Additive antitumor effects of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa), and the nonsteroidal antiandrogen, bicalutamide (Casodex), in prostate cancer cells *in vitro*

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Progression from an androgen-dependent to an androgen-independent state often occurs in patients with prostate cancer (PCa) who undergo hormonal therapy. We have investigated whether inhibition of the epidermal growth factor receptor (EGFR) signaling pathway affects the antitumor effect of a nonsteroidal antiandrogen. Gefitinib (Iressa), an EGFR tyrosine kinase inhibitor, and bicalutamide (Casodex), a nonsteroidal antiandrogen [androgen receptor (AR) antagonist], were administered alone and in combination to AR-positive human PCa cell lines. FACS analysis showed lower EGFR expression levels on AR-positive cells (LNCaP, CWR22, CWR22R 2152 and AR-transfected DU145 cell lines) compared with AR-negative cells (DU145, PC3 and TSU-Pr1). Moreover, in AR-transfected DU145 cells, chronic treatment with bicalutamide increased EGFR expression to levels similar to androgen-independent DU145 cells. All AR-positive PCa cell lines were sensitive to gefitinib (IC$_{50}$ = 0.1–0.6 μM), whereas higher concentrations of bicalutamide were needed to reduce AR-positive PCa cell line proliferation (IC$_{50}$ = 0.8–2.0 μM). Low doses of gefitinib increased the antitumor effects of bicalutamide by strongly reducing the IC$_{50}$ of bicalutamide (approximately 10-fold). Similarly, bicalutamide increased the antiproliferative effects of gefitinib by reducing the IC$_{50}$ of gefitinib (approximately 5-fold). Taken together, our data suggest that in androgen-dependent cell lines, addition of gefitinib in combination with bicalutamide results in concurrent dual inhibition of AR and EGFR/HER2 pathways. This causes a significant delay in the onset of EGFR-driven androgen independence.
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Key words: prostate cancer; hormonal therapy; EGFR; androgen-independent tumors

In industrialized countries, prostate cancer (PCa) is the most frequently occurring malignancy in men, representing the second highest cause of cancer-related death. PCa usually begins as an androgen-sensitive, androgen-dependent (AD), nonmetastatic cancer, which without intervention may follow a gradual transition into a highly metastatic and then androgen-insensitive (AI) variety. The androgen receptor (AR) is a nuclear receptor that cooperates with multiple proteins to exert its biologic function. Upon binding to the ligand testosterone/5α-dihydrotestosterone (DHT), the AR can bind to the androgen response element (ARE) on the 5′ promoter of target genes, resulting in the modulation of cell growth. Until relatively recent times, endocrine response pathways in PCa were described solely in terms of the intracellular pathways used by the androgens and the subsequent destructive effects exerted on AR signaling by antihormonal treatments.[1] It is widely accepted that androgens promote tumor growth by binding to ARs and acting as nuclear transcription factors that regulate the expression of genes involved in cell proliferation and survival mechanisms. In contrast, hormonal therapies promote tumor regression either by reducing the amount of androgens available to the tumor cells or by binding the AR to antagonize the cellular actions of androgens.[2]

However, a more modern view of the endocrine response pathways retains the concept that androgens acting through ARs are central to the development of PCa, but also recognizes that it is naive to consider AR signaling as being isolated from the rest of the signals governing the cancer cell biology. Indeed, an increasing number of elements within the PCa phenotype, notably peptide growth factors, have been recognized as factors able to modify AR signaling.[3] These factors have the capacity to influence the androgen sensitivity of PCa cells significantly. They are also likely to be critical in the mechanism of response to hormonal therapies and in the escape from hormonal control of growth during disease progression and relapse.[4]

Clinically, PCa is treated differently depending on the stage of the disease. At present, hormonal therapies are the only suitable systemic treatment for patients with androgen-dependent PCa, while current treatments for androgen-independent PCa offer small benefits and potentially have a serious impact on the quality of life. Hormonal therapy usually decreases the volume of primary and metastatic lesions by inducing apoptosis.[2] It is thought that tumor cells eventually circumvent the need for steroid hormones, allowing them to continue to grow and progress despite therapy. In fact, after this initial response, the tumors recur in an AI form that shows only a limited response to additional androgen withdrawal[5–7] and are partially resistant to cure by chemotherapy. As with the mechanism through which androgens exert their mitogenic effect, little is known about the ways in which prostate cells can bypass this requirement. In most established AI tumor cell lines, androgen receptor expression is actually lost.[8] A substantial body of literature suggests that the AR can be regulated directly or indirectly by growth factor signal transduction pathways, which may contribute to the development and progression of PCa.[4] The identification of biologic factors and pathways responsible for the development of endocrine-resistant conditions is therefore a fundamental goal in cancer research.

The accurate identification of primary hormonal resistance in advance of hormonal therapy may prevent the administration of an inappropriate therapy, thereby avoiding unnecessary morbidity in unresponsive patients, and limit the waste of financial resources associated with newer drugs such as docetaxel and zoledronic acid. Therapeutic regimes that may either prevent the evolution of hormone-resistant disease or delay their appearance would be highly desirable. Epigenetic changes leading to the overexpression of the *erbB1* and *erbB2/neu* receptors are also involved in tumor
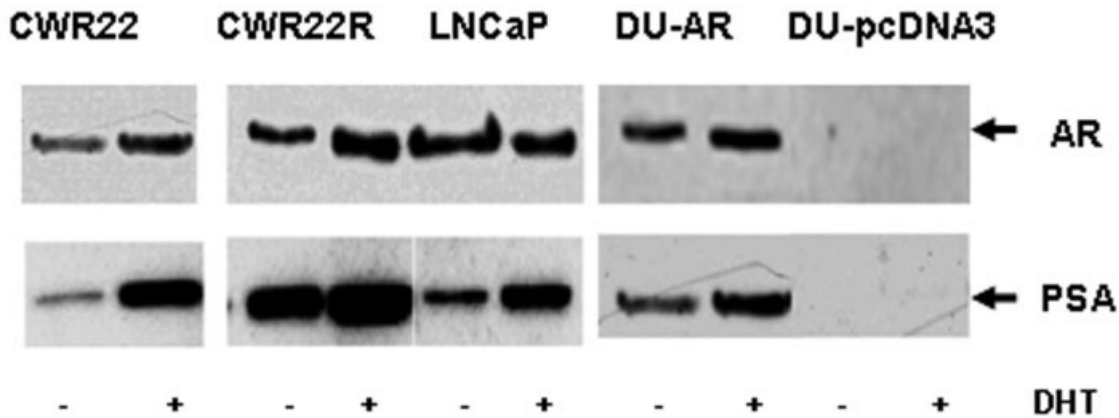
FIGURE 1 – Effects of 10 nM DHT on AR expression and PSA secretion in PCa cell lines and xenograft-derived PCa cells. For AR analysis, we used 40 μg/lane of protein by total cell lysates harvested from cells treated with 10 nM DHT for 24 hr. For PSA secretion, we used 20 μg/lane of protein by TCA-precipitated serum-free conditioned media from 1×10^6 cells obtained from cells cultured with 10 nM DHT for 48 hr. AR-transfected DU145 (DU-AR) cells were compared with mock-transfected DU145 (DU-pcDNA3).

progression[9] and in disease relapse and progression to androgen independence in human PCa.[10,11] Studies from many groups have demonstrated critical roles for a series of peptide growth factors and corresponding epithelium-expressed receptor tyrosine kinases (RTKs) in the normal prostate and/or in PCa. There are multiple lines of evidence indicating that deregulation of the EGFR signal transduction pathway plays a critical role in the processes of tumor pathogenesis, growth and metastasis.[12]

Several investigators have described amplifications of the EGFR gene and overexpression of the EGFR surface membrane protein in a large number of human cancers, including lung,[12] breast,[13] colon[14] and ovarian[15] carcinomas. This enhanced expression has been associated with increased proliferative activity and metastatic potential and has been identified as an independent determinant of poor prognosis for patients with malignant disease. Blockade of EGFR has been shown to inhibit the proliferation of human prostate tumor xenografts,[16] and in patients with PCa whose tumor samples initially showed no activation of MAP kinase, high levels of activated MAP kinase were found in recurrent tumor samples after hormonal therapy.[17]

In this report, the effects of a combination of an EGFR tyrosine kinase (EGFR-TK) quinazoline inhibitor, gefitinib (Iressa), with a nonsteroidal antiandrogen (androgen receptor antagonist), bicalutamide (BCLT; Casodex), were determined using 3 androgen-sensitive human prostate tumor cell lines.

