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Insulin-Like Growth Factor-1 Receptor Inhibition Induces a Resistance Mechanism via the Epidermal Growth Factor Receptor/HER3/AKT Signaling Pathway: Rational Basis for Cotargeting Insulin-Like Growth Factor-1 Receptor and Epidermal Growth Factor Receptor in Hepatocellular Carcinoma

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Abstract

Purpose: The insulin-like growth factor (IGF) signaling axis is frequently dysregulated in hepatocellular carcinoma (HCC). Therefore, we investigated whether the specific targeting of the IGF type 1 receptor (IGF-1R) might represent a new therapeutic approach for this tumor.

Experimental Design: Total and phosphorylated levels of IGF-1R were measured in 21 paired samples of human HCCs and adjacent nontumoral livers using ELISA. The antineoplastic potency of a novel anti–IGF-1R antibody, AVE1642, was examined in five human hepatoma cell lines.

Results: Overexpression of IGF-1R was detected in 33% of HCCs and increased activation of IGF-1R was observed in 52% of tumors. AVE1642 alone had moderate inhibitory effects on cell viability. However, its combination with gefitinib, an epidermal growth factor receptor (EGFR) inhibitor, induced supra-additive effects in all cell lines that were associated with cell cycle blockage and inhibition of AKT phosphorylation. The combination of AVE1642 with rapamycin also induced a synergistic reduction of viability and of AKT phosphorylation. Of marked interest, AVE1642 alone up-regulated the phosphorylated and total levels of HER3, the main partner of EGFR, and AVE1642-induced phosphorylation of HER3 was prevented by gefitinib. Moreover, the down-regulation of HER3 expression with siRNA reduced AKT phosphorylation and increased cell sensitivity to AVE1642.

Conclusions: These findings indicate that hepatoma cells overcome IGF-1R inhibition through HER3 activation in an EGFR-dependent mechanism, and that HER3 represents a critical mediator in acquired resistance to anti-IGF-1R therapy. These results provide a strong rational for targeting simultaneously EGFR and IGF-1R in clinical trials for HCC]. (Clin Cancer Res 2009;15(17):5445–56)

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third most common cause of mortality worldwide. Its incidence is increasing in Western countries, mainly due to hepatitis C virus and excessive alcohol consumption. At the time of diagnosis, most of the patients suffer advanced tumor disease and a curative treatment is possible only in a minority of patients and overall survival is poor (1). In this context, the recent development of molecular therapies targeting specific receptors and/or signaling proteins has opened promising perspectives for the treatment of advanced HCC (2, 3). However, until now, the targeting of vascular endothelial growth factor or epidermal growth factor receptor (EGFR) has shown disappointing results in HCC treatment (4) leading to the concept of multitarget therapies. This concept has been recently validated by the approval of sorafenib, a multikinase inhibitor, as a reference treatment of advanced HCC (5).

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Translational Relevance

Based on a recent phase III study (Llovet 2008), sorafenib, a multikinase inhibitor, has been approved in Europe and the United States as the reference treatment for patients with advanced hepatocellular carcinoma (HCC). However, despite the therapeutic advance allowed with sorafenib, medical need for new compounds is still important in this indication. Activation of insulin-like growth factor (IGF) signaling pathways is strongly implicated in the pathogenesis of HCC and, based on preclinical data, clinical trials using IGF type 1 receptor (IGF-1R) inhibitors are currently ongoing. Here, we show that the specific inhibition of IGF-1R using a monoclonal antibody induces a resistance mechanism to this drug in HCC cells via the activation of the epidermal growth factor receptor (EGFR)/HER3/AKT pathway, which could be prevented by a combination of EGFR and IGF-1R inhibitors. Therefore, these results together with our previous data showing cross-talks between EGFR and IGF-1R in HCC (Desbois-Mouthon 2006) provide a strong rationale for IGF-1R and EGFR cotargeting in future clinical trials.

Evidence has accumulated showing that the insulin-like growth factor (IGF) signaling axis is activated in HCC (6, 7): IGF-II may be overexpressed as a result of loss of promoter-specific imprinting and reactivation of fetal promoters; increased amounts of bioactive IGF-II are also the result of a reduced expression of IGF binding protein and/or inactivation of the type 2 IGF receptor, which mediates IGF-II degradation. IGF-II promotes proliferation, survival, and migration in hepatoma cells through binding to its receptor IGF type 1 receptor (IGF-1R; refs. 8–10). This receptor as well as its main substrates IRS-1 and IRS-2 may be also overexpressed in HCC and in experimental models of liver carcinogenesis (11–14).

The potential interest of targeting IGF-1R–mediated signaling pathways in HCC has been suggested both *in vitro* and *in vivo*: first, the blockade of IGF-II overexpression impairs HCC development in murine models (15, 16), and second, inhibition of IGF-1R with a monoclonal antibody (mAb; αIR-3; ref. 17) or with tyrosine kinase inhibitors (TKI; AG1024, NVP-AEW541) reduces HCC cell proliferation and/or increases apoptosis (9, 18). However, we and others recently reported that the combination of AG1024 with an EGFR TKI (erlotinib, gefitinib) resulted in synergistic antineoplastic effects in hepatoma cell lines (9, 19), suggesting that the blockage of either EGFR or IGF-1R may allow the other receptor to compensate for.

Having shown that IGF-1R was overexpressed or hyperactivated in human HCC tumors, the study was then designed to examine the antineoplastic effects of cotargeting IGF-1R and EGFR in HCC cells by using a novel mAb against IGF-1R (AVE1642), currently in phase I/II clinical trials (20), and gefitinib. Although AVE1642 alone had moderate inhibitory effects on cell viability, we observed that its combination with gefitinib exerted supra-additive effects in the five cell lines tested. We characterized the molecular mechanisms of this synergy by showing that (*a*) both the total and the phosphorylated levels of HER3 (erbB-3), the main partner of EGFR, were up-regulated in the presence of AVE1642; (*b*) gefitinib reduced the phosphorylation of HER3 induced by AVE1642, which was paralleled by a marked inhibition of AKT phosphorylation; and (*c*) the down-regulation of HER3 expression using a siRNA strategy also reduced AKT phosphorylation and significantly increased hepatoma cell sensitivity to AVE1642. These data highlight an original compensatory mechanism between IGF-1R and EGFR signaling pathways in hepatoma cells and provide a strong rational basis for targeting simultaneously EGFR and IGF-1R in advanced HCC to prevent the early development of EGFR-mediated resistance.

Materials and Methods

Reagents. AVE1642, a humanized version of the murine mAb EM164 (21), was provided by sanofi-aventis/Immunogen; cetuximab (Erbitux) was purchased from Merck KGaA; and gefitinib was provided by AstraZeneca. Bovine insulin and human recombinant IGF-I and IGF-II were purchased from Sigma-Aldrich.

Cell culture. HepG2 (HBs^{neg}, Rb^{wt}, p53^{wt}, β-catenin^{mut}), Hep3B (HBs^{pos}, undetected Rb, deleted p53, β-catenin^{wt}), and HuH7 (HBs^{neg}, Rb^{wt}, p53^{mut}, β-catenin^{wt}) cells were from the American Type Culture Collection. HuH6 (HBs^{neg}, p53^{wt}, β-catenin^{mut}), and PLC/PRF5 (HBs^{pos}, Rb^{wt}, p53^{mut}, axin^{mut}) were provided by Dr Christine Perret (Institut Cochin, Paris, France). Normal human hepatocytes were isolated from liver tissue obtained from patients undergoing partial liver resection for liver metastasis, and primary cultures were established as previously described (22). Pools of HCC cells resistant to gefitinib were generated as previously reported in non–small cell lung carcinoma cell lines (23). Briefly, HuH6 and HepG2 cell lines were continuously exposed to gefitinib (10 μmol/L) in routine culture medium that was replaced every 4 d. Initially, cell number was dramatically reduced, and for the next 2 mo, the surviving cells were passaged approximatively every 15 d. Cell proliferation slowly increased the next 2 mo and a stable growth rate was reached after a total of 5 mo.

