Combined targeting of epidermal growth factor receptor and hedgehog signaling by gefitinib and cyclopamine cooperatively improves the cytotoxic effects of docetaxel on metastatic prostate cancer cells

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Abstract
The epidermal growth factor receptor (EGFR) and hedgehog cascades provide a critical role in prostate cancer progression and contribute to the resistance to clinical therapies and disease relapse. Therefore, we evaluated, for the first time, the antiproliferative and cytotoxic effects induced by a combination of selective inhibitors of EGFR tyrosine kinase and smoothened hedgehog signaling element, gefitinib and cyclopamine, with a current chemotherapeutic drug used in the clinics, docetaxel, on some metastatic prostate cancer cell lines. Immunohistochemical analyses revealed that sonic hedgehog (SHH) expression was enhanced in 39% of primary prostatic adenocarcinomas (Gleason scores 4–10) compared with the corresponding normal tissues of the same prostate gland from 32 prostate cancer patients. The confocal microscopy and Western blot analyses have also indicated the high expression levels of SHH and EGFR in metastatic LNCaP, DU145, and PC3 cells. Moreover, the results revealed that the drugs, alone or in combination, at lower concentrations inhibited the growth of EGF plus SHH–stimulated and serum-stimulated androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81, DU145, and PC3 cells. Importantly, the combined docetaxel, gefitinib, and cyclopamine also caused a higher rate of apoptotic death of prostate cancer cells compared with individual agents. The cytotoxic effects induced by these drugs in PC3 cells seem to be mediated in part through the cellular ceramide production and activation of caspase cascades via a mitochondrial pathway and the release of cytochrome c into the cytosol. Additionally, the combined agents were more effective at suppressing the invasiveness of PC3 cells through Matrigel in vitro than the single drugs. These findings indicate that the combined use of inhibitors of EGF-EGFR and hedgehog signaling with docetaxel could represent a more promising strategy for treatment in patients with metastatic and androgen-independent prostate cancer. [Mol Cancer Ther 2007;6(3):967–78]

Introduction
Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths in men (1–3). Currently, chemical and surgical androgen ablation is the most effective therapy available for prostate cancer. Although the treatment of prostate cancers by radical prostatectomy and antiandrogen therapies has a high responsive rate in patients diagnosed with localized and androgen-dependent prostate cancers, the progression within 12 to 24 months to hormone-refractory prostate cancers (HRPC) and/or metastatic states is associated with disease relapse and poor patient survival (1–7). In this matter, several lines of evidence indicated that the persistence of androgen receptor–negative prostate cancer progenitor cells, also designated as prostate cancer stem cells, in primary and secondary neoplasms, which might be resistant to antihormonal therapy, may contribute at least in part to disease relapse (2, 8–13). More specifically, the activation of numerous autocrine and paracrine loops induced by distinct growth factor cascades, including epidermal growth factor receptor (EGFR) and hedgehog pathways, in androgen-independent prostate cancer cells may notably contribute to their sustained survival and growth in the absence or presence of low androgen levels (2, 11). Thus, nonhormonal systemic chemotherapy represents another option for patients with nonlocalized HRPC. The standard chemotherapeutic agents for patients with HRPC and clinical evidence of metastases include a combination of either mitoxantrone and prednisone or docetaxel and prednisone or estramustine (2, 4–7, 14). These drug combinations have been reported to improve the quality of life for patients, offering pain relief. These current chemotherapeutic treatments for HRPCs and/or
metastatic prostate cancer disease states, however, showed little survival benefits and are only palliative with a median survival rate of about 12 to 19 months after diagnosis and start of treatment (2, 4–7). This is associated with the development of mechanisms of resistance by metastatic and androgen-independent prostate cancer cells to current mitoxantrone- or docetaxel-based chemotherapeutic therapies. Therefore, these facts underline the importance of additional trials for optimizing the regimen options of conventional chemotherapeutic treatments against aggressive and metastatic HRPCs. Furthermore, numerous studies have indicated that blockade of the EGFR pathway by the anti-EGFR antibody or EGFR tyrosine kinase inhibitor resulted in cell cycle arrest in the G1 phase, an inhibition of invasion, and/or induced apoptosis in metastatic prostate cancer cells in vitro and in vivo (2, 15–23). Similarly, the inhibition of the hedgehog cascade, by using either smoothened signaling element inhibitor, cyclopamine alkaloid, or the anti–sonic hedgehog (SHH) antibody, has also resulted in an inhibition of the growth and invasion of metastatic prostate cancer cells in vitro and in vivo, whereas the normal prostate epithelial cells were insensitive to the cytotoxic effects of these agents (2, 11, 22, 24). Additionally, the stimulation of ceramide- and caspase-induced apoptotic cascades has also been associated with the cytotoxic effects induced by androgen ablation and diverse chemotherapeutic drugs, including docetaxel, paclitaxel, and etoposide, on metastatic androgen-responsive androgen-independent prostate cancer cells (25–27). Moreover, it has also been reported that the induction of de novo synthesis of the sphingolipid ceramide through the activation of enzyme ceramide synthase by diverse agents, such as activator of protein kinase Cα, 12-O-tetradecanoylphorbol 13-acetate, and anandamide, or the inhibition of acid ceramidase by using ceramide analogue, B13, induces apoptosis in metastatic prostate cancer cells in vitro and enhances their sensitivity to radiation-induced apoptosis in vivo (28–30). Of therapeutic interest, our recent works have also indicated that the use of lower doses of selective EGFR inhibitor, gefitinib or PD153035, alone or in combination with other cytotoxic agents, acting as activators of caspase and/or ceramide accumulation, including cyclopamine, protein kinase A inhibitor (Rp-cAMPs), sodium nitroprusside, tamoxifen, and/or etoposide, induced a growth arrest and apoptosis of diverse metastatic prostate cancer cells more effectively than the individual drugs (2, 19, 20, 22, 31).

