available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Epidermal growth factor receptor inhibitor (PD168393) potentiates cytotoxic effects of paclitaxel against androgen-independent prostate cancer cells

Yeong-Shiau Pu [a], *Min-Wei Hsieh* [b], *Chuang-Wei Wang* [b], *Guang-Yaw Liu* [c], *Chao-Yuan Huang* [a], *Chia-Chi Lin* [d], *Jing-Yi Guan* [b], *Shinne-Ren Lin* [e], *Tzyh-Chyuan Hour* [b,*]

[a] *Department of Urology, National Taiwan University College of Medicine, Taipei, Taiwan*
[b] *Institute of Biochemistry, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Institute of Immunology, Chung Shan Medical University, Taichung, Taiwan*
[d] *Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan*
[e] *Faculty of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung, Taiwan*

ARTICLE INFO

Article history:
Received 25 October 2005
Accepted 8 December 2005

Keywords:
Docetaxel
Synergism
Quinazoline
Chemotherapeutic agents
Hormone refractory

ABSTRACT

Recent data showed that epidermal growth factor receptor (EGFR) inhibitors, such as ZD1839, alone or in combination with chemotherapeutic agents for androgen-independent prostate cancer (AIPC) did not produce promising results in clinical settings. More effective regimens involving novel stronger inhibitor of EGFR and better combinations are needed. The anti-tumor activity of PD168393, an irreversible EGFR inhibitor, with or without chemotherapeutic agents for the treatment of AIPC was investigated in vitro. In results, both the androgen-independent cell lines PC-3 and DU145 expressed higher levels of EGFR than the androgen-dependent MDA PCa 2b and androgen-responsive LNCaP cells by Western blotting. DU145 was much more sensitive to PD168393 and ZD1839 than MDA PCa 2b. PD168393, but not ZD1839, significantly potentiated paclitaxel cytotoxicity against DU145 by MTT assay and median-effect analysis. The combination of PD168393 or ZD1839 with other cytotoxic agents including docetaxel and 5-fluorouracil, however, was either additive or antagonistic. Compared to paclitaxel alone, PD168393 significantly enhanced paclitaxel-induced DNA fragmentation, sub-G1 fraction accumulation, mitochondrial membrane dysfunction, cytochrome C release, caspase-3 activation and eventually apoptosis. These molecular events were accompanied by Bad up-regulation, p53 and $p21^{Waf1/Cip1}$ induction, ERK1/2 inactivation and inhibition of EGFR phosphorylation in the presence of PD168393. These effects did not involve significant alteration in the Akt1/2 and STAT3 signaling pathway. In conclusion, the combination of paclitaxel and PD168393 produced a profound synergistic growth inhibition of AIPC cells. Combining PD168393 with paclitaxel may have clinical benefits and warrants further investigation.

* *Corresponding author at.* Institute of Biochemistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. Tel.: +886 7 3211101x2242x12; fax: +886 7 3218309.
E-mail address: cliff@cc.kmu.edu.tw (T.-C. Hour).
0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.12.009

1. Introduction

Prostate cancer is the most commonly diagnosed internal malignancy and the second leading cause of cancer death in American men [1]. The incidence and mortality rate of prostate cancer have also increased rapidly in the past decade in Taiwan [2]. Progression from a benign prostate epithelium to an androgen-dependent tumor, and finally to androgen-independent prostate cancer (AIPC) upon androgen deprivation, is a multi-step process involving alterations in expression of proto-oncogenes and tumor suppressor genes [3]. Although the majority of patients with metastatic prostate cancer benefit from androgen deprivation therapy initially, most patients die of AIPC within a few years.

Numerous studies have revealed that epidermal growth factor receptor (EGFR) plays multiple roles in the developing prostate, the mature prostate, androgen-dependent prostate cancer, and AIPC [4–6]. It has been shown that EGFR activation mediates several key processes [6,7]. EGFR signaling can be activated by several mechanisms including overexpression of the mutated receptor, overexpression of its ligands (EGF and others), hetero-dimerization with other members of the erbB receptor family, and transactivation by other receptors [8]. Interestingly, autocrine EGFR activation has been shown to be associated with up-regulating cell motility, invasive potential, and survival in early tumorigenesis [9,10] but does not promote cellular proliferation [11].

The possibility of a link between EGFR signaling and the development of AIPC is still under investigation. However, results from several studies strongly suggest an association between the two. For example, EGFR expression is stronger in androgen-independent tumor cells than in hormone-naïve counterparts [7,12]. A switch from paracrine to autocrine activation may lead to the progression of an androgen-independent state [12–14]. Reports have noted that EGFR signaling may activate the androgen receptor in the absence of androgen [12,15]. Together, these data suggest that EGFR and other growth factors may activate alternative signaling pathways to mediate androgen-independent growth [5]. These results therefore provide strong evidence that suppression of the EGFR signaling pathway may be a promising strategy for AIPC treatment.

Recently, novel therapeutic approaches targeting the EGFR superfamily and their downstream pathways have been generated [5,16,17]. Approaches that show promise include enzyme inhibitors, antibodies, antisense oligonucleotides, and fusion proteins, all of which have been designed to suppress EGFR signaling activity. A major target is to suppress tyrosine kinase activity of the EGFR [5,17]. Three major types of molecules are involved in this effort: pyrrolopyrimides, pyridopyrimidines, and quinazolines [5,17]. These compounds vary in selectivity for members of the EGFR family, their physiochemical properties and their anti-tumor activity, as demonstrated in pre-clinical models. The quinazoline derivatives, ZD1839 (gefitinib or Iressa®, AstraZeneca, Macclesfield, UK) [18], PD168393 [19], CI-1033 (Parke-Davis Division of Warner Lambert Co., Ann Arbor, MI) [20], PKI-166 (Novartis AG, Basel, Switzerland) [21], and OSI-774 (OSI Pharmaceuticals, Uniondale, NY) [22] are promising in terms of clinical development.

The most advanced EGFR chemical inhibitors in development is ZD1839. ZD1839, a synthetic molecule, which targets the EGFR ATP binding site, is a very specific inhibitor of EGFR tyrosine kinase activity [23]. In phase I trials, responses were seen in advanced non-small cell lung cancer (NSCLC), and cutaneous toxicity and diarrhea were the most important side effects [24]. A large phase II study in second and third line has demonstrated a single agent activity of 18.5%. Another large phase II study in patients who received prior platinum and docetaxel obtained a response rate of 11%. There was no difference in response rate between the 250 and the 500 mg/day doses, but side effects were higher in patients who received the 500 mg dose [24]. A very similar small molecule, OSI-774, has also shown activity in this setting. However, recent data have shown that ZD1839 alone exhibits only minimal clinical activity against AIPC [25].

