Dual EGFR/HER2 inhibition sensitizes prostate cancer cells to androgen withdrawal by suppressing ErbB3

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STATEMENT OF TRANSLATIONAL RELEVANCE

The goal of these studies is to identify therapeutic strategies that prolong the effectiveness of androgen withdrawal therapy (AWT) in patients with metastatic prostate cancer (PCa). Inhibitors of ErbB kinases such as erlotinib, lapatinib and trastuzumab have been tested in patients with castration resistant prostate cancer (CRPC) and in hormone-naïve patients, with little effect. Here we present novel data demonstrating that, instead, dual ErbB inhibitors sensitize PCa to AWT, and are thereby likely to prolong its effects. We show that during AWT, HER2 and ErbB3 levels increase, resulting in significant ErbB-dependent survival advantage that allows progression to CRPC. However, dual EGFR/HER2 inhibition, which inhibits their dimerization partner ErbB3 as well, induced apoptosis in cells undergoing AWT, despite ineffectiveness in hormone-naïve cells and in cells that have already progressed to CRPC. Our data indicate that administration of dual EGFR/HER2 inhibitors in PCa patients undergoing AWT may impede the onset of CRPC.
ABSTRACT

Purpose: Patients with recurrent prostate cancer (PCa) are commonly treated with androgen withdrawal therapy (AWT); however, almost all patients eventually progress to castration resistant prostate cancer (CRPC), indicating failure of AWT to eliminate androgen-sensitive PCa. The overall goal of these studies is to determine whether dual inhibition of the receptor tyrosine kinases EGFR and HER2 would prolong the effectiveness of this treatment in PCa.

Experimental Design: We used androgen-dependent LNCaP cells and its CRPC sublines LNCaP-AI and C4-2. Additional data were collected in pRNS-1-1 cells stably expressing a mutant androgen receptor (AR-T877A), and in nude mice harboring CWR22 tumors. Studies utilized EGFR inhibitors erlotinib and AG1478, and HER2 inhibitors trastuzumab and AG879.

Results: Dual EGFR/HER2 inhibition induced apoptosis selectively in androgen-sensitive PCa cells undergoing AWT, but not in the presence of androgens, or in CRPC cells. We show that AWT alone failed to induce significant apoptosis in androgen-dependent cells, due to AWT-induced increase in HER2 and ErbB3, which promoted survival by increasing Akt phosphorylation. AWT-induced ErbB3 stabilized the AR and stimulated PSA, while it was inactivated only by inhibition of both its dimerization partners EGFR and HER2 (PCa cells do not express ErbB4); but not the inhibition of any one receptor alone, explaining the success of dual EGFR/HER2 inhibition in sensitizing androgen-dependent cells to AWT. The effectiveness of the inhibitors in suppressing growth correlated with its ability to prevent Akt phosphorylation.

Conclusions: These studies indicate that dual EGFR/HER2 inhibition, administered together with AWT; sensitize PCa cells to apoptosis during AWT.
INTRODUCTION

Androgen withdrawal therapy (AWT) is currently the standard of care for men with advanced prostate cancer (PCa) (1); however, it was found that in most patients its effects typically last 18-24 months, after which the patient developed resistance to such therapy (castration resistant prostate cancer; CRPC). Although some groups reported increased apoptosis in prostatic tissue following AWT (2, 3), others found no increase in apoptotic indices in the majority of tumors (4, 5), although proliferation indices were consistently suppressed (2, 4). These studies, therefore, concluded that “androgen deprivation may act through suppression rather than ablation of prostatic cancers” (5, 6). These reports indicate that failure to undergo apoptosis during AWT maybe a major cause of resistance of PCa cells to this therapy. Surviving cells likely undergo growth arrest and lie dormant following AWT, but will revive when an alternate growth stimulant comes to release it from this growth arrest, as was demonstrated in a CWR22 xenograft model (7, 8). Therefore, adjuvant therapy that causes apoptosis during AWT would impede the onset of CRPC.

Here we investigate the role of ErbB inhibitors in this effect. The ErbB family of four closely related type 1 transmembrane tyrosine kinase receptors include the epidermal growth factor receptor (EGFR/HER1/ErbB1), and related family members ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (9). The ErbB receptors are activated by ligand binding, dimerization and phosphorylation. EGFR, ErbB3, ErbB4, but not HER2, have specific ligands, such as EGF for EGFR and heregulins (HRG1-4) for ErbB3 and ErbB4 (9). However, ErbB3 lacks significant kinase activity; hence both HER2 and ErbB3 require heterodimerization, with each other or the other ErbB receptors, for phosphorylation and activation. Significantly, PCa cells typically lack ErbB4 expression, but express high levels of ErbB3 (10, 11).

