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Treatment of Breast Cancer Cells by IGF1R Tyrosine Kinase Inhibitor Combined with Conventional Systemic Drugs

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Abstract. *Aim: Insulin-like growth factor-1 receptor (IGF1R) is a tyrosine kinase receptor mediating cell growth and survival of cancer cells. We studied responses to IGF1R tyrosine kinase inhibitor NVP-AEW541 combined with conventional systemic drugs in breast cancer cell lines of different clinical subtype. Materials and Methods: Sensitivity to NVP-AEW541, single treatment and combinations with tamoxifen, trastuzumab, doxorubicin or paclitaxel, was tested in MCF7, SKBR3 and T47D cells. Cells were assayed for proliferation, cell death, cell cycle distribution and phosphorylation of proteins downstream of IGF1R. Results: Treatment of NVP-AEW541 resulted in reduced proliferation, G-1 cell cycle arrest and reduced phosphorylation of protein kinase B (AKT) and extracellular-signal-regulated protein kinase (ERK). Sensitivity to IGF1R tyrosine kinase inhibition was low in T47D cells, despite their high IGF1R expression. NVP-AEW541 combined with trastuzumab had synergistic cytotoxic effects in T47D cells, and additive effects were shown in MCF7 and SKBR3 cells. Also, combination with doxorubicin had antagonistic effects in T47D cells. Doxorubicin caused up-regulation of phosphorylated ERK in T47D cells, which was not inhibited by NVP-AEW541. Conclusion: Antagonistic effects should be anticipated when IGF1R inhibitors are combined with conventional systemic drugs in a subset of breast tumors. Development of functional biomarkers predicting tumor response to tailored IGF1R therapy is warranted.*

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Interaction of insulin-like growth factor 1 with its key receptor IGF1R gives strong proliferation and survival signals (1, 2). Ample evidence has implicated dysregulation of IGF1R signaling in tumor development and progression (3). Currently, development of drugs targeting the IGF1R as anticancer treatment is emerging. Antibodies targeting the IGF1R have been developed and their activity in multiple tumor types is now being tested in clinical trials (4). For example, single-agent activity of figitumumab, a humanized monoclonal antibody directed against IGF1R, has been successfully tested for treatment of Ewing's sarcoma (5). IGF1R functions as a ligand-activated tyrosine kinase receptor. Upon ligand binding, a conformational change induces activation of the kinase. Several docking proteins, such as SRC homology 2 domain-containing (SHC) protein and insulin receptor substrates (IRS1-4), are subsequently recruited to the phosphorylation sites in the cytoplasmic domain. Then the signal is propagated through the phosphatidylinositol-3-kinase (PI3-Kinase)/AKT and mitogen-activated protein (MAP) kinase pathways resulting in cell proliferation and inhibition of apoptosis (6). IGF1R signaling can also induce differentiation, malignant transformation and regulate cell–cell adhesion (2). A dynamic downstream signaling network of different phosphorylation sites of the receptor and cell context specific recruitment and activation of signaling molecules regulates these different functions (7-9). Furthermore, IGF1R can interact with steroid hormones and their receptors, other peptide growth factor receptors, such as human epidermal growth factor (HER) family of receptors, and is intimately connected to the metabolic functions of insulin through a shared structural homogeneity to the insulin receptor (IR),

allowing formation of IGF1R/IR hybrid receptors (10-15). Following successful introduction of small-molecular weight inhibitors of related receptor tyrosine kinases, such as erlotinib, gefitinib and lapatinib, for anticancer therapy (16), tyrosine kinase inhibitors are being developed selectively targeting IGF1R, among them NVP-AEW541 (17). Specificity of tyrosine kinase inhibitors of IGF1R versus IRs has been an issue. Previous studies showed that NVP-AEW541 inhibits tumor growth *in vitro* and *in vivo* in a panel of solid and hematological tumors, alone and in combination with chemotherapeutic drugs (17, 18). Studied efficacy of this agent has been extended to panels of breast cancer cell lines, however, translation of responses to IGF1R targeted tyrosine kinase inhibitors in various breast cancer cell lines into clinically applicable approaches has proven to be difficult (19-22). The mainstay of systemic adjuvant and non-adjuvant treatment for breast cancer nowadays includes anthracycline- and taxane-based chemotherapy, hormonal therapy with tamoxifen and/or aromatase inhibitors for hormone receptor-positive tumors, and targeted therapy, mostly with the HER2 antibody trastuzumab, in HER2-positive tumors (23). We studied responses to IGF1R tyrosine kinase inhibitor NVP-AEW541 in different clinical subtypes of breast cancer cell lines, expressing contrasting levels of the estrogen receptor, HER2 and IGF1R, and tested combinations with conventional systemic drugs.

Materials and Methods

Cell lines. The human breast carcinoma cell lines MCF-7, SKBR3 and T47D were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF7 and T47D are hormone receptor-positive cell lines. SKBR3 is an HER2-positive, hormone receptor-negative cell line. MCF-7 and T47D were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS), SKBR3 was grown in Dulbecco's modified eagle's medium (DME)-high supplemented with 10% FCS. The cells were routinely passaged twice a week. Cells grown in monolayer to 80% confluence were detached by 5 min trypsin treatment (Trypsin 0.025%, 1 mM EDTA) and resuspended in appropriate dilutions in culture medium. Assays were performed in culture medium with lower serum concentrations, 1% FCS for MCF-7 and SKBR3, 5% FCS for T47D.

