 4B). Clonogenic assays were used to assess the sensitivity of 786-0/EV cells to rapamycin, and it was noted that higher concentrations of this drug were required to inhibit cell survival compared with the low nanomolar levels shown to block signaling. Cell killing by rapamycin was enhanced following IGF1R depletion (Fig. 4C): mean IC₅₀ values for control-transfected and IGF1R siRNA–transfected cells were 340 ± 38 and 92 ± 26 nmol/L, respectively (P < 0.05), representing 3.7-fold sensitization. These findings indicate that IGF1R depletion prevented rapamycin-induced Akt activation, and sensitized CC-RCC cells to mTOR inhibition in vitro.

### Administration of Stabilized IGF1R siRNA Sensitizes RCC Xenografts to Rapamycin In vivo

Aiming to assess the effects of IGF1R depletion on the growth of CC-RCC xenografts, we generated modified siRNAs for in vivo delivery. It has been shown that siRNAs can accommodate stabilizing modifications at both base paired and non–base paired positions without significant effects on activity (47, 48). siRNA molecules with one or both strands consisting entirely of 2′-O-Me–modified residues were reported to show enhanced stability but were unable to induce gene silencing, whereas siRNAs incorporating alternate 2′-O-Me modification retained RNA
Similar results were obtained in two additional assays, and mean IC50 values in NSC and IGF1R siRNA.

β-actin loading and expressed relative to solvent-treated controls.

Figure 4. IGF1R depletion suppresses rapamycin-induced Akt activation and sensitizes RCC cells to rapamycin in vitro. A, subconfluent 786-O/EV cultures were treated with rapamycin or solvent for 6 h before lysis and immunoblotting for phosphorylated IRS-1, AKT, p70 S6 kinase, and S6. Similar results were obtained in two sets of independently prepared lysates. B, the 786-O/EV cells were transfected with NSC or IGF1R R4 siRNA. After 48 h, cultures were treated with rapamycin or solvent for 6 h before lysis and immunoblotting for IGF1Rβ and phosphorylated signaling intermediates as in A. B, Bottom, graph shows the analysis of three sets of independently prepared lysates, quantified by densitometry, in which Akt phosphorylation was corrected for β-actin loading and expressed relative to solvent-treated controls. Points, mean; bars, SE; continuous line, NSC; dotted line, R4 (*, P < 0.05; **, P < 0.01). C, forty-eight hours after transfection with control or IGF1R R4 siRNA, 786-O/EV cells were re-seeded for clonogenic assay and treated with rapamycin. The number of surviving colonies in triplicate dishes were expressed as a percentage of survival in solvent-treated dishes. Points, mean of three replicates from an individual experiment; bars, SE. The data were curve-fitted (continuous line, NSC; dotted line, R4) and IC50 values were calculated. Similar results were obtained in two additional assays, and mean IC50 values in NSC and IGF1R siRNA–transfected cultures were 340 ± 38 and 92 ± 26 nmol/L, respectively (P < 0.05).

interference activity and stability to nuclease digestion (47). Therefore, we adopted the strategy of replacement of alternate 2′-hydroxy groups of the ribose sugar ring with 2′-O-Me. We synthesized two variants of the R4 IGF1R siRNA, in which the 2′-O-Me groups were placed either in opposing (R4-1) or alternating (R4-2) positions on the sense and antisense strands of the siRNA (Fig. 5A). In order to assess serum stability, siRNAs were incubated in 100% FCS at 37°C, and were analyzed by agarose gel electrophoresis. As shown in Fig. 5B, the half-life of 2′-O-Me-modified IGF1R siRNA was ~24 hours, compared with ~4 hours for unmodified siRNA. This indicated that 2′-O-Me modification of IGF1R siRNA had significantly increased stability to serum nucleases, consistent with published data (47). It has been reported that modifications that enhance stability could impair RNA interference activity in vitro (47–49). Indeed, there was detectable reduction in efficacy of the modified siRNAs, most notably in the R4-1 duplex (Fig. 5A). The alternating pattern of modification in the R4-2 duplex seemed to be relatively well-tolerated; this sequence retained the ability to silence the IGF1R, and to inhibit clonogenic survival in vitro, comparable with the effect of the unmodified siRNA (Fig. 5C and D). This suggests that 2′-O-Me modification of IGF1R siRNA at specific nucleotides did not compromise the ability of this duplex to induce RNA interference, and therefore, this siRNA was used in subsequent experiments.

To assess the ability of 2′-O-Me–modified and unmodified IGF1R siRNAs to effect in vitro IGF1R gene silencing, 786-0/EV cells were grown as xenografts in immunodeficient mice. When tumors were 6 to 8 mm in diameter, animals were treated with a single i.p. injection of 50 μg unmodified or 2′-O-Me–stabilized control or IGF1R siRNAs, in complex with siPORT Amine delivery agent. After 48 hours, the mice were sacrificed and tumor IGF1R was analyzed by immunoblotting. There was no reduction in IGF1R levels in xenografts from animals treated with unmodified IGF1R siRNA (Fig. 6A). In these small groups of animals, there was some variation in IGF1R levels in xenografts of mice treated with 2′-O-Me–stabilized siRNAs; the mean IGF1R level in animals treated with 2′-O-Me–stabilized R4-2 IGF1R siRNA was ~55% of levels in control-treated xenografts (Fig. 6A). Therefore, we proceeded to evaluate the effects of repeated administration of siRNA, alone and in combination with mTOR inhibition.

