

to culture media on day 0. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was added to the cell culture media (0.5 mg/mL) and incubated for 4 hours. After solubilization of the precipitated formazan crystals, absorbance at 490 nm was determined.

Immunoprecipitation and immunoblotting analysis. Cells were washed with cold PBS and lysed in buffer [0.5 mol/L NaCl, 20 mmol/L HEPES (pH 7.3), 50 mmol/L sodium fluoride, 10% glycerol, 1% Triton X-100, 5 mmol/L EDTA, aprotinin (6 µg/mL), leupeptin (10 µg/mL), and 1 mmol/L sodium orthovanadate]. After clearing insoluble material with centrifugation, immunoprecipitation was done with following antibodies: for EGFR, EGFR #22, rabbit polyclonal antisera against the carboxyl-terminal domain of EGFR; for HER-2, mouse monoclonal antibody clone 9G6.10 (Lab Vision/NeoMarkers, Fremont, CA). After immunoprecipitates were separated by SDS-PAGE, proteins were transferred to a membrane and probed with antiphosphotyrosine antibody (PY20-H, Santa Cruz Biotechnology, Santa Cruz, CA) and detected with enhanced chemoluminescence. Alternatively, whole cell lysates, after gel electrophoresis and transfer, were probed with antibody against phospho- and pan-p44/42 (Cell Signaling Technology, Beverly, MA) or phospho- and pan-Akt (Cell Signaling) or prostate-specific antigen (PSA; Santa Cruz Biotechnology) or AR (Santa Cruz Biotechnology) as indicated.

Quantitation of prostate-specific antigen protein. LNCaP cells were seeded at a density of 10^6 cells per well in six-well plates and allowed to attach overnight. Cells were incubated in 2 mL serum-free media and dihydrotestosterone (2 nmol/L) or GW572016 (10 µmol/L) or ZD1839 (10 µmol/L) was added. After 24 hours of incubation, the amount of PSA protein in supernatant media was determined by an ELISA kit (ICN Pharmaceutical, Costa Mesa, CA).

Northern blot analysis. Total RNA was prepared from cells using the RNEasy kit (Qiagen, Valencia, CA) according to manufacturer's directions. Twenty micrograms RNA per lane were separated using an agarose gel and transferred to a nylon membrane. Northern blot analysis was done using a probe specific for PSA, as previously described (17).

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation analysis was done according to a published procedure (18) with the following modifications. LNCaP cells (5×10^6 cells) were incubated in steroid-depleted media containing 10% charcoal-stripped FCS for 4 days and then stimulated with dihydrotestosterone (10 nmol/L) for 16 hours. Cells were treated with 1% formaldehyde at room temperature for 10 minutes and then 0.125 mol/L glycine at 37°C for 5 minutes. Cell pellets were collected and lysed in 0.4 mL lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris (pH 8.1), 150 mmol/L NaCl] on ice for 10 minutes and then sonicated for 45 seconds total (sequential 1 second pulse followed by 1 second gap) using a Branson Sonifier 200 (Branson Sonifier, Danbury, CT). Insoluble debris was removed by centrifugation and the soluble chromatin was diluted 2-fold with a buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris (pH 8.1), and 167 mmol/L NaCl]. Two hundred microliters of the diluted solution were precleared with salmon sperm DNA/protein A agarose (Upstate Biotechnology, Lake Placid, NY) for 1 hour at 4°C. The supernatant was incubated overnight at 4°C with AR antibody (Santa Cruz Biotechnology) or acetylated histone H3 antibody (Upstate Biotechnology). Sixty microliters of salmon sperm DNA/protein A agarose were added and Sepharose beads were washed sequentially with a low salt buffer, a high salt buffer, a LiCl buffer, and a TE buffer (18). DNA was eluted from the beads, heated at 65°C for 6 hours, phenol/chloroform extracted, ethanol precipitated, and resuspended in 10 µL water. Two hundred microliters of diluted soluble chromatin were processed in the same way without immunoprecipitation and termed input DNA. The amount of immunoprecipitated DNA was determined by real-time PCR reaction using the ABI PRISM 7900HT system (Applied Biosystems, Branchburg, NJ). The primer and probe sequences amplifying the androgen responsive element III enhancer located 4.2 kb upstream of the transcription start site of the *PSA* gene were according to Jia et al. (18). One microliter of DNA was analyzed in each PCR reaction containing Taqman universal PCR master mix (Applied Biosystems). Serial dilutions of input DNA was run in the PCR reactions in parallel and the amount of immunoprecipitated DNA was normalized to the input DNA.

