Sequencing of Type I Insulin-Like Growth Factor Receptor Inhibition Affects Chemotherapy Response In Vitro and In Vivo

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Abstract Purpose: The aim of this study was to determine the optimal sequence of combining anti-type I insulin-like growth factor receptor (IGF1R) antibodies with chemotherapeutic drugs in cancer cells in vitro and in vivo.

Experimental Design: MCF-7 and LCC6 cells were treated with subcytotoxic concentrations of doxorubicin with or without anti-IGF1R antibodies (scFv-Fc or EM164 and its humanized version AVE1642). Treatments were given simultaneously, doxorubicin followed by anti-IGF1R antibody, or anti-IGF1R antibody followed by doxorubicin, with measurement of in vitro proliferation, apoptosis, and anchorage-independent growth. The effects of sequencing on LCC6 xenograft growth and metastasis were studied.

Results: Doxorubicin followed by anti-IGF1R antibody (scFv-Fc or EM164) was the most effective combination strategy to inhibit cell monolayer growth and anchorage-independent growth. This sequential combination triggered increased poly (ADP-ribose) polymerase cleavage compared with other treatment sequences. The reverse sequence, antibody followed by doxorubicin treatment, protected cells from chemotherapy by decreasing apoptosis, arresting cells in S phase, and inhibiting the level and activity of topoisomerase IIα. Finally, our in vivo data show that recovery of IGF1R prior to doxorubicin therapy resulted in the best therapeutic responses. Low doses of AVE1642 that allowed IGF1R expression to recover at one week were more effective in combination with doxorubicin than higher antibody doses.

Conclusion: The timing of IGF1R inhibition affects responses to chemotherapy. The optimal sequence was doxorubicin followed by anti-IGF1R antibody, whereas the opposite sequence inhibited doxorubicin effects. Thus, the dose and sequencing of anti-IGF1R therapies should be considered in the design of future clinical trials.

Although chemotherapy produces objective responses in patients with breast cancer, it is far from completely effective. Cytotoxic agents also may cause severe and dose-limiting systemic toxicities. Thus, identifying new methods to enhance the beneficial effects of chemotherapy while decreasing systemic toxicities are clearly needed (1, 2). Recent progress suggests that the combination of chemotherapy with targeted therapy is superior to either one alone. For example, patients with metastatic colon cancer treated with both the anti–epidermal growth factor receptor antibody cetuximab and the chemotherapy drug irinotecan have superior results, even if the patients progressed on irinotecan alone (3). In addition, trastuzumab (Herceptin), an anti–human epidermal growth factor receptor 2 antibody, is widely used to treat patients overexpressing human epidermal growth factor receptor 2 in combination with chemotherapeutic drugs (4, 5).

Among the new potential cancer targets, the type I insulin-like growth factor receptor (IGF1R) has emerged as a relevant pathway. Population, preclinical, and research findings suggest that the insulin-like growth factor (IGF) system functions to maintain the malignant phenotype in cancer (6). Disruption of IGF1R activation has been shown to inhibit cancer cell growth and motility in vitro and in vivo (7–9). Various approaches of disrupting IGF1R activity have been developed as potential interventions in the treatment of malignancies in the past several years. Antibodies that disrupt IGF1R function have been developed. scFv-Fc, a chimeric humanized single-chain antibody, causes initial receptor biochemical signaling followed by receptor down-regulation, and exhibits dose-dependent growth inhibition of some breast cancer cell lines (10, 11). EM164, a full antagonistic anti-IGF1R antibody, did not stimulate IGF1R autophosphorylation, but down-regulated IGF1R in vitro and in vivo. It also displays inhibitory activity against IGF-I- and IGF-II–induced survival of MCF-7 breast cancer cells (12). The humanized version of EM164, AVE1642 (Sanofi-aventis), is currently in clinical trials against various types of solid cancer. Several studies have shown that activation of IGF1R protects breast cancer cells from apoptosis induced by chemotherapy and radiation, and receptor activation mediates resistance to...
chemotherapy and radiation (13–15). Therefore, inhibiting IGF1R signaling may enhance the sensitivity of cancer cells to chemotherapy. Indeed, several groups have shown that combining anti-IGF1R antibody with chemotherapy enhances chemotherapy responses in human cancer cells (12, 16, 17).

Recent findings suggest that the combination of chemotherapy and targeted therapy may be sequence-dependent. A large cooperative group trial showed an estimated disease-free survival advantage of 18% for sequential rather than concurrent chemotherapy and tamoxifen treatment when given in the adjuvant setting (18). In addition, inhibition of human epidermal growth factor receptor 2 by trastuzumab first, but not in the reverse order, increased the paclitaxel resistance of ovarian cancer cells (19). As several anti-IGF1R antibodies are being evaluated in phase I/II/III clinical trials, some in combination with cytotoxic chemotherapy, it is important to determine the optimal schedule for the antibodies in combination with chemotherapy.