Western blotting and ELISA. The following antibodies were used to detect electrotransferred proteins: phospho-Tyr^{204} extracellular signal-regulated kinase (ERK)1/2 (E-4), ERK1 (C-16), polyADP-ribose polymerase (H-250), IRS-1 (C-20), insulin receptor (IR, β-subunit, C-19; all from Santa Cruz Biotechnology, Inc.), phospho-EGFR (Tyr^{992}), total EGFR, phospho-AKT (Ser^{473}), total AKT, phospho-IGF-1R (Tyr^{1131})/IR (Tyr^{1146}), total IGF-1R (β-subunit), phospho-HER3 (Tyr1289; 21D3), PTEN (138G6), phospho-tyrosine mouse mAb (P-Tyr-100; all from Cell Signaling Technology, Inc., IRS-2, total HER3 (clone 2F12; all from Upstate), and β-actin (clone AC-15; Sigma-Aldrich). Immune complexes were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc.). Total amounts of HER3 were quantified by ELISA according to manufacturer's instructions (R&D systems, Inc.). For HER3 immunoprecipitation, cell extracts (1,000-1,500 μg) were incubated overnight at 4°C with 4 μg of anti-HER3 antibody (clone 2F12) together with 30 μL of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were washed thrice with lysis buffer, resuspended in gel loading buffer, and analyzed by Western blotting.

Cell surface protein biotinylation. Biotinylation of surface proteins was done for 30 min at 4°C using the cell surface protein isolation kit (Pierce Biotechnology) and following instructions from the manufacturer.

Liver tissue specimens. Tumoral and nontumoral liver tissue specimens were obtained from 21 patients with HCC who underwent partial hepatectomy. Histologic analyses of nontumoral livers showed cirrhosis in all cases. Cirrhosis was related to HCV infection (14 cases), to HBV infection (3 cases), and to alcohol abuse (4 cases). Liver tissue samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. This study was done with informed consent of patients in accordance with the French legislation.

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Measurements of IGF-1R expression and phosphorylation in human liver tissue specimens. Liver tissue specimens were homogenized in cell extraction buffer (Biosource, Invitrogen) and centrifuged at 14,000× *g* for 15 min at 4°C. The amounts of phosphorylated (Y1135/Y1136) and total IGF-1R were quantified in supernatants using ELISA following manufacturer's instructions (Biosource, Invitrogen). The anti-phospho(Y1135/Y1136) IGF-1R antibody did not cross-react with phosphorylated IR.

Cell viability. Cells were seeded in 24-well plates (3×10^5 cells per well) and allowed to proliferate for 24 h. Then cells were incubated for a further 72 h in serum-free or in complete medium [10% fetal bovine serum (FBS)] containing or not AVE1642, gefitinib, rapamycin, and/or cetuximab. At the end of the treatment period, cell viability was measured using the MTT assay.

Flow cytometry analysis of cell cycle and apoptosis. Cells plated in 60-mm dishes were exposed to complete medium containing AVE1642 and/or gefitinib for 48 and 72 h. Both adherent and floating cells were collected, washed, fixed in ice-cold 70% ethanol at -20°C, and stained with 20 μg/mL propidium iodide in the presence of 100 μg/mL RNase A for 30 min at 37°C in the dark. DNA content was analyzed by flow cytometry (FACS Calibur, Becton Dickinson). Apoptotic cells with hypodiploid DNA staining were found in the ≪ sub-G_1 ≫ peak.

Reverse transcription-PCR analysis. Total RNA was extracted using RNA Plus lysis solution (MP Biomedicals) and reverse-transcribed as previously described (24). Transcripts coding for IGF-II and β-actin were analyzed by semiquantitative PCR as previously described (14). HER3 mRNA transcripts were quantified by real-time PCR using previously published primers (25). Each sample was normalized on the basis of its 18S mRNA content as reported (24). PCR was done on a LightCycler instrument (Roche Applied Science).

Immunofluorescence microscopy. Cells seeded on glass coverslips were incubated with a 1:50 dilution of a mouse monoclonal anti-HER3 antibody directed against the extracellular domain (Ab-4, clone H3.90.6, Thermo Fisher Scientific) or of a control IgG (Jackson ImmunoResearch Laboratories, Inc.) in HBSS for 1 h at 4°C. Cells were then rinsed and fixed with 4% paraformaldehyde. Cells were blocked and incubated with a 1:200 dilution of FITC-conjugated goat anti-mouse antibody [Alexa Fluor 488 F(ab′)$_2$; Invitrogen] in 1% bovine serum albumin in PBS for 1.5 h at room temperature. The slides were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) for nuclei detection. Fluorescence was visualized using a Leica differentially methylated region immunofluorescence microscope with DFC300 FX digital camera.

Down-regulation of HER3 expression using a siRNA strategy. HER3 expression was down-regulated by using a mixture of four siRNAs targeting human HER3 (ON-TARGET*plus* SMARTpool; Dharmacon). Control siRNA was a pool from Dharmacon (siGENOME nontargeting siRNA pool). Cells plated in 35-mm dishes (35×10^4 cells/dish) or in 24-well plates (15×10^4 cells per well) were cultured for 24 h to 40% confluency and then transfected with 100 nmol/L siRNA using Lipofectamine 2000 (InVitrogen). Twenty-four h later, cells were stimulated or not with AVE1642 for further 48 to 72 h before being harvested for protein extraction or analyzed for cell viability in a MTT assay.

Statistical analysis. Results are given as means ± SEM. Statistical comparison of mean values was done using the Student's *t* test.

Results

The IGF-II/IGF-1R signaling axis is dysregulated in human HCC cell lines and tissue. Five human hepatoma cell lines (HepG2, Hep3B, HuH7, HuH6, and PLC/PRF5) were used in the present study. All these cell lines overexpressed IGF-1R and its substrates IRS-1 and IRS-2 compared with normal human hepatocytes (Fig. 1A). The heterogeneity of IGF-1R bands between cell lines has been reported by others (26) and may reflect differential phosphorylations and glycosylations. IGF-II was more expressed in Hep3B, HepG2, and HuH7 cells compared with HuH6 and PLC/PRF5 cells (Fig. 1B). The overexpression of both IGF-II and IGF-1R suggested the existence of an autocrine stimulating loop in these cells.

IGF-1R status was examined in frozen tumors. The amounts of total and phosphorylated IGF-1R were quantified by ELISA in paired tumoral and nontumoral liver specimens from 21 patients with HCC. The ratio of phospho-IGF-1R to total IGF-1R was calculated to evaluate the activation level of IGF-1R. We observed increased expression of IGF-1R in 7 tumors (33%; Fig. 1C, *left*) and increased activation of IGF-1R in 11 tumors (52%; Fig. 1C, *right*). Of note, phospho-IGF-1R levels did not correlate well with overall IGF-1R levels. These observations fully justified the need for subsequent investigations examining the effects of the specific targeting of IGF-1R in HCC cells.

The anti–IGF-1R antibody AVE1642 specifically inhibits IGF but not insulin signaling in hepatoma cells. As a first step, experiments were conducted in HepG2, HuH7, and Hep3B cells to ensure the specificity of the anti–IGF-1R antibody AVE1642 against IGF signaling. IGF-1R/IR phosphorylation was examined by Western blotting using a dual phospho-IGF-1R (Y1131)/IR (Y1146) antibody. Similar results were obtained in the three cell lines but, for the clarity of the presentation, only data obtained in Hep3B cells are shown in Fig. 2A. In control cells, IGF-I, IGF-II, and insulin (5×10^{-9} mol/L; 15 min) increased the phosphorylation of both IGF-1R/IR and Akt. In the presence of AVE1642 (0.5 μg/mL), the stimulatory effects of IGF-I and IGF-II were markedly inhibited, whereas those of insulin were fully maintained. In addition, we observed that long-term treatment (48 and 72 h) with AVE1642 decreased IGF-1R but not IR expression in HepG2 and Hep3B (Fig. 2B). Therefore, besides its neutralizing effect on IGF-1R, AVE1642 can also promote IGF-1R down-regulation and degradation in hepatoma cells as previously reported in other cancer cell types (21, 27). Altogether, these results show that AVE1642 specifically inhibits IGF but not insulin induction of receptor and AKT phosphorylation.