Results

Inactivation of HER-2 signaling by expression of intracellular antibody against HER-2. To investigate the role of HER-2 signaling in AR function, we inhibited endogenous HER-2 activity by stable intracellular expression of single-chain antibody against HER-2 protein. This antibody scFv-5R is fused with a peptide signal for retention in the endoplasmic reticulum, thereby preventing mature HER-2 protein from reaching the cell membrane. This abolishes downstream signaling by HER-2 and leads to inhibition of the HER-2-driven transformed phenotype (14, 15). We utilized the LNCaP cell line to study the functional interaction between AR and HER-2, because LNCaP has been extensively characterized as a model of AR signaling in prostate cancer and does not overexpress HER-2. LNCaP cells stably expressing the HER-2 single-chain antibody were derived by transduction with retrovirus encoding the antibody followed by antibiotic selection. In vector control cells, both EGF and heregulin treatment activated HER-2 phosphorylation, presumably signaling through the EGFR/HER-2 or HER-2/HER-3 heterodimer. In LNCaP-scFv-5R cells expressing the HER-2 antibody, HER-2 phosphorylation was not induced in response to heregulin or EGF treatment. However, EGF-induced phosphorylation of EGFR remained intact in scFv-5R cells (Fig. 1A), demonstrating that only HER-2 signaling is selectively impaired in scFv-5R cells. Furthermore, heregulin did not induce p44/42 MAPK phosphorylation in scFv-5R cells, whereas heregulin induced p44/42 activation in vector control cells (Fig. 1B). Akt signaling was constitutively activated in these cells because of loss of the PTEN tumor suppressor. Heregulin treatment did not further activate Akt in control cells, and expression of the scFv-5R antibody had no effect on Akt signaling (Fig. 1B). These data show that expression of the scFv-5R antibody leads to selective inhibition of HER-2 signaling in LNCaP cells.

Inhibition of androgen receptor function by intracellular HER-2 antibody. We characterized the effect of inhibition of HER-2 signaling on AR function. Whereas addition of dihydrotestosterone to the steroid-depleted culture media robustly induced proliferation of vector control LNCaP cells, scFv-5R LNCaP cells proliferated poorly in response to dihydrotestosterone (Fig. 1C). We examined androgen-stimulated expression of a well-characterized androgen-regulated gene *PSA*, as a marker of AR-induced transcription in these cells. In vector control cells, dihydrotestosterone treatment increased intracellular PSA protein expression, as detected by immunoblotting. In contrast, dihydrotestosterone-stimulated PSA protein expression in scFv-5R cells was markedly decreased (Fig. 2A). Quantitative measurement of PSA protein secreted into culture media showed that induction of PSA synthesis in response to increasing doses of dihydrotestosterone was severely impaired in scFv-5R cells, compared with vector control cells (Fig. 2B). Northern blot analysis showed that dihydrotestosterone-stimulated up-regulation of PSA mRNA expression was likewise inhibited in scFv-5R cells (Fig. 2C). Therefore, the inability to induce PSA synthesis in scFv-5R cells was most likely at the transcriptional level. Taken together, these data are consistent with the idea that HER-2 inhibition impairs AR transcriptional function.

Inhibition of epidermal growth factor receptor and HER-2 by GW572016 (lapatinib), a dual kinase inhibitor. We were interested in alternative approaches to HER-2 inhibition that are amenable to therapeutic application in prostate cancer. GW572016 is a novel small molecule kinase inhibitor with selectivity for both EGFR and HER-2 (11, 12). To verify that GW572016 inhibits EGFR