The work presented here describes the in vitro and in vivo activity of anti-IGF1R antibody in combination with several chemotherapeutic drugs delivered concurrently or sequentially in human cancer cell lines. We have determined the optimal sequence of anti-IGF1R antibodies in combination with commonly used chemotherapeutic drugs. Our results support the idea that sequencing of anti-IGF1R therapy with chemotherapy can optimize the antitumor effect and have significant implications for the clinical development of this strategy.

Translational Relevance
Disruption of type I insulin-like growth factor receptor (IGF1R) signaling has emerged as an important target in cancer therapy. Whereas interruption of receptor signaling may have single-agent activity, combining this strategy with other cytotoxic agents may have appeal. These data show that the sequencing of IGF1R blockade plays a critical role in determining efficacy. Optimal anticancer effects were seen when chemotherapy was given prior to exposure to an anti-IGF1R monoclonal antibody. Moreover, lower doses of antibody, which resulted in transient receptor suppression in vivo, were more effective than continuous receptor suppression caused by higher antibody doses. Thus, sequencing of cytotoxic chemotherapy with IGF1R blockade should be considered in the design of clinical trials.

Materials and Methods

Reagents. All reagents and chemicals were purchased from Sigma, and cell culture reagents were from Invitrogen/Life Technologies, Inc. unless otherwise noted. IGF-I was purchased from NovoZyme. The anti-IGF1R antibody scFv-Fc was engineered and purified as described previously (20). EM164 and AVE1642 (a humanized EM164) antibody were previously reported (12). Antibodies against extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 were purchased from Cell Signaling. The polyclonal antibodies against IGF1R α and β subunits were from Santa Cruz Biotechnology, Inc. The anti-β-actin was from Sigma-Aldrich. Anti-topoisomerase IIA antibodies were from TopoGEN. Anti-rabbit and antimuscle secondary antibodies conjugated to horseradish peroxidase were from GE Biosciences.

Cell lines and culture. MCF-7 cells were originally obtained from Dr. C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were routinely maintained in Iscove’s modified essential medium (IMEM) with 10% FBS, 100 units/mL penicillin, and 50 μg/mL streptomycin. LCC6 cells were obtained from Dr. Robert Clarke (Georgetown University, Washington D. C.). LCC6 cells were routinely maintained in DMEM with 10% FBS, 11.25 nmol/L human insulin, 50 units/mL penicillin, and 50 μg/mL streptomycin. LCC6 cells were transfected with pCMV-MCS plasmid vector to express scFv-Fc (30 μg/mL) or EM164 (15 μg/mL) for 48 h; (c) doxorubicin and antibody simultaneeously for 24 h; (d) pretreatment with doxorubicin for 24 h followed by antibody treatment for 48 h; (e) doxorubicin and antibody for 24 h followed by doxorubicin treatment for another 48 h. Cell number was estimated using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay as described previously (21). Sixty microliters of 5 mg/mL MTT reagent in PBS were added to each well and plates were incubated for 3 h at 37 °C. Wells were aspirated and 0.5 mL of solubilizing solution (95% DMSO plus 5% IMEM) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a 670-nm differential filter.

Anchorage-independent growth. Anchorage-independent growth assays were done as follows. A bottom agar was prepared by solidifying 1 mL of 0.8% SeaPlaque agarose (BioWhitaker) in 2% FBS-containing growth media in each well of a 6-well plate. The bottom agar was overlaid with 800 μL of a 0.25% top agar mixture containing 10,000 cells from EM164, LCC6, scFv-Fc or doxorubicin and anti-IGF1R antibody. The plates were incubated at 37°C for 24 h, then growth media with or without doxorubicin and antibody were added to the top of agar. After 9 to 10 d, colonies were counted using a light microscope with an ocular grid. Only colonies larger than two thirds of a grid square were counted. Five random fields were counted for each well and the average number of colonies per well is shown. Results are representative of one experiment done in triplicate for each treatment.