Significant enthusiasm has thus moved towards EGFR inhibitors and their potential to enhance other anti-tumor therapies. In particular, combined treatments involving EGFR inhibitors plus conventional chemotherapy have been tested in vitro [23] and in animal models [26,27], showing significant improvements in anti-tumor effects [28]. However, combinations of ZD1839 and chemotherapeutic agents fail to show additional anti-tumor benefits compared to standard chemotherapy alone, as recently reported for NSCLS [29,30] and AIPC [25]. Recently, docetaxel has been shown to significantly prolong the survival of patients with AIPC, albeit the median life extension was less than 3 months [31]. It is apparent that more effective regimens, involving better combinations and novel agents, are needed. In this study, we investigated the effects of combined treatments, using two EGFR inhibitors and three commonly used chemotherapeutic agents, in a clinical setting in order to identify superior combinations. Molecular events underlying the synergistic cytotoxic effects observed have also been dissected.

2. Materials and methods

2.1. Cell culture

The DU145 cell line was cultured in DMEM medium and PC-3 and LNCaP cell lines were cultured in RPMI-1640 medium. MDA PCa 2b (an androgen-dependent prostate cancer cell line [32], kindly provided by Dr. N.M. Navone from the U.T.M.D. Anderson Cancer Center, Houston, TX) was cultured in BRFF-HPC1 medium (BRFF Brand™ Media Products, Baltimore, MD). LNCaP and MDA PCa 2b represent androgen-responsive and androgen-dependent cells, respectively. DU145 and PC-3 are androgen-independent cells. All media contained 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

2.2. Chemical reagents and antibodies

ZD1839 was kindly provided by AstraZeneca as a sample to be used for research purposes only. PD168393 was purchased from Calbiochem Corporation (San Diego, CA). PD168393 (4-[(3-Bromophenyl) amino]-6-acrylamidoquinazoline) is a spe-

cific and irreversible inhibitor of EGFR tyrosine kinase activity through a specific, covalent modification of the cysteine-733 residue present in the catalytic domain of the ATP binding pocket [19]. Paclitaxel (Bristol-Myers Squibb, Princeton, NJ), docetaxel (Aventis Pharma, Dagenham, England), and 5-fluorouacil (ICN Switzerland AG, Birstfelden, Switzerland) were from clinical preparations. Antibodies against p53, p21, Akt, cytochrome C, EGFR, and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bad monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Mouse antiserum raised against α-tubulin was obtained from Oncogene Research Products (San Diego, CA). Anti-ERK1/2 and anti-phosphorylated ERK1/2 (T202/Y204) antibodies were purchased from R&D Systems Inc. (Minneapolis, MN). Monoclonal antibody against active caspase-3 was purchased from Imgenex (Imgenex Corp., San Diego, CA). Anti-phosphotyrosine monoclonal antibody was obtained from Upstate Biotech (Lake Placid, NY).

2.3. Western blot analysis

Cells (3×10^6) scraped from a 100 mm Petri dish were re-suspended in 100 μl of Gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 100 μM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% sodium dodecyl sulfate, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml leupeptin) on ice for 30 min. The lysate was then centrifuged at 18,000 × *g* for 30 min at 4 °C to collect supernatant for protein concentration determination with BCA Protein Assay Reagent (Pierce Life Science Co., Rockford, IL) and for Western blot analysis. Protein extracts (50 μg) were separated on 10% SDS-polyacrylamide gels and transferred to microporous polyvinylidene difluoride membranes. After blocking with TBST buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) plus 1% bovine serum albumin, membranes were incubated with various primary antibodies at 4 °C for 12 h. Membranes were then washed three times with TBST buffer (20 min each) and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. After three washes with TBST buffer, proteins were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

2.4. Combined treatment of chemotherapeutic agents and EGFR inhibitors

Cellular chemosensitivity was determined by using a modified 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (MTT, Sigma Chemical Co., St. Louis, MO) assay in vitro [33]. In brief, cells in 100 μl culture medium were seeded into 96-well microplates and incubated at 37 °C for 24 h prior to drug exposure. Cell numbers were titrated to keep control cells growing in the exponential phase throughout the 72 h incubation period. For the combined treatment, cells were treated with EGFR inhibitor and chemotherapeutic agent (each in 100 μl of culture medium) simultaneously and incubated for 72 h. At 72 h, 50 μl of MTT (2 mg/ml in RPMI medium) was added to each well and incubated for 2.5 h. Blue formazan crystals that formed were pelleted to the bottom of the well by centrifugation, separated from the supernatant, and dissolved in 150 μl of dimethylsulfoxymide. The optical density at 492 nm was determined by absorbance spectrometry using a microplate reader (MRX-2, Dynex Technologies Inc., Chantilly, VA). Three separate experiments with triplicate data were performed to obtain mean cell viability. Drug concentration that inhibited cell growth by 50% (IC50) was determined by the dose-effect analysis model as previously described [34] and presented as mean ± standard standard error of the means (S.E.M.).

2.5. Median-effect analysis of combined effects

In our experiments, to determine the combined effects of EGFR inhibitor and chemotherapeutic agent, we have to perform three sets of experiments:

(1) Drug-free medium + chemotherapeutic agent → MTT (chemotherapeutic agent alone)
(2) Drug-free medium + EGFR inhibitor → MTT (EGFR inhibitor alone)
(3) EGFR inhibitor + chemotherapeutic agent → MTT (PD168393 + paclitaxel)

We then input the growth inhibition data (MTT data) to compute combination indices for each combined treatment. For each combined treatment, there will be two figures generated: extent of growth inhibition (say 30% growth inhibition or fraction affected of 0.3) from the MTT result and a combination index (say 0.8) from the median-effect analysis. A plot with combination indices against fractions affected can then be made. By combining EGFR inhibitors and chemotherapeutic agents at graded concentrations, numerous combined effects on growth inhibition were determined by median-effect analysis with the mutually non-exclusive model as previously described [35]. The calculation was carried out by using the computer software Calcusyn® (Version 1.1.1, 1996, Biosoft Inc., Cambridge, United Kingdom). Control experiments were performed, where either active agent was replaced with drug-free medium. For a given degree of growth inhibition (known as the fraction affected in the analysis), there is a corresponding combination index (CI) that reflects the combined effect. The combined effect is displayed in fraction affected-combination index plots, where a combination index of <1, $=1$, and >1 indicates synergism, additivism, and antagonism, respectively. Synergism or antagonism of varied degrees may occur in different fraction affected ranges with the same combination.