Quantification of IGF1R, HER2 and epidermal growth factor receptor (EGFR) membrane expression. Cells were seeded in 3 ml culture medium supplemented with 10% FCS in 6-well plates. At day 1, the culture medium was replaced by low serum-containing medium (see above) with or without NVP-AEW541 (0.5-5 μM, stock solution 10 mM in DMSO) (gift from Novartis Pharma AG, Basel, Switzerland) and incubated for another 24 or 72 hours. Cells were harvested at 70-80% confluency, resuspended and assayed by fluorescence-activated cell sorting. Analysis of IGF1R, HER2, and EGFR membrane expression was performed using a flow cytometer (Epics Elite; Coulter Electronics, Hialeah, FL, USA; and FACScalibur; Becton Dickinson Medical Systems, Sharon, MA, USA). For IGF1R,

phycoerytrocin (PE)-conjugated mouse anti-human IGF1Rα (BD Biosciences Pharmingen, San Jose, CA, USA) was used, and PE-conjugated mouse IgG$_1$,κ (BD Biosciences Pharmingen) was taken as an isotype control. For HER2 detection trastuzumab (humanized IgG1 monoclonal antibody against HER2; Genentech, San Francisco, CA, USA) and for EGFR detection cetuximab (humanized monoclonal antibody against EGFR; Merck, Darmstadt, Germany) were used as primary antibodies, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Fc-specific) antibody. FITC-conjugated anti-human IgG antibody alone was taken as an isotype control. Membrane expression was determined by subtracting the mean fluorescent intensity (MFI) of the isotype control from the MFI in presence of the detection antibody. Comparisons were made by paired Student's *t*-test.

Cytotoxicity assay. Sensitivity of MCF-7, SKBR3 and T47D to NVP-AEW541 was tested with 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Prior to the experiments, the optimal cell density was assessed to ensure exponential growth of cells at 96 hours in 96-well microculture plates in conditions of low serum culture used for the experiments. For the experiments, MCF-7 and T47D cells were seeded at 5,000 cells per well and SKBR3 at 7,500 cells per well into 96-well flat-bottom cell culture plates. NVP-AEW541 (1-10 μM), as single treatment, or combined with trastuzumab (1-100 μg/ml), paclitaxel (1-4 nM), or doxorubicin (5-50 nM), was added after attachment of the cells to the culture plate. Tamoxifen (0.5-2 μM) was combined with NVP-AEW541 for MCF-7 and T47D cells and cetuximab (0.5-50 μg/ml) was combined with NVP-AEW541 for T47D and SKBR3 cells. Furthermore, cells were treated with IGF1 (1-500 ng/ml; Abcam, Cambridge, UK) and IGF1R neutralizing (mouse monoclonal) antibody αIR-3 (0.125-1.0 μg/ml, Abcam). In cases of combined treatment, both drugs were added simultaneously and cells were exposed continuously to the drugs. All experiments were performed in quadruplicates and confirmed in at least three independent experiments. The percentage of cell survival was calculated by dividing the mean of the treated samples by the mean of the untreated sample, after subtraction of background extinction. The concentration of NVP-AEW541 single treatment yielding 50% inhibition (IC$_{50}$ values) were calculated. Treatment effects of combined treatments were calculated as follows: Enhancement ratio (ER)=(Survival(modulator+NVP-AEW541)/Survival(modulator)×100)/Survival (NVP-AEW541). Outcomes of ER≤0.8 were considered synergistic, ER≥1.2 antagonistic, and intermediary values, additive effects (24).

Trypan blue cell counts and analysis of apoptosis. In parallel, cells were seeded and treated as described for caspase activity assay and trypan blue cell counts (0.5 ml total volume, 24-well plates). At the designated time points, cell numbers and viability of harvested cells, resuspended in the collected supernatant, were assessed by trypan blue exclusion. This experiment was performed in duplicate and every sample was counted twice. Results are expressed relative to the number of initially seeded cells. Activity of caspase-3 and -7 after exposure to NVP-AEW541 for 24 and 72 hours was determined using the fluorescence peptide substrate Ac-DEVD-AFC (Sigma-Aldrich, Zwijndrecht, the Netherlands), according to the manufacturer's instructions. Relative caspase-3/-7 activity was expressed as the ratio of that of treated to untreated cells. Staining with acridine orange was used as well to distinguish apoptotic cells from vital cells in SKBR3 and T47D cells.

Cell cycle analysis. Exponentially growing cells were harvested and washed in phosphate buffered saline (PBS), and then fixed and permeabilized by ice-cold ethanol. Cells were stored at 4°C for up to 48 hours. Prior to the addition of propidium iodide (PI), the cells were washed and incubated with RNAse for 15 min at room temperature. After PI incubation, the DNA content of at least 5,000 cells was measured by flow cytometry and analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA). Differences in cell cycle distribution between treated and untreated samples were analyzed by Chi-square test.