Based on these observations, it seems that the inclusion of selective inhibitors of distinct oncogenic signaling pathways, including EGF-EGFR and hedgehog cascades, which can induce an activation of ceramide- and/or caspase-induced apoptotic death in metastatic and androgen-independent prostate cancer cells, may represent a novel therapeutic approach for improving the current clinical treatments of metastatic HRPCs. In the present investigation, we hypothesized that the simultaneous blockade of EGFR and hedgehog pathways by using gefitinib and cyclopamine might improve the cytotoxic effects induced by docetaxel on metastatic and androgen-sensitive and androgen-independent prostate cancer cells. Therefore, the antiproliferative, anti-invasive, and apoptotic effects induced by these cytotoxic agents, alone or in combination, were determined on the metastatic and androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81, DU145, and PC3 prostate cancer lines stimulated by EGF plus SHHNp or serum. Additionally, the molecular mechanisms involved in the cytotoxic effects of drugs were studied in highly metastatic PC3 cells.

Materials and Methods

Materials

The human PZ-HPV-7 nonmalignant prostate epithelial cells and LNCaP-FGC (LNCaP), DU145, and PC3 prostate cancer cell lines were originally purchased from the American Type Culture Collection (Manassas, VA), whereas androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81 cells were developed at the University of Nebraska Medical Center (Omaha, NE; ref. 32). The prostate cancer cells were maintained routinely in RPMI 1640 culture medium containing 10% fetal bovine serum, 26 mmol/L NaH2CO3 (pH 7.4), 1% L-glutamine, and antibiotics (100 IU/mL penicillin-100 µg/mL streptomycin) in a 37°C incubator supplied with 5% CO2. PZ-HPV-7 cells were cultured in keratinocyte serum-free medium supplemented with 1-glutamine, bovine pituitary extract, and EGF according to the instructions of the American Type Culture Collection. RPMI 1640 and all other culture materials were from Life Technologies (Carlsbad, CA). Docetaxel, fumonisin B1, catalase, 3,3-dihexyloxacarbocyanine iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and EGF were purchased from Sigma-Aldrich (St. Louis, MO), and broad caspase inhibitor Z-VAD-fmk was from Calbiochem Corp (San Diego, CA). The NH2-terminal peptide of recombinant human SHH (SHHNp) was obtained from R&D Systems (Minneapolis, MN). The gefitinib was synthesized according to a modification of a described procedure (33). The rabbit polyclonal anti-SHH antibody (H-160), rabbit polyclonal anti-EGFR antibody (1005), and goat polyclonal p-Tyr1173, EGF were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The amounts of proteins were estimated by using a detergent-compatible protein assay kit from Bio-Rad Laboratories, Inc. (Hercules, CA). The Vectastain avidin-biotin complex method peroxidase kit and 3,3'-diaminobenzidine substrate kit were purchased from Vector Laboratories (Burlington, CA).