Material and methods

Reagents

All the materials for tissue culture were purchased from Hyclone (Cramlington, NE). Plastic ware was obtained from Nunc (Roskilde, Denmark). EGF was purchased from Sigma (St. Louis, MO). Gefitinib and bicalutamide were obtained from AstraZeneca (London, U.K.). Hydroxyflutamide (HF) and cyproterone acetate (CPA) were obtained from Schering-Plough (Italia, Milan, Italy). Charcoal-stripped fetal calf serum (CT-FCS) was obtained from Gibco (Gaithersburg, MD). Antibodies were purchased from Santa Cruz (Santa Cruz, CA) unless otherwise indicated. Antibodies against phosphorylated forms of EGFR, HER2 and ERK1/2 were obtained from Biosource International (Camarillo, CA).

Cell lines and xenograft-derived cell cultures

We used several cell lines in these studies: DU145, an invasive and metastatic androgen-independent human PCa cell line, either transfected with the wild-type (wt) human androgen receptor (hAR) or with a control neomycin-resistant plasmid DNA[18];

TABLE I – BASAL EXPRESSION OF EGFR AND HER2 AS MEAN FLUORESCENCE INDEX EVALUATED IN PCA CELLS BY 3 DIFFERENT FACS ANALYSES
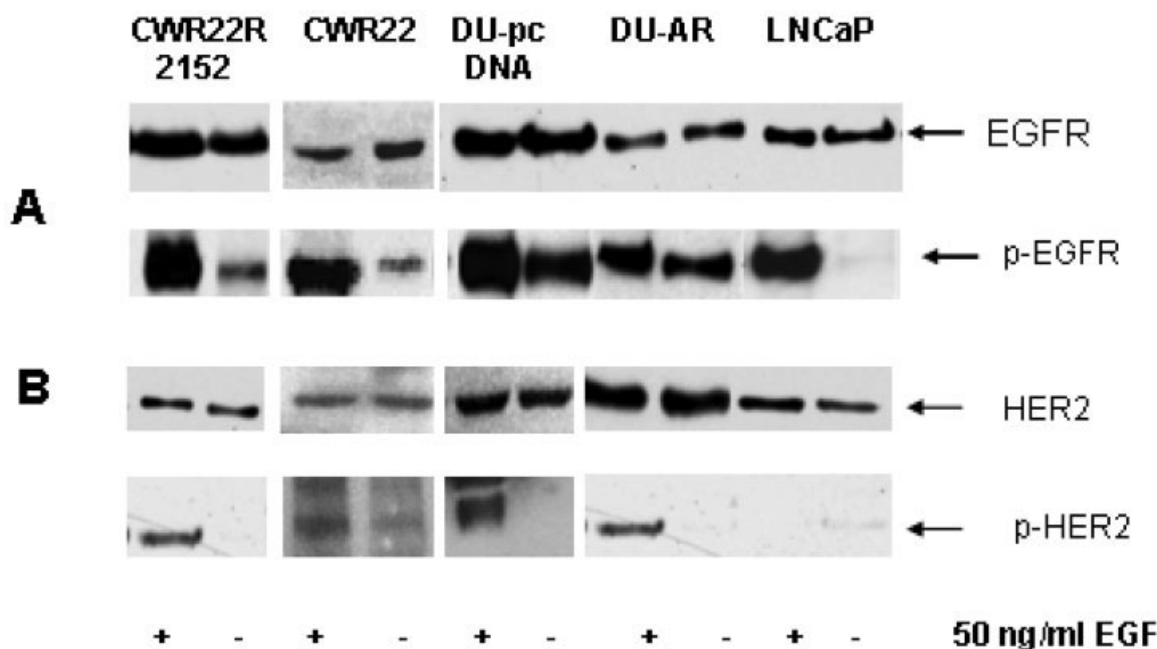
	EGFR	HER2
PC3	5.0 ± 0.6	2.0 ± 0.1
TSU-Pr1	2.9 ± 0.1	1.5 ± 0.1
DU145	9.0 ± 0.3	4.0 ± 0.3
DU-pcDNA	7.5 ± 0.3	3.5 ± 0.2
DU-AR	3.0 ± 0.1	3.0 ± 0.1
CWR22	2.5 ± 0.1	2.0 ± 0.1
CWR22R	6.0 ± 0.3	6.5 ± 0.3
LNCaP	4.0 ± 0.2	3.0 ± 0.1

LNCaP cells, which express mutant AR[19] that has been identified frequently in PCa patients who have AI disease[20]; and CWR22R-2152 xenograft-derived cells.[21,22] Despite having mutant AR, CWR22R-2152 and LNCaP ARs have been shown to bind androgens and to maintain their ligand-dependent transactivational activity. The AR-PC3, DU145 and TSU-Pr1 cell lines were used as controls (obtained from ATCC, Rockville, MD) and from Dr. S. Kanoh (TSU-Pr1).[23]

The CWR22 primary human CaP xenograft and relapsed CWR22R tumors were propagated and serially transplanted in nude mice as previously described.[21,22] CWR22 and CWR22R cells were cultured in 10% fetal bovine serum DMEM. In addition, we stably transfected the DU145 cell line with the human wt AR as previously described.[18] Briefly, we used 1 μg of pcDNA3-hAR expressing plasmid DNA to obtain DU-AR and 1 μg of pcDNA3 to obtain mock-transfected cells. Stable clones were cultured in complete medium supplemented with CT-FCS. Cells were allowed to grow in these conditions for 3 days and then 400 μg/ml of geneticin was added to maintain the stably transfected clones. Cells were maintained in the presence of geneticin, repeatedly grown at low density (using different cell dilutions) and then screened for AR expression using Western blots. A clone expressing high levels of AR was selected and used throughout the study.

Growth assays

Cells were seeded at a density of 2×10^4 cells on 50 mm petri dishes. Cells were left to attach and grow in 5% FCS DMEM for 24 hr. After this time, cells were maintained in culture medium containing androgens or subjected to androgen depletion. The following day, 3 dishes were analyzed (time 0) in order to measure the baseline cell number, while the remaining dishes had their medium changed. Morphologic controls were performed every

FIGURE 2 – EGFR and Her2 expression in PCa cell lines and xenograft-derived PCa cells. (*a*) Western blot for EGFR and p-EGFR. (*b*) Western blot for HER2/neu and p-HER2/neu. We used 40 µg/lane of total cell lysate protein harvested from cells treated with 50 ng/ml EGF for 30 min.

day with an inverted phase-contrast photomicroscope (Nikon Diaphot, Tokyo, Japan) before cell trypsinization and counting. All other cells were treated with either 10 ng/mL EGF or different doses of gefitinib, or with 1 nM DHT and different doses of bicalutamide for 24 hr. Cells trypsinized and resuspended in 20 ml saline were counted by a hemocytometer every 24 hr (LabRecyclers, Gaithersburg, MD) and 5 independent counts were performed for each dish. All experiments were conducted in triplicate.

In order to calculate the inhibitory concentrations at 50% (IC_{50}) of bicalutamide or gefitinib, 2,500 cells were cultured in 96-well plates for 24–96 hr. After adhesion (16 hr), cells were grown in the different culture conditions. After 48–96 hr, the cells were exposed for 4 hr to thyazol blue (MTS; Promega, Madison, WI). The 96-well culture plates were then placed on a microplate shaker for 5 min and the absorbance of the converted dye was measured at the wavelength of 490 nm using a Bio-Rad multiscan plate reader (Bio-Rad, Richmond, CA). Usually, 5 replicate wells were used for each group. Inhibition curves were performed by means of values obtained by OD percentages *versus* control for each concentration. IC_{50} values were calculated by the GraFit method (Erithacus Software, Staines, U.K.) considering the slopes of inhibition curves obtained for each group of tests.