AVE1642 exerted a moderate antitumoral effect in hepatoma cells. The blockade of IGF-1R by AVE1642 may impact both autocrine and serum-induced cell growth. To examine this point, cell viability was examined in cells cultured for 72 h in serum-free medium or in complete medium (10% FBS) containing or not AVE1642 (0.5 μg/mL). In serum-free conditions (Fig. 2C, *top*), AVE1642 decreased by 41% the viability in HepG2 cells, whereas its effects were less potent in Hep3B, PLC/PRF5, and HuH7 cells (24.5%, 17.9%, and 12% decrease, respectively). AVE1642 was without effect in HuH6 cells. In complete medium (Fig. 2C, *bottom*), similar results were obtained, HepG2 cells being the most sensitive cell line (45% inhibition). Of note, even higher concentrations of AVE1642 (up to 2 μg/mL) did not further inhibit hepatoma cell viability (data not shown). AVE1642 was without any significant effect on viability in normal hepatocytes (Fig. 2D). These findings show that AVE1642 by itself has an inhibitory effect, even if moderate, on both autocrine and serum-induced cell growth in almost all hepatoma cell lines.

Combining AVE1642 to gefitinib induces a synergistic inhibition of proliferation in hepatoma cell lines. Our previous study showing cross-talks between EGFR and IGF-1R (9) led us to examine whether the antineoplastic effect of AVE1642 might be influenced by EGFR in hepatoma cells. To this aim, cell lines were cultured in complete medium and treated with AVE1642 (0.5 μg/mL) in presence or absence of gefitinib. A 2-μmol/L concentration of gefitinib was used for these experiments because in

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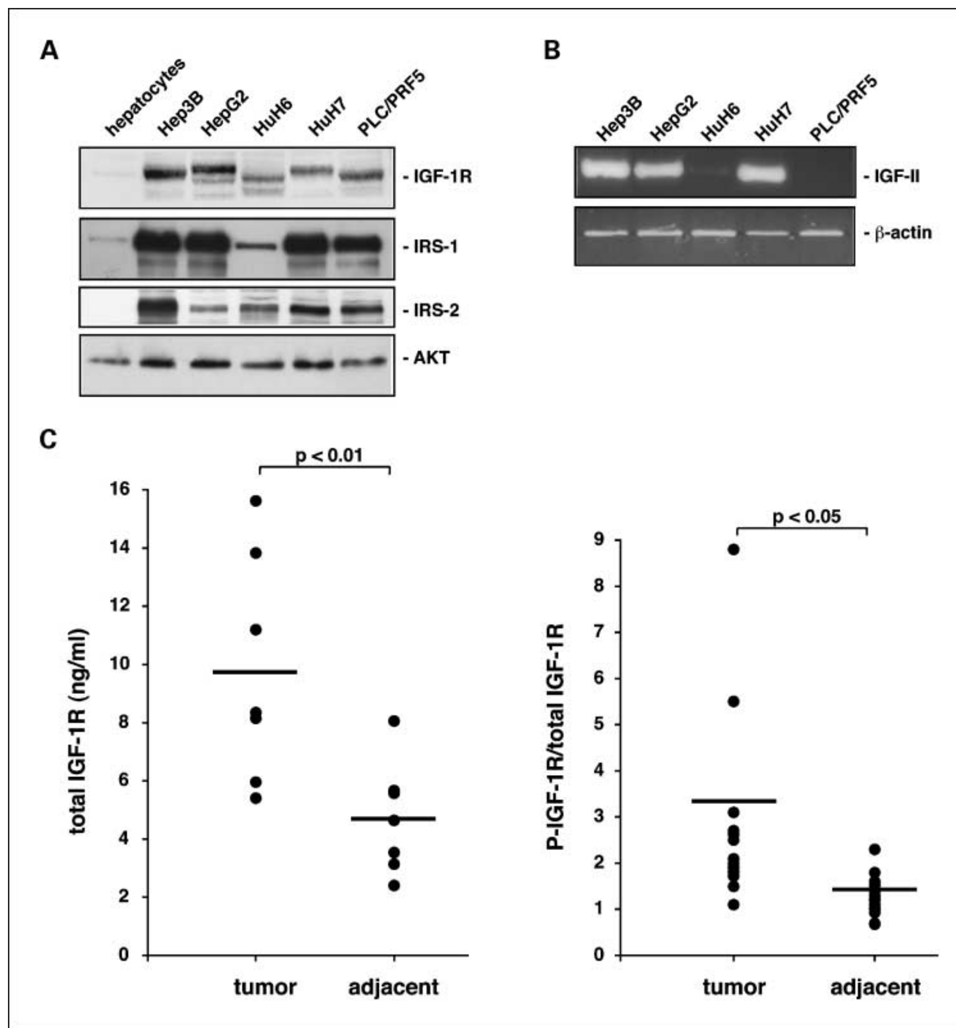

Fig. 1. Expression of components of the IGF-II/IGF-1R signaling axis in human HCC cell lines and tissue specimens. *A,* whole-cell extracts obtained from normal human hepatocytes and HCC cell lines were analyzed by Western blotting for IGF-1R, IRS-1, and IRS-2 expressions. The membrane was reblotted with an AKT antibody to ensure equivalent loading. *B,* total RNA (2 μg) extracted from the five hepatoma cell lines were analyzed by semiquantitative reverse transcription-PCR using human IGF-II primers. β-Actin PCR products were run in parallel to ensure that equivalent amounts of cDNA were amplified. *C,* the levels of phosphorylated (Y1135/Y1136) and total IGF-1R were examined in 21 paired tumoral (*tumor*) and nontumoral (*adjacent*) liver specimens using ELISA.

preliminary studies, this concentration was found to be submaximal on cell viability (data not shown). In addition, this concentration approaches the serum concentrations reported in patients orally treated with gefitinib (400-600 mg/day). We observed that the combination of AVE1642 with gefitinib induced a supra-additive inhibition of cell growth in all cell lines, even in HuH6 cells in which AVE1642 exhibited no effect by its own (Fig. 3A). Similar synergistic effects were obtained when AVE1642 was combined with a submaximal dose of cetuximab, an EGFR-neutralizing antibody (Fig. 3B).

To discriminate whether the synergistic reduction in cell viability induced by AVE1642 *plus* gefitinib resulted from reduced proliferation and/or to increased cell death, we did flow cytometric analyses. Forty-eight-hour treatment with the AVE1642/gefitinib combination decreased the percentage of HepG2 cells in the S phase and arrested cells in the G_0-G_1 phase of the cell cycle (Fig. 3C). No increased rate of apoptosis (% of sub-G_1 cells after 48 and 72 hours of incubation) was evidenced in these experimental conditions (data not shown; Fig. 3C). In addition, cell incubation with a nontoxic dose (10 μmol/L) of the pancaspase inhibitor ZVAD-fmk did not alter the synergistic effect of AVE1642 combined to gefitinib on cell viability (data not shown). Altogether, these findings indicate that the combination of AVE1642 and gefitinib synergistically inhibits hepatoma cell viability through cell cycle inhibition without apoptosis induction.