2.6. Sub-G1 fraction analysis by DNA flow cytometry

The sub-G1 fraction that has nuclear DNA content less than normal represents the cell population that undergoes apoptosis. DU145 cells (3×10^5) were treated with the combination of 10 μM PD168393 and 1 μM paclitaxel for varied time intervals (12, 24, and 48 h) in 6-well plates. Cells were harvested by trypsinization, washed with 1× PBS, re-suspended in 200 μl PBS, and fixed in 800 μl of ice-cold 100% ethanol at −20 °C. After an overnight incubation, cell pellets

were collected by centrifugation, re-suspended in 1 ml of hypotonic buffer (0.1% Triton X-100 and 50 μg/ml RNase A), and incubated at 37 °C for 30 min. One millilitre of propidium iodide solution (50 μg/ml) was added and the mixture was incubated on ice for 30 min. Nuclear DNA content was analyzed with flow cytometry (FACScan, Becton-Dickinson, San Jose, CA).

2.7. Induction of internucleosomal DNA fragmentation

DU145 cells (3×10^5) were cultured in 10 cm Petri dishes with the combination of 10 μM PD168393 and 1 μM paclitaxel. They were harvested after 12 and 24 h of culture, re-suspended in 100 μl lysis buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% sarcosyl, and 0.5 μg/ml proteinase K) and incubated at 50 °C for 3 h. The mixture was incubated with RNase A (500 μg/ml) at 50 °C for 1 h. DNA fragments were extracted with phenol:-chloroform:isoamyl alcohol (25:24:1) and the supernatant was collected by centrifugation for 30 min at 14,000 × *g*. DNA fragments were separated by electrophoresis in 1.8% agarose gels and stained with ethidium bromide.

2.8. Mitochondrial membrane potential by flow cytometry

Relative mitochondrial membrane potential (MMP) was determined using the cationic, lipophilic fluorescent dye DiOC6(3) that specifically accumulates within the mitochondrial compartment in an MMP-dependent manner. Relative MMP was determined by flow cytometric analysis. Briefly, DU145 cells (3×10^5) were treated with 1 μM paclitaxel, with or without 10 μM PD168393, for 0, 18, and 24 h. Next, 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6(3), Molecular Probes, Eugene, OR) was incubated with the cells for 15 min at 37 °C in order to stain the cells. DiOC6(3) anchors onto the inner surface of the mitochondrial membrane. The amount of dye anchorage is positively proportional to the membrane potential. Loss of the mitochondrial membrane potential is associated with a reduction of dye anchorage; reduction of green fluorescence is detectable using flow cytometry with a 525 nm band pass filter. The magnitude of reduction in mitochondrial membrane potential was calculated by [1 − the ratio of mean fluorescence intensity of treated cells over that of control cells] × 100%.

2.9. Caspase-3 activity

After combined treatment with 1 μM paclitaxel and 10 μM PD168393 for 12, 18, 24, and 36 h, cells (3×10^5) were collected, washed with 1× PBS, and re-suspended in 50 μl cell lysis buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin). Caspase-3 activity was determined using Promega's CaspACE Assay System™ kit (Promega, Madison, WI). Cell lysates were centrifuged at 12,000 rpm for 5 min, and aliquots of clear lysate containing 50 μg of protein were incubated with 50 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) as the substrate at 30 °C for 1 h. Upon cleavage by activated caspase-3, the substrate releases a yellow-green fluorescent compound, 7-amino-4-methyl coumarin (AMC) that is detectable with a spectrofluorometer (Hitachi F-4500, Hitachinaka-Shi, Japan), with excitation and emission at 360 and 460 nm, respectively. Fluorescence intensity is proportional to caspase-3 activity. Three separate experiments were performed and the results were expressed as mean ± standard error of the means (S.E.M.) and compared with the Student's *t*-test.

2.10. Data analysis and statistical methods

The above experiments were repeated three times and the results were presented as mean ± standard error of the means (S.E.M.) and compared with the Student's *t*-test.

3. Results

3.1. EGFR expression in prostate cancer cells

EGFR expression was significantly higher in the two androgen-independent cell lines PC-3 and DU145 than in the androgen-dependent cell line MDA PCa 2b or the androgen-responsive cell line LNCaP. This suggests that androgen-independent tumors may depend more on the EGFR signaling pathway. EGFR expression in DU145 was over 20-fold higher than in MDA PCa 2b, as indicated by densitometric scanning (Fig. 1)

3.2. Chemosensitivity of prostate cancer cells to EGFR inhibitors

DU145 cells that have higher EGFR expression were more sensitive to ZD1839 and PD168393 than MDA PCa 2b cells that have lower EGFR expression (Fig. 2A). The ZD1839 IC50s of DU145 and MDA PCa 2b cells were 16.9 and 44.0 μM, respectively, with the latter being 2.6-fold higher than the former (Fig. 2A). The difference between PD168393 IC50s of the two cells was even larger than that between ZD1839 IC50s. The PD168393 IC50 of DU145 was only 13.2 μM. In contrast, PD168393 was minimally toxic to MDA PCa 2b up to 30 μM (Fig. 2B).

3.3. Combined cytotoxic effects by EGFR inhibitors and chemotherapeutic agents

Median-effect analysis revealed that a combined treatment of paclitaxel and PD168393 generated profound synergistic cytotoxic effects in DU145 cells (Fig. 3A). Of particular note, the higher the fraction affected (or degree of growth inhibition), the more profound was the synergism with the two-drug combination. For the androgen-dependent MDA

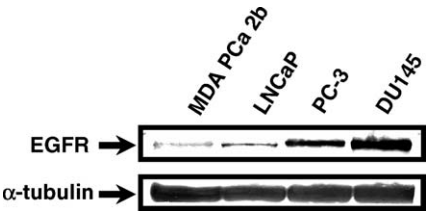

Fig. 1 – EGFR expression in four prostate cancer cell lines by Western blot analysis. MDA PCa 2b and LNCaP are androgen-dependent and androgen-responsive prostate cancer cells. PC-3 and DU145 are androgen-independent cells. α-tubulin served as an internal control.