Annexin V analysis of apoptosis. The Annexin V-APC-labeled Annexin V Detection Kit I (BD Biosciences) was used to detect apoptosis by flow cytometry according to the manufacturer’s instructions. LCC6 cells were treated with either doxorubicin and antibody simultaneously for 48 h; (e) doxorubicin 24 h followed by scFv-Fc or EM164 24 h; (f) pretreated with scFv-Fc or EM164 for 48 h; (g) doxorubicin and antibody for 24 h followed by doxorubicin treatment for another 48 h. Cell number was estimated using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay as described previously (21). Sixty microliters of 5 mg/mL MTT reagent in PBS were added to each well and plates were incubated for 3 h at 37°C. Wells were aspirated and 0.5 mL of solubilizing solution (95% DMSO plus 5% IMEM) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a 670-nm differential filter.

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Timing of IGFIR Inhibition and Chemotherapy Response

Proliferation assays. MCF-7 cells were plated in triplicate in 6-well plates with or without doxorubicin or anti-IGF1R antibody. The plates were incubated at 37°C for 24 h, then growth media with or without doxorubicin and antibody were added to the top of agar. After 9 to 10 d, colonies were counted using a light microscope with an ocular grid. Only colonies larger than two thirds of a grid square were counted. Five random fields were counted for each well and the average number of colonies per well is shown. Results are representative of one experiment done in triplicate for each treatment.

Western blotting. LCC6 cells were grown to 70% confluence, incubated in serum-free medium overnight, and treated with the same schedule as described for MTT assays. Cells were lysed in TNE/SDS buffer and 40 μg of total cellular proteins were separated by SDS-PAGE on 8% gels. Proteins were transferred to nitrocellulose and immunoblotted with the anti-poly (ADP-Ribose) polymerase (PARP) antibody (Santa Cruz). Enhanced chemiluminescence was done according to the manufacturer’s instructions (Pierce). For studies on the effect of combining the anti-IGF1R antibody with doxorubicin on topoisomerase IIA protein levels, nuclear extracts were prepared from LCC6 cells treated with AVE1642, doxorubicin, or AVE1642 combined with doxorubicin concurrently or sequentially. Nuclear extracts were purified by lysis of cells with a high-salt buffer (100 mmol/L NaCl, 20 mmol/L KCl, 20 mmol/L Tris, and 0.5 mmol/L Na2HPO4 (pH 7.4)) containing 0.5% Triton X-100 and 1% NP40. Nuclei were pelleted, and the nuclear proteins were solubilized by sonication in 1% SDS. After quantitation, 50 μg of nuclear proteins were loaded and separated by 8% SDS-PAGE, transferred onto nitrocellulose, and incubated with an antihuman topoisomerase IIA rabbit polyclonal antibody (TopoGEN, Inc.).
24 h and followed by doxorubicin for another 24 h. Cells were harvested in cold PBS after treatment. Heat-shocked cells (cells were incubated in 56°C water bath for 1 min) were used as positive control. Cells were then resuspended at a density of 1 × 10^6 cells/mL in 1 × binding buffer (HEPES, 10 mmol/L, pH 7.4, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mol/L MgCl2, and 1.8 mml/L CaCl2) and stained simultaneously with APC-labeled Annexin V (25 ng/mL) and 7-AAD (50 ng/mL). 7-AAD was used as a cell viability marker. Cells were analyzed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson), and the data were analyzed with CellQuest software.

**Cell cycle analysis.** Confluent LCC6 cells were trypsinized and plated at a density of 5 × 10^5 cells/mL per 60 mm dish. After 24 h, cells were switched to serum-free medium for 24 h. Cells were then treated as described above. Cells were collected on ice in 1 mL of 1 × PBS, and stained with propidium iodide (Sigma-Aldrich Corp.). Cells were analyzed for phase of the cell cycle by flow cytometry. Results were analyzed using Modfit software.

**Topo II decatenation assay.** The assay was done according to the manufacturer’s instructions (TopoGen, Inc.). LCC6 cells were treated with doxorubicin, AVE1642, or doxorubicin with AVE1642 simultaneously or sequentially. Nuclear extracts were prepared as noted above. The total reaction volume was held at 20 µL. Assay buffer (120 mmol/L KCl, 50 mmol/L Tris-HCl, 10 mmol/L MgCl2, 0.5 mmol/L dithiothreitol, 0.5 mmol/L ATP, and 30 µg/mL bovine serum albumin) containing 100 ng of catenated kinetoplast DNA (kDNA) and 2 µg LCC6 nuclear extract was incubated for 30 min at 37°C. The reaction was stopped upon addition of 4 µL of stop buffer (4% glycerol, 1% sarkosyl, and 0.025% bromphenol blue), and then analyzed by 1% agarose gel electrophoresis.