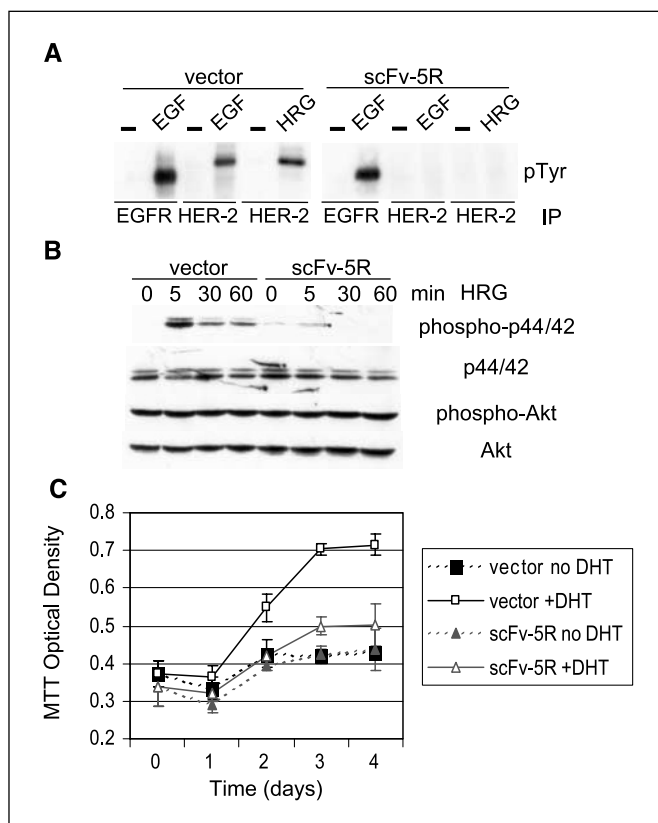


Figure 1. Stable expression of intracellular antibody against HER-2 leads to inactivation of HER-2 signaling and inhibition of androgen-stimulated proliferation of LNCaP cells. *A*, vector control cells or scFv-5R cells stably expressing intracellular antibody against HER-2 were treated with EGF (100 ng/mL) or heregulin (HRG; 10 ng/mL) for 15 minutes. Cell lysates were immunoprecipitated with antibody specific for EGFR or HER-2 and then immunoblotted with antibody specific for phosphotyrosine (pTyr). *B*, vector control or scFv-5R cells were treated with heregulin (50 ng/mL). At indicated times, cells were harvested and cell extracts were analyzed by immunoblotting with antibody against phospho-p44/42 MAPK, pan-p44/42 MAPK, phospho-Akt, or pan-Akt. *C*, equal numbers of vector control or scFv-5R cells in 96-well plates were treated with dihydrotestosterone (DHT, 2 nmol/L) and relative cell number was determined daily with the colorimetric dye MTT. Points, mean of the absorbance readings of triplicate wells; bars, SD. Similar results were obtained from three independent experiments.

and HER-2 kinases, LNCaP cells were pretreated with increasing doses of GW572016 and the amount of tyrosine-phosphorylated receptor after stimulation with EGF or heregulin was determined. GW572016 abrogated EGF-induced phosphorylation of EGFR in a dose-dependent manner (Fig. 3A). GW572016 potently inhibited heregulin-induced phosphorylation of HER-2 at a relatively low dose and was capable of inhibiting basal activation of HER-2 at a higher dose.

Inhibition of androgen-induced prostate-specific antigen expression by GW572016. Having established that GW572016 inhibits EGFR and HER-2 kinase activity, we characterized the effect of GW572016 on androgen-regulated expression of PSA in LNCaP cells. Treatment of cells with GW572016 completely abolished androgen-stimulated PSA protein expression, as assessed by immunoblot analysis (Fig. 3B). Because GW572016 is capable of inhibiting both EGFR and HER-2, we also utilized ZD1839 (gefitinib), which is a more selective inhibitor of EGFR over HER-2 (13). Our dose-titration analysis of ZD1839 on inhibition of ligand-induced EGFR and HER-2 activation also confirmed that ZD1839

inhibits EGFR more potently than HER-2 (19). Treatment of cells with ZD1839 had only a minor effect on dihydrotestosterone-stimulated intracellular PSA expression (Fig. 3B). In agreement with the above results, GW572016 markedly reduced the amount of PSA protein secreted into the culture supernatant after dihydrotestosterone stimulation, whereas ZD1839 had only a modest effect on dihydrotestosterone-induced PSA production (Fig. 3C). These results are consistent with the hypothesis that GW572016, similar to the intracellular HER-2 antibody, impairs AR function through inhibition of HER-2 kinase.