EGFR and HER2 are known to regulate cell proliferation, differentiation, angiogenesis and survival (12); however, in clinical trials for patients with CRPC, studies using selective and
specific inhibitors of individual receptors did not show any significant effect (13-17). In recent times, a number of dual EGFR/HER2 inhibitors have been developed, and were found to be more effective against PCa cells and animal models compared to the single inhibitors (18, 19). Tyrosine phosphorylation of HER2 and ErbB3, transactivation of the androgen receptor (AR), and cell proliferation induced by heregulin were more potently inhibited by the EGFR/HER2 dual tyrosine kinase inhibitor GW572016 (lapatinib) than the EGFR-specific inhibitor gefitinib (20, 21). Despite the success of the pre-clinical studies, in phase II single-agent clinical trials, lapatinib was fairly well-tolerated and resulted in stable disease for 12 weeks but evidenced no decrease in prostate specific antigen (PSA), an AR transcriptional target, in patients with hormone sensitive PCa (22) or in unselected patients with CRPC, as measured by PSA (23).

Here, we concentrate on the effects of dual EGFR/HER2 inhibitors and the conditions under which they are effective. It is known that AR function at low levels of androgen is mediated not by EGFR, but by the heterodimerization of HER2 with ErbB3 (18). Sergina et al demonstrated that ErbB3 was upregulated and provided compensatory signaling precisely in response to EGFR/HER2-directed tyrosine kinase inhibitor (TKI) treatment (24). Indeed, ErbB3-directed RNA inhibition duly restored the pro-apoptotic effects of TKIs (24). These reports suggested that the failure of EGFR and HER2 inhibitors may be due to the activation of ErbB3 in these tumors. Studies conducted in vitro (25, 26), in animal models (6), and in clinical specimens (27) indicate an increase in Akt phosphorylation during AWT which promotes cell survival. Based on these reports we investigated whether dual EGFR/HER2 inhibitors were effective when they downregulated ErbB3 and/or Akt phosphorylation, and whether they impede PCa progression to CRPC by inducing cell death during AWT.
MATERIALS AND METHODS

Cell Culture and Pharmacological Treatments Androgen-dependent LNCaP prostate cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), and C4-2 cells were obtained from UroCor (Oklahoma City, OK). Castration resistant clones of LNCaP cells (LNCaP-AI cells) have been described by us elsewhere (11, 25). pRNS-1-1 cells were also described earlier (11, 28). Recombinant human epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) were obtained from Invitrogen, (Carlsbad, CA), recombinant human heregulin 1 (HRG1) was from PeproTech INC. (Rochy Hill, NJ). AG1478 and AG879 were from Calbiochem, EMD Chemicals, Inc. (Gibbstown, NJ). Erlotinib (Tarceva) was provided by OSI Pharmaceuticals, Inc. (Melville, NY), and also was obtained from LC Laboratories (Woburn, MA), while trastuzumab (Herceptin) was a gift from Genentech, Inc. (South San Francisco, CA). Bicalutamide (Casodex) was kindly provided by AstraZeneca (Cheshire, UK), while lapatinib was purchased from LC Laboratories (Woburn, MA). Rabbit polyclonal EGFR, HER2, ErbB3, β-actin and AR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-Akt (Ser 473), anti-phospho-EGFR (Y1068), anti-phospho-HER2 (Y1248), phospho-ErbB3 (Y1289), α-tubulin and Akt antibodies were from Cell Signaling Technology (Beverly, MA). Transfections and plasmids used have been described earlier (11). Human Akt1 siRNA was obtained from Santa Cruz Biotechnology, Santa Cruz, CA against the sequence: 5’-ACGAGGGGAGUACAUCAAGAC-3’.

Mouse Studies: 4-5-week old Balb/c athymic nude-Foxn1nu (nu/nu) male mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Suspensions of CWR22 cells were mixed in 50% Matrigel solubilized basement membrane (BD Biosciences, Bedford, MA) and xenografts were established by subcutaneous injections of 2.5 x 10⁶ cells/site into the flanks. When palpable tumors were observed, animals were treated with (i) vehicle or (ii) a combination of erlotinib (0.8 mg/Kg, 100 μl per dose, 5 times per week by oral gavage) and trastuzumab (20
mg/Kg, 90 μl per dose, 2 times per week by i.p. injection), dissolved in a solution of phosphate buffered saline (PBS) and 0.5% Tween 20. 3 days after start of drug regimen, the animals were castrated by bilateral scrotal excision, following isoflurone-anesthetization. Control animals were sham-operated by opening the animals surgically, but no tissues were removed. Drug administration was continued post-surgery, but after 8 days, the mice were euthanized, tumors were collected and divided into sections for paraffin-embedding and snap-freezing in liquid nitrogen. Mice were weighed and blood was collected periodically and PSA levels measured by a standard ELISA kit (Fitzgerald Industries Intnl., Acton, MA).

