

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

Yuanbo Liu,¹ Samarpan Majumder,¹ Wesley McCall,¹ Carolyn I. Sartor,^{1,2} James L. Mohler,^{1,3,4,7} Christopher W. Gregory,^{1,3} H. Shelton Earp,^{1,5,6} and Young E. Whang^{1,5}

¹Lineberger Comprehensive Cancer Center; Departments of ²Radiation Oncology, ³Pathology and Laboratory Medicine, ⁴Surgery, ⁵Medicine, and ⁶Pharmacology, University of North Carolina, Chapel Hill, North Carolina; and ⁷Department of Urologic Oncology, Roswell Park Cancer Center, Buffalo, New York

Abstract

Advanced prostate cancer invariably recurs despite androgen deprivation therapy. The androgen receptor (AR) likely plays a key role in this progression and in the continued survival and proliferation of prostate cancer cells in the low androgen environment. Cross-talk with growth factor receptors, such as epidermal growth factor receptor (EGFR) family, has been postulated as a potential mechanism to activate AR in recurrent prostate cancer. We have investigated the role of HER-2/neu (ErbB-2) tyrosine kinase in AR function by characterizing the effect of inhibiting endogenous HER-2 activity in LNCaP cells. We used two independent methods, expression of intracellular single-chain antibody against HER-2 and treatment with a novel dual EGFR/HER-2 kinase inhibitor GW572016 (lapatinib). Expression of intracellular HER-2 antibody scFv-5R and treatment with GW572016 inhibited HER-2 signaling. This HER-2 inhibition led to impairment of AR-mediated functions, such as androgen-stimulated growth and the induction of endogenous prostate-specific antigen (PSA) mRNA and protein. Androgen-stimulated recruitment of AR and histone acetylation at the androgen responsive enhancer of the PSA gene, detected by chromatin immunoprecipitation analysis, were impaired by HER-2 inhibition. GW572016 was more potent in its ability to inhibit PSA expression and AR recruitment and histone acetylation than the EGFR-selective kinase inhibitor ZD1839 (gefitinib), consistent with the HER-2 kinase playing the major role in AR regulation. These results show that HER-2 signaling is required for optimal transcriptional activity of AR in prostate cancer cells and suggest that HER-2 inhibition may provide a novel strategy to disrupt AR function in prostate cancer. (Cancer Res 2005; 65(8): 3404-9)

Introduction

Prostate cancer in advanced stage is a fatal disease because of the eventual failure of androgen deprivation therapy. Although prostate cancer typically regresses following surgical or medical castration of the host, prostate cancer will recur and progress despite low levels of circulating androgen. Current evidence implicates the androgen receptor (AR) as playing a key role in the hormone refractory progression of prostate cancer. Inactivation of AR by antibody,

ribozyme, or RNA interference results in inhibition of proliferation or tumorigenicity of hormone refractory prostate cancer cells (1, 2). Potential mechanisms by which AR may be reactivated in the low androgen environment include mutation in AR, amplification of the AR gene, increased expression of steroid hormone receptor coactivators, and stimulation by growth factors and cytokines (3). The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, such as EGFR and HER-2/neu (ErbB-2), has been postulated to modulate AR transcriptional function. Gregory et al. (4) showed that epidermal growth factor (EGF) increased AR transactivation in recurrent prostate cancer cells by promoting phosphorylation of the p160 family coactivator transcription intermediary factor 2/glucocorticoid receptor interacting protein 1 (TIF2/GRIP1) and increasing its interaction with AR. Other groups have reported that overexpression of HER-2 induced androgen-independent growth of prostate cancer cells and activated AR transcriptional function through the mitogen-activated protein kinase (MAPK) pathway or the Akt pathway (5–7). However, published data on the frequency of overexpression of HER-2 in primary prostate cancer specimens are conflicting (8–10), and the *in vivo* role of HER-2 in prostate cancer and AR function remains unclear. Therefore, in this study, we investigated the effect of inhibiting HER-2 using an intracellular antibody and a novel kinase inhibitor GW572016. Our results show that HER-2 inhibition leads to impairment of AR transcriptional function at the level of recruitment of AR to the androgen responsive enhancer on chromatin.