Combined drug effects were analyzed by the median effect method. In brief, cells were treated with serial dilutions of each drug individually or with both drugs simultaneously or sequentially at a fixed ratio of doses that typically corresponded to 1/2, 5/8, 3/4, 7/8, 1.0, and 1.5 times the individual IC_{50} values. The fractional survival (f) was calculated by dividing the number of cells in drug-treated plates by the number of cells in control plates. Log $[(1/f) - 1]$ was plotted against log [drug dose]. From the resulting graphs, the x intercept (log IC_{50}) and slope m were calculated for each drug and for the combination by the method of least squares. The doses of the individual drugs and the combination required to produce varying levels of cytotoxicity were calculated according to the following equation: $dose_f = dose\ IC_{50}[(1 - f)/f]^{1/m}$.

Because the 2 drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be divided into the component doses $(D)_1$ and $(D)_2$ of drugs 1 and 2, respectively. For each level of cytotoxicity, the combination
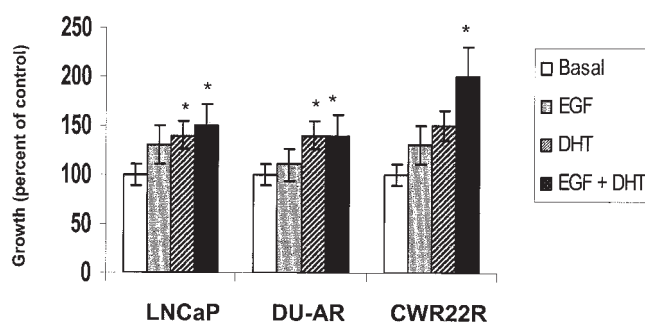

FIGURE 3 – Proliferative effects of 10 nM DHT and 10 ng/ml EGF alone or in combination after 48 hr of incubation in LNCaP, DU-AR and CWR22R cells. Asterisk, $p < 0.05$ compared with baseline.

index (CI) was then calculated, with synergy indicated by CI < 1, additivity indicated by CI = 1 and antagonism indicated by CI > 1. Unless otherwise indicated, experiments were repeated until 3 replicates yielded correlation coefficients of $1 \geq 0.9$ for all 3 median effect lines. Results of multiple experiments are summarized by indicating the mean ± SD of the CI at the indicated level of colony inhibition.

Determination of human kallikrein 2 activity

The kallikrein 2 activity was measured using H-D-Pro-Phe-Arg-pNA (S-2302) as substrate. Briefly, samples (40 µl) were incubated with 50 µl of Trasylol (20 KIU/ml) in Tris, 50 µl of 0.2 M Tris (pH 8.2) and 10 µl of S-2302 in 96-well plates for 60 min at 37°C, and the reaction was stopped using 10 µl 50% acetic acid. Plates were read at 405 nm. The substrate S-2302 is also sensitive to plasmin and by testing with 2 mmol/l S-2251 it was possible to check whether plasmin is present in the sample; S-2251 is not sensitive to kallikrein.
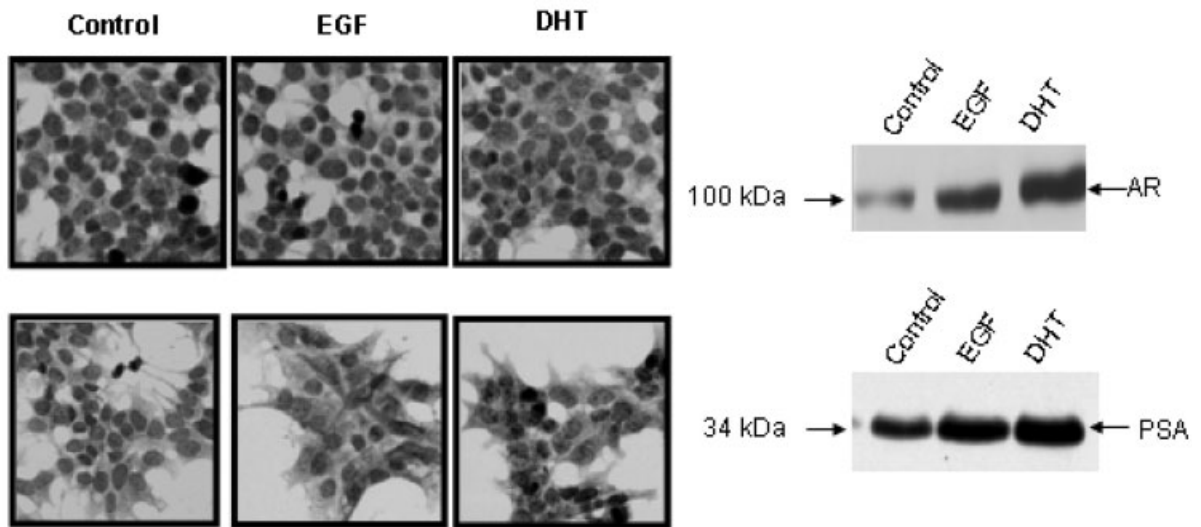
FIGURE 4 – (*a*) AR nuclear translocation and (*b*) PSA expression in CWR22R-2152 xenograft-derived cells after 48 hr of culture with 10 nM DHT and 50 ng/ml EGF treatment. Both AR and PSA expression were analyzed by immunocytochemical and Western blot analyses. For Western blot analysis we used 40 μg/lane of protein by total cell lysates and for PSA secretion 20 μg/lane of protein by TCA-precipitated serum-free conditioned media from 1×10^6 cells.
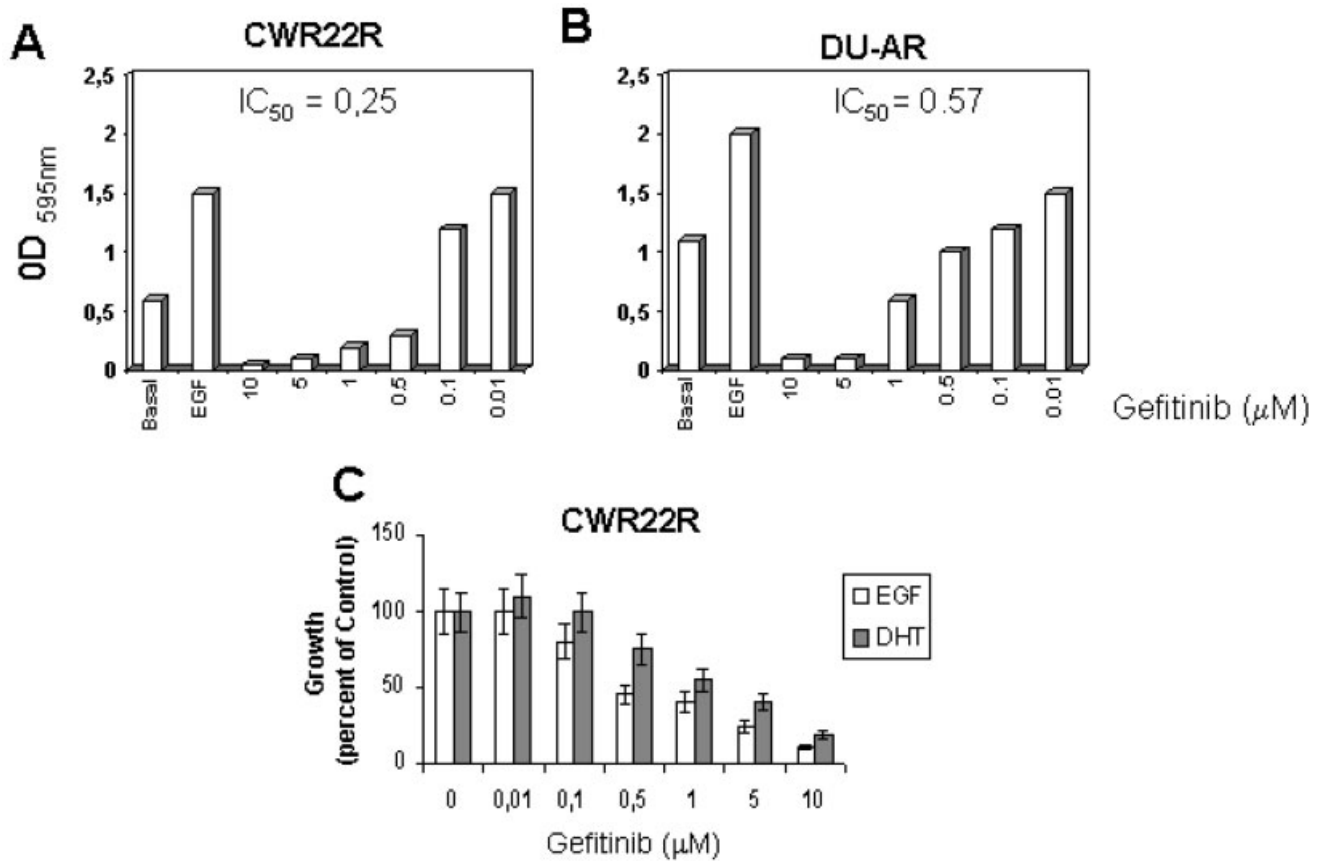
FIGURE 5 – Dose response of antiproliferative effects of gefitinib in (*a*) CWR22R and (*b*) DU-AR. Cells (1×10^4) were cultured in 24-well plates, and after adhesion cells were grown under different culture conditions in triplicate. After 48 hr, cells were fixed with methanol and stained with 10% crystal violet. Single wells were treated with acid methanol in order to extract crystal violet. Crystal violet (200 μl) solution was transferred in flat-bottomed 96-well culture plates and absorbance was analyzed at 595 nm. (*c*) Protective effects of DHT in CWR22R cells treated with increasing doses of gefitinib.