Intracellular localization of androgen receptor after HER-2 inhibition. To characterize the mechanism underlying inhibition of androgen-induced gene expression by HER-2 inhibition, we characterized androgen-induced stabilization of AR protein in cells after HER-2 inhibition. In both scFv-5R cells and in cells treated with GW572016, dihydrotestosterone-stimulated increase in the AR protein level was comparable with control cells (Figs. 2A and 3B). We next characterized intracellular localization of AR in scFv-5R cells and cells treated with GW572016 by immunofluorescence staining. In cells without dihydrotestosterone treatment, AR staining was weak and AR was present in the nuclear and

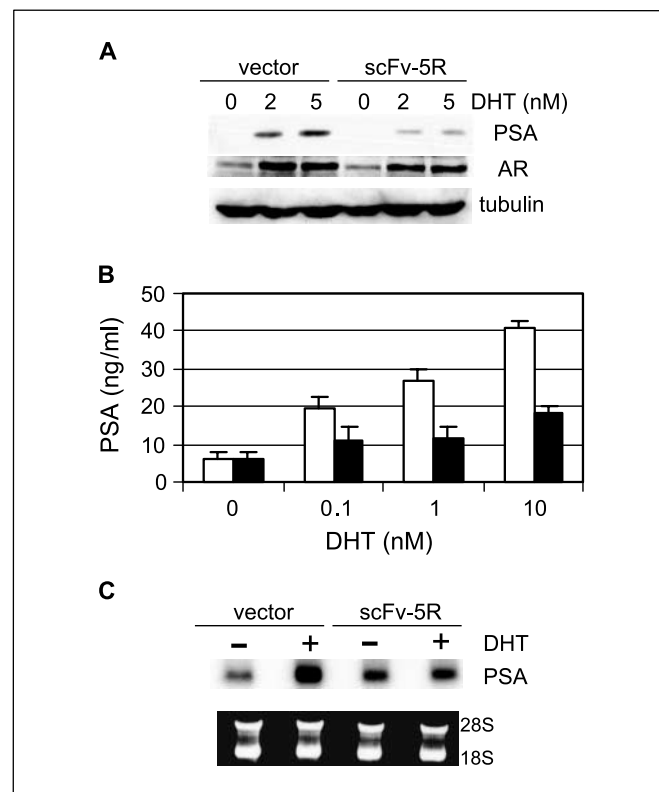


Figure 2. Functional inactivation of HER-2 leads to inhibition of androgen-stimulated PSA production. *A*, vector control or scFv-5R cells were stimulated with dihydrotestosterone at indicated concentrations. Cells were harvested after 16 hours. Intracellular PSA and AR protein expression was determined by immunoblotting with antibody specific for PSA and AR. The membrane was stripped and reprobed with antibody against tubulin to confirm equal loading. *B*, vector control or scFv-5R cells were stimulated with dihydrotestosterone at indicated concentrations. The culture supernatant was collected after 24 hours. The amount of PSA protein secreted into culture media was determined by an ELISA assay. Columns, mean of three determinations; bars, SD. *C*, vector control or scFv-5R cells were stimulated with dihydrotestosterone (2 nmol/L) for 16 hours. Total RNA was prepared and Northern blot analysis for PSA mRNA expression was done. Ethidium bromide-stained 28S and 18S RNA bands confirmed equal loading.