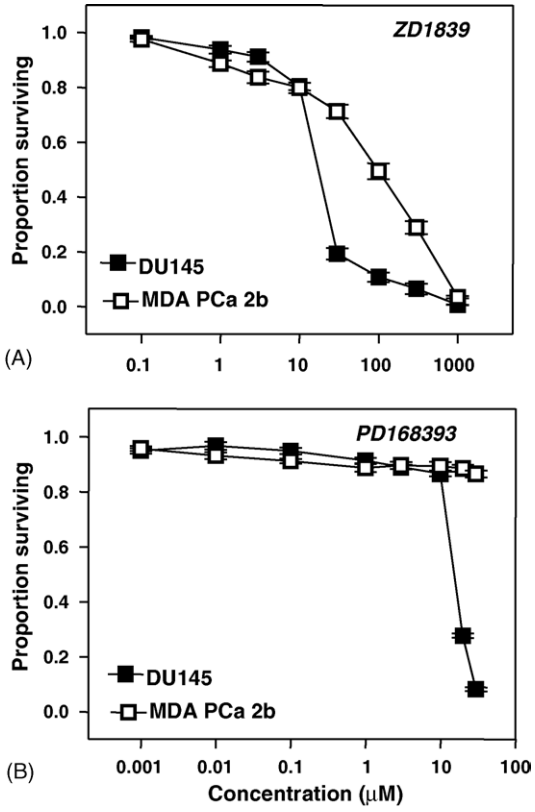

Fig. 2 – Chemosensitivity of prostate cancer cells to EGFR inhibitors as determined by the MTT assay. (A) The ZD1839 IC50s of DU145 and MDA PCa 2b cells were 16.9 and 44.0 μM, respectively. (B) The PD168393 IC50 of DU145 was 13.2 μM. PD168393 was minimally toxic to MDA PCa 2b up to 30 μM.

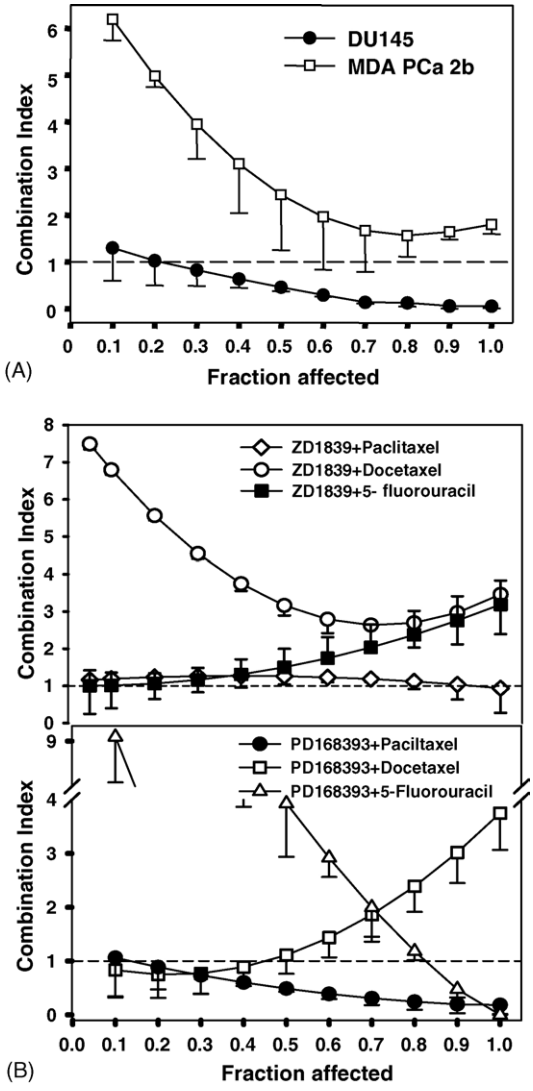

Fig. 3 – Combined effects of EGFR inhibitors and chemotherapeutic agents in prostate cancer cells as determined by median-effect analysis. A combination index of <1, =1, and >1 indicates synergistic, additive, and antagonistic effect, respectively. (A) Evident synergistic effects were seen in the combined treatment of PD168393 and paclitaxel in DU145 cells. Marked antagonism was seen in MDA PCa 2b cells treated with the same combination. (B) The combination of ZD1839 and paclitaxel in DU145 cells was additive in cytotoxicity. Yet, the combination of ZD1839 with docetaxel or with 5-fluorouracil was antagonistic (upper panel). Combining PD168393 with paclitaxel generated strong synergistic cytotoxic effects. The combination effect of docetaxel and 5-fluorouracil was additive, to antagonistic, and grossly antagonistic, respectively (lower panel).

PCa 2b cells, where only minimal EGFR expression was detected, marked antagonism was observed with the same combination. This indicates that PD168393 may potentiate paclitaxel cytotoxicity in AIPC cells. In contrast, EGFR inhibitors do not play a role in the treatment of androgen-dependent prostate cancer if they do not rely on the EGFR pathway for growth or survival.

Additionally, we evaluated other combinations of EGFR inhibitors and chemotherapeutic agents. The combined cytotoxic effects of ZD1839 and paclitaxel in DU145 cells were additive only, according to the median-effect analysis. More so, the combinations of ZD1839 with docetaxel or with 5-fluorouracil were evidently antagonistic (Fig. 3B). The combination of PD168393 with docetaxel was additive and antagonistic at low and high fraction affected ranges, respectively. Although synergism was observed at a small range of very high fraction affected (≥0.9), marked antagonism was observed in the combination of PD168393 and 5-fluorouracil (Fig. 3B).

3.4. *PD168393 sensitizes DU145 cells to paclitaxel-induced apoptosis*

Next, we compared the extent of apoptosis induced by 1 μM paclitaxel alone or in combination with 10 μM PD168393 in DU145 cells. More prominent as well as earlier internucleosomal DNA fragmentation (Fig. 4A) and sub-G1 fraction accumulation (Fig. 4B) were seen with the combined treatment than with paclitaxel alone.