	LNCaP	DU-AR	CWR22R
− DHT	0.15 ± 0.04	0.53 ± 0.12	0.25 ± 0.07
+ DHT	0.42 ± 0.08	0.84 ± 0.08	1.00 ± 0.14

*Preparation of cell lysates, nuclear extracts and Western
blot analysis*

Following treatments, cells were washed with cold PBS and
immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5,
150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA,
1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM
p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM
phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml
leupeptin). The nuclear extracts were collected as described; cells
were scraped in 1 ml PBS-EDTA and centrifuged at 400*g*. Pellets
were resuspended in 1 ml harvest buffer containing 10 mM
HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA and
0.5% Triton X100 and incubated on ice for 5 min. Cells were pel-
leted at 80*g* in a table top swinging microfuge, washed and resus-
pended in 1 ml 10 mM HEPES, pH 7.9, containing 10 mM KCl,
0.1 mM EDTA and 0.1 mM EGTA. Nuclear extract was pelleted
at 80*g* in a swinging microfuge, and after removal of supernatant,
400 μl 10 mM HEPES, pH 7.9, containing 0.5 M NaCl, 0.1 mM
EDTA, 0.1 mM EGTA and 0.1% NP40 was added. Pellets were
vortexed for 15 min at 4°C and centrifuged at 16,000*g* for 10 min
at 4°C before protein content was measured. Lysates and nuclear
extracts were electrophoresed in 7% SDS-PAGE, and separated
proteins were transferred to nitrocellulose and probed with the
appropriate antibodies using the conditions recommended by the
suppliers. The EGFR or the ERK activation status was analyzed
using phosphospecific antibodies; blots were stripped and
reprobed with the appropriate antibody for assessment of total
ERK or EGFR expression.

*Immunoperoxidase staining and immunofluorescent analysis by
flow cytometry*

Cells were cultured in Lab-Tek Chamber slides (Nalgene Nunc)
and treated as described in the "growth assays" section above.
After 24–48 hr of cell culture, cells were washed in PBS and fixed
either in 4% PBS-paraformaldehyde for 5 min for immunostaining
of membrane-associated antigens (EGFR/HER2) or in cold 1:1
acetone:methanol mixture in ice for 2 min for immunostaining of
cellular or nuclear antigens (PSA and AR). Primary and secondary
antibodies were used according to the manufacturer's protocols.
Quantification of EGFR/HER2-positive cells was performed by
flow cytometric analysis (FACScan; Becton Dickinson, Mountain
View, CA). Cells were trypsinized and left at 37°C
for 1 hr in DMEM/10% FCS in polypropylene tubes in order to
reconstitute cellular external membrane. Cells were washed in sal-
ine buffer and 1 × 10^6 cells were fixed in a 3.7% paraformalde-
hyde-buffered solution, washed twice and then treated with about
10 μg/ml of primary antibodies. After 1 hr at 4°C, cells were
washed twice in PBS and FITC antirabbit and antimouse secon-
dary antibodies (about 1 μg/ml) were added to the fixed cells.
After 30 min of incubation at 4°C, cells were washed twice and
resuspended in PBS at 1 × 10^6 cells/ml before analysis using Cell
Quest software (Becton Dickinson).

Apoptosis analysis by flow cytometry

The adherent cells were trypsinized, pooled with the culture
supernatant containing the apoptotic cells already detached from
the dish and centrifuged. Cells (1 × 10^6) were washed in PBS and
fixed for 30 min by the addition of 1 ml of 70% ethanol. After 30
min, the cells were pelleted by centrifugation (720 *g*; 5 min),
resuspended in 1 ml of DNA staining solution (PBS containing
200 mg/ml RNase A, 20 mg/ml propidium iodide plus 0.1% Triton
X-100) and stained by incubation at room temperature for 60 min.
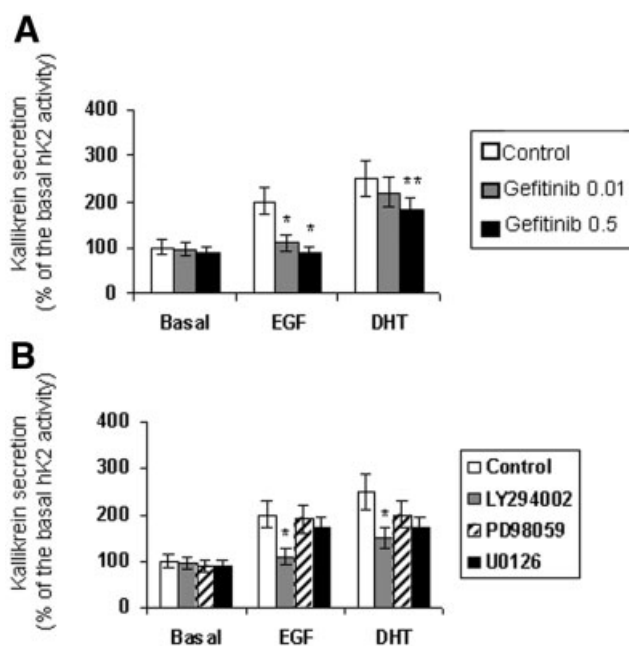
FIGURE 6 – Secretion of kallikrein by PCa cells. PCa cells secreted
hK2 at about 3–5% of the amount of PSA. Usually one-chain, mature
PSA and hK2 obtained when cells were grown in the presence of fetal
bovine serum had no enzymatic activity, but were active when the
cells were grown in the absence of serum. Using enzymatically active
recombinant hK2, it was possible to activate pro-PSA. Usually we
obtained 5 ng/10^4 LNCaP cells, 8 ng/10^4 CWR22R cells and 2 ng/10^4
DU-AR cells, while we obtained 0.25, 0.8 and 0.15 ng hK2/10^4 cells,
respectively. Gefitinib decreased EGF-mediated kallikrein secretion
but only partially decreased DHT-mediated kallikrein secretion. In
(*a*), we show the effects of gefitinib in CWR22R-2152 cells. Kallik-
rein activity was measured using urinary kallikrein as international
units/10^6 cells. The values of hK2 activity were expressed as percent
of the basal activity. EGF induces an increment of hK2 of 2-fold and
DHT of 2.5-fold. Gefitinib reduced dose-dependently the hK2 activity.
In (*b*), we show the role of PI-3K in both the DHT- and the EGF-
mediated hK2 secretion. Similar inhibitions were obtained analyzing
PSA secretion (also a product of an AR-dependent gene). Asterisk,
*p* < 0.01; double asterisk, *p* < 0.05 compared to baseline.