**Tumor growth in athymic mice and tumor extract analyses.** Four-week-old female athymic mice were used for in vivo study. To study the effect of AVE1642 dose on IGF1R down-regulation in vivo, 5 × 10^5 LCC6 cells were injected into the mammary fat pads on each side of each mouse. When tumor volume reached 100 mm^3, AVE1642 was injected i.p. Tumors from the left side of all mice were surgically resected and snap-frozen in liquid nitrogen 2 d after AVE1642 treatment. The tumors on the right side of the mouse were collected and frozen after another 7 d. Tumors were homogenized and blotted for IGF1Rβ as noted above.

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**Fig. 1.** Sequence-dependent antiproliferation effect of combining scFv-Fc or AVE1642 with doxorubicin in MCF-7 cells. MCF-7 cells grown in 5 nmol/L IGF-I (A, C) or 1% FBS condition (B, D) were treated with different concentrations of doxorubicin, scFv-Fc (scFv; 30 µg/mL) or AVE1642 (AVE; 15 µg/mL) simultaneously or sequentially as follows: (1) doxorubicin alone for 72 h; (2) doxorubicin and antibody simultaneously for 72 h; (3) pretreatment with doxorubicin for 24 h followed by antibody treatment for 48 h; (4) pretreatment with antibody for 24 h followed by doxorubicin treatment for another 48 h. MTT assays were done over 72 h. Cell survival is displayed as a percentage of growth over non-doxorubicin-treated control. The experiment was repeated three times; a representative experiment is shown.
For the tumor growth study, $5 \times 10^6$ LCC6 cells in serum-free IMEM were injected into the mammary fat pad of the mice. Tumor growth was measured every 3 d and tumor volume was estimated from bidirectional measurements using the formula length × breadth$^2$/2. When the tumors reached volume of $\approx 80$ mm$^3$, the mice were randomized into 5 groups of 5 animals each. The dose of AVE1642 was based on the findings from the previous experiment. Mice were treated with PBS, doxorubicin (3 mg/kg body weight/wk), AVE1642 (50 μg/ wk), doxorubicin followed 24 h later by AVE1642 (50 μg/wk) or doxorubicin followed 24 h later by AVE1642 (800 μg/wk) by i.p. injection. Treatment was given weekly for 4 wk. Twenty-four hours after the final treatment, the mice were sacrificed. The lungs of the mice were harvested, fixed, embedded, sectioned, and stained with H&E, and examined microscopically for micrometastatic deposits. In the second set of experiments, the mice were sacrificed 1 wk after the final treatment. Tumors were harvested, snap-frozen, and homogenized. Tissue homogenates were suspended in 500 μL TNE SV lysis buffer. One hundred micrograms of each tumor extract were subjected to 8% SDS-PAGE followed by immunoblotting for the level of IGF1R. Total levels of mitogen-activated protein kinase were used as loading control. All the animal protocols were approved by the University of Minnesota’s Institutional Animal Care & Use Committee.

**Statistical tests.** Statistical significance between means of control and antibody in combination with doxorubicin was assayed using Student’s $t$ test; 95% confidence interval ($P < 0.05$) was considered significant. For comparisons between more than two treatments in the xenograft experiments, one-way ANOVA analysis was done. If not otherwise indicated, error bars in all experiments represent the SD.

**Results**

**Sequence-dependent antiproliferation effects of combining scFv-Fc or AVE1642 with doxorubicin in MCF-7 cells.** To assess whether the IGF1R antibody scFv-Fc can synergize or inhibit the cytotoxicity of doxorubicin, the IC$_{50}$ of doxorubicin were...
that were apoptotic). A representative experiment is shown for cells of apoptosis (percentage of all cells analyzed by flow cytometry. The experiment was repeated three times with similar results; a representative experiment is shown for cells of apoptosis (percentage of all cells that were apoptotic). * P < 0.05 versus doxorubicin alone.

studied in monolayer growth using MTT assays (Fig. 1). These assays were done in the absence (1% FBS) or presence of IGF-I (5 nmol/L). Whether in the presence of IGF-I or in the 1% FBS condition, simultaneous treatment with scFv-Fc and doxorubicin or the sequence of scFv-Fc followed by doxorubicin, decreased cytotoxicity was observed (Fig. 1A and B). The IC_{50} of doxorubicin was increased when antibody was given before or simultaneously with the drug. Interestingly, doxorubicin followed by scFv-Fc resulted in enhanced doxorubicin cytotoxicity and the IC_{50} 2 to 4 times lower in MCF-7 cells.