Commitment to apoptosis is largely a mitochondrial event controlled by proteins in the Bcl-2 family [36]. Compared to paclitaxel treatment alone, the pro-apoptotic protein Bad was

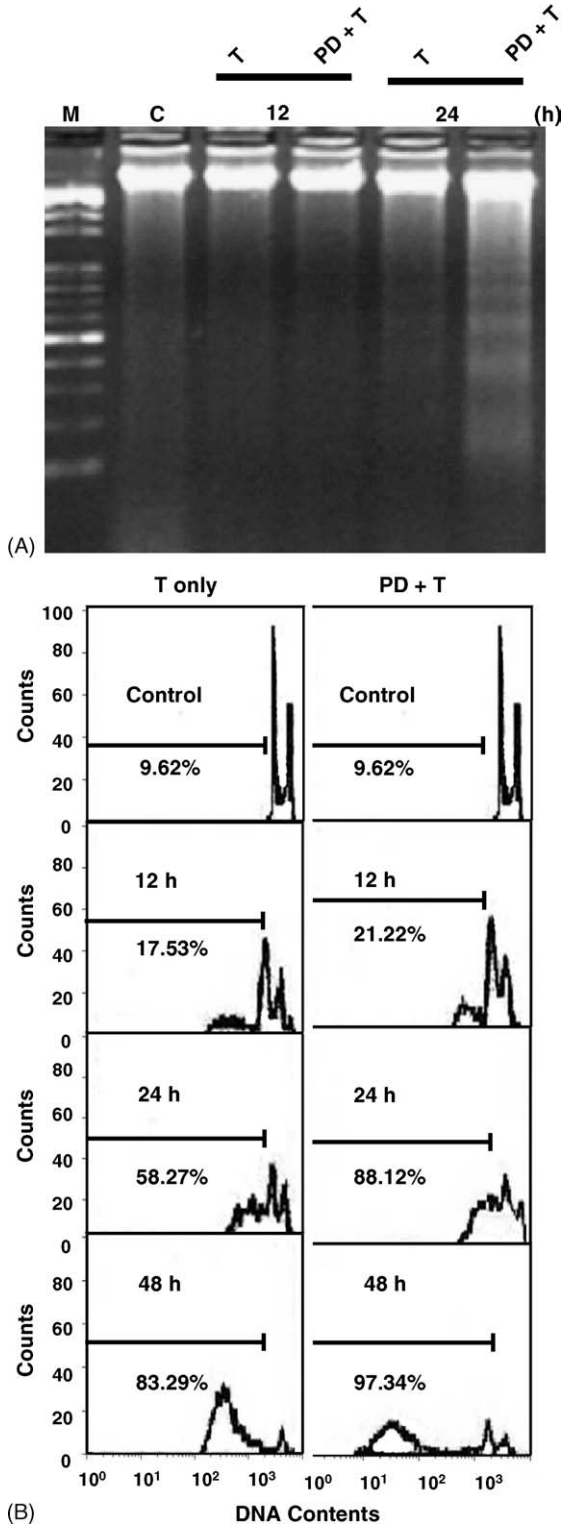

Fig. 4 – Apoptosis of DU145 cells treated with paclitaxel (T) with or without PD168393 (PD). (A) Internucleosomal DNA fragmentation was more evident in cells treated with PD + T than T only at 24 h. M, marker (100 bp increments); C, control without any drug treatment. (B) Sub-G1 fraction analysis by flow cytometry. Higher sub-G1 fractions were seen in cells treated with PD + T than with T only.

significantly up-regulated in cells incubated with the combined treatment (Fig. 5A). Western blots, however, did not detect expression of anti-apoptotic protein Bcl-2 in DU145 cells under identical conditions (data not shown).

To determine whether or not the apoptotic effect induced by paclitaxel alone or in combination with PD168393 was through the mitochondrial signaling pathway, relative MMP was determined. The combined treatment induced a significant MMP reduction after 24 h of continuous treatment compared to paclitaxel alone (Fig. 5B). The reduction was also accompanied by a substantial amount of cytochrome C release into the cytosol as well as caspase-3 activation, as shown by western blot analysis and fluorescence caspase-3 activity assay (Fig. 5C and D). These results indicate that MMP loss, cytochrome C release, and caspase-3 activation are all involved in the paclitaxel-mediated apoptotic pathway in DU145 cells and that the extent of apoptosis can be significantly amplified in the presence of PD168393.

3.5. *Impact on EGFR signaling pathway*

We further characterized the impact of paclitaxel treatment with or without PD168393 on the EGFR signaling pathway in DU145 cells. Western blot analysis showed that the combined treatment significantly blocked EGFR phosphorylation (EGFR-P) in DU145 cells compared to paclitaxel alone (Fig. 6A). Although the level of ERK1/2, a downstream EGFR signaling regulator, remained unchanged in the two treatments, the level of phosphorylated (or activated) ERK1/2 (designated as ERK1/2-P) was significantly abolished in the presence of PD168393 (Fig. 6B).

In addition, we examined the effects of the combined treatment on two EGFR-related survival molecules, STAT3 and Akt. The level of STAT3 remained grossly unchanged between the two treatments. Akt1/2 expression was only slightly reduced in the combined treatment at 36 h compared to paclitaxel alone (Fig. 6C). These data indicate that paclitaxel-induced apoptosis in DU145 cells may not involve the STAT3 or Akt pathways (Fig. 6C).

3.6. *Alteration of p53-dependent pathway*

The p53 tumor suppressor and its signaling pathway play an important role in regulating cell apoptosis. Compared to paclitaxel treatment alone, the combined treatment induced a significant amount of $p21^{Waf1/Cip1}$ expression at 12 h and p53 expression at 18–24 h in DU145 cells. Both $p21^{Waf1/Cip1}$ and p53 expression appeared earlier and was more profound with the combined treatment than with paclitaxel alone, indicating that there was an enhanced paclitaxel-mediated apoptosis in the presence of PD168393 (Fig. 6D).