All cells were then measured on a FACScan flow cytometer (Bec-
ton Dickinson) with an argon laser at 488 nm for excitation and
analyzed using Cell Quest software (Becton Dickinson). All the
flow cytometric measurements were done using the same instru-
ment settings, and at least 10,000 cells were measured in each
sample. Apoptotic cells were detected by a quantifiable peak in
sub-G1 phase corresponding to the red fluorescence light emitted
by subdiploid nuclei of cells, and the results were expressed as
the percentage of death by apoptosis induced by a particular
treatment.

Statistics

Data are expressed as the mean ± SEM of at least 3 independ-
ent experiments. Statistical analysis was performed using an
unpaired Student's *t*-test. *p*-values < 0.05 and < 0.01 were consid-
ered statistically significant.

Results

In order to verify the crosstalk between AR and EGFR/HER2
pathways, we used one cell line with wt AR, DU145 transfected
with wt AR and 2 cell lines expressing different mutant forms of
AR that have been frequently identified in CaP patients who have
AI disease: LNCaP and CWR22R-2152 *in vitro* xenograft-derived
cells. Despite these mutations, the LNCaP and CWR22R-2152
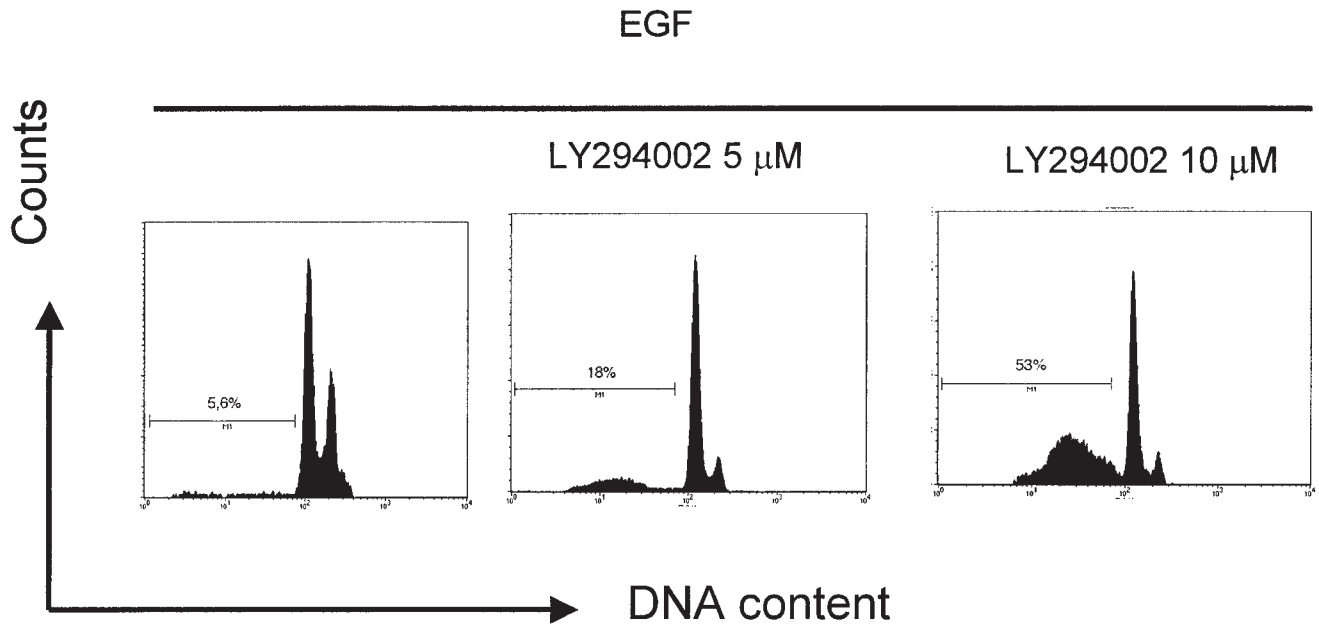
FIGURE 7 – Induction of cell death by LY294002. LY294002, a PI-3K inhibitor, renders cells apoptosis-prone. We verified cell cycle effects and apoptosis in AR^{+} cells and we observed that in all EGF-treated cells, LY294002 induced G1 cell arrest, and at time >24 hr it induced apoptosis. Here we show that 5 and 10 μM LY294002 induces cell death in 18% and 53% of CWR22R cells, respectively.
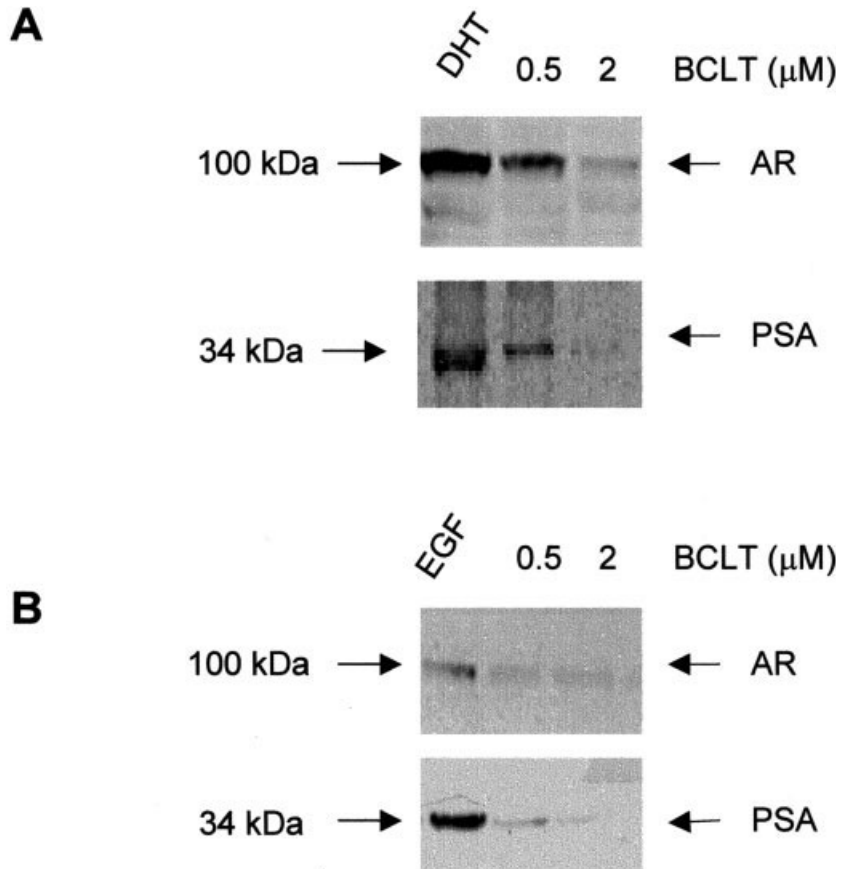
FIGURE 8 – Effects of (*a*) DHT and (*b*) EGF on AR nuclear translocation and function. DHT and EGF induce AR nuclear translocation and function. DHT showed a higher effect compared to EGF. Bicalutamide reduced nuclear translocation and function of AR at 0.5 and 2.0 μM; these concentrations reduce CWR22R growth by 20% and 50%, respectively. The IC$_{50}$ for CWR22R is 0.8 μM. Each lane was loaded with 40 μg of total lysates (AR) and 20 μg of TCA-precipitated serum-free conditioned media harvested from subconfluent cells cultured for 48 hr.

ARs have been shown to bind androgens and to maintain their ligand-dependent transactivational activity with DHT-mediated PSA secretion as illustrated in Figure 1. DU-AR cells were compared with mock cells, obtained by transfection with a control neomycin-resistant plasmid DNA (DU-pcDNA3) or with DU145 wt. CWR22R-2152 xenograft-derived cells were compared with those obtained from a nonrelapsed CWR22 xenograft. We successively compared the expression of EGFR and p-EGFR in these

cell systems and in AR-negative cell lines (DU145, PC3 and TSU-Pr1) using cytofluorimetric analysis and Western blot (Table I, Fig. 2*a*). The cytofluorimetric data (Table I) demonstrated that the AR-transfected DU145 cells expressed lower levels of EGFR compared with AR-negative mock-transfected DU145 (about 3-fold) and that AI AR-positive CWR22R-2152 cells expressed higher levels compared with AD CWR22 cells (about 1.5-fold). These results are in agreement with several observations demonstrating that EGFR overexpression is correlated with androgen unresponsiveness.[7,11,17,24] Western blot analysis confirmed the fluorescence data and showed that both basal and EGF-mediated phospshorylated EGFR levels were higher in AR-negative DU145 cells, AR-positive and androgen-sensitive DU-AR and CWR22R cells compared with androgen-dependent LNCaP and xenograft-derived CWR22 cells.