It was evident that the magnitude of the effect of sequencing was different between the two culture conditions. Because scFv-Fc stimulated biochemical activity of the receptor, the effects were less marked in cells cultured in the presence of IGF-I as the agonist effect of scFv-Fc was masked by the presence of IGF-I. In order to address whether the sequence-dependent inhibition was affected by the agonistic properties of scFv-Fc, we examined the effect of doxorubicin in combination with EM164. This antibody does not stimulate IGF1R biochemical activation and displayed potent inhibitory activity against IGF-I, IGF-II, and serum-stimulated proliferation of diverse cancer cell lines (12). As shown in Fig. 1C and D, similar results were obtained, as the sequence of doxorubicin followed by EM164 significantly inhibited the cell proliferation in MCF-7 cells. These results suggest that the cytotoxic effects of combining doxorubicin with scFv-Fc or EM164 are sequence-dependent with doxorubicin followed by antibody showing the optimal antiproliferative effect.

Doxorubicin, but not 5-fluorouracil, followed by scFv-Fc or EM164, significantly inhibited the anchorage-independent growth of LCC6 cells. In MCF-7 cells, IGF1R activation stimulates proliferation, complicating the interpretation of this type of sequencing experiment (22). In order to further evaluate the effects of inhibition of IGF1R on chemotherapy and confirm the sequence-dependent inhibition effects, without the added complexity of the growth regulatory effects of IGF1R on the cell, we concentrated on the LCC6 cell line in anchorage-independent growth assays. This cell line, derived from the MDA-MB-435 cell line, expresses IGF1R yet is not growth-regulated by its activation (23). Recently, this cell line has been shown to originate from a malignant melanoma cell (24), but still is an excellent model for studying the effects of IGF1R inhibition on cancer cell biology.

In this experiment, we used a dose of doxorubicin (100 ng/mL) that by itself did not inhibit colony formation. Cells were either treated simultaneously with doxorubicin and scFv-Fc or treated sequentially. As shown in Fig. 2A, similar to the result of the MTT assay in MCF-7 cells, the sequential treatment of doxorubicin followed by scFv-Fc significantly inhibited colony growth compared with the other sequences (Fig. 2A). Because scFv-Fc is a partial agonistic antibody of IGF1R, we examined the effect of doxorubicin in combination with EM164 in anchorage-independent growth. As shown in Fig. 2B, similar results were obtained, as the sequence of doxorubicin followed by EM164 significantly inhibited the colony growth in LCC6 cells.

Rochester et al. showed that silencing of the IGF1R gene enhances sensitivity to DNA-damaging agents in human prostate cancer cells, but not with other cytotoxic agents (25). Doxorubicin is a topoisomerase II inhibitor and also intercalates into DNA to cause DNA damage (26). To investigate whether the sequence-dependent inhibition of doxorubicin in combination with anti-IGF1R antibodies was specific to DNA-damaging drugs, the effect of combining anti-IGF1R antibodies with etoposide and 5-fluorouracil were examined. Etoposide is another topoisomerase II inhibitor and induces double-strand DNA breaks (27), whereas 5-fluorouracil is an antimetabolite that undergoes biotransformation to 5-fluoro-2'-deoxyuridine-5'-phosphate and forms a covalently bound ternary complex with the enzyme thymidylate synthase and its cofactor to inhibit DNA synthesis (28). As expected, etoposide followed by scFv-Fc or EM164 significantly inhibited colony growth (data not shown). In contrast, sequence-dependent inhibition was not observed with 5-fluorouracil in combination with scFv-Fc or EM164 (Fig. 2C and D).

Doxorubicin followed by scFv-Fc or AVE1642 increased PARP cleavage and pretreatment with scFv-Fc or EM164 rendered cell resistant to doxorubicin-induced apoptosis. IGF-I protects MCF-7 breast cancer cells from doxorubicin and paclitaxel by induction of proliferation and inhibition of apoptosis (22). In LCC6 cells, monolayer cell growth is not affected by IGF-I; we used these cells to examine whether anti-IGF1R antibody scFv-Fc or AVE1642 sequencing affected doxorubicin-induced apoptosis in LCC6 cells. LCC6 cells were treated with doxorubicin and scFv-Fc or AVE1642 simultaneously or sequentially. After treatment, both adherent and nonadherent
cells were collected and subjected to Western blotting with antibodies to PARP, a caspase substrate that is cleaved in cells undergoing apoptosis. In Fig. 3A, doxorubicin followed by scFv-Fc or AVE1642, but not in other sequences, significantly increased doxorubicin (500 ng/mL)-induced PARP cleavage. Neither antibody by itself induced PARP cleavage.