4. Discussion

We have demonstrated that synergistic anti-tumor activity can be generated by combining paclitaxel and the irreversible EGFR inhibitor PD168393. The synergism may be associated with several facts, including: (a) DU145 cells express moderate levels of EGFR that may be responsible for the observed

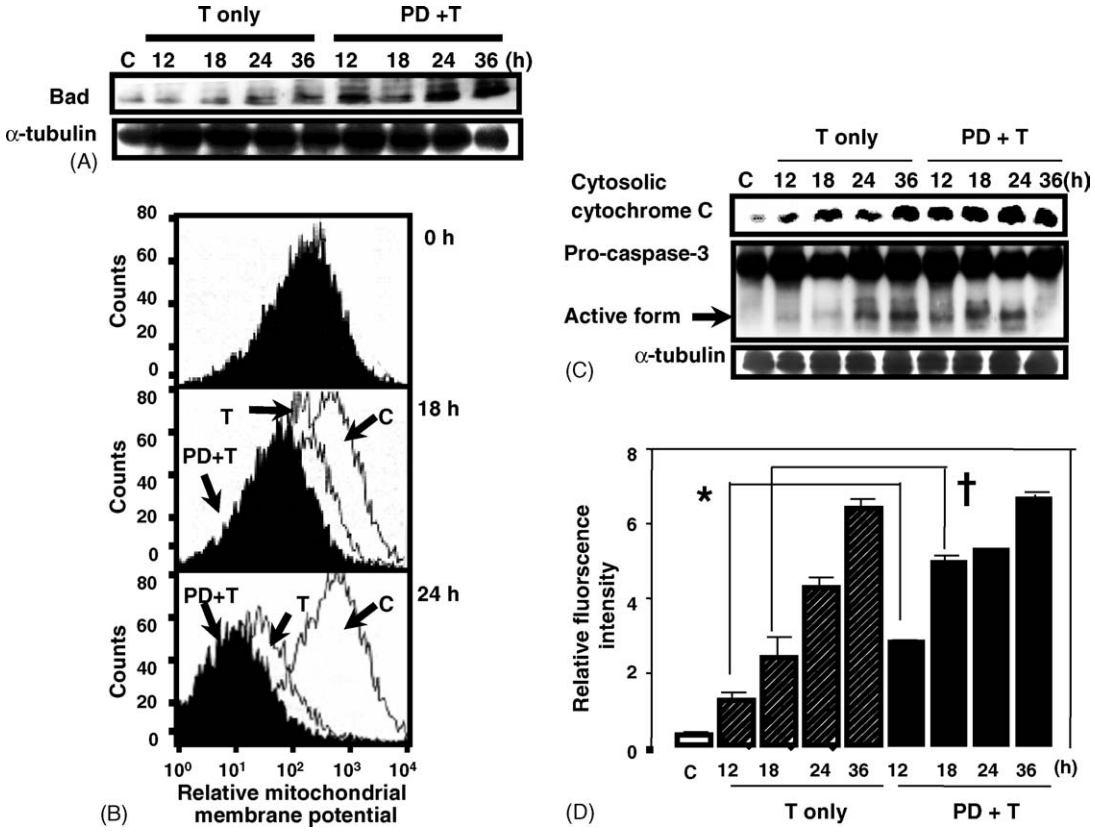

Fig. 5 – Serial apoptotic effects induced by 1 μM paclitaxel (T) with or without 10 μM PD168393 (PD) in DU145 cells. (A) The pro-apoptotic protein, Bad, was significantly up-regulated by PD + T compared to T only treatment in a time-dependent manner. (B) Flow cytometric analysis of relative levels of mitochondrial membrane potential. C, control treatment with drug-free medium. Control and PD + T treatment induced the least and most reduction of the membrane potential in a time-dependent manner. (C) Western blot of cytosolic, cytochrome C, and caspase-3 indicating protein levels in whole cell extracts. PD + T induced greater cytochrome C release and caspase-3 activation than T only. α-tubulin served as a protein loading control. (D) Caspase-3 activity, as shown by relative fluorescence intensity determined by the extent of cleavage of the substrate Ac-DEVD-AMC by activated caspase-3. Caspase-3 activity increased approximately two-fold in cells treated with PD + T compared to T only treatment at 12 (*, $P = 0.001$) and 18 h (†, $P = 0.018$), respectively. Data are presented as mean ± standard error of the means of three separate experiments.

androgen-independent growth and thus may be vulnerable to EGFR targeting therapy and (b) PD168393 significantly suppresses activation or phosphorylation of the EGFR signaling pathway. Disappointing results have been noted in clinical trials that employed either paclitaxel or ZD1839 alone for the treatment of hormone-refractory prostate cancer [25]; however, the new combination of paclitaxel and PD168393 may provide new hope in this and other scenarios.

Numerous reports suggest that EGFR plays a key role in the progression of prostate cancer from androgen-dependent to independent growth. One report cites evidence that expression and activity levels of EGFR are significantly higher in hormone-refractory tumor cells than in hormone-naïve cells [7,12]. Our results showed that both the androgen-independent cells PC-3 and DU145 expressed higher levels of EGFR than the androgen-dependent MDA PCa 2b and androgen-responsive LNCaP cells. The characteristics of EGFR as a promising molecular target for cancer therapy have prompted an extensive drug development effort for inhibiting EGFR signaling [4]. Our data supports the concept that EGFR is an ideal target for treatment. Indeed, DU145 was more sensitive to EGFR inhibitors alone than MDA PCa 2b, and combined treatments of EGFR inhibitors and chemotherapeutic agents generated greater cytotoxicity in DU145 than in MDA PCa 2b cells.

Since EGFR inhibitors, such as ZD1839, by themselves have not to date produced promising clinical results against AIPC, significant interest has now emerged towards increasing anti-tumor effects by using combinations of EGFR inhibitors and other anti-tumor modalities, particularly conventional chemotherapy [23]. Although combinations of EGFR inhibitors and cytotoxic agents were extensively studied in many cancer models [27,37,38], the combined effects have rarely been scrutinized in a mathematical way to better define the interaction between two drugs. Theoretically, combinations that yield synergistic effects are more desirable than additive or antagonistic ones. Synergistic combinations may implicate stronger effects and/or lower toxicity. Currently, only two small-scale phase II trials have been performed, evaluating the combined effects of ZD1839 and cytotoxic agents for the