Western blot analysis. To determine phosphorylation status of the IGF1R and downstream proteins AKT and ERK, cells were cultured in 6-well plates to a confluence of 70% in culture media supplemented with 10% FCS. Cells were then serum starved for 8 hours and treated with IGF1R inhibitor NVP-AEW-514 (2.5 μM) and the antibody αIR-3 (1 μg/ml) for 10 min and 6 hours. Five minutes before harvesting, the cells were stimulated with 100 ng/ml IGF1. Combination effects of doxorubicin and NVP-AEW541 were determined by adding NVP-AEW541 at 2.5 μM 8 hours before or 8 hours after treatment with doxorubicin at 0.5 μM in culture media containing 1 and 5% FCS, respectively. Cells were harvested on ice, washed three times in ice-cold PBS and cell lysates were prepared with lysis buffer consisting of mammalian protein extraction reagent (M-PER) with 1:100 Protease Inhibitor Cocktail and 1:100 Phosphatase Inhibitor (Pierce, Rockford, IL, USA). After determination of protein concentrations by Bradford analysis, cell lysates clarified of insoluble components were dissolved in sodium dodecyl sulfate (SDS)-sample buffer with 10% 2-β-mercaptoethanol to a final concentration of 1 μg/μL protein and boiled for 5 min. The samples were stored at –20°C until analysis. Equivalent amounts of cell lysate were separated by 10% SDS-polyacrylamide gel electrophoresis (1 hour 180 V; BioRad, Veenendaal, the Netherlands) and transferred to a polyvinylidene difluoride (PVDF) membrane (1 hour 250 mA; Immobilon-P; Millipore, Billerica, MA, USA). After appropriate washing and blocking of the membrane (TBS-tween-milk; Sigma Aldrich), focus proteins were detected by the respective antibody directed towards phospho p44/42 MAPK (ERK) (thr202/204) E10 (mouse monoclonal, 1:2000 in TBS-t-milk), phospho AKT (ser473) (rabbit; 1:1000 in BSA), AKT (rabbit, 1:1000 in BSA), phosphorylated IGF1R (tyr1135/1136)/IR (tyr1150/1151) (monoclonal rabbit, 1:1000 in BSA) (all: Cell Signaling, Danvers, MA, USA) and ERK-1 (k-23):sc-94 (polyclonal rabbit, 1:800 in BSA; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with overnight incubation at 4°C with 1:1000 chlorohexidine. A secondary horseradish peroxidase (HRP)-conjugated antibody directed to mouse (rabbit-anti-mouse) or rabbit (goat-anti-rabbit) (1:1500 in TBS-t-milk; DakoCytomation, Glostrup, Denmark) was used and staining was performed with Lumilight Plus (western blotting substrate; Roche, Almere, the Netherlands) or for actin staining BM chemiluminescence blotting substrate (POD; Roche).

Results

Sensitivity to IGF1R tyrosine kinase inhibition was tested in three breast cancer cell lines with different membrane expression levels of IGF1R, HER2 and EGFR. MCF7 and T47D are hormone receptor positive cell lines, expressing high levels of IGF1R. Whereas MCF7 expresses very low levels of

HER2 and EGFR, T47D expresses intermediate levels of HER2 and EGFR; SKBR3 is an HER2-positive, hormone receptor-negative cell line, expressing low levels of IGF1R and high levels of EGFR (Figure 1). IGF1 stimulation enhanced proliferation in MCF7 (60% at 100 ng/ml), but not in SKBR3 and T47D. NVP-AEW541 reduced proliferation as measured by MTT assays in all tested cell lines in a dose-dependent manner (IC_{50} MCF7=2.9±0.6 μM, SKBR3=4.9±0.4 μM, T47D=5.6±0.4 μM) (Figure 2). This was confirmed by trypan blue cell counts; however, no significant effect on caspase activity as an indicator of apoptosis was shown. By acridine orange staining, small numbers of apoptotic cells were found: 12% in SKBR3 cells treated with 5 μM NVP-AEW541. Cell cycle distribution changed significantly on treatment with NVP-AEW541 with reduction of the S-phase fraction and increase of the G-0/G-1 fraction (Figure 3A). IGF1R membrane expression levels were significantly reduced by 20% after 24-hour treatment with 2.5 μM NVP-AEW541 in all cell lines. After 72-hour treatment, however, persistent receptor down-regulation was only seen in T47D cells (Figure 4). The neutralizing anti-IGF1R antibody αIR-3 did not have any substantial effect on cell survival of any of the cell lines.

NVP-AEW541 combined treatment. NVP-AEW541 combined with trastuzumab had synergistic cytotoxic effects in T47D cells (ER=0.78±0.07, at doses of 5 μM and 100 μg/ml, respectively). This combination also synergistically reduced the S-phase fraction (Figure 3B). Additive effects were shown in SKBR3 and MCF7 cells. NVP-AEW541 combined with doxorubicin had antagonistic effects in T47D cells (ER=1.75±0.26, at doses of 6 μM and 25 nM, respectively), irrespective of treatment sequence (Figure 5). Additive effects for NVP-AEW541 and doxorubicin were shown in SKBR3 and MCF7 cells. All other combinations tested revealed additive effects. On protein level (Figure 6), phosphorylated IGF1R/IR level was not measurable in SKBR3 and T47D cells (below detection limit), but was evident in MCF-7 cells after IGF1 stimulation. We found AKT phosphorylation was up-regulated by IGF1 stimulation both in MCF-7 and T47D cells, as well as complete down-regulation of IGF1 induced phosphorylated AKT in both cell lines in response to NVP-AEW541 and αIR-3 after longer incubation times. In SKBR3 cells AKT was constitutively activated and not further up-regulated by IGF1 stimulation, yet in response to NVP-AEW541, AKT was down-regulated. The ERK antibody detects pERK1 at p44 and, to a lesser extent, pERK2 at p42. In MCF7 cells both phosphorylated variants of ERK are up-regulated in response to IGF1, and this up-regulation is only partially blocked by NVP-AEW541. In T47D and SKBR3 cells, an amount of constitutively active ERK2 exists. Upon IGF1 stimulation, strong up-regulation of phosphorylation of both variants of ERK occurs in T47D and only slightly in SKBR3 cells. In addition, in T47D cells NVP-AEW541 more
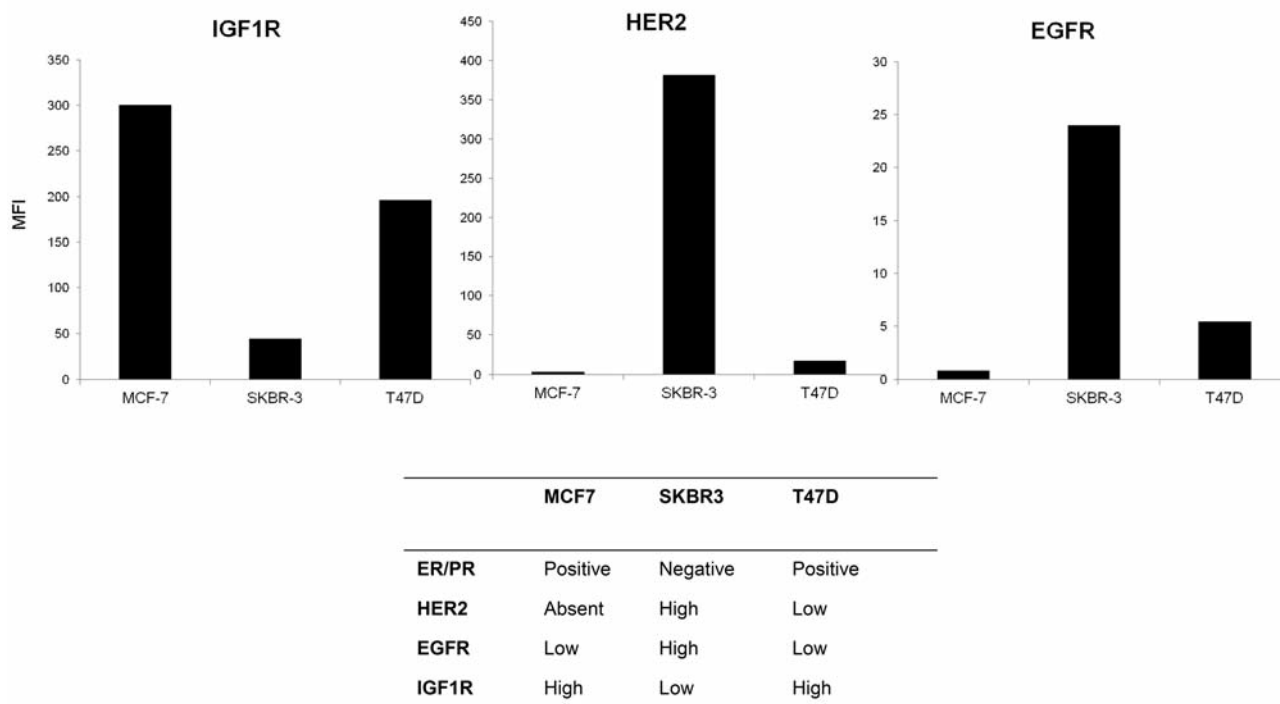
	MCF7	SKBR3	T47D
ER/PR	Positive	Negative	Positive
HER2	Absent	High	Low
EGFR	Low	High	Low
IGF1R	High	Low	High