To address whether these changes in PARP cleavage reflected an increase in induction of apoptosis by chemotherapeutics, we measured Annexin-V staining by flow cytometry analysis. LCC6 cells were treated with doxorubicin in combination with scFv-Fc or EM164 concurrently or sequentially for 48 hours, and the adherent and nonadherent cells were analyzed. In this assay, pretreatment with EM164 significantly decreased the doxorubicin-induced apoptosis (Fig. 3B). We did not see doxorubicin followed by EM164 increase cell apoptosis in this experiment. These data indicate, however, that if IGF1R was inhibited prior to doxorubicin exposure, then cells were protected from the effects of doxorubicin.

**Pretreatment with scFv-Fc or AVE1642 followed by doxorubicin arrested LCC6 cells in S phase.** To investigate the potential mechanism of this sequence-dependent effect of doxorubicin in combination with anti-IGF1R antibodies, we studied the cell cycle profile of LCC6 cells treated with doxorubicin and scFv-Fc or AVE1642. In these cells, the antibodies did not affect cell cycle distribution of LCC6 cells (Fig. 4). Consistent with previous report, doxorubicin-treated cells were arrested in G2-M phase (29). Simultaneous exposure to scFv-Fc/AVE1642 and doxorubicin, doxorubicin followed by scFv-Fc or AVE1642, showed similar effects to doxorubicin alone. In contrast, cells pretreated with antibody followed by doxorubicin decreased G2-M arrest with an increase in G0-G1 and S-phase fraction (Fig. 4A and B). These data suggested that inhibition of IGF1R prior to doxorubicin exposure blunted the effects of doxorubicin as reflected by effects on the cell cycle.

**Pretreatment of AVE1642 followed by doxorubicin decreased topoisomerase II activity and protein level.** Because the effects of sequencing were seen with drugs that affect topoisomerase II, such as doxorubicin and etoposide, we hypothesized that blockade of IGF1R may affect topoisomerase II activity. We measured topoisomerase II activity in nuclear extracts by the decatenation of kDNA assay. This assay measures the ability of topoisomerase II to catalyze strand passage between two double-stranded DNA segments. Topoisomerase II decatenation activity (per identical amounts of nuclear extract proteins) was approximately 5-fold lower in the sequence AVE1642 followed by doxorubicin extracts as compared with doxorubicin alone (Fig. 5A, lane 6 versus lane 9). In contrast, nuclear extracts from cells, with sequential treatment of doxorubicin followed by AVE1642, had significantly increased topoisomerase II activity (Fig. 5A, lane 6 versus lane 8). To determine if the altered topoisomerase II activity was related to the level of topoisomerase II protein, we determined topoisomerase II levels in cellular extracts by immunoblotting. AVE1642 followed by doxorubicin caused a 2-fold decrease in topoisomerase II levels (Fig. 5B). Taken together, these data suggest that decreased topoisomerase II activity by pretreatment with AVE1642 led directly to a decreased sensitivity of LCC6 cells to topoisomerase II poisons like doxorubicin. In contrast, post-doxorubicin blockade of IGF1R increased the activity of topoisomerase II and increased sensitivity of LCC6 cells to doxorubicin.

**Sequential treatment of doxorubicin followed by a low dose of AVE1642 significantly inhibited xenograft tumor growth and metastasis.** Our in vitro data suggest that blockade of IGF1R after doxorubicin was the most effective combination. Moreover, inhibition of IGF1R prior to doxorubicin might interfere with the cytotoxic effects of topoisomerase II inhibitors. Because antibodies have a very long half-life in vivo, we next explored whether low doses of antibody would allow IGF1R to recover over the course of a week. Mice bearing two xenograft tumors implanted in the mammary fat pad of opposite sides were studied. When xenograft tumors were formed, mice were treated with varying doses of AVE1642 by i.p. injection. Tumors were removed from mice at 2 and 7 days after AVE1642 treatments. Figure 6A shows that all doses of AVE1642 suppressed IGF1R levels at 2 days after treatment. Low doses of antibody (25 and 50 μg/mouse) allowed IGF1R to recover to pretreatment levels at 7 days. Whereas some individual
reported to metastasize from the mammary fat pad to the lungs of mice (23). We investigated whether the sequential combination doxorubicin with AVE1642 influenced lung metastasis \textit{in vivo}. At the end of the treatment period, lungs were harvested and examined by histologic examination for metastases. Figure 6C shows that the numbers of pulmonary metastases were reduced in the animals treated with doxorubicin plus low-dose AVE1642.