Materials and Methods

Cell culture and reagents. LNCaP cells (American Tissue Type Collection, Manassas, VA) were cultured in phenol red-free RPMI medium supplemented with 10% FCS. Dihydrotestosterone (Sigma, St. Louis, MO) and EGF (Becton Dickinson, Bedford, MA) were obtained commercially. Heregulin was a gift from Genentech (South San Francisco, CA). GW572016 (*N*-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]-amino}methyl)-2-furyl]-4-quinazolinamine; lapatinib; ref. 11, 12) and ZD1839 [4-(3-chloro-4-fluorophenylamino)-7-methoxy-6 (3-(4-morpholinyl-propoxy)-quinazoline; gefitinib; ref. 13) were provided by Dr. Tona M. Gilmer (GlaxoSmithKline, Research Triangle Park, NC).

Construction of cell lines stably expressing intracellular antibody against HER-2. The single-chain fragment variable (scFv)-5R construct cloned into the retrovirus vector pBabePuro was kindly provided by Dr. Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland; refs. 14, 15). The empty vector and scFv-5R were amphotropically packaged (16), and LNCaP cells were infected with retroviral supernatant in the presence of polybrene (8 µg/mL, Sigma). Cells resistant to puromycin (0.75 µg/mL) were pooled and used for subsequent analysis.

Cell proliferation assay. Vector control or scFv-5R cells (5×10^3) were seeded per well in 96-well plates in triplicate in steroid-depleted media containing 10% charcoal-stripped FCS (HyClone, Logan, UT). Cells were allowed to attach overnight and dihydrotestosterone (2 nmol/L) was added

Note: Y. Liu and S. Majumder contributed equally to this work. C.W. Gregory is currently at Voyager Pharmaceutical Corporation, Raleigh, NC 27615.

Requests for reprints: Young E. Whang, Lineberger Comprehensive Cancer Center, University of North Carolina, 102 Mason Farm Road, CB #7295, Chapel Hill, NC 27599-7295. Phone: 919-966-8645; Fax: 919-966-8212; E-mail: ywhang@med.unc.edu.

©2005 American Association for Cancer Research.

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

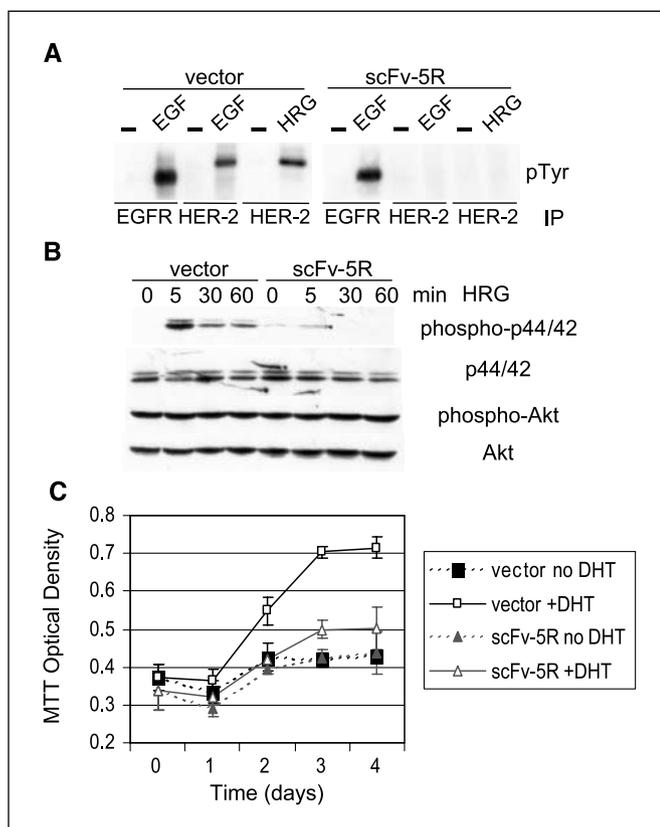


Figure 1. Stable expression of intracellular antibody against HER-2 leads to inactivation of HER-2 signaling and inhibition of androgen-stimulated proliferation of LNCaP cells. **A**, vector control cells or scFv-5R cells stably expressing intracellular antibody against HER-2 were treated with EGF (100 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