The synergistic effect of AVE1642 combined with gefitinib routes through the down-regulation of AKT signaling. As a next step, we analyzed the molecular mechanisms whereby AVE1642 and gefitinib had a synergistic inhibitory effect on hepatoma cell proliferation. Cells were treated for 48 hours in the presence of AVE1642 combined or not with gefitinib and then stimulated for 20 minutes with 10% FBS. Similar results were obtained in the five cell lines. When used alone, AVE1642 slightly decreased serum-induced AKT phosphorylation (by about 1.5- to 2-fold), whereas gefitinib had no effect (Fig. 4A). When AVE1642 was combined with gefinitib, serum-induced AKT phosphorylation was markedly reduced (by about 3- to 5-fold) compared with the effects of each drug used alone. In contrast, the combination of drugs had no effect on serum-induced ERK phosphorylation (Fig. 4A). This strongly suggested that the inhibition of AKT signaling rather than the inhibition of ERK signaling contributed to the growth inhibitory effect of AVE1642 combined with gefitinib. Down-regulation of AKT phosphorylation upon treatment with the AVE1642/gefitinib combination did not result from the modulation of PTEN expression (Fig. 4A). The combination

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of AVE1642 with rapamycin (1 nmol/L), a specific inhibitor of mammalian target of rapamycin (a downstream target of AKT), also induced a synergistic inhibition of cell viability in hepatoma cells (Fig. 4B). Previous studies conducted in diverse cancer cell types (28–30) have shown that rapamycin triggers a rapid feedback mechanism resulting in AKT activation and which may be dependent on IGF-1R signaling. We examined whether such a mechanism also occurred in HCC cells and, if so, whether AVE1642 had the potential to block it. Rapamycin alone (1 and 10 nmol/L) markedly increased the phospho-AKT content in HCC cell lines and this effect was abolished by AVE1642 (Fig. 4C). These findings indicate that rapamycin up-regulates the AKT pathway in HCC cells through an IGF-1R–dependent pathway.

AVE1642 increases HER3 phosphorylation and expression in hepatoma cell lines. AKT activation by EGFR is due to the ability of this receptor to dimerize with and to transphosphorylate the kinase-inactive receptor HER3 (ErbB-3), leading to the recruitment

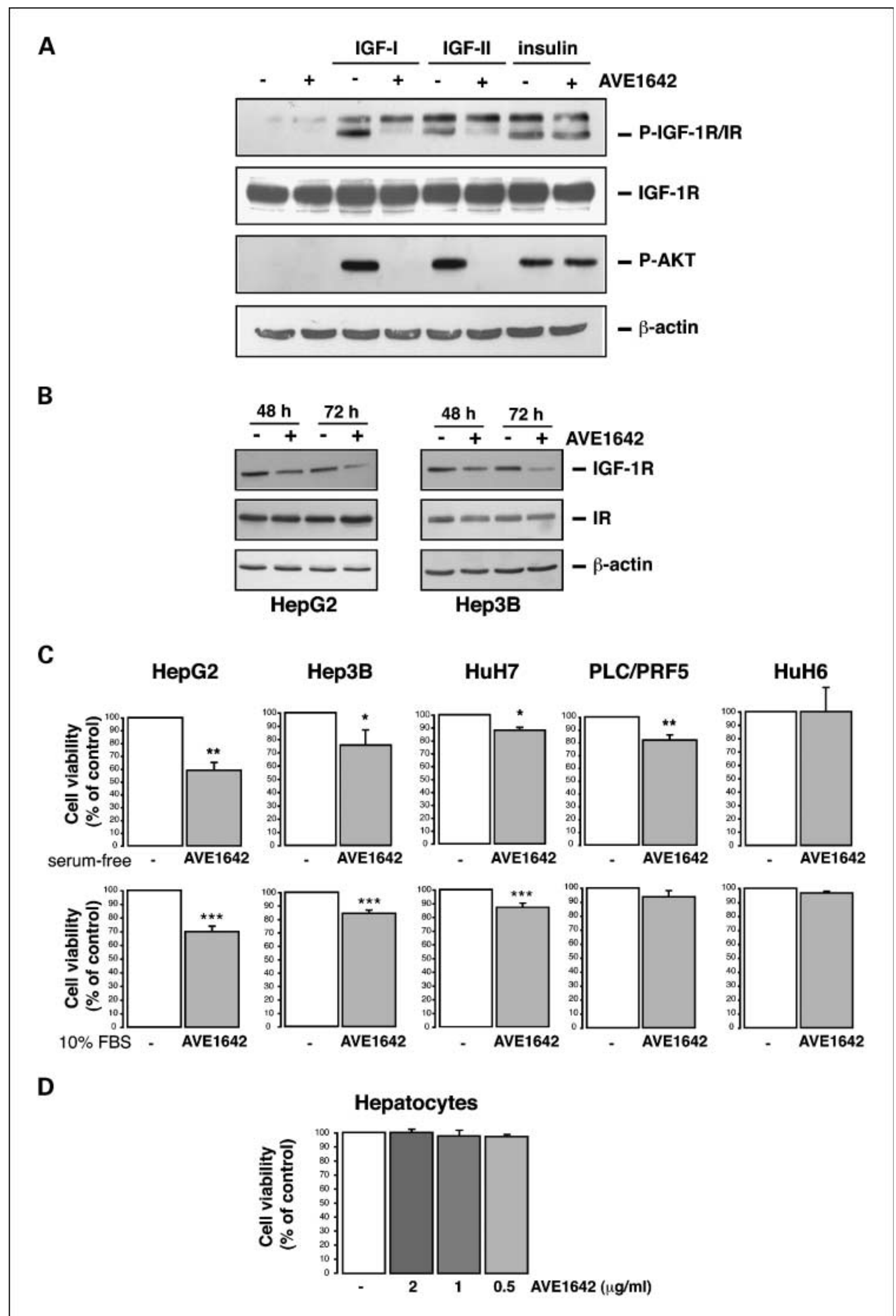

Fig. 2. Effects of the anti–IGF-1R antibody AVE1642 on IGF and insulin signaling in hepatoma cells. *A,* Hep3B cells were treated for 1 h with or without AVE1642 (0.5 μg/mL) and then stimulated for 15 min with IGF-I, IGF-II, or insulin (10^{-9} mol/L). Whole-cell extracts were analyzed by Western blotting for levels of phosphorylated and total IGF-1R/IR and AKT. *B,* HepG2 and Hep3B cells were treated with AVE1642 (0.5 μg/mL) for 48 and 72 h and total expression levels of IGF-1R and IR were assessed by Western blotting. Blots are representative of two independent experiments. *C,* HCC cell lines were treated for 72 h with or without AVE1642 (0.5 μg/mL) in serum-free (*top*) or in complete (*bottom*) medium and then cell viability was measured. *D,* normal hepatocytes were treated for 72 h with increasing concentrations of AVE1642 (0-2 μg/mL) and cell viability was measured. *Columns,* means of three to four independent experiments done in quadruplet; *bars,* SEM. *, $P < 0.05$; **, $P < 0.002$; ***, $P < 0.001$ compared with untreated cells.