Figure 1. *Membrane expression levels of insulin-like growth factor-1 receptor (IGF1R), human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) in breast cancer cell lines MCF7, SKBR3 and T47D (mean fluorescent intensity (MFI)).*

profoundly blocked IGF1 induced ERK activation than in SKBR3 cells. Constitutive levels of pERK were not blocked. IGF1 induced ERK activation was blocked by NVP-AEW541 more effectively in T47D compared to MCF7 cells (Figure 6). Doxorubicin caused a slight up-regulation of phosphorylated ERK in T47D cells and NVP-AEW541 did not inhibit this up-regulation. No significant effect of single treatment with doxorubicin or combined with NVP-AEW541 on phosphorylated AKT levels were seen (Figure 7). Adding doxorubicin resulted in an enormous increase of cells arrested in G-2/M and S phases, to a degree in which proper fitting became inaccurate. Adding NVP-AEW541 resulted in a slight increase of cells in the G-0/G-1 phase compared to single treatment with doxorubicin (Figure 3C).

Discussion

The aim of this work was to show tumor responses to NVP-AEW541 in breast carcinoma cell lines of different clinical subtypes and test the combination with conventional systemic drugs. Selection of our cell lines was based on the histologic parameters which direct current selection of breast cancer therapy. The SKBR3 cell line represents HER2-overexpressing breast tumors. MCF7 and T47D represent luminal hormone receptor-positive breast cancer. Because
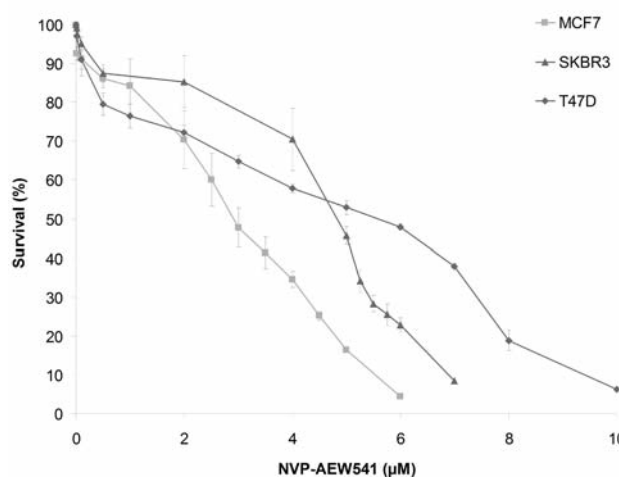

Figure 2. *Sensitivity to NVP-AEW541 as measured by 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, mean±SEM (n=4).*