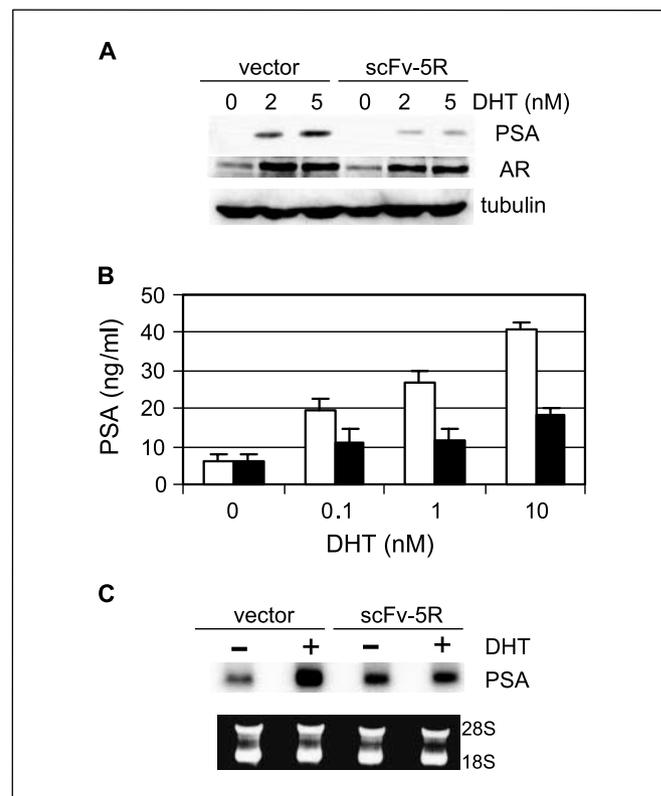


Figure 2. Functional inactivation of HER-2 leads to inhibition of androgen-stimulated PSA production. **A**, vector control or scFv-5R cells were stimulated with dihydrotestosterone at indicated concentrations. Cells were harvested after 16 hours. Intracellular PSA and AR protein expression was determined 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.