Immunohistochemistry

Immunohistochemical studies on the localization of SHH in normal and malignant prostate tissues were done as described previously (31). Briefly, the immunostaining was carried out on 32 pairs of AccuMax array tissue sections (Petagen, Inc., Shinchon-dong, Seoul, Korea) from patients with primary prostatic adenocarcinoma (Gleason scores 4–10) with their corresponding normal adjacent tissues from the same patients. Sections were deparaffinized with...
EZ-DeWax (BioGenex, San Ramon, CA) and rehydrated using graded ethanol solutions. After washing the slides thrice with PBS for 5 min, tissue sections were submerged in microwave antigen retrieval solution consisting of 0.01 mol/L citrate buffer (pH 6.0) and subjected to microwave irradiation thrice for 3 min. The nonspecific immunostaining was blocked using diluted Vectastain normal horse serum (Vector avidin-biotin complex method kit) for 10 min, and the slides were then incubated with a 1:50 dilution of primary anti-SHH antibody in a humidified chamber for 1 h at room temperature. After washing with PBS, the slides were incubated with biotinylated universal secondary antibody for 30 min and rewarshed with PBS. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol/PBS (1:1) for 10 min. After an additional wash, the slides were incubated with avidin-biotin complex method Vectastain solution for 30 min. The tissue sections were submerged in a staining solution containing 3,3′-diaminobenzidine substrate kit substrate as indicated in the manufacturer’s instructions and rinsed thrice in water. A reddish brown color precipitate observed on tissue sections indicates a positive immunoreactivity with the tested primary antibody. The slides were counterstained with hematoxylin, dehydrated, and permanently mounted with VectaMount permanent mounting medium (Vector Laboratories). Images, which were captured on a Nikon Eclipse E400 microscope (Nikon Corp., Tokyo, Japan) at different magnifications, are representative of analyzed samples. For each tissue section, the intensity of immunoreactivity for SHH was semiquantitatively graded by a urologic pathologist (S.L.J.) on a 0 to +3 scale (0 = no staining, 1+ = week staining, 2+ = moderately strong, and 3+ = strong staining). The staining intensity of SHH in prostate adenocarcinoma samples was scored and compared with the corresponding nonneoplastic prostate tissues, and the value was considered enhanced if the staining intensity was higher by one or more points.

Confocal Microscopy

The prostate cancer cells were grown at a low density on sterilized coverslips for 24 h in the absence or presence of tested agents, washed with PBS buffer, and fixed in ice-cold methanol at −20°C for 2 min. The cells were blocked in 10% goat serum for 30 min and incubated with rabbit polyclonal anti-SHH antibody or rabbit polyclonal anti-EGFR antibody plus goat polyclonal p-Tyr1173-EGFR antibody diluted in PBS for 1 h at room temperature. Cells were washed with PBS and then incubated with FITC-conjugated donkey anti-goat and/or Texas red–conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Cells were washed again and mounted on glass slides in antifade Vectashield mounting medium (Vector Laboratories). Immunofluorescence staining was observed under a confocal laser scanning microscope (LSM 410, Zeiss, Göttingen, Germany).

Western Blot Analyses

Prostate cancer cells were maintained in medium containing 10% fetal bovine serum for 48 h, and the conditioned medium (supernatants) was harvested and cells were extracted in lysis buffer as described previously (22, 31). The expression levels of SHH precursor and its NH2-terminal product SHH-N were estimated by Western blot in conditioned medium, and lystate samples prepared from prostate cancer cells by using rabbit polyclonal anti-SHH antibody. Moreover, the expression levels of β-actin were estimated in cell extracts using mouse monoclonal anti-β-actin antibody.

Cell Culture and Growth Assays

All prostate cancer cells were maintained in RPMI 1640 culture medium as mentioned previously (19, 20, 22, 28, 31). For growth assays, the cells were seeded on 96-well plates at a density of approximately 3 × 10^4 to 5 × 10^5 per well in a total volume of 200 μL of medium containing 10% fetal bovine serum. After 2 days, the cell culture medium was changed to serum-free medium and the cells were starved for 24 h for the synchronization. The cell growth assays were then done in the serum-free medium containing 10 ng/mL EGF plus 40 nmol/L SHH-Np or 10% fetal bovine serum. Different concentrations of docetaxel, gefitinib, and cyclopamine, alone or in combination, were also added to culture medium. After incubation for 48 h, the rate of cell growth was estimated by a colorimetric test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (34).