The cells also expressed HER2 (Fig. 2*b*) and phosphorylation occurred after a 30-min treatment with 50 ng/ml EGF. The cytofluorimetric data (Table I) demonstrate that the AR-transfected DU145 cells expressed lower levels of HER2/neu than AR-negative DU145 or mock-transfected DU145 (about 1.3-fold) and that AI AR-positive CWR22R-2152 cells expressed higher levels than AD CWR22 cells (about 1.5-fold). Western blot studies confirmed the fluorescence analyses and showed that the basal and EGF-mediated levels of phosphorylated were also higher in AR-negative DU145 cells, AR-positive and androgen-sensitive DU-AR and CWR22R cells compared with androgen-dependent LNCaP and xenograft-derived CWR22 cells. Similar to untransfected DU145 cells, DU-AR cells expressed detectable levels of phosphorylated forms of EGF and HER2 under basal conditions, suggesting that the new expression of AR does not modify the autocrine activation of these receptors.

In LnCaP cells, HER2 seems not to be phosphorylated after EGF treatment. Although EGFR and HER2 are present in detectable levels in these cells, the lack of HER2 activation deserves further studies because HER2 could either heterodimerize with EGFR family members which are not activated by EGF, or may need the action of coactivators including for example the binding to extracellular matrix components.

Effects of DHT and EGF on prostate cell growth and PSA expression

Exposure to EGF resulted in a significant increase in cell proliferation in a dose-dependent manner, reaching maximum stimulation at 10 ng/ml EGF. Interestingly, the total stimulatory effect produced by 10 ng/ml EGF was comparable to that induced by 10 nM DHT (Fig. 3) and was equally present in cells cultured in the presence or absence of charcoal-stripped serum (CSS)-containing culture medium. DHT and EGF showed additive proliferative effects in CWR22R-2152 cells, and partially additive effects were seen in DU145-AR and LNCaP cell lines. DHT induced nuclear translocation of AR. In Figure 4(*a*), we show the DHT-mediated AR nuclear translocation in CWR22R-2152 xenograft-derived cells. EGF also induced ligand-independent nuclear translocation of AR and ligand-independent PSA expression. Increased immunostaining for PSA was observed after exposure of CWR22R-2152 cells to EGF (as assessed by immunostaining or Western blot; Fig. 4*b*). Western blot analysis revealed a single band with a molecular mass of approximately 30 kDa that was clearly enhanced after treatment of CWR22R-2152 cells with either EGF or DHT.

Gefitinib inhibited EGF-mediated cell growth

Phosphorylation of EGFR and Her2 was inhibited by gefitinib in a dose-dependent manner as previously shown.[25] The blockade of EGFR/HER2 signaling pathways by gefitinib was able to reduce cell proliferation. In Figure 5, we show the dose-dependent effects of gefitinib on DU-AR and CWR22R cell growth (IC_{50} = 0.57 and 0.25 μM, respectively). However, when the cells were treated with 10 nM DHT, their proliferation was scarcely inhibited
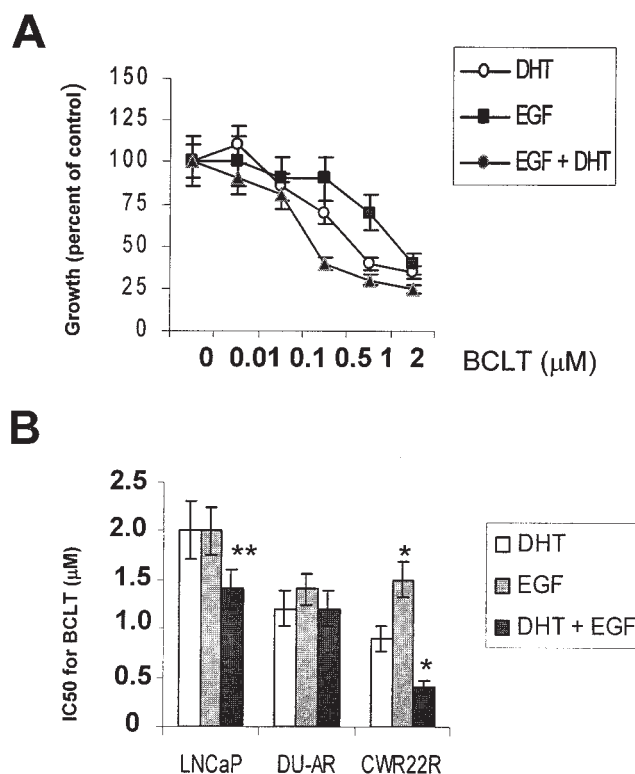
FIGURE 9 – (*a*) Dose-response of antiproliferative effects of BCLT in DU-AR cells. (*b*) IC_{50} values of bicalutamide in the presence of DHT, EGF, or their combination in LNCaP, DU-AR and CWR22R. Asterisk, $p < 0.01$; double asterisk, $p < 0.05$ compared to baseline.

by gefitinib, and the IC_{50} values increased by 40% (DU-AR) and 5-fold (LNCaP and CWR22R). In Figure 5(*c*), we show the protective effects of DHT in CWR22R cells and in Table II the increased IC_{50} after the addition of DHT.

Gefitinib inhibits AR nuclear translocation and expression of related genes: involvement of PI-3K

EGFR signaling induces an increase in phosphatidylinositol-3-kinase (PI-3K) activity. It has been shown that the inhibition of PI-3K activity with LY294002 dramatically decreased both basal and DHT-induced AR nuclear translocation as well as PSA secretion in LNCaP cells.[26] We determined whether these phenomena were sensitive to the inhibition of EGFR/HER2 pathway. We demonstrated that EGF induced kallikrein (K2) secretion and that the exposure to nontoxic concentrations of gefitinib (0.01–0.5 μM) strongly inhibited EGF-mediated (androgen-independent) and partially inhibited DHT-mediated (androgen-dependent) K2 secretion in all AR^+ cell lines. In Figure 6(*a*), we show the data observed using the CWR22R-2152 cells. The K2 production was dependent on PI-3K but not on MAPK activation because LY294002 (20 μM), but not PD98059 or U0126, was able to inhibit both EGF- and DHT-mediated K2 secretion (Fig. 6*b*). In fact, LY294002 but not PD98059 was able to reduce both DHT- and EGF-mediated AR nuclear translocations in these cells (data not shown).