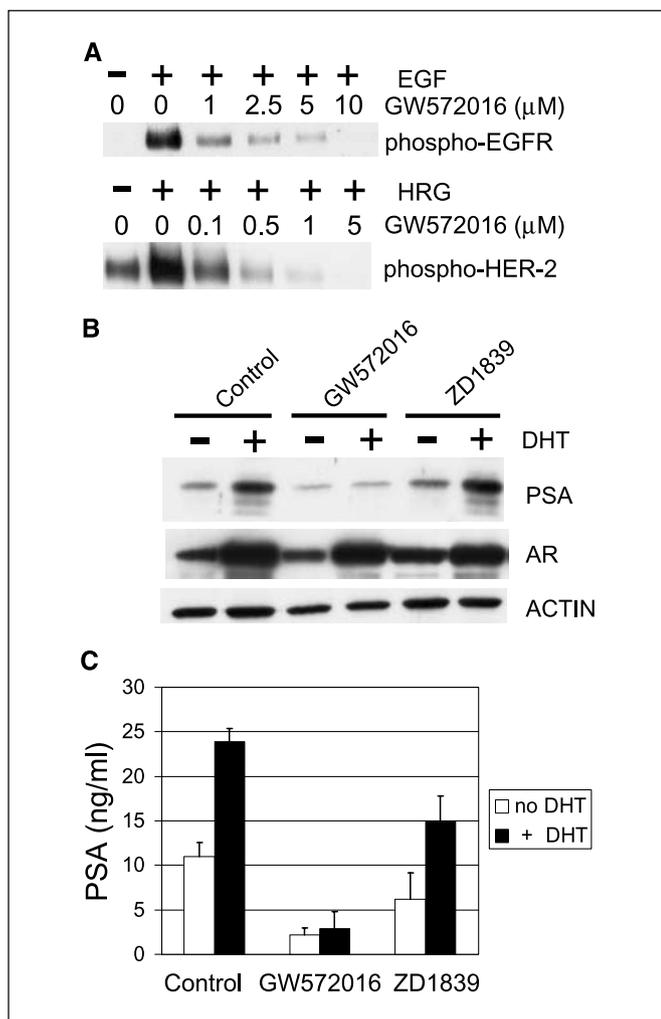


Figure 3. GW572016, a dual kinase inhibitor of EGFR and HER-2, inhibits androgen-stimulated PSA expression in LNCaP cells. *A*, cells were pretreated with indicated doses of GW572016 for 45 minutes, followed by treatment with EGF (100 ng/mL; *top*) or heregulin (10 ng/mL; *bottom*) for 15 minutes. Cell 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 μmol/L) or ZD1839 (10 μmol/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 μmol/L) or ZD1839 (10 μmol/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 ~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

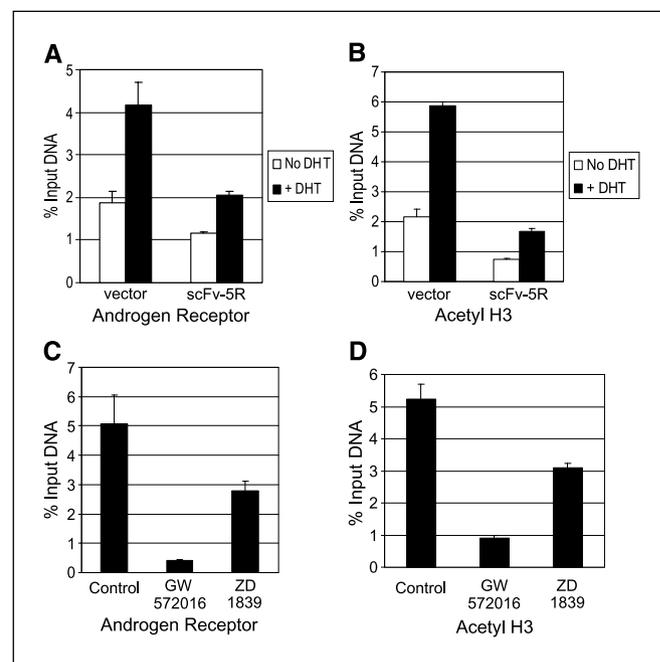


Figure 4. Androgen-stimulated recruitment of androgen receptor and histone acetylation at the androgen responsive PSA enhancer are impaired in cells with HER-2 inhibition. Vector control LNCaP cells or scFv-5R cells were treated with 10 nmol/L dihydrotestosterone for 16 hours or left untreated (*A* and *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 μmol/L) or ZD1839 (10 μmol/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.

Acknowledgments

Received 12/1/2004; revised 1/24/2005; accepted 2/10/2005.

Grant support: U.S. Army Medical Research and Materiel Command grant DAMD17-02-1-0110 (C.W. Gregory) and NIH grants CA81503 (H.S. Earp), CA83753 (C.I. Sator), CA85772 (Y.E. Whang), CA100700 (Y.E. Whang), and P01-CA77739 (J.L. Mohler).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank O. Harris Ford for androgen receptor staining.