MCF7 is strongly ER positive and HER2 negative, it may represent luminal A breast cancer, whereas T47D might represent luminal B breast cancer, because this cell line is ER positive and weakly HER2 positive. No cell line
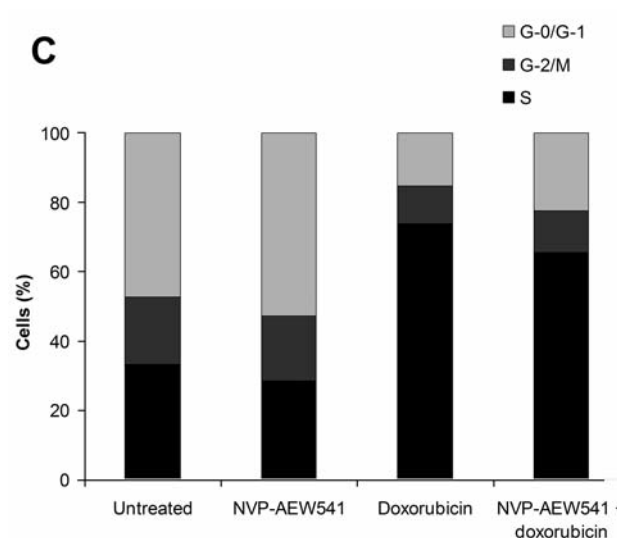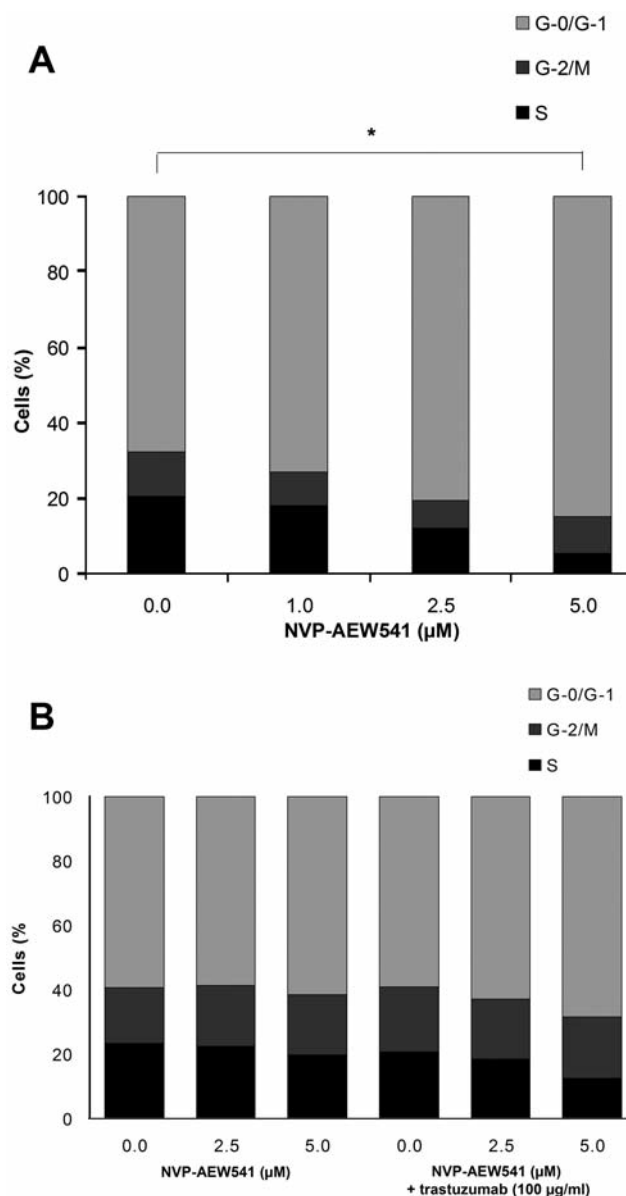
Figure 3. *A: Dose-dependent reduction of S-phase fraction in MCF7 cells after 72 hour treatment with NVP-AEW541 (n=3). *p-value 0.002, χ^2-test. B: Synergistic reduction of S-phase fraction by concurrent treatment of T47D cells with NVP-AEW541 and trastuzumab (100 μg/ml). C: Effect on cell cycle distributions of concurrent treatment of T47D cells with NVP-AEW541 (5 μM) and doxorubicin (25 nM).*

representing the triple negative or basal-like subtype was included in the present study. Best responses to IGF1R-directed therapy are anticipated in high IGF1R-expressing cells. However, the magnitude of effect of NVP-AEW541 in our experiments was not proportional to IGF1R membrane expression. T47D cells, although expressing high levels of IGF1R, showed resistance to IGF1R-targeted drugs in comparison to high IGF1R-expressing MCF7 cells, and was even less sensitive to NVP-AEW541 than low IGF1R-expressing SKBR3 cells. These data may indicate that luminal B type tumors may be resistant to IGF1R-targeted therapy in contrast to the luminal A type, irrespective of IGF1R expression. NVP-AEW541 induces G-0/G-1 arrest in breast cancer cell lines grown in monolayer and inhibits proliferation, while apoptosis is not a prominent effect. Comparison of previous studies suggests more dramatic effects of IGF1R inhibition in anchorage-independent growth conditions (anoikis) compared to monolayer (proliferation) (25). Our results in monolayer culture are consistent with the results of others by IGF1R inhibition with αIR-3 (26). We further show IGF1R down-regulation by NVP-AEW541. IGF1R antibodies are known to cause receptor down-regulation by internalization and degradation of the receptor by endocytosis (27-29). Mechanisms by which receptor down-regulation occurs with tyrosine kinase inhibition of the receptor are as yet unclear. Receptor down-regulation may contribute to the effects of therapeutic drugs on IGF1R functioning, or may be merely a manifestation of a low proliferative state. Our assays may indicate the latter, as we saw a similar effect on receptor down-regulation occurring at a later timepoint in untreated cells. Cells might well have been beyond their exponential growth phase at this later timepoint. AKT and ERK are both critical components of two respective major downstream proliferation and survival pathways of IGF1R. In the presence of IRS1, IGF1R signaling through phosphorylation of receptor phosphorylation sites induces recruitment and phosphorylation of IRS proteins, resulting in recruitment of p110, the catalytic subunit of PI3K, generation of phosphoinositol trisphosphate, and subsequent activation of
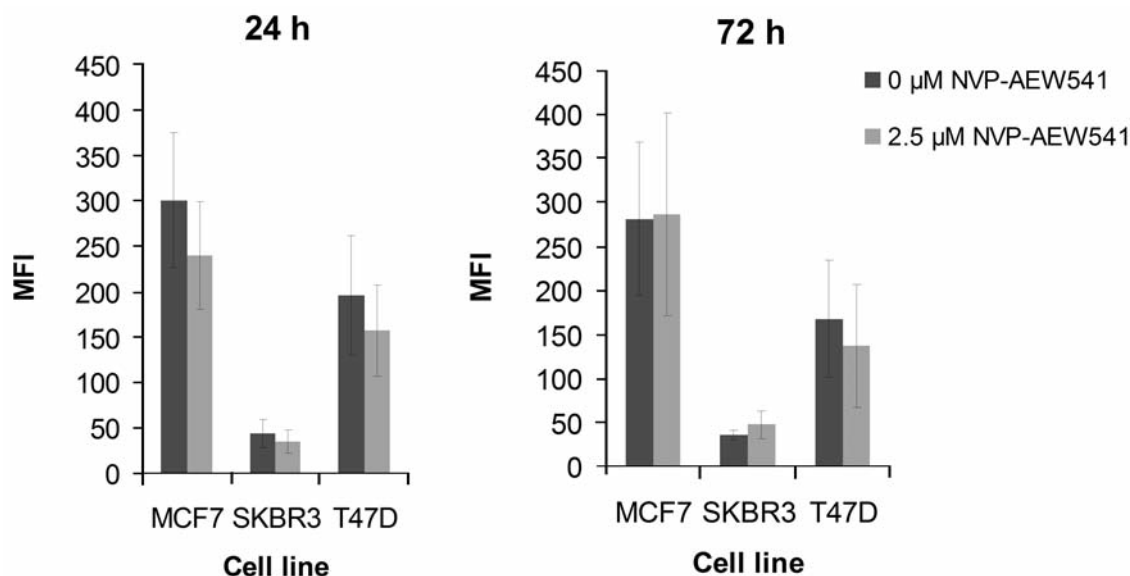
Figure 4. *Insulin-like growth factor-1 receptor (GF1R) membrane expression (mean fluorescent intensity (MFI)) after 24-hour and 72-hour treatment with 2.5 μM NVP-AEW541 (n=4).*