The PI-3K inhibitor LY294002 renders cells apoptosis-prone. In CWR22R-2152 cells, blockade of PI-3K activity by LY294002 inhibited cell proliferation and induced G1 cell cycle arrest, with dose- and time-dependent apoptosis. Figure 7 shows the dose-dependent apoptosis in CWR22R-2152.
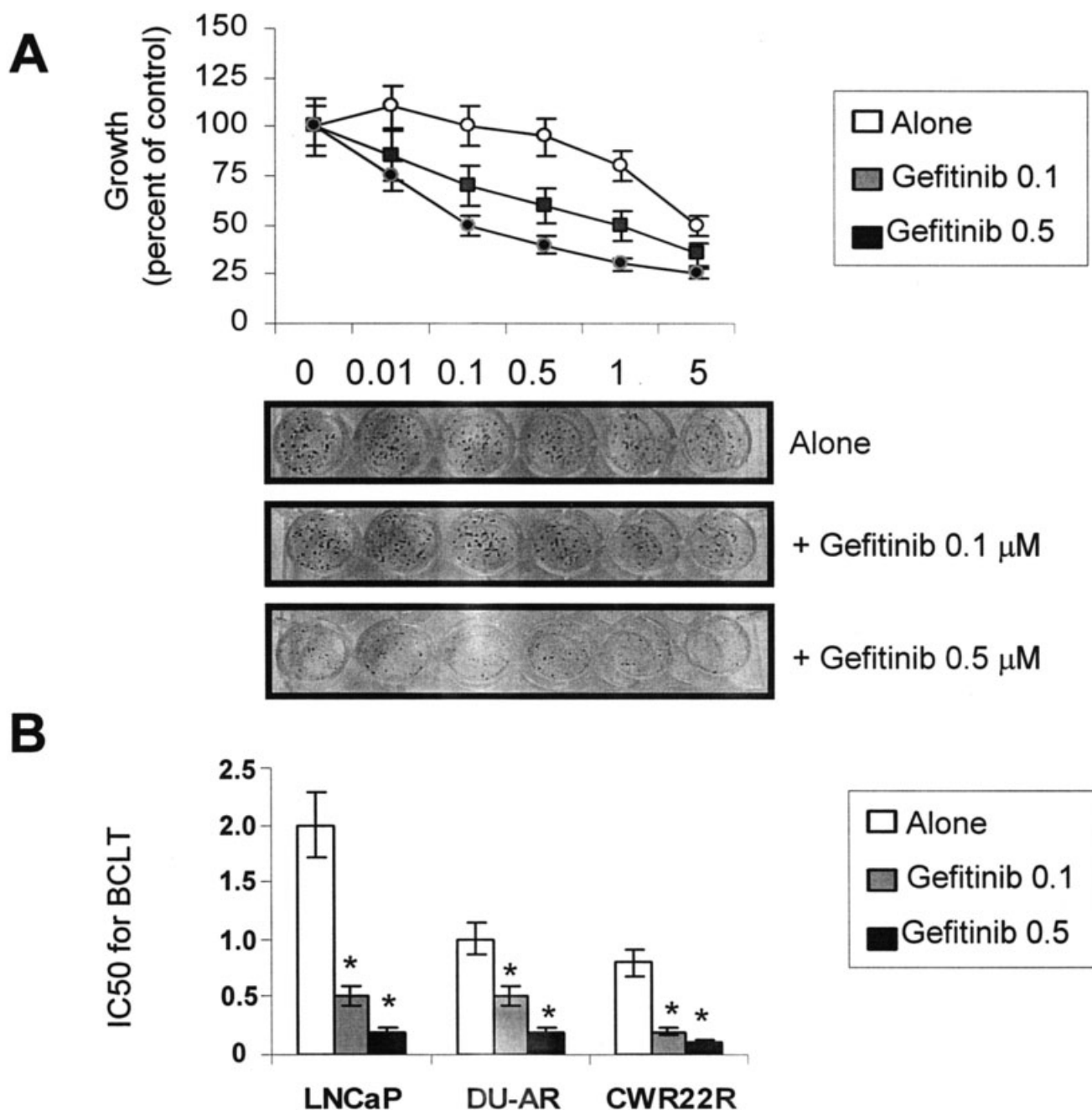
FIGURE 10 – (a) Dose-response of BCLT after treatment with 0.1 and 0.5 μM gefitinib in CWR22R cells. A total of 1×10^4 cells were cultured in 24-well culture plates after adhesion; cells were grown in the different culture conditions in triplicate. After 48 hr, cells were methanol-fixed and stained with 10% crystal violet. Single plates were photographed with a digital camera. Single wells were treated with acid methanol in order to extract crystal violet. Crystal violet (200 μl) solution was transferred in flat-bottomed 96-well culture plates and absorbance was analyzed at 595 nm. Gefitinib increased the antiproliferative effects of bicalutamide. Gefitinib at 0.1 and 0.5 μM was added to cells treated with increasing doses of bicalutamide. (b) Increased antiproliferative effects of gefitinib (0.1–0.5 μM) in CWR22R, LNCaP and DU-AR cells. Asterisk, $p < 0.01$ compared to baseline.

Bicalutamide inhibited both DHT- and EGF-mediated
AR nuclear translocation and PSA secretion

As expected, treatment with 0.5–1 μM of bicalutamide was able to reduce AR nuclear translocation and PSA secretion both in DHT- and in EGF-treated cells. In Figure 8, we show the effects of bicalutamide in DHT-treated and EGF-treated CWR22R cells. These experimental data strongly reinforce the idea that EGFR/HER2 signaling pathways modulate AR activity, primarily through PI-3K (and only partially through MAPK).

Bicalutamide inhibited both DHT- and EGF-mediated
cell proliferation

In the presence of DHT, bicalutamide inhibited cellular proliferation with IC_{50} values of > 2 (LNCaP), 0.8 (CWR22R-2152) and 1.0 (DU-AR) μM. Potentiation of these effects was significantly greater with bicalutamide compared with CPA and HF using the AR-transfected DU145 cells. However, bicalutamide reduced not only the DHT-mediated cell growth (Fig. 9) but also the EGF-mediated cell growth. The comparisons of IC_{50} values indicated
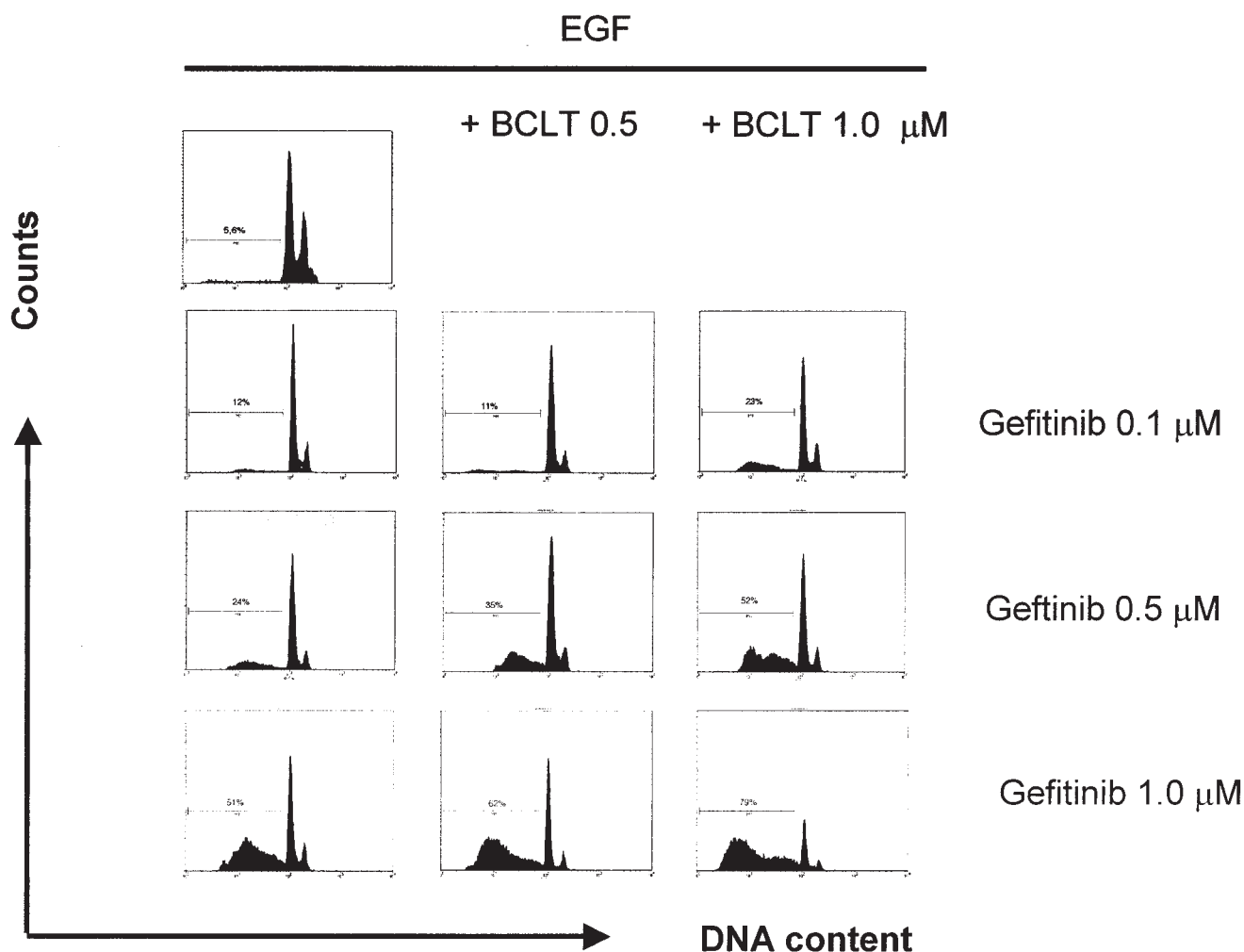
FIGURE 11 – Cytofluorimetric analyses in CWR22R cells treated with 0.1, 0.5, 1.0 μM gefitinib with or without 0.5 and 1.0 μM BCLT for 24 hr. Combination with bicalutamide amplified the apoptotic events in cells treated with gefitinib.