Anti-IGF1R antibodies commonly down-regulate receptor levels and it is felt that this is a common mechanism of action (10). To determine if our dosing scheme affected IGF1R levels in a manner we expected, we examined tumor IGF1R levels one week after treatment. Figure 6D shows that IGF1R levels were not affected in control and doxorubicin-treated animals; 50 μg AVE1642 only mildly inhibited the IGF1R levels 7 days after treatment and doxorubicin followed by 800 μg AVE1642 dramatically inhibited the IGF1R for at least 7 days. Because the sequence of doxorubicin followed by 50 μg AVE1642 showed a better antitumor effect compared with 800 μg AVE1642, chronic down-regulation of IGF1R by high doses of antibody may not be the optimal strategy when combining with DNA-damaging agents.

Discussion

Several lines of experimental evidence validate IGF1R signaling blockade as an important anticancer target that may prove clinically useful in combination with chemotherapy. Treatment with antiIR3 has been shown to enhance the effect of chemotherapeutic agent doxorubicin and vincristine against Ewing’s sarcoma cells (33). An almost complete inhibition of non–small lung cancer cells (A549) xenograft growth was observed when mice were treated with anti-IGF1R antibody H7C10 combined with vinorelbine (17). Another human anti-IGF1R antibody, CP-751,871, was shown to enhance the antitumor growth effect of chemotherapy in several different cell lines (16). Hug et al. reported that tamoxifen protected cells from the cytotoxicity of both chemotherapy agents 5-fluorouracil and doxorubicin (34). Data from recent randomized trials also showed a clinically antagonistic effect of tamoxifen on concurrent cytotoxic chemotherapy and beneficial effect of sequential chemotherapy and tamoxifen (18, 35). Similarly, inhibition of human epidermal growth factor receptor 2 by trastuzumab followed by paclitaxel increased resistance (19). These preclinical and clinical studies showed that sequencing needs to be considered when combining the chemotherapy with target therapy. Several anti-IGF1R antibodies are currently being evaluated in phase I or II clinical trials (36). Although it is possible that combination of anti-IGF1R antibodies with cytotoxic chemotherapy might be more beneficial than single agents alone, it is also possible that disruption of IGF1R signaling might interfere with the cytotoxic effects. Thus, the optimal schedule for the combination therapy remains to be established.

Our study assesses the effect of two anti-IGF1R antibodies, scFv-Fc and EM164 (humanized version, AVE1642), given concurrently or sequentially, in combination with several widely used cytotoxic drugs. Our study shows several significant considerations for combination therapy. First, there are important sequence-dependent effects when combining some types of chemotherapy with anti-IGF1R antibodies, with drug variation was seen at day 2 (200 μg dose), higher doses of antibody (100, 200, and 400 μg/mouse) suppressed IGF1R levels for the entire 7 days.

Because low doses of AVE1642 suppressed IGF1R expression immediately after dosing, but allowed recovery after 7 days, we used a low and high dose of antibody to model our \textit{in vitro} findings showing that IGF1R suppression at the time of doxorubicin was deleterious. As shown in Fig. 6B, doxorubicin and AVE1642 alone had little effect on LCC6 xenograft growth. A low dose (50 μg/mouse) and a high dose (800 μg/mouse) of AVE1642 were used in combination with doxorubicin. This high dose of AVE1642 was based on our previously published results showing tumor inhibition of MCF-7 cells (30). Doxorubicin followed with the low dose of AVE1642 (four repeated weekly cycles) best resembled the \textit{in vitro} optimal sequence and was most effective \textit{in vivo}. Because high dose of AVE1642 (>100 μg/mouse) resulted in sustained IGF1R down-regulation, repeated four cycles of doxorubicin followed by 800 μg/mouse AVE1642 mimicked the \textit{in vitro} sequence of antibody pretreatment followed by doxorubicin. This higher dose of antibody was superior to doxorubicin alone, but less effective than the low dose of AVE1642.

It has been reported that IGF1R is involved in invasion and metastasis in several tumor types (23, 31, 32). LCC6 has been...
followed by anti-IGF1R antibody as the best sequence. Second, low doses of antibody may be more effective by simulating this sequence-dependent effect in vivo. Third, inhibition of IGF1R prior to exposure to doxorubicin may interfere with the cytotoxic effects of the drug.