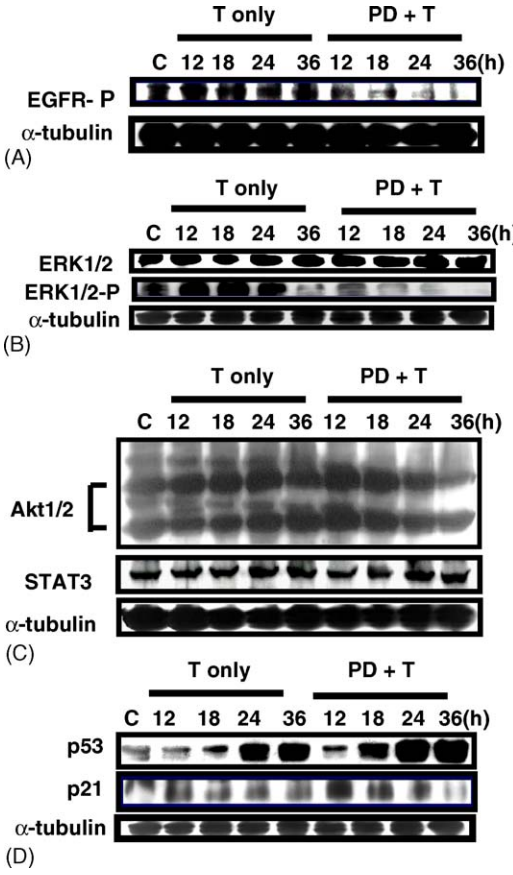

Fig. 6 – EGFR signaling in DU145 cells treated with the combination of paclitaxel (T) and PD168393 (PD) as shown by western blot. (A) T only treatment resulted in minimal alteration of EGFR phosphorylation (EGFR-P), whereas PD significantly reduced EGFR-P at 12 h and beyond, indicating an efficient blockade of EGFR signaling. (B) ERK1/2 levels were similar between T and PD + T treatments. T only treatment induced significant ERK1/2 phosphorylation (ERK1/2-P). However, ERK1/2-P was drastically diminished by PD in a time-dependent manner. (C) Levels of Akt1/2 and STAT3, the EGFR-associated survival proteins. Akt1/2 was only slightly reduced in cells treated with PD + T compared to T only. There was no difference in STAT3 levels between the two treatments. (D) Total p53 and $p21^{Waf1/Cip1}$ expression was induced earlier and was more profound with PD + T than T treatment, indicating enhanced apoptosis by PD + T treatment. α-tubulin served as a loading control.

treatment of AIPC [39,40]. Anti-tumor effects were modest at best and no better than the cytotoxic agents alone, as shown by 24–33% of PSA response (over 50% reduction lasting for ≥4 weeks). The lack of clinical efficacy of ZD1839 in combination with cytotoxic agents may merely reflect an additive or even antagonistic interaction between them. If either agent in a two-drug combination has only minimal activity, an additive or antagonistic combination may only result in disappointing outcomes. The reason why ZD1839 yielded such an outcome remains to be determined. However, its reversible nature [41] and incomplete inhibition of EGFR tyrosine kinase function may be responsible. It has also been shown that maximizing EGFR inhibition by multiple targeting of different EGFR functions may improve therapeutic effects [42]. Similarly, irreversible inhibition and better blockade of EGFR tyrosine kinase activity by PD168393 may explain the more pronounced anti-tumor effects observed in the current study [19].

The combination of PD168393 and chemotherapeutic agents has barely been explored and has never been studied in prostate cancer. Among all combinations tested in the study, only PD168393 and paclitaxel resulted in an evident synergistic combination. The synergism of the combination was so profound and desirable, with the combination index getting smaller (more synergistic) at high fraction affected ranges (0.8–1.0), indicating that the combination generated more profound synergism at maximal growth inhibition. This further implicates that the combination of PD168393 and paclitaxel may generate more synergistic anti-tumor activity at more intensive doses, a result that suggests a highly advantageous application in a clinical setting.

Previous studies have shown that EGFR signaling may go through two major kinase pathways, MEK/ERK and PI3K/Akt [9,10]. Intrinsic activation of these pathways can potentially circumvent EGFR inhibition. We demonstrated that the potentiation of paclitaxel-induced cytotoxicity in DU145 cells by PD168393 may be associated with the evident inhibition of EGFR and MEK/ERK1/2 phosphorylation but may be unrelated to the Akt and STAT3 pathways. Evidence suggests that persistent ERK activation may protect cells from apoptosis induced by EGFR-targeting agents [4]. Our results indicated that phosphorylation or activation of ERK by paclitaxel alone was not inhibited until 36 h. Phosphorylation, however, was obviously diminished by the combination of paclitaxel and PD168393 at 12–18 h, indicating that the paclitaxel-induced survival signaling in DU145 cells can be abolished in the presence of PD168393. If ERK activation cannot be diminished effectively, cancer cells may escape apoptosis. These data were in agreement with previous studies that have shown that ERK activation may be associated with reduced apoptosis in ZD1839-treated prostate cancer cells [23,27].

In conclusion, we have demonstrated that AIPC cells overexpress EGFR and are more vulnerable to EGFR inhibition compared to androgen-dependent cells. Among all combinations of EGFR inhibitors and cytotoxic agents tested, only the combination of paclitaxel and PD168393 produced a profound synergistic growth inhibition in DU145 cells. The enhanced cytotoxicity may result from a more effective inhibition of EGFR and ERK phosphorylation. Paclitaxel-induced apoptosis in DU145 cells was p53- and p21-dependent and involves reductions in mitochondrial membrane potential, cytochrome C release, caspase-3 activation, and internucleosomal DNA breakdown. PD138393 appears to be a promising agent against prostate cancer. Its combination with paclitaxel warrants a more in-depth investigation for use in pre-clinical and clinical settings.

Acknowledgment

This study was supported by NSC 91–2314–B–037–337 from the National Science Council, Executive Yuan, Taiwan, ROC.