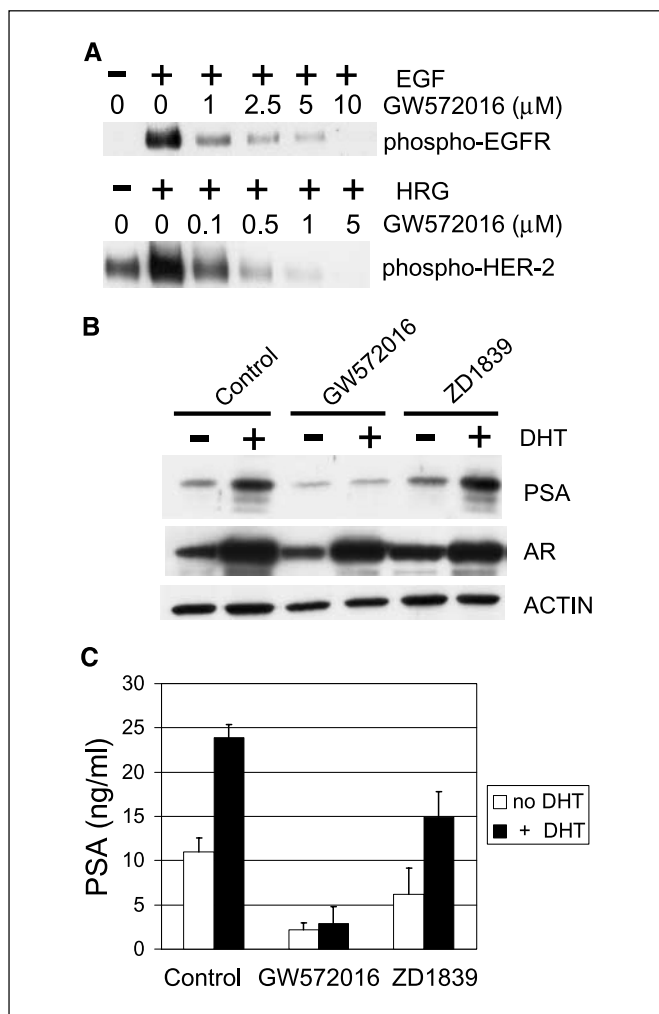


Figure 3. GW572016, a dual kinase inhibitor of EGFR and HER-2, inhibits androgen-stimulated PSA expression in LNCaP cells. *A*, cells were pretreated with indicated doses of GW572016 for 45 minutes, followed by treatment with EGF (100 ng/mL; *top*) or heregulin (10 ng/mL; *bottom*) for 15 minutes. Cell lysates were prepared and immunoprecipitated with antibody against EGFR (*top*) or HER-2 (*bottom*), and then immunoblotting was done with antiphosphotyrosine antibody. *B*, cells were treated with dihydrotestosterone (2 nmol/L) in combination with GW572016 (10 μ mol/L) or ZD1839 (10 μ mol/L). Cells were harvested after 16 hours. Cell lysates were immunoblotted with antibody specific for PSA, AR, or actin (for loading control). *C*, cells were treated with dihydrotestosterone (2 nmol/L) in combination with GW572016 (10 μ mol/L) or ZD1839 (10 μ mol/L). The culture supernatant was collected after 24 hours. The amount of PSA protein secreted into culture media was determined by ELISA. *Columns*, mean of three determinations; *bars*, SD.

cytoplasmic compartments. After dihydrotestosterone treatment, AR staining was intense and strongly nuclear without a cytoplasmic component. This dihydrotestosterone-dependent nuclear localization was not altered in scFv-5R cells or in cells treated with GW572016 (data not shown).

Chromatin immunoprecipitation analysis of the prostate-specific antigen enhancer. To characterize the nature of inhibition of transcriptional function of AR by HER-2 inhibition, we assessed the recruitment of AR to the regulatory region of the *PSA* gene and the histone modification status at this locus by chromatin immunoprecipitation analysis. In this procedure, cells are treated with formaldehyde to cross-link proteins to DNA. Chromatin is isolated, sheared, and immunoprecipitated using AR

antibody or acetylated histone H3 antibody. The amount of DNA immunoprecipitated by the antibody is quantitated by real-time PCR. Androgen stimulation led to increased recruitment and binding of AR to the distal androgen responsive enhancer (androgen responsive element III) region of the endogenous *PSA* gene (Fig. 4*A*) in agreement with previous reports (18, 20). Androgen treatment resulted in increased acetylation of histone H3 (Fig. 4*B*), reflecting transcriptional activation as a consequence of the assembly of the multiprotein transcriptional complex containing histone acetylase activity following the AR recruitment. However, in scFv-5R cells, androgen-stimulated AR recruitment to the *PSA* enhancer was reduced by \sim 2-fold (Fig. 4*A*). Concomitantly, androgen-stimulated histone acetylation at the *PSA* enhancer in scFv-5R cells was decreased (Fig. 4*B*). We also did the same assay after pretreatment of cells with GW572016 and ZD1839. GW572016 inhibited AR binding at the *PSA* enhancer and histone acetylation (Fig. 4*C* and *D*). ZD1839 also inhibited AR recruitment and histone acetylation at the *PSA* enhancer, but less than GW572016. The degree of inhibition of AR recruitment and histone acetylation at the *PSA* enhancer by GW572016 and ZD1839 paralleled the degree of inhibition of PSA expression by these agents (Fig. 3*C*). These data suggest that inhibition of HER-2 leads