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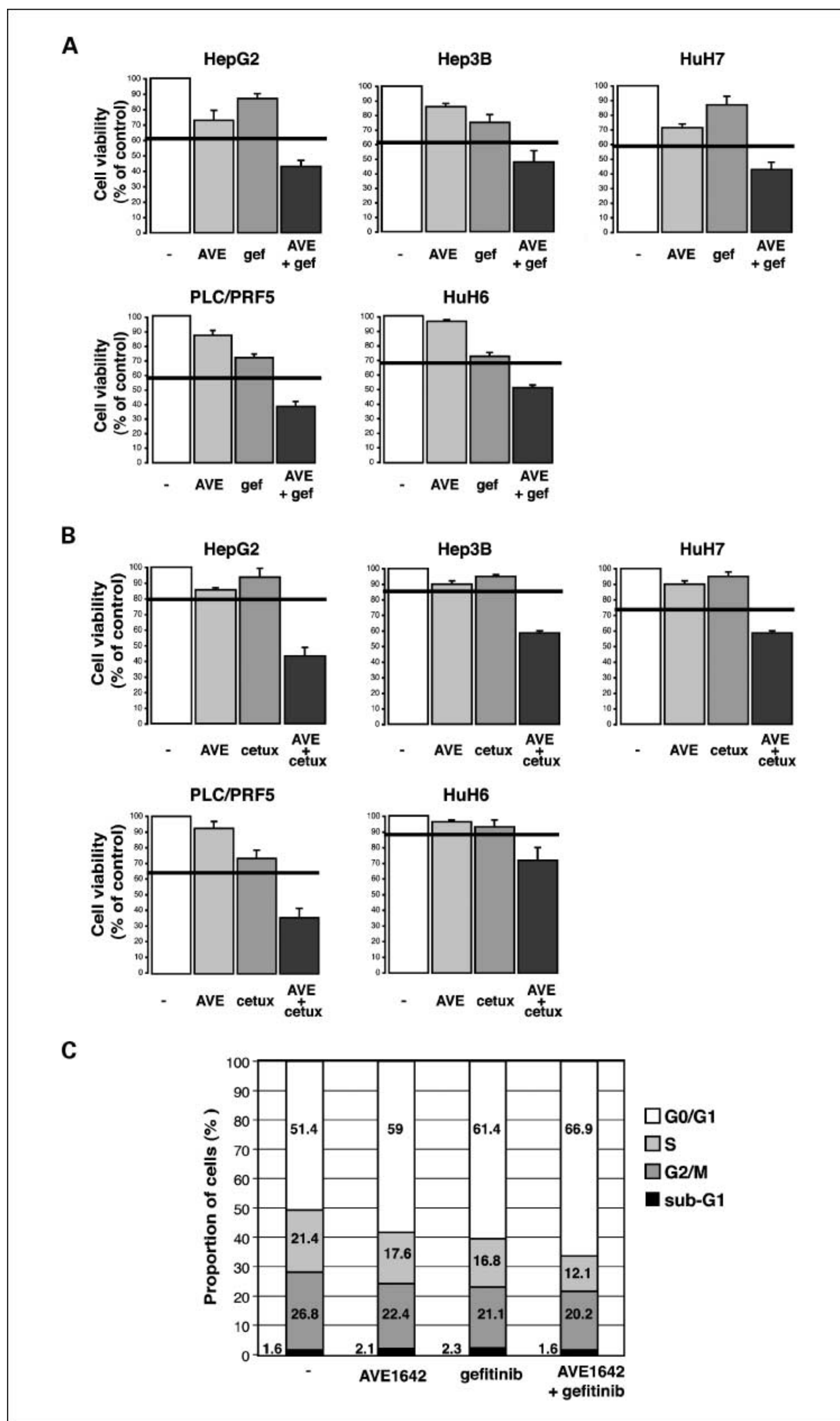

Fig. 3. Antitumoral effects of AVE1642 alone or in combination with gefitinib in hepatoma cell lines. *A,* cell lines were treated for 72 h with or without AVE1642 (*AVE*; 0.5 μg/mL) and/or gefitinib (*gef*; 2 μmol/L) in complete medium, and then cell viability was measured. *B,* the same experiments were done with cetuximab (*cetux*; 1-2 μg/mL) instead of gefitinib. *Columns,* means of three independent experiments done in quadruplet; *bars,* SEM. Black lines, the additivity threshold. *C,* HepG2 cells were treated for 48 h with or without AVE1642 (0.5 μg/mL) and/or gefitinib (2 μmol/L) and analyzed for cell cycle repartition by flow cytometry. A representative experiment out of two is shown.

of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) to HER3 and subsequent activation of PI3K (31). Because AKT phosphorylation was almost completely abrogated in the presence of AVE1642 and gefitinib but not in the presence of AVE1642 alone, we examined by Western blot analyses whether the EGFR/HER3/AKT pathway may be modulated by AVE1642. All hepatoma cell lines expressed EGFR and HER3, whereas HER-2 (ErbB-2) expression was barely detected (data not shown). HepG2

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and HuH7 cell stimulation with 10% FBS induced the tyrosine phosphorylation of both EGFR and HER3 and these effects were inhibited by gefitinib (Fig. 5A). In the presence of AVE1642 alone, the phosphorylation of EGFR and more especially that of HER3 were further increased. The stimulatory effects of AVE1642 on both EGFR and HER3 were strongly inhibited by gefitinib (Fig. 5A). AVE1642 increased HER3 protein expression, although it did not modify the amounts of EGFR (Fig. 5A). These findings were also observed in Hep3B and PLC/PRF5 cells. Increased tyrosine phosphorylation of HER3 parallel to increased expression of HER3 was also seen in HER3 immunoprecipitates obtained from HCC cells treated with AVE1642 (Supplementary Fig. S1). An accurate measurement of HER3 protein amounts done by ELISA showed that AVE1642 increased HER3 by 1.5-, 2.7-, and 4.6-fold in HepG2, HuH7, and PLC/PRF5 cells, respectively (Fig. 5B). Immunofluorescence microscopy (Fig. 5C) and surface protein biotinylation (Fig. 5D) showed that AVE1642 increased the amount of membrane-associated HER3. No increase of HER3 expression was observed in normal hepatocytes treated with increasing doses of AVE1642 (Supplementary Fig. S2). Altogether, these findings show that, in all tested hepatoma cells, the specific targeting of IGF-1R stimulates a cross-talk toward the EGFR signaling pathway via the up-regulation of the total HER3 cellular content. Interestingly, a reciprocal up-regulation of IGF-1R signaling consecutive to EGFR inhibition was not observed in HCC cell lines after short-term and long-term treatments with gefitinib. Thus, a 48-hour treatment with gefitinib (5 and 10 μmol/L) modified neither the phosphorylated nor