AKT. The IRS1/PI3K/AKT pathway is considered a preferential pathway of IGF1R (30). The second major survival pathway results from Rat sarcoma (RAS) signaling after recruitment of adaptor proteins to the activated IGF1R and subsequent activation of transcriptional programs downstream of the ERKs, also referred to as the MAPK pathway. The requirement for activation of AKT or ERK in IGF1R-mediated (survival) signals appears to be context dependent and has not yet been fully determined. Moreover, other receptor tyrosine kinases, such as HER2 and EGFR, share the same downstream signal transduction (16). Our breast cancer cell lines of different clinical subtypes exhibited differential phosphorylation levels of both AKT and ERK in low serum conditions, upon stimulation with IGF1, and blocking by NVP-AEW541, respectively. A previous study comparing MCF7 to T47D cells, expressing almost identical IGF1R levels, has suggested that reduced sensitivity/resistance to NVP-AEW541 in T47D cells is determined by a lack of IGF1R signaling through downstream proteins IRS1 and PI3K/AKT (22). In contrast to this report, we found AKT phosphorylation by IGF1 stimulation both in MCF-7 and T47D cells, as well as down-regulation of phosphorylated AKT in both cell lines in response to NVP-AEW541. Caution should be exercised with comparing data of the same putative cancer cell lines yet cultured in separate laboratories, as these may differ considerably due to genetic instability and digression. However, our data seem to confirm that T47D cells do not depend on the IRS1/PI3K/AKT pathway for survival and proliferation, whether or not the pathway is functional,