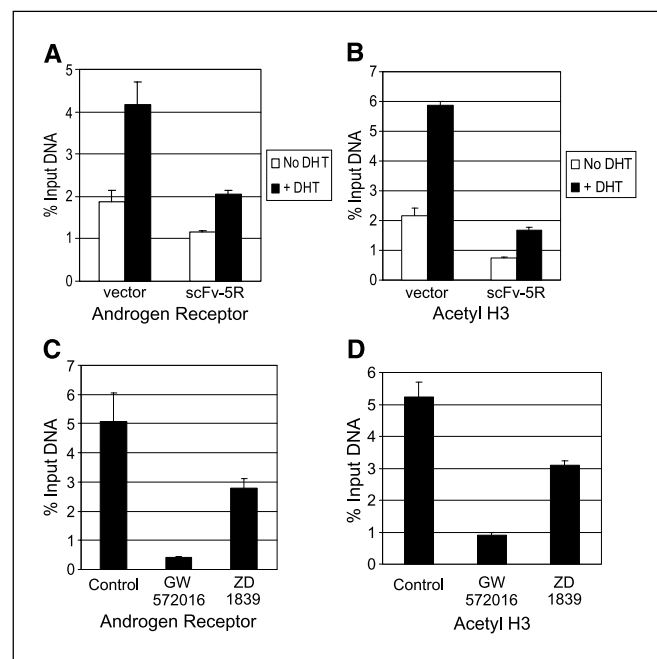


Figure 4. Androgen-stimulated recruitment of androgen receptor and histone acetylation at the androgen responsive *PSA* enhancer are impaired in cells with HER-2 inhibition. Vector control LNCaP cells or scFv-5R cells were treated with 10 nmol/L dihydrotestosterone for 16 hours or left untreated (*A* and *B*). Chromatin immunoprecipitation assays using antibody specific for AR (*A*) or acetylated H3 histone (*B*) were done. Quantitative PCR using primers amplifying the androgen responsive element III enhancer located 4.2 kb upstream of the transcription initiation site of the *PSA* gene (18) was used to determine the amount of DNA precipitated by the antibody, relative to the total input DNA. *Columns*, mean of triplicate PCR reactions; *bars*, SD. Data are representative of three independent experiments that yielded similar results. LNCaP cells were pretreated with GW572016 (10 μ mol/L) or ZD1839 (10 μ mol/L) or untreated (*C*, *D*). After 30 minutes, dihydrotestosterone (10 nmol/L) was added and incubated for 16 hours. Chromatin immunoprecipitation assays using antibody specific for AR (*C*) or acetylated H3 histone (*D*) were done. Quantitative PCR for the *PSA* enhancer region was done as above. Data shown are representative of three independent experiments that yielded similar results.

to inhibition of AR transcription function primarily at the level of AR recruitment to the regulatory region of AR-regulated genes on chromatin.

Discussion

In this report, we investigated the role of endogenous HER-2 signaling in AR function in LNCaP prostate cancer cells. We inhibited HER-2 kinase activity by the means of intracellular antibody expression or a novel kinase inhibitor GW572016 and showed that HER-2 inhibition impaired AR-mediated functions, including androgen-stimulated growth and transcriptional activation of the *PSA* gene. These results suggest that HER-2 signaling is required for optimal transcriptional activity of AR in LNCaP cells.

Our data show that initial steps in AR signaling (such as protein stabilization and nuclear localization) in cells with loss of HER-2 activity seem to be largely intact. However, we uncovered at least one mechanism of AR functional impairment in these cells, a defect in the androgen-stimulated recruitment of AR to the *PSA* gene enhancer, which leads to decreased histone acetylation and decreased production of *PSA* mRNA. This finding is similar to a recent report by Mellinghoff et al. (21). The decreased binding of AR to the androgen responsive enhancer on chromatin may be due to reduced DNA-binding affinity of AR protein without input from HER-2. An alternative possibility is that initial AR binding to the specific DNA sequences on the enhancer site occurs normally, but AR binding (or subsequent assembly of the transcription complex) is unstable in the absence of HER-2 signaling. Binding of steroid receptors, such as estrogen receptor and AR, to the enhancer and subsequent transcription activation are a dynamic process that involves coordinated cycles of transient association and dissociation of the receptor and many interacting proteins in the transcriptional complex, composed of coactivators, histone modifying enzymes, and basal transcription factors (22–24). Therefore, a relatively small change in the affinity between AR protein and its interacting partners or DNA may lead to a substantial defect in the recruitment and binding of AR protein to the enhancer site detected by chromatin immunoprecipitation analysis.