**Immunohistochemistry and Statistical Analysis:** We used rabbit polyclonal anti-ErbB3 (C-17) (1:100 dilution) antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, Ki67 was from DAKO (Carpinteria, CA), while TUNEL kit was from Millipore (Billerica, MA). For negative controls we used a Universal Rabbit IgG control (DAKO) in place of the primary antibody. Diaminobenzidine (DAB) was used as a chromogen, and counterstaining was with hematoxylin. Only the epithelial cells were scored. The extent of staining was scored 0-3, where 0 represented no staining, +0.5 represents low (<20% staining), +1 represent intermediate (30-50%), +1.5 (50-70%) and +2 represent high staining (>80%). To evaluate the differences in staining expression in the three diagnostic groups, we used t-tests with a Welch approximation. Columns represent the mean ± standard deviation of samples from each group.

**Flow cytometry and MTT assay:** MTT and flow cytometric analysis was carried out as described earlier (11, 29, 30). Proliferation was estimated in propidium iodide stained ethanol-fixed cells by MODFIT (Verity software, Topsham, ME), while the rate of apoptosis induction was estimated in live cells staining with Annexin V by CellQuest V3.1 (Becton-Dickinson, Franklin Lanes, NJ).
RESULTS

Dual EGFR/HER2 inhibition sensitized androgen-dependent prostate cancer cells, but not castration resistant lines, to apoptosis by androgen withdrawal. We first compared the individual effects of the HER2 inhibitor trastuzumab (21 μg/ml), and the EGFR inhibitor erlotinib (10 μM), to dual inhibition with both drugs in androgen dependent LNCaP PCa cells. The drug combination caused cell cycle arrest in LNCaP cells following 48 hours of treatment in FBS medium (Figure 1A, upper). Culture in CSS, where androgen levels are significantly lower, also induced cell cycle arrest, but very little apoptosis, in these cells. However, the combination of trastuzumab and erlotinib, but not the individual drugs, induced 10-fold higher apoptosis in LNCaP cells in CSS-containing media (Figure 1A, lower). The overall effect is that, in FBS, dual EGFR/HER2 inhibition prevented cell number increase, whereas upon culture in CSS, additionally, there was a decrease in cell numbers indicating cell death (Supplemental Figure 1A). Unlike LNCaP cells, however, its CRPC sublines C4-2 (Figure 1A, lower) or LNCaP-AI (Supplementary Figure 1B), which have higher AR transcripational activity (25), did not respond to dual inhibition of EGFR and HER2 even in CSS. Similarly, LNCaP cells underwent apoptosis in response to the dual EGFR/HER2 inhibitor lapatinib in CSS, but not in FBS, while its CRPC subline C4-2 cells were resistant to apoptosis by this drug (Supplementary Figure 2). Dual EGFR/HER2 inhibition prevented cell growth in FBS in AR-negative pRNS-1-1 cells stably transfected with vector only, but not those expressing AR(T877A), an androgen-sensitive active mutation found in LNCaP cells (Figure 1B). However, in CSS, where AR was inactive, this treatment inhibited growth, despite the presence of the AR(T877A) mutant (Figure 1B). These results indicate that AR activity suppresses the effects of ErbB inhibitors.

Androgen withdrawal stimulates, while dual EGFR/HER2 inhibition suppresses, ErbB3 levels. 48 hour treatment with erlotinib (10 μM), but not trastuzumab (21 μg/ml) inhibited EGF-
stimulated EGFR phosphorylation, whereas trastuzumab, but not erlotinib, affected the expression of HER2 (Figure 2A, left). On the other hand, the combination, but not the individual drugs, inhibited ErbB3 phosphorylation, and reduced ErbB3 levels (Figure 2A, right) also (Supplementary Figure 3A). Since PCa cells do not express ErbB4 (Supplementary Figure 3B) (10), we examined the effects of AWT on the levels of the other ErbB receptors. There was no significant change in EGFR levels upon culture in CSS, however, both HER2 and ErbB3 levels increased significantly as AR levels declined (Figure 2B, upper panels) (also Supplementary Figure 3C). Consistent with previous findings (6, 26), we saw a concomitant increase in Akt phosphorylation (Ser 473) in LNCaP (Figure 2B, upper). However, AWT caused no change in ErbB3 in LNCaP-AI cells, which expressed both higher AR (11) and ErbB3 (Figure 2B, lower panels). Comparison of LNCaP vs LNCaP-AI showed that the latter expressed higher levels of HER2 and ErbB3, and also higher ErbB3 phosphorylation (Figure 2C). Taken together, these results indicate that in LNCaP cells, but not its CRPC subline, ErbB3 levels increase during AWT whereas it is suppressed by dual EGFR/HER2 inhibition.