Flow Cytofluorometric Analyses

All prostate cancer cells were grown at a density of about 5 × 10^4 on 25 cm² dishes as described previously. The EGF plus SHHNp–stimulated cells were treated with different concentrations of docetaxel, gefitinib, and cyclopamine, alone or in combination, in the absence or presence of Z-VAD-fmk, catalase, or fumonisin B1 for 4 days. In all experiments, the cells were kept at subconfluent to avoid contact inhibition. Moreover, to determine the influence of drugs on the cellular cycle progression of EGF plus SHHNp–stimulated PC3 cells, the cytometric analyses by fluorescence-activated cell sorting (FACS) were done 48 h after the addition of different concentrations of docetaxel, gefitinib, and cyclopamine, alone or in combination. The DNA content analysis of each sample was done by FACS analyses essentially as described previously (19, 20, 22, 28, 31). In addition, the percentage of cell death was also estimated by the amount of trypan blue–stained cells.

Estimation of Mitochondrial Transmembrane Potential and Cytosolic Cytochrome c Release

To determine whether the apoptotic effect induced by docetaxel, gefitinib, and cyclopamine, alone or in combination, in PC3 cells is mediated via a mitochondrial pathway, the mitochondrial membrane potential (MMP) and the amount of cytosolic cytochrome c were estimated as described previously (19, 20, 22, 28, 31). Briefly, PC3 cells were untreated (control) or treated with 2 nmol/L docetaxel, 1 μmol/L gefitinib, and 2 μmol/L cyclopamine, alone or in combination, in the absence and presence of irreversible and broad caspase cascade inhibitor, Z-VAD-fmk, for 4 days. MMP measurements were done by FACS analyses with the cationic, lipophilic, and fluorescent dye...
3,3′-dihexyloxacarbocyanine iodide, which specifically accumulates within the mitochondrial compartment in a MMP-dependent manner (35). Moreover, the amounts of cytochrome c present in the cytosolic extracts were estimated following the method described in the ELISA kit from Zymed Laboratories Inc. (San Francisco, CA) with a human anti–cytochrome c antibody.

**In vitro Invasion Assays**

The invasive potential of prostate cancer cells was estimated by their ability to penetrate a Matrigel invasion chamber with a Falcon cell culture insert 8-μm pore size polyethylene terephthalate membrane, with a thin layer of Matrigel matrix acting as a basement membrane in vivo, and allowing us an estimate of the metastatic potential of tumor cells in vitro as described previously (22). Briefly, PC3 cells were untreated (control) or pretreated with tested drugs, alone or in combination, for 24 h and during cell invasion assay for an additional 24 h. For each experiment, serum-free growth medium supplemented with EGF plus SHH-Np was added to the bottom well and 5 × 10⁴ PC3 cells per well in the medium without drugs (control) or containing the drugs were loaded into the top of the BD BioCoat Matrigel cell invasion chamber or control insert chamber without Matrigel according to the manufacturer’s instructions from Becton Dickinson Labware (San Jose, CA). After incubation for 24 h at 37°C, the invasive cells reaching the lower chamber were stained with a Diff-Quick stain set from Dade Behring, Inc. (Deerfield, IL), and counted using a hemocytometer by phase-contrast microscopy. The percentage of cell invasion was calculated by dividing the mean of cell numbers invading through the Matrigel insert membrane by the mean of cell numbers migrating through the control insert chamber.

**Statistical Analysis**

Statistical analyses were done using the Student’s *t* test to compare the results, with *P* values of <0.05 indicating statistically significant differences.

**Results**

**Immunohistochemical, Confocal Microscopy, and Western Blot Analyses of SHH and EGFR/p-Tyr1173** - EGFR Expression Levels in Normal and Malignant Prostate Tissues and Different Prostate Cancer Cell Lines

The expression levels of SHH were examined by immunohistochemical stains on normal and malignant human prostate tissues (Fig. 1). In nonmalignant prostate tissues, SHH protein expression was variable in basal cells as indicated by faint cytoplasmic staining, whereas very weak immunostaining was detected in the luminal epithelial cells. In contrast, SHH immunostaining varied from moderate to strong within the cytoplasm and near the cell surface in malignant epithelial cells localized in the intermediate and luminal compartments in a subset of primary adenocarcinomas as shown in Fig. 1. The staining intensity associated with the SHH protein expression was enhanced in 39% of 32 primary adenocarcinoma samples compared with the corresponding nonneoplastic tissues.