References

1. Zegar-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* 2002;62:1008-13.
2. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004;10:33-9.
3. Grossmann ME, Huang H, Tindall DJ. Androgen receptor signaling in androgen-refractory prostate cancer. *J Natl Cancer Inst* 2001;93:1687-97.
4. Gregory CW, Fei X, Ponguta LA, et al. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. *J Biol Chem* 2004;279:7119-30.
5. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med* 1999;5:280-5.
6. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A* 1999;96:5458-63.
7. Wen Y, Hu MC, Makino K, et al. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000;60:6841-5.
8. Calvo BF, Levine AM, Marcos M, et al. Human epidermal receptor-2 expression in prostate cancer. *Clin Cancer Res* 2003;9:1087-97.
9. Osman I, Scher H, Drobnjak M, et al. Her2/neu (p185neu) protein expression in the natural or treated history of prostate cancer. *Clin Cancer Res* 2001;7:2643-7.
10. Signoretti S, Montironi R, Manola J, et al. Her-2-neu expression and progression toward androgen independence in human prostate cancer. *J Natl Cancer Inst* 2000;92:1918-25.
11. Rusnak DW, Lackey K, Affleck K, et al. The effects of the novel, reversible epidermal growth factor receptor/ ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines *in vitro* and *in vivo*. *Mol Cancer Ther* 2001;1:85-94.
12. Wood ER, Truesdale AT, McDonald OB, et al. A unique structure for epidermal growth factor receptor bound to GW572016 (lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res* 2004;64:6652-9.
13. Wakeling AE, Guy SP, Woodburn JR, et al. ZD1839 (Iressa): An orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* 2002;62:5749-54.
14. Beerli RR, Wels W, Hynes NE. Intracellular expression of single chain antibodies reverts ErbB-2 transformation. *J Biol Chem* 1994;269:23931-6.
15. Graus-Porta D, Beerli RR, Hynes NE. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol Cell Biol* 1995;15:1182-91.
16. Sartor CI, Zhou H, Kozlowska E, et al. HER4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells. *Mol Cell Biol* 2001;21:4265-75.
17. Gregory CW, Hamil KG, Kim D, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* 1998;58:5718-24.
18. Jia L, Kim J, Shen H, Clark PE, Tilley WD, Coetzee GA. Androgen receptor activity at the prostate specific antigen locus: steroidal and non-steroidal mechanisms. *Mol Cancer Res* 2003;1:385-92.
19. Gregory C, Whang Y, McCall W, et al. Heregulin-induced activation of HER2 and HER3 increases androgen receptor transactivation and CWR-R1 human recurrent prostate cancer cell growth. *Clin Cancer Res*. In press 2005.
20. Jia L, Choong CS-Y, Ricciardelli C, Kim J, Tilley WD, Coetzee GA. Androgen receptor signaling: mechanism of interleukin-6 inhibition. *Cancer Res* 2004;64:2619-26.
21. Mellinghoff IK, Vivanco I, Kwon A, Tran C, Wongvipat J, Sawyers CL. HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* 2004;6:517-27.
22. Metivier R, Penot G, Hubner MR, et al. Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 2003;115:751-63.
23. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002; 9:601-10.
24. Kang Z, Pirskanen A, Janne OA, Palmivo JJ. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. *J Biol Chem* 2002;277:48366-71.
25. Gioeli D, Ficarro SB, Kwiek JJ, et al. Androgen receptor phosphorylation: regulation and identification of the phosphorylation sites. *J Biol Chem* 2002;277: 29304-14.
26. Lin D-L, Whitney MC, Yao Z, Keller ET. Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. *Clin Cancer Res* 2001;7:1773-81.
27. Ueda T, Bruchoovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J Biol Chem* 2002;277:7076-85.
28. Lee MS, Igawa T, Yuan TC, Zhang XQ, Lin FF, Lin MF. ErbB-2 signaling is involved in regulating PSA secretion in androgen-independent human prostate cancer LNCaP C-81 cells. *Oncogene* 2003;22:781-96.
29. Lara PN, Chee KG, Longmate J, et al. Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. *Cancer* 2004;100:2125-31.
30. Agus DB, Akita RW, Fox WD, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2002;2:127-37.
31. Mellinghoff IK, Tran C, Sawyers CL. Growth inhibitory effects of the dual ErbB1/ErbB2 tyrosine kinase inhibitor PKI-166 on human prostate cancer xenografts. *Cancer Res* 2002;62:5254-9.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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

Yuanbo Liu, Samarpan Majumder, Wesley McCall, et al.

Cancer Res 2005;65:3404-3409.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/65/8/3404>

Cited articles This article cites 30 articles, 20 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/65/8/3404.full#ref-list-1>

Citing articles This article has been cited by 22 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/65/8/3404.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/65/8/3404>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.