Dual EGFR/HER2 inhibition suppresses ErbB3 and PSA levels in CWR22 xenografts in nude mice. CWR22 xenografts were established in 4-5 month old male nude mice, and when the tumors were palpable, the animals were treated with vehicle only or with erlotinib (0.8 mg/Kg, 5 times per week) and trastuzumab (20 mg/Kg, 2 times per week) in combination. The animals were castrated, or sham operated, 3 days after the drugs were started, but drug treatments were continued until the end. The animals were divided as: (a) vehicle only, sham operated (n=6), (b) vehicle only, castrated (n=6) and (c) drug-treated, castrated (n=6). CWR22 tumors shrink rapidly following castration, hence to obtain sizable tumors that can be analyzed; the animals were sacrificed 8 days after the procedure. Serum levels of prostate specific antigen (PSA), a clinical indicator of AR activity in the prostate, were analyzed in blood drawn (i) at the

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beginning of the study, (ii) on the day of castration/sham operation, and (iii) at the end of the study (Figure 3A, upper). In vehicle-treated, sham operated animals, PSA levels increased significantly with time (p=0.049), whereas in castrated animals, the change in PSA was not significant. In those treated with the drug combination, PSA levels decreased three-fold. At the end of the study, the difference between PSA levels from castrated animals that were vehicle treated (16.3 ± 8.3 ng/ml) vs drug treated (4.3 ± 3.2 ng/ml) was significant (p=0.02), whereas the difference between sham-operated (29.8 ± 7.9 ng/ml) vs control animals were not (p>0.05).

Staining for ErbB3 in the formalin-fixed and paraffin-embedded (FFPE) sections showed weak staining in the sham operated mice (n=6) whereas the castrated and vehicle treated mice showed strong staining (n=6), which was eliminated in the castrated mice treated with the drug combination (n=5; one of the tumors was too small for analysis) (Figure 3B). Quantitation of the staining levels showed a significant increase in ErbB3 levels from sham operated, vehicle treated (0.63 ± 0.43) to castrated, vehicle treated tumors (1.33 ± 0.26) (p=0.009), which was reduced 40% in tumors treated with the drugs in castrated animals (0.8 ± 0.45) (p=0.05) (Figure 3C). Castration suppressed proliferation and induced apoptosis in these animals, as indicated by Ki67 and TUNEL staining (Supplementary Figure 4), respectively, whereas both effects were enhanced by treatment with the drug combination (Figure 3D). These results confirm that dual EGFR/HER2 inhibition reduce ErbB3 levels and reduces serum PSA levels.

ErbB3 overexpression stabilizes androgen receptor levels and promotes castration resistant cell growth mediated by Akt. LNCaP cells overexpressing ErbB3 grew at a much faster rate compared to parental LNCaP cells (Figure 4A, upper) and were not growth inhibited by the AR antagonist bicalutamide (Casodex) even at 10 μM (Figure 4A, middle) indicating androgen-independent cell growth. Flow cytometric analysis revealed this to be due to an increase in the percentage of cells entering the cell cycle (increased S-phase) which was not
impeded by bicalutamide (Figure 4A, lower). Although culture in CSS-containing medium causes a decrease in the levels of the AR in LNCaP cells, increased expression of ErbB3 in the same cells maintained AR levels (Figure 4B). Since ErbB3 is a known inducer of Akt phosphorylation (29), we examined the role of Akt in ErbB3-mediated cell growth. Increased ErbB3 stimulated Akt phosphorylation (Figure 4C), while downregulation of Akt expression by siRNA suppressed ErbB3-induced proliferation in LNCaP cells (Figure 4D), thereby indicating that Akt phosphorylation mediated the regulation of LNCaP cell growth by ErbB3.