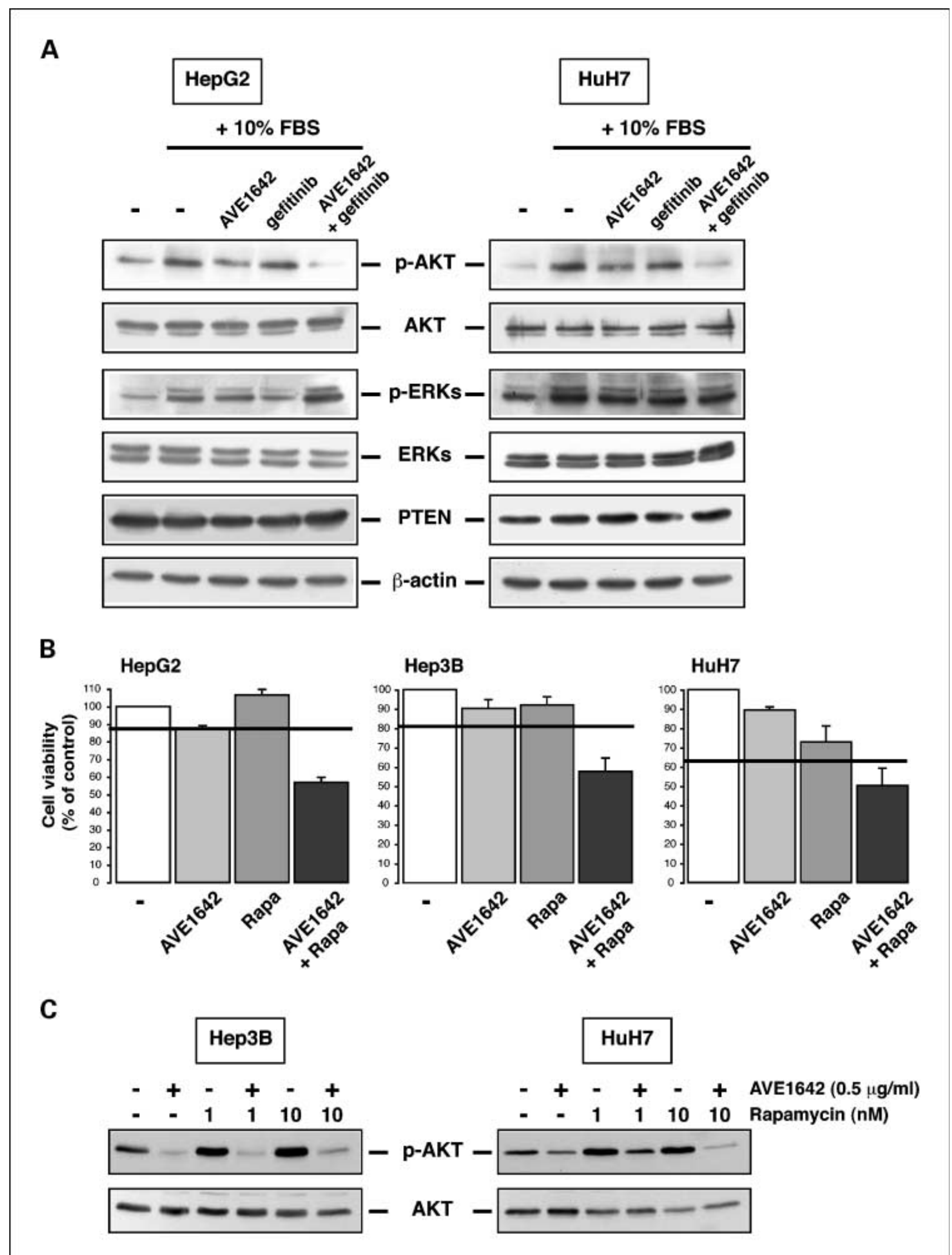

Fig. 4. Effects of AVE1642 combined with gefitinib on AKT and ERK signaling in hepatoma cells. *A,* serum-starved HepG2 and HuH7 cells were treated for 48 h with or without AVE1642 (0.5 μg/mL) and/or gefitinib (2 μmol/L) and then stimulated with 10% FBS for 20 min. Whole-cell extracts were analyzed by Western blotting for phosphorylated and/or total AKT, ERKs, and PTEN. *B,* HepG2, Hep3B, and HuH7 were treated for 72 h with or without AVE1642 (0.5 μg/mL) and/or rapamycin (rapa; 1 nmol/L) in complete medium, and cell viability was measured. *Columns,* mean of three independent experiments done in quadruplet; *bars,* SEM. Black lines, additivity threshold. *C,* Hep3B and HuH7 cells were pretreated for 2 h with or without AVE1642 (0.5 μg/mL) and then incubated or not with rapamycin (1 and 10 nmol/L; 3 h). Whole-cell extracts were analyzed by Western blotting for phosphorylated and/or total AKT levels. Blots are representative of two independent experiments.

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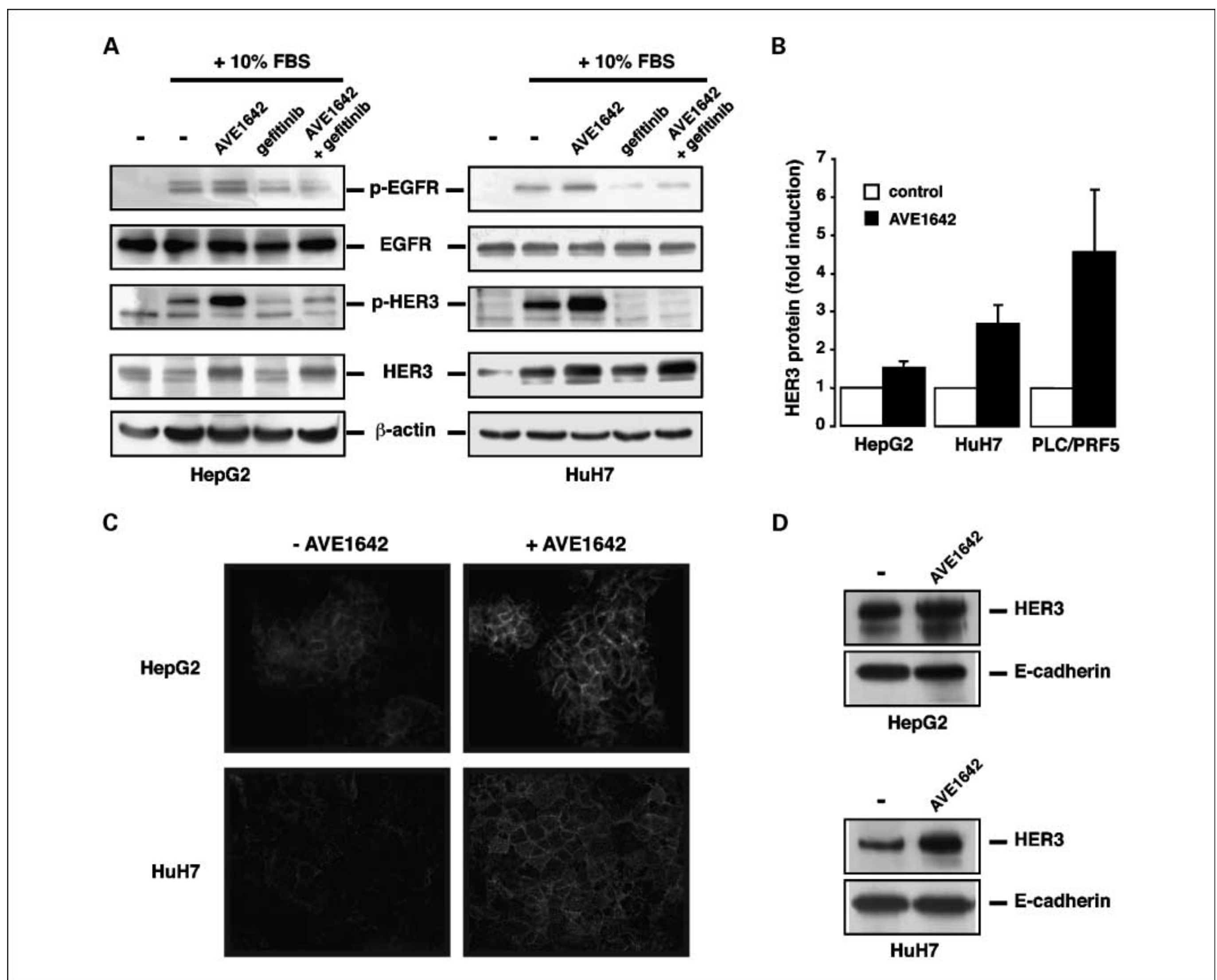

Fig. 5. Effects of AVE1642 on EGFR/HER3 signaling in hepatoma cells. *A,* serum-starved HepG2 and HuH7 cells were treated for 48 h with or without AVE1642 (0.5 μg/mL) and/or gefitinib (2 μmol/L) and then stimulated with 10% FBS for 20 min. Whole-cell extracts were analyzed by Western blotting for phosphorylated and total expression levels of EGFR and HER3. *B,* serum-starved HepG2, HuH7, and PLC/PRF5 cells were treated for 48 h with or without AVE1642 (0.5 μg/mL) and the levels of total HER3 were quantified for by ELISA. *Columns,* mean of three independent experiments; *bars,* SEM. *C,* HepG2 and HuH7 cells treated or not with AVE1642 for 24 h were incubated with an anti-HER3 antibody at 4°C to minimize internalization and to enhance cell surface labeling of receptors. Cells were then fixed and receptor-antibody complexes were visualized with a secondary antibody conjugated with FITC. Parallel experiments conducted with a control IgG showed no labeling (data not shown). *D,* cells treated or not with AVE1642 for 24 h were processed for cell surface protein biotinylation. Biotinylated proteins were purified and analyzed for HER3 expression by Western blot analysis. E-cadherin detection was used as a loading control.

the total levels of IGF-1R (Supplementary Fig. S3A). Moreover, HepG2 and HuH6 cells continuously exposed to 10 μmol/L gefinitib for 5 months (resistant cells) showed no increased levels of either phosphorylated or total IGF-1R (Supplementary Fig. S3B) nor elevated sensitivity to growth inhibition by AVE1642 (Supplementary Fig. S3C) compared with the untreated counterparts (parental cells). Rather, a decreased expression of IGF-1R together with a decreased IGF-1R phosphorylation was observed in HepG2 cells chronically incubated with gefitinib (Supplementary Fig. S3B).