causing resistance to NVP-AEW541. Contrary to expectations, in SKBR3 cells, levels of constitutively activated AKT, which did not further change upon IGF1 stimulation, were nonetheless down-regulated in response to NVP-AEW541. This may suggest constitutively activated IGF1R, or, more likely, non-IGF1R-specific effects of NVP-AEW541. Non-IGF1R-specific effects of NVP-AEW541 are supported by reported dosage-dependent specificity of the drug. The IC50 of inhibition of IGF1R autophosphorylation by NVP-AEW541 is much lower (0.086 μM) than the effective doses in our study (2.9-5.6 μM). The reported IC_{50} of inhibition of IR autophosphorylation by NVP-AEW541 is 2.3 μM (17). Although we did not exclude co-targeting of both IGF1R and IR by NVP-AEW541 in our study, the measured phosphorylated receptor levels by an antibody directed against both phosphorylated forms of IGF1R and IR showed no activity in SKBR3 cells. Another possibility may be that heterodimeric or heterotrimeric complexes of IGF1R with HER1-4 receptors are formed and co-targeted by NVP-AEW541 (15, 31). Several lines of evidence suggest synergistic augmentation of trastuzumab effects by NVP-AEW541 in HER2-positive breast cancer with or without acquired trastuzumab resistance (19, 20). IGF1 induced ERK phosphorylation in MCF7 cells was only partly blocked by NVP-AEW541, possibly due to remaining activity of non-tyrosine kinase domains, such as serine clusters. Constitutive levels of pERK in SKBR3 and T47D cells were not blocked, suggesting constitutive ERK activation independently of the IGF1R. Whereas combinations of NVP-AEW541 with cytostatic agents had additive effects in MCF7 and SKBR3
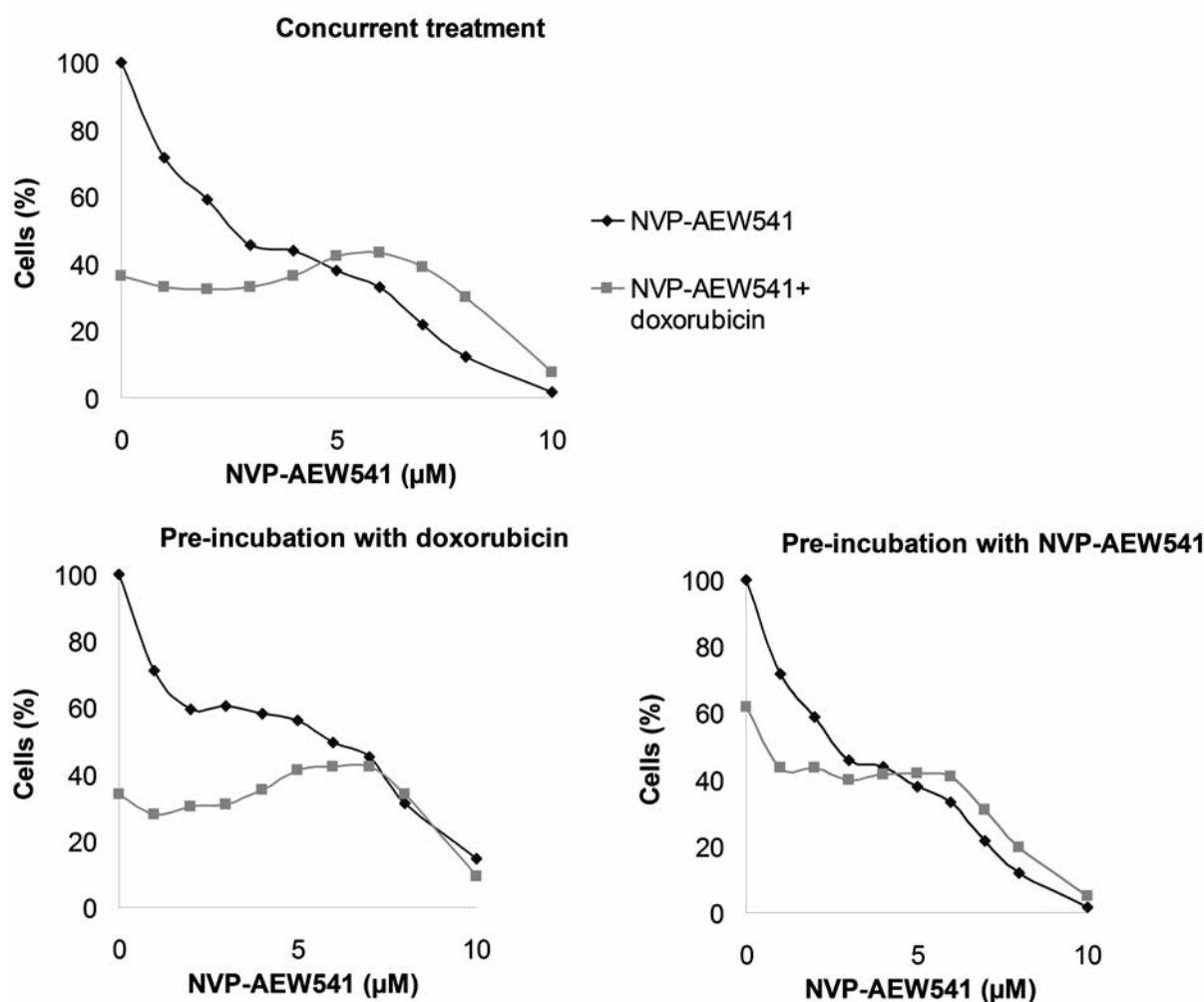
Figure 5. *Antagonistic effects of doxorubicin (25 nM) and NVP-AEW541 combinations in a 96-h MTT assay of T47D cells.*

cells, comparable to responses seen by other study groups (32-34), we found an antagonistic effect of the combination of NVP-AEW541 with doxorubicin in T47D cells. Varying sequences of NVP-AEW541 and doxorubicin administration did not alter the antagonistic effects in our hands. Timing of IGF1R-targeted therapy by antibodies has previously been shown to affect responses to chemotherapy both *in vitro* and *in vivo* (35). The optimal sequence proved to be doxorubicin followed by anti-IGF1R antibody. Clearly, these results do not apply to IGF1R tyrosine kinase inhibition in the same way. This could be explained by different time-effect curves of antibodies vs. tyrosine kinase inhibition, for instance NVP-AEW541 blocked IGF1R activity more quickly than the antibody αIR-3. No plausible explanation was found for the antagonistic effect in T47D cells. Up-regulation of phosphorylated ERK levels after single treatment with doxorubicin, which NVP-AEW541 did not inhibit, suggests