REFERENCES

[1] Bostwick DG, Cooner WH, Denis L, Jones GW, Scardino PT, Murphy GP. The association of benign prostatic hyperplasia and cancer of the prostate. Cancer 1992;70:291–301.
[2] Pu YS. Prostate cancer in Taiwan: epidemiology and risk factors. Int J Androl 2000;23(Suppl. 2):34–6.
[3] Bostwick DG. Prostatic intraepithelial neoplasia (PIN): current concepts. J Cell Biochem 1992;16H(Suppl.):10–9.
[4] Barton J, Blackledge G, Wakeling A. Growth factors and their receptors: new targets for prostate cancer therapy. Urology 2001;58:114–22.
[5] Ye D, Mendelsohn J, Fan Z. Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor $p27^{Kip1}$ and costimulate proliferation of MDA PCa 2a and MDA PCa 2b prostate cancer cells. Clin Cancer Res 1999;5:2171–7.
[6] Kim HG, Kassis J, Souto JC, Turner T, Wells A. EGF receptor signaling in prostate morphogenesis and tumorigenesis. Histol Histopathol 1999;14:1175–82.
[7] Gil-Diez DM, Salomon L, Colombel M, Abbou CC, Bellot J, Thiery JP, et al. Modulation of cytokeratin subtype, EGF receptor, and androgen receptor expression during progression of prostate cancer. Hum Pathol 1998;29: 1005–12.
[8] Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. Nat Rev Mol Cell Biol 2001;2:127–37.
[9] Xie H, Turner T, Wang MH, Singh RK, Siegal GP, Wells A. In vitro invasiveness of DU-145 human prostate carcinoma cells is modulated by EGF receptor-mediated signals. Clin Exp Metastas 1995;13:407–19.
[10] Lin J, Adam RM, Santiestevan E, Freeman MR. The phosphatidylinositol 3′-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. Cancer Res 1999;59: 2891–7.
[11] Jenster G. Ligand-independent activation of the androgen receptor in prostate cancer by growth factors and cytokines. J Pathol 2000;191:227–8.
[12] Kumar VL, Majumder PK, Gujral S, Kumar V. Comparative analysis of epidermal growth factor receptor mRNA levels in normal, benign hyperplastic and carcinomatous prostate. Cancer Lett 1998;134:177–80.
[13] De Bellis A, Ghiandi P, Comerci A, Fiorelli G, Grappone C, Milani S, et al. Epidermal growth factor, epidermal growth factor receptor, and transforming growth factor-alpha in human hyperplastic prostate tissue: expression and cellular localization. J Clin Endocrinol Metab 1996;81: 4148–54.
[14] De Miguel P, Royuela. Bethencourt R, Ruiz A, Fraile B, Paniagua R. Immunohistochemical comparative analysis of transforming growth factor alpha, epidermal growth factor, and epidermal growth factor receptor in normal, hyperplastic and neoplastic human prostates. Cytokine 1999;11:722–7.
[15] Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474–8.
[16] Ciardiello F, Damiano V, Bianco R, Bianco C, Fontanini G, De Laurentiis M, et al. Anti-tumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. J Natl Cancer Inst 1996;88:1770–6.
[17] Ciardiello F. Epidermal growth factor receptor tyrosine kinase inhibitors as anticancer agents. Drugs 2000;60(Suppl. 1):25–32.
[18] Barker AJ, Gibson KH, Grundy W, Godfrey AA, Barlow JJ, Healy MP, et al. Studies leading to the identification of ZD1839 (Iressa): an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor targeted to the treatment of cancer. Bioorg Med Chem Lett 2001;11:1911–4.
[19] Fry DW, Bridges AJ, Denny WA, Doherty A, Greis KD, Hicks JL, et al. Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. Proc Natl Acad Sci USA 1998;95:12022–7.
[20] Smaill JB, Showalter HD, Zhou H, Bridges AJ, McNamara DJ, Fry DW, et al. Tyrosine kinase inhibitors. 18. 6-Substituted 4-anilinoquinazolines and 4-anilinopyrido[3,4-d]pyrimidines as soluble, irreversible inhibitors of the epidermal growth factor receptor. J Med Chem 2001;44:429–40.
[21] 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.
[22] Hidalgo M, Siu LL, Nemunaitis J, Rizzo J, Hammond LA, Takimoto C, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. J Clin Oncol 2001;19:3267–79.
[23] Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, et al. Anti-tumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin Cancer Res 2000;6:2053–63.
[24] Janmaat ML, Giaccone G. The epidermal growth factor receptor pathway and its inhibition as anticancer therapy. Drugs Today (Barc) 2003;39(Suppl. C):61–80.
[25] Canil CM, Moore MJ, Winquist E, Baetz T, Pollak M, Chi KN, et al. Randomized phase II study of two doses of gefitinib in hormone-refractory prostate cancer: a trial of the National Cancer Institute of Canada–Clinical Trials Group. J Clin Oncol 2005;23:455–60.
[26] Kim SJ, Uehara H, Karashima T, Shepherd DL, Killion JJ, Fidler IJ. Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. Clin Cancer Res 2003;9:1200–10.
[27] Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin Cancer Res 2000;6:4885–92.
[28] Mendelsohn J, Fan Z. Epidermal growth factor receptor family and chemosensitization. J Natl Cancer Inst 1997;89:341–3.
[29] Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial–INTACT 2. J Clin Oncol 2004;22:785–94.
[30] Normanno N. Gefitinib and cisplatin-based chemotherapy in non-small-cell lung cancer: simply a bad combination? J Clin Oncol 2005;23:928–30.
[31] Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502–12.
[32] Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, et al. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 1997;3:2493–500.
[33] Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated

colorimetric assay: assessment of chemosensitivity testing. Cancer Res 1987;47:936–42.
[34] Chou TC. Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. J Theor Biol 1976;59:253–76.
[35] Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22: 27–55.
[36] Bruckheimer EM, Cho S, Brisbay S, Johnson DJ, Gingrich JR, Greenberg N, et al. The impact of bcl-2 expression and bax deficiency on prostate homeostasis in vivo. Oncogene 2000;19:2404–12.
[37] Magne N, Fischel JL, Tiffon C, Formento P, Dubreuil A, Renee N, et al. Molecular mechanisms underlying the interaction between ZD1839 (Iressa) and cisplatin/5-fluorouracil. Br J Cancer 2003;89:585–92.
[38] Vicentini C, Festuccia C, Gravina GL, Angelucci A, Marronaro A, Bologna M. Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in vitro on human cell lines and primary cultures. J Cancer Res Clin Oncol 2003;129:165–74.
[39] Miller K, Rabbe N, Trachtenberg J, Trump D, Wilding G, Das-Gupta A. ZD1839 an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), is well tolerated in combination with mitoxantrone and prednisone in patients with hormone-refractory prostate cancer (HRPC). Ann Oncol 2002;13(Suppl. 5):91.
[40] Soulié P, Trump D, Wilding G, Small E, Das-Gupta A. ZD1839 an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in combination with docetaxel and estramustine in patients with hormone-refractory prostate cancer (HRPC). Ann Oncol 2002;13(Suppl. 5):91.
[41] Roskoski Jr R. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004;319:1–11.
[42] Huang S, Armstrong EA, Benavente S, Chinnaiyan P, Harari PM. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. Cancer Res 2004;64:5355–62.