To further characterize the mechanisms whereby AVE1642 increased HER3 expression in HCC cells, we compared HER3 mRNA expression in control and AVE1642-treated cells by quantitative real-time PCR. AVE1642 increased the levels of HER3 transcripts by 1.6-, 1.9-, and 3.8-fold in HepG2, HuH7, and PLC/PRF5 cells, respectively (Fig. 6A). Given that rapamycin synergized with AVE1642 to reduce cell viability (Fig. 4B), we examined whether rapamycin could affect AVE1642 induction of HER3 expression and phosphorylation. In HepG2 (Supplementary Fig. S4), HuH7, and Hep3B cells (data not shown), the stimulatory effect of AVE1642 on HER3 remained unchanged in the presence of rapamycin suggesting that AVE1642 increases HER3 expression and phosphorylation through a mammalian target of rapamycin (mTOR)-independent pathway.

HER3 limits the antitumoral action of AVE1642 in hepatoma cells. We examined whether the up-regulation of HER3 expression interfered with the antitumoral effect of AVE1642. To address this issue, HepG2 and PLC/PRF5 cells were transiently

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transfected with HER3-specific siRNAs or with control siRNAs. As shown in Fig. 6B, HER3-specific siRNAs induced a partial knock-down of HER3 expression (approximately 50% and 80% reduction in HepG2 and PLC/PRF5 cells, respectively) both in unstimulated and in AVE1642-treated cells compared with cells transfected with the control siRNAs. HER3 down-regulation was accompanied by a subsequent decrease in the phosphorylation state of HER3. AKT phosphorylation was also reduced but only in cells treated with AVE1642. When transfected cells were analyzed in an MTT assay, we observed that the antitumoral effect of AVE1642 was significantly increased in cells in which HER3 was down-regulated compared with control cells (Fig. 6C). This result confirms the major role of HER3 in the regulation of hepatoma cell sensitivity to the anti–IGF-1R antibody. Taken as a whole, our findings show that hepatoma cell lines may adapt to IGF-1R inhibition by inducing early compensatory inputs leading to the activation of an EGFR/HER3/AKT-dependent pathway (Fig. 6D).

Discussion

The dysregulation of the IGF signaling axis, notably via an increased IGF-II availability, has been well documented in human HCC (6). However, data concerning the IGF-1R status in HCC tumors are limited and conflictory. A study analyzing 15 paired samples of HCC and adjacent nontumoral liver tissue showed no significant increase of IGF-1R mRNA expression in tumors (32). A recent study showed by Western blot analysis

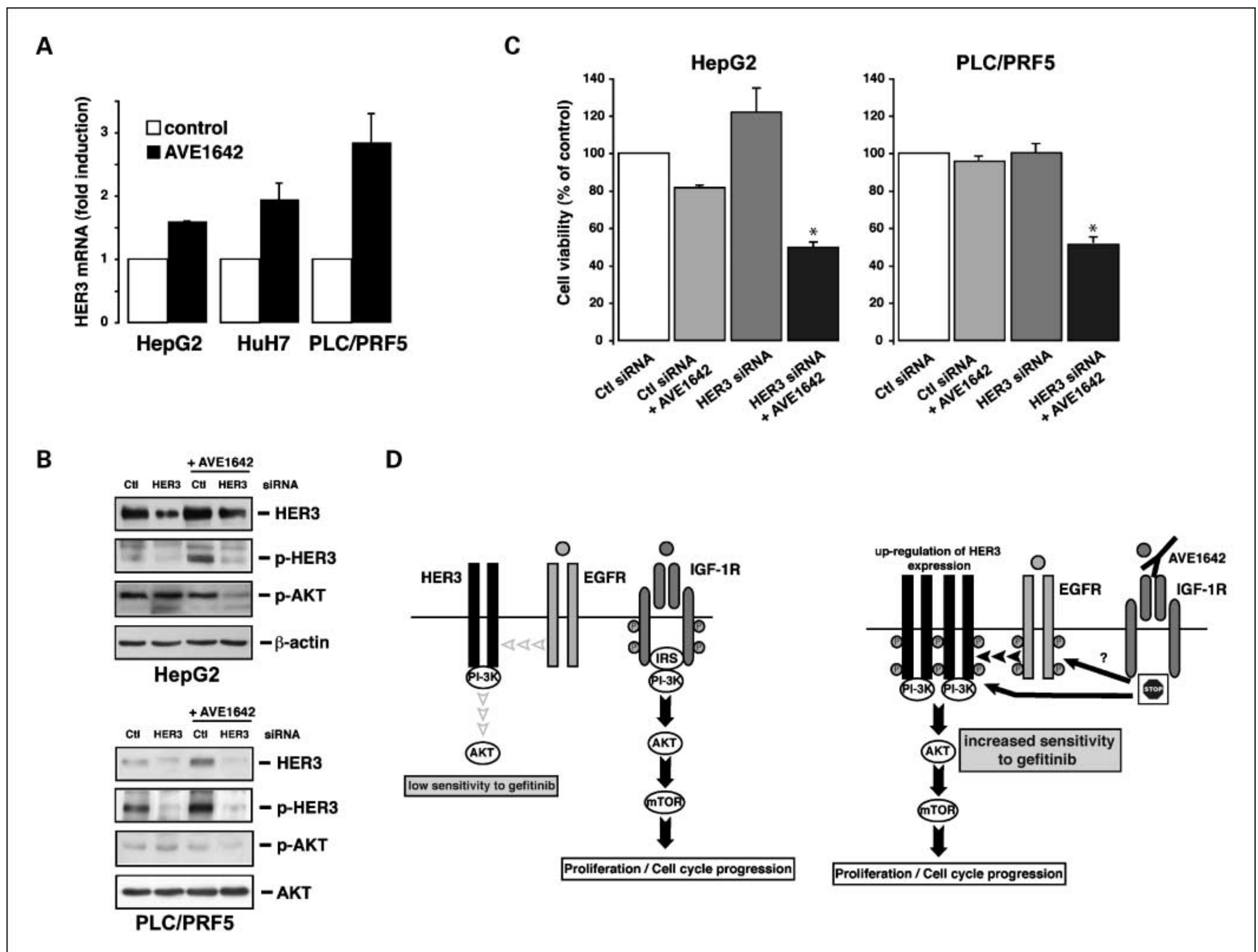

Fig. 6. Contribution of HER3 to hepatoma cell sensitivity to AVE1642. *A,* serum-starved HepG2, HuH7, and PLC/PRF5 cells were treated for 48 h with or without AVE1642 (0.5 μg/mL), and HER3 mRNA transcripts were quantified by real-time PCR. *B* and *C,* HepG2 and PLC/PRF5 cells were transiently transfected with HER3 siRNA or with control (*ctl*) siRNA and analyzed for HER3 and AKT expression by Western blotting after 48 h of treatment with AVE1642 (*B*), and for cell viability after 72 h of treatment with AVE1642 (*C*). Representative blots of three independent experiments are shown. *Columns,* means of three independent experiments; *bars,* SEM. *, $P < 0.001$ compared with cells transfected with the control siRNA and treated with AVE1642. *D,* proposed mechanism for the synergistic antitumoral effect of AVE1642 combined with gefitinib. *Left*, IGF-1R is a strong activator of the AKT pathway in hepatoma cells, which may contribute to the proliferative effect of IGF-II. In contrast, EGFR is a poor activator of AKT in these cells, maybe due to a nonefficient coupling between EGFR and HER3. As a result, hepatoma cells are poorly sensitive to gefitinib. *Right*, in the presence of AVE1642, IGF-1R is blocked and an escape mechanism is induced, resulting in the up-regulation of HER3 expression. EGFR, which is also activated consecutively to IGF-1R inhibition probably by ligand-dependent mechanism, homodimerizes with and *trans*-phosphorylated HER3, thus restoring an efficient coupling to the PI3K/AKT pathway. Under this condition, hepatoma cell sensitivity to gefitinib is increased.