ERK up-regulation occurs downstream of the IGF1R, and upstream inhibition may therefore be in vain. On the other hand, such a mechanism is unlikely to cause contrary effects. Cell cycle distribution analysis suggests additive effects of doxorubicin and NVP-AEW541. Consistent with previous reports, doxorubicin alone arrests cells in the G-2/M phase by interacting with DNA synthesis and transcription by DNA intercalation (36). Due to the NVP-AEW541 induced G-0/G-1 arrest, G-2/M arrest by doxorubicin is reduces, but the overall S-phase fraction is not altered. The biological response to IGF1R inhibitors in cancer cells is diverse and may be dependent on multiple factors, according to the literature. Many studies have indicated not only IGF1R expression, but interaction with related receptors, such as estrogen receptor, HER2 family of receptors and the IR, as well as functionality of downstream proteins, such as IRS1 and -2, and AKT, on determining functional responses of the
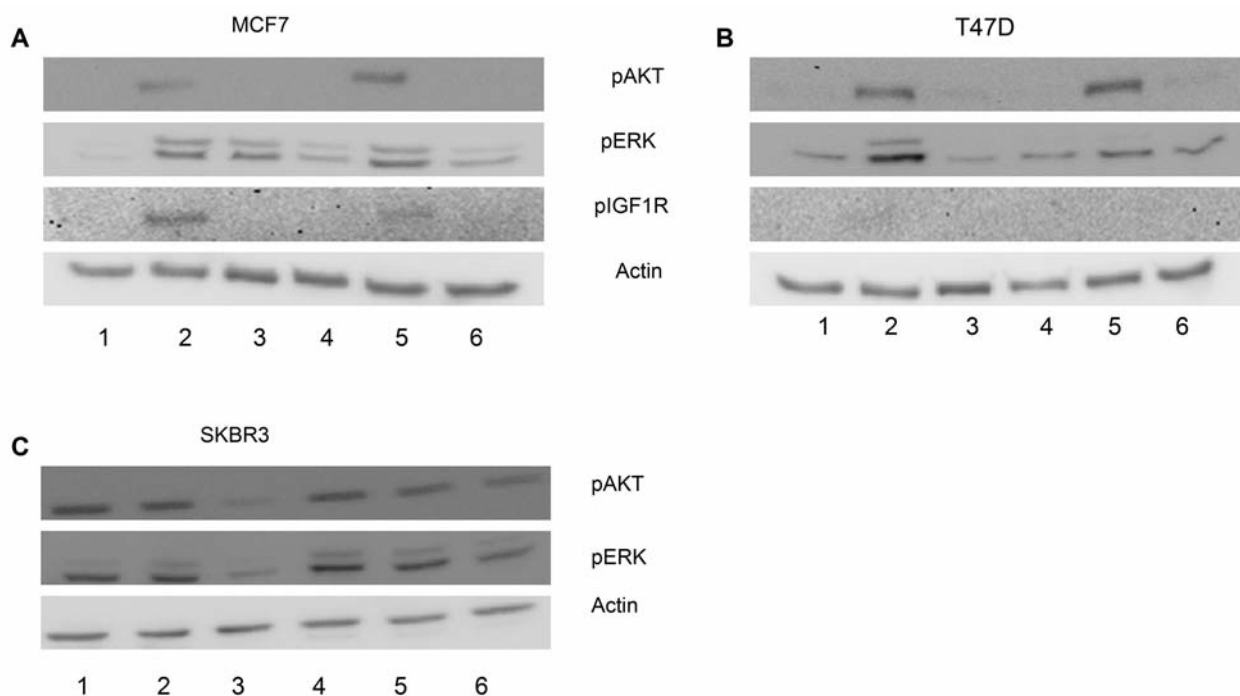
Figure 6. *The effect of aIR-3 and NVP-AEW541 on phosphorylated IGF1R and downstream AKT and ERK in serum-starved MCF7 (A) T47D (B) and SKBR3 (C) cells. 1: untreated cells; 2: IGF1 (100 ng/ml) for 5 min; 3: NVP-AEW541 (2.5 µM) for 10 min followed by IGF1 (100 ng/ml) for 5 min; 4: NVP-AEW541 (2.5 µM) for 6 h followed by IGF1 (100 ng/ml) for 5 min; 5: aIR-3 (1 µg/µl) for 10 min followed by IGF1 (100 ng/ml) for 5 min; 6: aIR-3 (1 µg/µl) for 6 h followed by IGF1 (100 ng/ml) for 5 min; Actin was used as a control for loaded protein concentrations.*
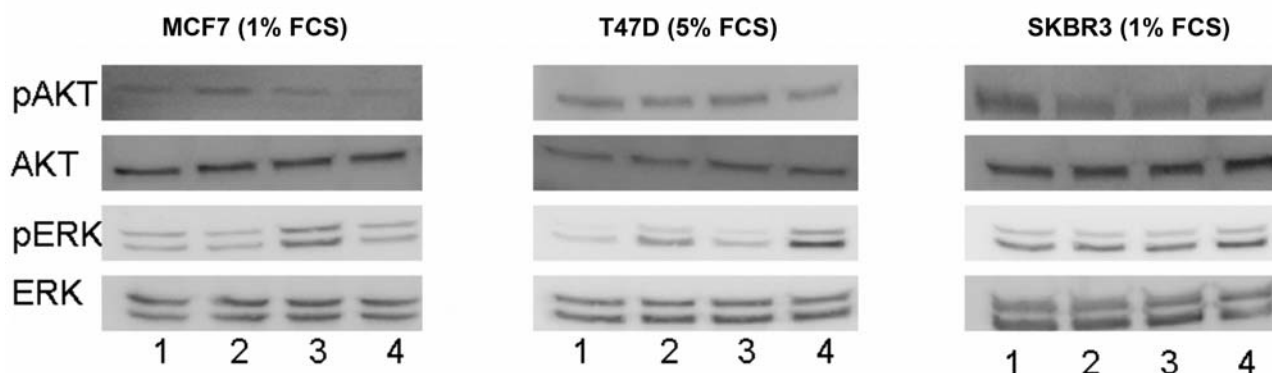

Figure 7. *The effect of doxorubicin and NVP-AEW541 on MCF7 (left), T47D (center) and SKBR3 (right). Lanes: 1: untreated cells; 2: doxorubicin 0.5 µM 24 h; 3: 2.5 µM NVP-AEW541 followed by doxorubicin 0.5 µM 24 h; 4: doxorubicin 0.5 µM 24 h followed by 2.5 µM NVP-AEW541.*

IGF1R and sensitivity to anti-IGF1R agents. Our study confirms the results of many other studies and we see conventional results in a majority of our cell lines and drug combinations. However, more problematic issues arise when we look in further detail into the minority of unexplained contrary effects. Neither in our study, nor in the existing literature, did we find satisfactory clarifications of unpredicted responses, which raises the possibility of

unexpected and contrary effects in clinical use. Analogous to the cell lines MCF7 and T47D, responses to IGF1R inhibitory drugs may be unpredictable in patients with high IGF1R-expressing and hormone-responsive breast cancer. It also may be possible that combining chemotherapy with IGF1R tyrosine kinase inhibitors may be beneficial in certain patients in combination with certain chemotherapeutic drugs, but disadvantageous in others. Tyrosine kinase inhibitors are

generally considered less specific compared to monoclonal antibodies and may be more prone to having upredictable clinical effects. In order to address such issues, we believe new techniques and biomarkers are needed to overcome the shortcomings of the studies to date.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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