Tumor-bearing mice were treated with intraperitoneal 2′-O-Me–modified control or R4-2 IGF1R siRNAs, or with PBS. The administration of siRNA with delivery agent had a
minor (nonsignificant) inhibitory effect on tumor growth compared with PBS-treated controls (Fig. 6B). After 1 week, there was evidence of modest tumor growth delay in the group that received 2'-O-Me–stabilized IGF1R siRNA compared with the control siRNA–treated group (P < 0.05). In the second week, all siRNA-treated animals also received daily rapamycin. This intervention had no detectable effect on the growth of control siRNA–treated tumors, but induced the cessation of growth in the group treated with 2'-O-Me–modified IGF1R siRNA (P < 0.01; Fig. 6B). On the day following the final treatment, all animals were sacrificed, and tumors were analyzed for IGF1R levels and Akt-mTOR pathway activation. IGF1R levels in tumors from control siRNA–treated animals were not significantly different from those in PBS-treated mice. As after a single siRNA administration (Fig. 6A), there was considerable variation in IGF1R levels in tumor tissue from animals treated with 2'-O-Me–IGF1R siRNA, and mean IGF1R levels were 52 ± 10% of levels in control siRNA–treated animals (P < 0.05; Fig. 6C and D). This provides some evidence of in vivo gene silencing, but effects were variable and probably limited by delivery, which is a major issue for the in vivo use of siRNA-based therapeutics (50). There was evidence that rapamycin had blocked mTOR activity, with reduction in phosphorylation of S6 in the tumor tissue of all rapamycin-treated animals (Fig. 6C and D). Rapamycin induced higher levels of Akt phosphorylation in the NSC siRNA–treated animals than were detected in PBS-treated controls, consistent with rapamycin-induced loss of the negative feedback that limits PI3K-Akt activation. However, levels of phosphorylated

Figure 5. IGF1R gene silencing and inhibition of clonogenic survival by 2'-O-Me–modified IGF1R siRNA. A, the sequences of NSC, R4-1, and R4-2 siRNAs, with 2'-O-Me–modified bases underlined. B, to measure serum stability, siRNAs were incubated with 100% FCS at 37°C for the indicated times, and analyzed by agarose gel electrophoresis. Images from three independently prepared sets of samples were quantified, and the results expressed as siRNA remaining as a percentage of the 0 h time point. Points, mean; bars, SE; continuous line, unmodified siRNA; dotted line, 2'-O-Me–modified siRNA. C, the 786-0 cells were transfected with 2'-O-Me–modified NSC, unmodified R4 IGF1R siRNA, or 2'-O-Me–modified R4-1 or R4-2 IGF1R siRNAs. Cells were lysed 48 h later to assess IGF1R levels by immunoblotting. M, mock-transfected; UT, untransfected. D, forty-eight hours after transfection with 100 nmol/L of unmodified or 2'-O-Me–modified control (C) or R4 or R4-2 IGF1R siRNA, cells were re-seeded for clonogenic assay, and remaining cells were lysed for immunoblotting. Left, IGF1R quantification from three sets of independent lysates (individual immunoblot, inset), corrected for loading and expressed as a percentage of IGF1R (columns, mean; bars, SE) in NSC siRNA transfecteds. Black column, NSC siRNA; open column, IGF1R siRNA R4 or R4-2. For both unmodified and modified siRNAs, mean IGF1R levels were lower in cells treated with IGF1R siRNA (*, P < 0.001). The effect was slightly greater in cells transfected with unmodified R4 siRNA, compared with 2'-O-Me–modified R4-2, but this difference was not significant. Right, results of clonogenic assay. Columns, mean colony counts from two independent experiments, each in triplicate (six data points); bars, SE. Both modified and unmodified IGF1R siRNAs inhibited clonogenic survival compared with NSC siRNA (*, P < 0.001), with no significant difference between results for unmodified (R4) and 2'-O-Me–modified (R4-2) IGF1R siRNAs by ANOVA.
Akt were significantly lower in rapamycin-treated animals that received IGF1R-siRNA compared with NSC-treated animals ($P < 0.05$). This suggests that IGF1R depletion sensitized to rapamycin in vivo, and suppressed rapamycin-induced Akt activation, consistent with the in vitro data (Fig. 4). These findings support the evaluation of novel IGF1R-inhibitory drugs in combination with rapamycin analogues in patients with CC-RCC.

In summary, human CC-RCC cells that lack functional VHL showed IGF1R up-regulation, and the survival of these cells seemed more heavily dependent on the IGF1R than were isogenic cells expressing functional VHL. IGF1R depletion enhanced the sensitivity of CC-RCC cells to 5-FU and etoposide, with significantly greater sensitization in cells expressing mutant inactive VHL. Finally, IGF1R gene silencing sensitized RCC cells to rapamycin in vitro. The relatively modest degree of IGF1R depletion achieved in vivo had only a minor effect on tumor growth. However, the combination of IGF1R depletion with rapamycin was capable of arresting the growth of CC-RCC xenografts, at least in part by suppressing the rapamycin-induced Akt activation that followed loss of the negative feedback loop operating via IRS-1. The results of this study validate IGF1R as a therapeutic target in CC-RCC, and highlight the importance of selecting treatment combinations with reference to the biological properties of the target.
Disclosure of Potential Conflicts of Interest

V. Macaulay: Pfizer and Roche expert panel; trials collaboration, Sanofi-Aventis, OSI-Pharmaceuticals. No other potential conflicts of interest were disclosed.

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References


17. Yeh AH, Bohula EA, Macaulay VM. Human melanoma cells expressing V66DE B-RAF are susceptible to IGF1R targeting by small interfering RNAs. Oncogene 2006;25:6574–81.


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