Resistance to growth inhibition by dual EGFR/HER2 inhibition correlates with the ability of the inhibitors to suppress Akt phosphorylation. LNCaP-AI cells expressed higher levels of Akt phosphorylation compared to parental LNCaP cells (Figure 5A, upper). Treatment with the combination of trastuzumab and erlotinib, but not the individual drugs, significantly inhibited heregulin 1β (HRG1)-induced Akt phosphorylation in LNCaP cells, but not in LNCaP-AI (Figure 5A, lower). Similarly, the same combination inhibited Akt phosphorylation in parental pRNS-1-1 cells which lack a functional AR, whereas in cells that express AR(T877A), the drug combination failed to inhibit Akt activity (Supplementary Figure 5A). These results correlate Akt phosphorylation with the growth inhibitory effects of the combination of trastuzumab and erlotinib. In addition, the tyrphostins AG1478 (EGFR inhibitor) and AG879 (HER2 inhibitor) (Figure 5B, upper), in combination, inhibited Akt phosphorylation in CSS-, but not in FBS-containing medium (Figure 5B, lower). Similar to trastuzumab and erlotinib, the combination of AG1478 and AG879, but not the individual drugs, suppressed growth of pRNS-1-1(ART877A) cells in CSS-containing medium, whereas they had little or no effect on cell growth in FBS-containing medium (Figure 5C). On the other hand, LNCaP-AI cells were not growth arrested by the latter combination (Supplementary Figure 5B). These results indicate that suppression of cell growth by the drug combination correlates with inhibition of Akt phosphorylation.
Suppression of Akt phosphorylation sensitizes castration resistant prostate cancer cells to dual EGFR/HER2 inhibition. Finally, we investigated methods of overcoming the resistance of PCa cells to ErbB inhibitors. Since LNCaP-AI are not sensitive to dual inhibition of EGFR and HER2, and expressed higher ErbB3 compared to LNCaP, we investigated whether the increase in ErbB3 contributed to this resistance. Similar to the effects of a combination of erlotinib and trastuzumab, the combination of AG1478 and AG879 impeded the increase in cell numbers but did not reduce them below initial levels in LNCaP cells cultured in FBS (Figure 6A, upper), indicating growth arrest but not cell death. However, when the same cells were cultured in CSS, there was a 50% decrease in cell numbers indicating cell death (Figure 6A, lower). On the other hand, culture in CSS failed to have a similar effect in LNCaP cells overexpressing ErbB3 (Figure 6B), indicating that ErbB3 increase induced resistance to this drug combination. In support of a role for Akt phosphorylation in this process, LNCaP cells cultured in CSS experienced increasing Akt phosphorylation over a period of 5 days when exposed to vehicle alone whereas when they were exposed to the combination of AG1478 and AG879, Akt phosphorylation was significantly impeded (Figure 6C, upper). On the other hand, in LNCaP-AI cells resistant to this drug combination (Supplementary Figure 5B), the increase in Akt phosphorylation in response to CSS exposure was not affected (Figure 6C, lower). The fact that Akt phosphorylation increased upon CSS treatment in LNCaP-AI cells whereas ErbB3 levels did not (Figure 2B) indicates that other factors also contribute to Akt phosphorylation in CRPC. Our results indicated that, failure of dual EGFR/HER2 inhibition to induce apoptosis resulted from a failure of the same drugs to downregulate Akt phosphorylation. In support, AG1478 and AG879 in combination was not effective in inducing apoptosis in LNCaP-AI cells in the presence of control siRNA (9.89% in control siRNA vs 13.25% in control siRNA + AG1478 + AG879), whereas Akt siRNA alone induced a significant increase in Annexin V staining (28.28%) which was further increased in the presence of the drugs (44.65%) (Figure 6D).
**DISCUSSION**

Previous studies showed that the dual EGFR/HER2 inhibitor lapatinib evidenced no decrease in PSA in patients with hormone sensitive PCa (22) or in unselected patients with CRPC (23). The goal of this study was to determine whether dual EGFR/HER2 inhibition has any role in the prevention of disease progression in PCa. We demonstrate that androgen-dependent PCa cells with low ErbB activity do not show substantial response to ErbB inhibitors, whereas during AWT, ErbB2 and ErbB3 levels increase, which regulates Akt phosphorylation and also cell survival. Hence, during this period, if the increase in these receptors is inhibited by dual EGFR/ErbB2 inhibition, which also inhibits ErbB3 phosphorylation, the increase in Akt phosphorylation and survival can be prevented. However, once ErbB3 levels have increased, the same drugs fail to affect the levels of Akt phosphorylation, thereby indicating that they can inhibit *de novo* activation of ErbB3 but cannot dephosphorylate the receptor after it is activated.