that LNCaP and DU-AR had similar susceptibility to bicalutamide inhibition in both DHT- and EGF-mediated cell growth, whereas CWR22R cells showed higher IC_{50} values for EGF compared to DHT (Fig. 9b). However, CWR22R cells, and partially LNCaP cells, were more potently inhibited by bicalutamide when EGF and DHT were present in culture medium. This was due to the additive effects of both growth factors on these cells.

Gefitinib increased antiproliferative effects of bicalutamide

As illustrated in Figure 10, the efficacy of bicalutamide was increased by low doses of gefitinib. The addition of both 0.1 and 0.5 μM gefitinib was able to reduce significantly the IC_{50} for bicalutamide by 2- to 4-fold and 5- to 10-fold, respectively, in our cell systems. Similarly, the efficacy of gefitinib was increased by bicalutamide. The addition of both 0.5 and 1.0 μM bicalutamide was able to reduce significantly the IC_{50} for gefitinib by about 4- and 5-fold, respectively, in all cell systems.

Effects of gefitinib alone or in combination with bicalutamide on cell cycle and apoptotic events

Gefitinib was able to block the cell cycle in G1/S phase and to induce cell apoptosis in a dose- and time-dependent manner according to the cell type used. In Figure 11, we show the cytofluorimetric data obtained for CWR22R-2152 cells treated with different doses of gefitinib with or without 0.5–1.0 μM bicaluta-
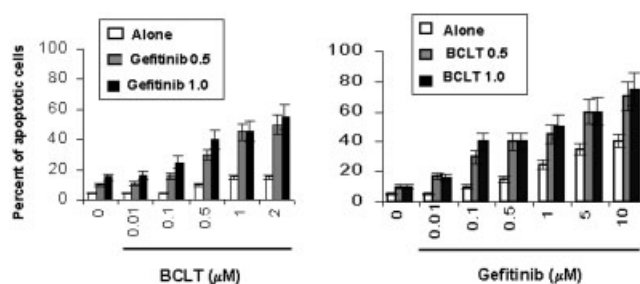
FIGURE 12 – Cytofluorimetric analyses in CWR22R, DU-AR and LNCaP cells treated with 0.1, 0.5, 1.0 μM gefitinib with or without 0.5 and 1.0 μM BCLT for 24 hr. Combination with bicalutamide amplified the apoptotic events in gefitinib-treated cells. The increment of apoptotic cell number was statistically significant ($p < 0.01$) in all combinations tested when compared to the single drug alone.

mide. Similar increases in apoptotic events were obtained when nontoxic gefitinib concentrations were added to different doses of bicalutamide. In Figure 12, we show the summarized cytofluorimetric data obtained with drug combinations for DU-AR, CWR22R and LNCaP cells.

Discussion

A major clinical problem with hormonal therapy of PCa is the progression of androgen-sensitive tumors to androgen independence. Constitutively high AR expression has been found in hormone refractory tumors, but lower expression of the AR was noted during regression of hormonally treated PCa.[21,22,27–30] AR amplification may contribute to its own activation in a relative absence of androgens; moreover, AR appears to undergo structural and functional alterations during PCa progression. We have previously observed that bicalutamide is able to inhibit significantly and dose-dependently the proliferation of established AR-positive human PCa cell lines and of primary cultures of PCa cells obtained directly from clinical samples.[31] In the present report, we have obtained a homogeneous inhibition response by bicalutamide only in the absence of EGF (produced *in vivo* by autocrine/paracrine modality and operating via EGFR), suggesting that in some patients, therapy additional to bicalutamide may be beneficial. In the absence of androgens, EGFR may increase the enzymatic activities of effectors (including PI-3K and MAP kinase) involved in the maintenance of cell survival. A recent study showed that the level of activated MAP kinase was elevated with increasing Gleason score and tumor stage of PCa and patients whose tumor samples originally showed no activation of MAP kinase before hormonal therapy exhibited high levels of activated MAP kinase in their recurrent state after such therapy.[17] We have also demonstrated that ERK1 and ERK2 phosphorylation was considerably higher in AR-transfected DU145 (DU-AR) cells treated with HF compared with the cells treated with DHT[24] and that the activation of ERK1 and ERK2 in response to EGF was totally neutralized when an EGFR inhibitor was used.[24] In addition, we have also demonstrated that EGFR levels are downregulated by androgens, whereas a prolonged androgen deprivation increases EGFR protein levels. This suggests that low EGFR/HER2 expression in androgen-sensitive cells result from an active mechanism by which androgens maintain low levels of these receptors. Thus, the interactions between EGFR and AR pathways may serve to modulate as well as to induce growth. Our data support the idea that, while maintained in the presence of androgens, the androgen-sensitive PCa cell lines remain growth-regulated by androgens. However, upon long-term abrogation of androgen action by the use of antiandrogens, the cells gradually adapt and use alternative transduction pathways, developing an ability to grow without androgens until they can eventually proliferate at the same rate as they had done previously in the presence of androgens.

In this study, we verify the molecular mechanism through which gefitinib inhibits the AR nuclear translocation. We indicated phosphatidylinositol-3-kinase inhibition by LY294002 reduces AR activity, as measured by the decreased nuclear translocation of AR and by the expression of AR-regulated genes such as PSA or kallikrein, and may induce cell death. Then, PKB/Akt, the key effector of the PI-3K, and AR may cooperate with each other to promote the PCa cell survival and growth as well as to regulate each other to maintain homeostasis of PCa cells.[32–35] Murillo *et al.*[33] show that androgen ablation can increase PI-3K-Akt activity, implying that enhanced Akt activity may rescue cells from impairment of androgen withdrawal. Manin *et al.*[34] show that LY294002 treatment could decrease AR protein and PSA levels in LNCaP cells, whereas Sharma *et al.*[35] find that LY294002 treatment did not suppress the AR protein level in LNCaP cells, but 4 hr of LY294002 treatment decreased the PSA mRNA level.

In presence of bicalutamide, EGF had the capacity to effectively rescue cell growth. We have also investigated the possibility that bicalutamide could interfere with EGFR activity. The increase in growth stimulation suggests that bicalutamide-treated cells become more sensitive to exogenous growth factors and this feature can allow the cells to bypass the requirement for androgens. We observed that a combination treatment with bicalutamide and gefitinib strongly reduced the IC_{50} values as compared with single treatments, suggesting the existence of additive effects in AR expressing PCa cells. Similar data were observed in primary cultures derived from prostate cancer tissues (data not shown). The molecular mechanisms involved in the cooperative effects of bicalutamide and gefitinib appear to indicate an AR transactivation mediated by EGFR/HER2 signaling both through PI-3K-mediated Akt/PKB activation (since it may be blocked by 30 μg/ml LY294002) and through ERK-mediated AR phosphorylation (since it may be blocked by 10 μM PD98059). These results are in agreement with other experimental data obtained in CWR22 and CWR22R models[36,37] and with a recent study of ours.[24] In conclusion, our experiments suggest that combination therapy of anti-EGFR and nonstreroidal antiandrogen might block tumor cell growth and that it is possible to raise the maximal effect in the combination of bicalutamide with low doses of gefitinib. Therefore, these data can be considered for the design of future trials, since the side effects of gefitinib can be avoided, suggesting the use of low doses of this drug in combination with antiandrogens.

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