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that IGF-1R was frequently overexpressed in a subgroup of tumors associated with a low copy number of HBV (7 of 9 tumors; ref. 12). In addition, some HCC tumors associated with hepatitis C virus and increased proliferation presented frequently with IGF-1R phosphorylation by immunohistochemistry analysis (11 of 23 tumors; ref. 33). In our panel of HCC, ELISA measurements revealed that the levels of IGF-1R expression and activation were increased in approximately 30% and 50% of tumors compared with matched cirrhotic liver tissues, respectively. As we did not find a clear correlation between total and phosphorylated levels of IGF-1R, our data suggest that both phosphorylated and total levels of IGF-1R might be predictive of IGF-1R signaling dependency and of potential sensitivity to IGF-1R targeting in HCC cells.

Two classes of IGF-1R inhibitors are currently under clinical development for cancer therapy: (*a*) mAbs such as AVE1642 that specifically bind to the extracellular domain of IGF-1R, block ligand/receptor interaction, and promote IGF-1R down-regulation and degradation and (*b*) small-molecule TKIs. However, due to the high sequence homology between IGF-1R and IR (84%), these latter may be not fully specific for IGF-1R. AVE1642 is the humanized version of the murine mAb EM164, which has been shown to potently and specifically inhibit IGF-1–stimulated proliferation in different cancer cell lines including breast, lung, colon, and myeloma cells (21, 27). Accordingly, we observed that, in hepatoma cells, AVE1642 inhibited signaling in response to exogenously added IGF-I, IGF-II, but not to insulin. In MTT assays, the antitumoral potency of AVE1642 was comparable in the presence or absence of serum, especially in cell lines with strong IGF-II production (i.e, HepG2, HuH7, and Hep3B), suggesting that autocrine IGF-II is an important driver for hepatoma cell growth. The strongest effect of AVE1642 on cell viability was observed in HepG2 cells, whereas HuH6 was the most resistant cell line. There was no correlation between cell sensitivity to AVE1642 and IGF-II mRNA expression in serum-free conditions because AVE1642 induced the same inhibition of cell viability in PLC/PRF5 (low IGF-II mRNA expression levels) and HuH7 (high IGF-II mRNA expression levels) cells. Otherwise, it has been shown that the antiproliferative effect of AVE1642 was not correlated to IGF-1R expression in a panel of cancer cells.[5]

We observed that the combination of AVE1642 with gefitinib or with cetuximab induced a supra-additive inhibition of viability in all cell lines compared with drugs alone. These findings suggest that ligand-dependent activation of EGFR limits the antineoplasic action of AVE1642 in hepatoma cells, irrespective of their phenotype (i.e, wild-type or mutated p53, β-catenin, and/or Rb), their viral status (HBs^{neg} or HBs^{pos} antigen), or their own sensitivity to AVE1642. We show that the synergy between AVE1642 and anti-EGFR therapy is a general phenomenon and not a peculiarity of a few cell lines. The synergy between AVE1642 and gefitinib led mainly to a cytostatic effect with growth arrest in G_0-G_1 without apoptosis. The analysis of the underlying mechanisms revealed that AVE1642 increased the amounts of total and phosphorylated HER3. Therefore, our findings indicate that, following blockade of IGF-1R signaling, hepatoma cells are able to switch from IGF-1R to EGFR dependency to maintain cell growth and AKT phosphorylation. During the preparation of this article, an adaptive up-regulation of the EGFR pathway in response to IGF-1R inhibition with small TKIs has been reported in ovarian and colon cancer cells (34, 35). A reciprocal regulation seems not to occur in hepatoma cells because we did not observe any stimulating effect of gefitinib on IGF-1R signaling after short-term and even long-term exposures to this drug. Such a regulation had been reported in breast, prostate, and non–small cell lung cancer cells in which EGFR TKIs induced a significant activation of IGF-1R signaling through different mechanisms: up-regulation of IGF-1R activity (36), increased availability of IRS-1 (37), formation of EGFR/IGF-1R heterodimers (38), or loss of IGF binding protein expression (39).

HER3 is a peculiar member of the EGFR family because it lacks a functional intrinsic tyrosine kinase activity and is able to directly bind PI3K and activate the donwstream AKT pathway (31). HER3 functions through heterodimerization with EGFR or HER-2. In EGFR/HER3 and HER-2/HER3 heterodimers, the transphosphorylation of HER3 allows the recruitment of the p85 regulatory subunit of PI3K. A growing body of literature has recently emerged showing that HER3 is a crucial sensor of sensitivity of cancer cells to EGFR TKIs. First, there is a strong correlation between the amounts of phosphorylated and total HER3 and response to gefitinib or to erlotinib in lung, pancreas, and liver cancer cells (40–43). Second, it has been shown that cancer cells that rely mainly on the EGFR/HER3 pathway for AKT activation displayed exquisite sensitivity to gefitinib and erlotinib (44, 45). In this context, the increased expression of phosphorylated HER3 observed in hepatoma cells exposed to AVE1642 might be the main mechanism accounting for the synergy between AVE1642 and gefitinib in these cells. Arguments for this hypothesis are the following: first, all hepatoma cell lines that express wild-type EGFR (9) were poorly sensitive to gefitinib alone in terms of inhibition of cell viability (IC_{50}, approximately 8-10 μmol/L in all cell lines; data not shown) and of AKT phosphorylation; second, gefitinib, by preventing AVE1642-induced phosphorylation of HER3, led to the down-regulation of AKT phosphorylation; and third, counter-acting the effect of AVE1642 on HER3 expression using siRNAs sensitized hepatoma cells to AVE1642. Of note, there was no correlation between basal levels of HER3 and cell response to the bitherapy AVE1642/gefitinib. Moreover, HER3 expression did not correlate with IGF-II expression (data not shown), suggesting that baselines of HER3 and IGF-II are not predictive markers of HCC response to the bitherapy.

AVE1642 synergized not only with gefitinib but also with rapamycin to reduce cell viability. In both combinations, a marked down-regulation of AKT phosphorylation was observed but the underlying mechanisms seemed to be different. Indeed, contrasting with what was observed in the presence of gefitinib, rapamycin did not prevent the induction of HER3 phosphorylation by AVE1642. Nevertheless, AVE1642 had the potential to impede the induction of AKT phosphorylation by rapamycin. These latter findings corroborate with those obtained in other cancer cell types showing that in HCC cells rapamycin is able to up-regulate the AKT pathway through an IGF-1R-dependent pathway (28–30). The inhibitory effect of AVE1642 on this process may contribute to the antitumoral effect of the rapamycin/AVE1642 combination in HCC cells.

It is highly probable that the up-regulation of HER3 expression consecutive to IGF-1R inhibition may be responsible for the increase of HER3 phosphorylation. Indeed, this could favor the formation of EGFR/HER3 heterodimers and the subsequent

[5] Unpublished results.

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trans-phosphorylation of HER3 by EGFR. This could also be enhanced by the fact that AVE1642 promoted a slight but reproducible increase of EGFR phosphorylation. Otherwise, AVE1642 did not seem to modulate the availability of specific ligands for HER3 because HER3 neutralization with a specific antibody was without effect on cell response to AVE1642 (data not shown).

In summary, we present evidence for the interest of targeting IGF-1R in HCC because this receptor may be overexpressed and/or activated in tumors. However, we also show that the antitumoral efficiency of a specific antibody against IGF-1R is counteracted in HCC cells due to its ability to stimulate the EGFR/HER3/AKT signaling pathway. These results provide therefore a strong rational for IGF-1R and EGFR cotargeting in human HCC to maximize antitumoral effect and to prevent the early development of resistance. Clinical studies are currently ongoing to validate this combinatory approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Insulin-Like Growth Factor-1 Receptor Inhibition Induces a Resistance Mechanism via the Epidermal Growth Factor Receptor/HER3/AKT Signaling Pathway: Rational Basis for Cotargeting Insulin-Like Growth Factor-1 Receptor and Epidermal Growth Factor Receptor in Hepatocellular Carcinoma

Christèle Desbois-Mouthon, Aurore Baron, Marie-José Blivet-Van Eggelpoël, et al.

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