Although individual EGFR and HER2 inhibitors had differential effects on PCa cells, the overall effect of dual inhibition was similar. The difference between various inhibitors of the same receptor may be attributed to the strength of the binding of these inhibitors to the receptor. We see that in both cases, the drug combinations resulted in a decrease in Akt phosphorylation. Since ErbB4 is lost in PCa, the ErbB dimers formed in this disease include EGFR homodimers and EGFR-HER2, HER2-ErbB3 and EGFR-ErbB3 heterodimers (discussed in details in (31)). All contribute to survival of PCa cells; hence inhibition of only one receptor will not prevent downstream signaling. Our data shows that inhibition of both EGFR and HER2 is required to prevent ErbB3 signaling, likely by preventing its dimerization. Since only ErbB3 but not EGFR or HER2 have p85 PI3K binding sites (9), the majority of the Akt signaling may be downstream of ErbB3 dimerization with EGFR or HER2, which will be inhibited only upon dual inhibition. ErbB3 monoclonal antibodies such as MM-121 are currently in development (32), and are also likely to succeed in combination with other ErbB inhibitors such as lapatinib.
We show that in cells expressing high AR, either hormone-naïve cells never exposed to AWT, or in CRPC cells that have high AR transcriptional activity, dual ErbB inhibition is unable to inhibit Akt phosphorylation and cell survival. In a previous study, we had shown that in hormone-naïve cells, the AR suppresses ErbB3 levels by transcriptionally regulating the ErbB3 inhibitor Nrdp1 (11). Since ErbB3 is capable of inducing AR-independent cell growth, this is likely an attempt by the AR to suppress AR-independent signaling. Hence, in androgen-dependent cells growing in the presence of high androgen levels, cell-survival is AR-dependent and not ErbB3-dependent. Therefore, inhibition of ErbB3 or its binding partners will not affect cell growth or survival. On the other hand, when AR levels decreased during AWT, ErbB3 levels rebound and cell growth becomes dependent on signal transduction downstream of this receptor. Therefore, if at this time, ErbB3 signaling is suppressed, cell survival is impacted.

ErbB3 increase during AWT likely as an attempt to prevent AR decrease. In this study, we show that ErbB3 stabilize AR levels; thereby preventing its decrease in low-androgen medium. Further studies are required to see whether this is the mechanism by which ErbB3 promotes androgen-independent cell growth, but if so, it will explain why, in some CRPC cells, growth is still AR dependent, but not androgen dependent, as has been demonstrated by other labs (33, 34). Despite this, it appears that the ErbB3-stabilized AR is incapable of downregulating ErbB3 (which is reasonable, if it requires that ErbB3 to stabilize it), as we previously showed (11). Furthermore, once the cell progresses to a CRPC phenotype, it is no longer capable of responding to dual EGFR/HER2 inhibition to downregulate Akt phosphorylation downstream of ErbB3. Hence, dual EGFR/HER2 inhibition does not affect cell survival or even cell growth in CRPC cells.

In CRPC cells, the effects of ErbB receptors and the AR are compounded by high Akt phosphorylation (29). Akt is induced by other factors including IGF, hence in CRPC cells, which are associated with multiple changes in cell signaling pathways (see (35) and references
within), it is likely that the cells have become adept at kinase switching, resulting in activation of multiple cell survival pathways. As a result, in these cells, dual EGFR/HER2 inhibition will not prevent all aberrant Akt phosphorylation. Therefore, our goal is to prevent the increase in aberrant Akt phosphorylation, and PSA progression, indicative of relapse, following AWT, by using the dual inhibitors during and not after this treatment. The clinical and therapeutic consequences of such a treatment could be quite profound. A 2009 study of 1,078 patients with hormone-sensitive PCa enrolled in SWOG trial 9346, where PSA progression (PSA-P) was defined as an increase of ≥25% over nadir, median subsequent overall survival was shown to be 10 months in patients experiencing PSA-P within 7 months of hormone treatment, vs 44 months for those who did not have PSA-P during this period (36). Therefore, it is likely that if co-administration of dual EGFR/HER2 inhibitors delays PSA-P beyond 7 months, we would see a significant increase in PSA progression.

In conclusion, our data indicate that dual EGFR/HER2 inhibition is an effective tool for sensitizing androgen-dependent PCa cells to apoptosis during AWT, likely preventing PCa progression to CRPC following AWT treatment, but is not effective in CRPC cells expressing high Akt phosphorylation. However, this strategy may find utility with the advent of new therapeutic agents such as abiraterone acetate, a CYP17 inhibitor that blocks steroid biosynthesis (37), and MDV3100, a more potent AR inhibitor (38). In post-docetaxel patients, abiraterone increased survival by 3.9 months over controls (37) and it would be of interest to see whether this leads to an increase in ErbB3/HER2 as well, and whether prevention of this increase, if any, would further prolong survival. It is clear from the current study, that the window of opportunity for using ErbB inhibitors in PCa is when ErbB3 is rising and not when it is stable. The study also demonstrates that potentially effective drugs if utilized in the wrong clinical setting may be prematurely judged to be ineffective.
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FIGURES AND LEGENDS:

FIGURE 1. Androgen withdrawal sensitized prostate epithelial cells to apoptosis by the combination of trastuzumab and erlotinib. (A) LNCaP cells were cultured in the presence of FBS or CSS for 48 hours together with trastuzumab (21μg/ml), erlotinib (10μM), or combinations thereof. The cells were collected and analyzed by flow cytometry to determine (upper) the fraction of cells in S-phase (which indicates proliferation) and (lower) those undergoing apoptosis (data presented represent fold changes over control cells treated with DMSO alone, 1.1% in LNCaP 3.48% in C4-2 cells). (B) (upper panel) MTT assay was used to determine the cell growth rate of parental pRNS-1-1 cells with the combination of Erlotinib (10 /g541M) and/or Trastuzumab (21 μg/ml) for 24 hours. (Lower panels) MTT assay to determine the effect of Erlotinib and/or Trastuzumab in the presence of (middle panel) medium containing FBS (lower panel) or CSS in pRNS-1-1 cells transfected with mutant AR (T877A). Data represents mean ± S.D. for three independent experiments.