The AR protein is modified posttranscriptionally by phosphorylation (25). Regulation of AR phosphorylation by HER-2, directly or through downstream kinases, is a possible mechanism of AR regulation by HER-2. It seems unlikely that HER-2 directly phosphorylates the AR protein, because characterization of AR protein phosphorylation in LNCaP cells showed that tyrosine residues are not phosphorylated (25). Several studies have reported that HER-2 may induce AR phosphorylation through the downstream MAPK or Akt kinases (6, 7). However, detailed analysis of endogenous AR protein in LNCaP cells showed that these putative sites are not phosphorylated *in vivo* (25). Therefore, the significance of AR protein phosphorylation through these pathways remains unclear. Because constitutive activated Akt is not affected by HER-2 signaling (Fig. 1B), the Akt pathway is unlikely to play a significant role in AR regulation by HER-2 in LNCaP cells, consistent with another report (21). The role of MAPK or other downstream pathways in mediating the effect of HER-2 on AR is currently unclear and will require further investigation.

HER-2 may also regulate the phosphorylation status of proteins that interact with AR. It is known that phosphorylation of the steroid hormone receptor coactivators TIF2/GRIP1 induced by EGFR signaling through MAPK enhances binding between TIF2/

GRIP1 and AR (4). It is possible that activation or inhibition of HER-2 kinase modulates phosphorylation of TIF2/GRIP1 or other coactivators and AR binding proteins and thereby regulates the assembly of the AR transcriptional complex. The effect of HER-2 inhibition on the recruitment of AR seems somewhat different from interleukin (IL)-6, which also inhibits androgen-stimulated *PSA* expression in LNCaP cells (18). Other groups, however, have reported that IL-6 stimulates AR activity (26, 27). Jia et al. (18, 20) showed that androgen-induced recruitment of AR to the *PSA* enhancer takes place normally in the presence of IL-6, but subsequent histone acetylation is inhibited by IL-6. In contrast, we show that HER-2 inhibition impairs both the initial recruitment of AR and subsequent histone acetylation. More work is necessary to understand in detail the molecular basis of HER-2 modulation of AR function.

Although data presented in this report were obtained in LNCaP cells, which are a classic androgen responsive cell line, we have investigated the effect of HER-2 activation by heregulin as well as inhibition of HER-2 by GW572016 on androgen-mediated growth and AR transcriptional function in the recurrent prostate cancer cell line CWR-R1. Results obtained in CWR-R1 cells are similar to those presented here (19). Lee et al. (28) investigated the role of HER-2 in the regulation of the *PSA* gene in the androgen-independent C-81 subclone of LNCaP cells and showed that increased HER-2 activity stimulated *PSA* expression. Taken together, these results support the notion that HER-2 function is necessary for optimal AR function in both androgen-dependent and “androgen-independent” recurrent prostate cancer cells.

Because of the postulated role of HER-2 in prostate cancer, a clinical trial of patients with advanced prostate cancer using trastuzumab (Herceptin), the HER-2 specific antibody, was initiated, but could not be completed because of the infrequent occurrence of HER-2 overexpression (29). Alternative approaches to HER-2 inhibition that do not require HER-2 overexpression are necessary to test the *in vivo* role of HER-2 in prostate cancer. One report showed that the antibody pertuzumab (Omnitarg or 2C4) that blocks ligand-induced heterodimerization and activation of HER-2 potentially inhibited growth of both androgen-dependent and androgen-independent prostate xenograft tumors, thereby implicating the necessity of continued HER-2 function in propagation of prostate cancer tumors (30). Another group reported that PKI-166, a dual EGFR/HER-2 kinase inhibitor, inhibited AR function through its effect on DNA binding and protein stability and inhibited growth of prostate cancer xenograft tumors *in vivo* (21, 31). Our data presented in this report show that AR function is dependent on continued HER-2 signaling and that inhibition of endogenous HER-2 kinase impairs AR transcriptional activity. Collectively, these results suggest that HER-2 inhibition may represent a novel therapeutic strategy for disruption of AR function in prostate cancer that warrants further clinical testing.

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Inhibition of HER-2/neu Kinase Impairs Androgen Receptor Recruitment to the Androgen Responsive Enhancer

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