Doxorubicin exerts its effects on cancer cells via two different mechanisms: first, it intercalates between the bases of DNA and blocks DNA synthesis and transcription; second, it inhibits the activity of topoisomerase II and leads to breaks in the genomic DNA (37). Topoisomerase II has two isoforms, IIA and IIB, and topoisomerase IIB exhibits cell cycle regulation of its expression that is elevated during S phase and that peaks at G2/M phase (38). Furthermore, tumor cell sensitivity to topoisomerase II inhibitors has been positively correlated with increased nuclear topoisomerase IIA levels (39, 40). Our cell cycle analysis revealed that, when cells were treated with doxorubicin at subtoxic concentration, most cells accumulated in the G2-M phase, reflecting the mechanism of action of doxorubicin; in contrast, pretreatment with scFv-Fc and AVE1642 caused an increase in G0/G1 and S phase accumulation (Fig. 4). Because topoisomerase IIA expression is regulated by cell cycle and relates to drug sensitivity, we measured the topoisomerase IIA expression and activity of different groups. Our experiments showed that pretreatment with anti-IGF1R antibodies decreased both topoisomerase IIA level and enzymatic activity, and decreased sensitivity to doxorubicin. In contrast, the sequence doxorubicin followed by AVE1642 increased the topoisomerase II activity and was associated with increased sensitivity of doxorubicin (Fig. 5). The sequence of pretreatment with anti-IGF1R antibodies resulted in fewer cells in G2/M phase compared with other sequences, consistent with the reduction in topoisomerase IIA levels. Son et al. showed that the activity of topoisomerase II was reduced in a doxorubicin-resistant human stomach cancer cell line (41). Another report showed that the cytotoxicity of doxorubicin was inhibited by anti-ErbB2 antibody trastuzumab and caused by a decrease of topoisomerase II protein and activity (42). These data underscore the correlation of topoisomerase II protein level with doxorubicin sensitivity; increases in topoisomerase IIA expression are associated with sensitivity to the doxorubicin presumably due to increased target on which the drug acts. Although a direct mechanism between IGF signaling and topoisomerase II has not been shown, our results suggest that alteration in topoisomerase II activity may be one possible mechanism to explain the sequence-dependent effect in combination of anti-IGF1R antibody with doxorubicin.

![Fig. 6](image_url)
Down-regulation of IGF1R expression by monoclonal anti-bodies has been shown in vivo and in vitro (30). Our in vitro data suggested that down-regulation of the receptor before chemotherapy would attenuate the cytotoxicity of doxorubicin. Extending these findings to an in vivo mouse model, LCC6 xenograft growth was significantly inhibited with doxorubicin followed by AVE1642 compared with either treatment alone (Fig. 6B). As predicted by the in vitro data, a lower dose of AVE1642 was more effective in combination with doxorubicin than the higher dose. Our data show that transient down-regulation of receptor by the low dose of antibody was associated with a more substantial tumor inhibition in combination with doxorubicin. Previous data from our laboratory showed that inhibition of IGF1R may prevent metastases to lung in LCC6 cells xenograft tumor. Here, we tested the effectiveness of combined therapy against lung metastasis. Similar to the growth of the xenograft tumor, the best results were obtained with doxorubicin followed by transient down-regulation of IGF1R. One hypothesis to explain the lack of benefit for the sustained down-regulation of receptor might be the decreased topoisomerase II protein and activity in vivo seen in these experimental conditions. Our data show that sustained down-regulation of IGF1R impairs sensitivity of doxorubicin by lowering topoisomerase IIα level. The status of topoisomerase II in combination therapy also needs further in-depth investigation. It is noteworthy that not all chemotherapies show this sequence dependency. Furthermore, it is uncertain if cells dependent on IGF1R for proliferation would also have similar sequence-dependent effects. Cohen at al. showed that low doses of a monoclonal antibody were ineffective at suppressing the growth of a mouse fibroblast cell line transfected with IGF1R (16). These data have been taken to suggest that continuous suppression of IGF1R is necessary for single-agent activity, but lower doses of IGF1R inhibitors with chemotherapy have not been studied in IGF1R-dependent cells. Further in vivo modeling of these sequence effects with additional classes of chemother-apy and cell lines need to be pursued.

In summary, our studies have important implications for IGF1R-targeted cancer therapy. First, down-regulation of IGF1R may not affect tumor growth but could sensitize cells to apoptotic insults. Second, the sequencing of IGF1R inhibition with chemotherapy may distinguish between synergy and inter-ference. This observation is important, as several anti-IGF1R antibodies including AVE1642 are being evaluated in phase I/II clinical trials as single agents and in combination with chemotherapy. The antibody dosages used in these trials will likely result in sustained down-regulation of IGF1R. Although this should clearly extinguish IGF1R signaling, the effects on the response to DNA-damaging agents might be counterproductive. Third, given the potential toxicity on glucose metabolism of chronic IGF1R inhibition, it is possible that low doses of anti-bodies might have reduced toxicity. Our study suggests that careful consideration of sequencing and dose is necessary when considering combining anti-IGF1R strategies with chemotherapy.

Disclosure of Potential Conflicts of Interest

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