FIGURE 2. ErbB3 inhibition by the combination of erlotinib and Trastuzumab, and its stimulation by AWT, in LNCaP cells. (A) Western blots demonstrating the effect of erlotinib (10 μM) and Transtuzumab (21 μg/ml) on ErbB receptor tyrosine kinases. (left) LNCaP cells were serum starved in the presence of erlotinib and/or trastuzumab for 48 hours, followed by further treatment with 10 ng/ml EGF for 5 mins. Lysates were blotted with anti-phospho EGFR (Y1068) (1st Panel), anti-EGFR (2nd Panel), or anti-HER2 (3rd Panel) antibodies. (right) Alternately, the cells were stimulated with 50 ng/ml HRG1 to induce ErbB3 phosphorylation, and immunoblotted with anti-phospho ErbB3 (1st panel) and anti-ErbB3 (2nd panel). (B) Western blots demonstrating that AWT causes increased HER2 and ErbB3 expression and phosphorylation of Akt. LNCaP cells were cultured in FBS-containing medium up to 75% confluence and then switched to CSS-containing medium for the indicated period of time. Cell
lysate was collected and immunoblotted with antibodies to anti-AR (1st panel), anti-EGFR (2nd Panel), anti-HER2 (3rd panel), anti-ErbB3 (4th Panel), anti-phospho Akt (Ser 473) (5th panel), anti-Akt (6th Panel), and anti-β-actin (7th Panel). In contrast to LNCaP, its CRPC subline LNCaP-AI did not experience a similar increase in ErbB3 following AWT (8th panel). (C) Comparison of the activation and expression of the ErbB receptors expressed in LNCaP cells and its CRPC subline LNCaP-AI. The cells were serum starved for 48 hours and then EGF (10 ng/ml) (left), or HRG (50 ng/ml) (right) were added for the times indicated.

**FIGURE 3** The combination of EGFR and HER2 inhibitors inhibited PSA and ErbB3 levels in CWR22 xenograft-bearing nude mice. (A) (upper panel) Serum PSA was measured in weekly blood draws from the three groups of animals: sham operated/vehicle treated (n=6), castrated/vehicle treated (n=6) and castrated/drug treated (n=5), (lower panel) while their body weight was monitored to determine overall health. (B) Representative ErbB3 stainings of tumors extracted from (upper) sham operated (this section was scored +1), (middle) castrated/vehicle treated (this section scored +2) and (lower) castrated/trastuzumab+erlotinib treated (this section scored +0.5) mice (20X). (C) The scores from each group were statistically analyzed to determine overall effects. Castrated/vehicle treated mice had a significant overall increase in mean ErbB3 levels (1.33 ± 0.26, n=6) compared to sham operated animals (0.63 ± 0.43, n=6), p=0.009; which decreased again (0.8 ± 0.45, n=5) in castrated/drug treated mice (p=0.05). (D) Ki67 and TUNEL staining to determine levels of proliferation and apoptosis in CWR22 xenograft tumors in the same three groups. There was a significant decrease in nuclear staining for both Ki67 (p=0.0027) and TUNEL (p=0.0037) in cells from tumors extracted from the castrated+trastuzumab+erlotinib group compared to the sham castrated (intact) group (n=6).
FIGURE 4. Increased ErbB3 levels induce castration resistant cell growth mediated by Akt and androgen receptor stabilization. (A) (upper) MTT assay showing the growth of LNCaP cells transfected with an empty vector or with pcDNA3-ErbB3 cultured in FBS over 6 days. (middle) MTT assay showing the growth rate of LNCaP-ErbB3 cells cultured with DMSO (control) or 10 μM bicalutamide (Casodex). All data in this series is representative of three independent experiments. (lower) Flow cytometric analysis showing that LNCaP cells expressing pcDNA3 alone were responsive to bicalutamide-induced growth arrest whereas those expressing high ErbB3 levels did not. **: p=<0.05. (B) LNCaP cells transfected with vector (pCDNA3) or overexpressing ErbB3 were cultured in FBS-containing medium until 70% confluent, then switched to medium containing CSS and collected after the periods shown. AR and tubulin (loading control) levels were determined by Western blotting. (C) LNCaP cells were stably transfected with vector alone or with a plasmid expressing ErbB3, and demonstrates an increase in ErbB3 levels in the latter cells as well as an increase in Akt phosphorylation. (D) ErbB3 mediated cell growth was dependent on Akt activation. LNCaP cells transfected with vector alone or ErbB3 plasmid were subjected to treatment with control or Akt siRNA. The effect of Akt siRNA on Akt levels are shown in the inset. Growth rates were estimated after 4 days of treatment by MTT assay.

FIGURE 5. Akt phosphorylation at Ser 473 correlates with the ability of ErbB inhibitors to impede cell growth. (A) (upper) LNCaP-AI cells experience increased levels of Akt phosphorylation (Ser 473) compared to LNCaP. LNCaP-AI cells were serum starved and then treated with 10 ng/ml IGF-1 for various times as shown. Note the increase in Akt phosphorylation at Ser 473 with time. (lower) Western blots demonstrating the effect of erlotinib (10 μM) and trastuzumab (21 μg/ml) on LNCaP and LNCaP-AI cells. Cells were grown to 75% confluence, and then serum starved for 48 hours in the presence of erlotinib or trastuzumab or
both. The cells were then further treated with 50 ng/ml HRG1 for 15 mins, to stimulate Akt phosphorylation downstream of ErbB3 activation, cell lysates collected and immunoblotted with antibodies to anti-phospho Akt (Ser 473) (1st, 3rd Panels), and total Akt (2nd, 4th Panels). (B) Western blots demonstrating the specificity and selectivity of AG1478 and AG879 on the activation of EGFR and HER2 respectively. Serum starved LNCaP cells were treated with vehicle (DMSO), 5 μM AG1478 or 2 μM AG879 for 48 hours followed by further treatment with PBS or 10 ng/mls EGF for 5 mins. EGF induced the phosphorylation of both EGFR (Tyr1068) and HER2 (Tyr1248). (lower) LNCaP cells cultured in FBS or CSS were treated with the two drugs for 3 or 5 days. Western blotting shows that in the presence of FBS, there was no effect of the drugs, alone or in combination, on Akt phosphorylation whereas in CSS, Akt phosphorylation at Ser 473 was significantly affected. (C) MTT assay was used to determine the cell growth rate with the combination of AG879 (2 μM) and AG1478 (5 μM) of pRNS1-1 cells stably transfected with a T877A mutant AR grown in medium containing FBS (left panel) or medium containing CSS (right panel). Data represents mean ± S.D. of three independent experiments.

FIGURE 6. ErbB3 overexpression induces resistance to dual EGFR/HER2 inhibition in CRPC, which can be overcome by Akt downregulation. (A,B) LNCaP cells expressing vector alone, or overexpressing erbB3, were treated with 2 μM AG879, 5 μM AG1478, or both, were cultured in medium containing FBS or CSS. MTT assays were conducted to determine the effects of the drug combination on cell growth. (A, upper) In medium containing FBS, where control cells experienced a 2.25-fold increase in cell number after four days of treatment, those treated with a combination of AG1478 and AG879 failed to grow (p<0.0001), but showed no decrease in cell numbers. (A, lower) LNCaP cells transfected with vector only showed a decrease in cell numbers upon culture in CSS. (B) FBS- or CSS-cultured, ErbB3-transfected
LNCaP cells demonstrated comparable increase in growth rates (2.5-fold increase in growth in 4 days), but dual treatment with AG1478 and AG879 prevented growth (p=0.004), but did not decrease cell numbers. Data represents mean ± S.D. of three independent experiments for each point. (C) LNCaP and LNCaP AI cells were cultured in FBS then switched to CSS-containing medium in the presence of vehicle (DMSO) or a combination of AG1478 and/or AG879. Cells were harvested after the indicated period of time, and cell lysates run on 10% SDS-PAGE, immunoblotted and the blots stained with rabbit polyclonal anti-phospho-Akt (Ser 473) antibody. (D) Flow cytometric analysis of LNCaP-AI cells following 48 hour treatment with siRNA duplexes against a scrambled sequence or Akt1 siRNA. Propidium iodide and Annexin V-FITC stained cells were then analyzed by flow cytometry to determine the fraction of cells undergoing apoptosis.
FIGURE 1

A

Parental, FBS

T877A, FBS

T877A, CSS

%7.7%

%6.7%

%9.2%

Fold Change

Fold Change

Fold Change

Time (days)

GO/G1 Phase
S Phase
G2/M Phase

LNCaP

Annexin V stain

Fold Change

% Cells (% staining)
FIGURE 4

A

B

C

D

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