In addition, the higher expression levels of SHH as well as EGFR and its phosphorylated and activated form, p-Tyr1173, EGFR, were also detected in cytoplasm and at the cell surface by confocal microscopy in metastatic and androgen-independent LNCaP-C81, DU145, and PC3 cells than in androgen-responsive LNCaP-C33 cells (Fig. 2A). Furthermore, Western blot evaluation of the expression levels of SHH was also done in lysates and/or conditioned medium (supernatant) samples prepared from prostate cancer cell lines maintained in 10% fetal bovine serum. As shown in Fig. 2B, greater expression levels of SHH precursor protein and its NH₂-terminal autocleavage product (SHH-N; ~20 kDa) were detected in lysate samples prepared from androgen-independent LNCaP-C81, DU145, and PC3 cells relative to LNCaP-C33 cells and nonmalignant PZ-HPV-7 prostate epithelial cells. Furthermore, an amount of secreted NH₂-terminal fragment SHH-N produced by prostate cancer cells was also detected in conditioned medium samples (Fig. 2B).

**Antiproliferative Effect Induced by Docetaxel, Gefitinib, and Cyclopamine in the Different Prostate Cancer Cells**

To evaluate the antiproliferative effect of drugs on metastatic prostate cancer cells, the growth-inhibitory effects of drugs, alone or in combination, were evaluated on the EGF plus SHH-Np–stimulated and serum-stimulated prostate cancer cells. In preliminary experiments, we established the concentration-response curves for each drug to determine the lowest concentration that can induce a significant antiproliferative effect on the growth of EGF plus SHH-Np–stimulated or serum-stimulated prostate cancer cells. The concentrations of docetaxel, gefitinib, or cyclopamine, which produced about 15% to 20% inhibition, were used in experiments in a combination study with other drugs. As shown in Fig. 3A, docetaxel, gefitinib, or cyclopamine inhibited the growth of EGF plus SHH-Np–stimulated androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81, DU145, and PC3 cells. Moreover, the bicomination of 2 nmol/L docetaxel plus 0.1 μmol/L gefitinib or 1 μmol/L cyclopamine induced a supra-additive growth-inhibitory effect on the EGF plus SHH-Np–stimulated prostate cancer cells. Similarly, EGF plus SHH-Np–induced prostate cancer cell growth was also inhibited more markedly by combined docetaxel, gefitinib, and cyclopamine exposure compared with a bicomination of drugs (Fig. 3A). As shown in Fig. 4, the inhibition of EGF plus SHH-Np–stimulated PC3 cell growth induced by the 1 or 2 nmol/L docetaxel after 2 days was accompanied by an increase in the cell population in the G2 phase of the cell cycle. In contrast, 0.1 μmol/L gefitinib or 1 μmol/L cyclopamine induced a blockade of the PC3 cells in the G1 phase of the cell cycle concomitant with a reduction of PC3 cell populations in the S and G2-M phases (Fig. 4). Of particular interest, the combined use of 2 nmol/L docetaxel, 0.1 μmol/L gefitinib, plus 1 μmol/L cyclopamine caused an arrest of the cell growth via a blockade in the G1 and G2 phases of the cell cycle. In addition, the combination of 2 nmol/L docetaxel, 5 μmol/L gefitinib, and 2 μmol/L
cyclopamine also induced a greater antiproliferative effect on serum-stimulated prostate cancer cells compared with individual agents and the inhibitory effect induced by the three drugs was also higher compared with the bicombination of these agents at the same concentration (Fig. 3B).

**Apoptotic Effect Induced by Docetaxel, Gefitinib, and Cyclopamine in the Different Prostate Cancer Cells**

The flow cytometric analyses were made to determine the percentage of apoptotic cell death induced by drugs, alone or in combination, and the number of apoptotic cells in the sub-G1 phase was quantified. As shown in Fig. 5, the treatment of the EGF plus SHHNP-stimulated prostate cancer cells for 4 days with different drug concentrations caused an increase in the apoptotic cell population compared with untreated cells (control). In particular, 2 nmol/L docetaxel, 1 μmol/L gefitinib, or 2 μmol/L cyclopamine induced a significant rate of apoptotic death in EGF plus SHHNP-stimulated LNCaP-C33, LNCaP-C81, DU145, and PC3 cells. In addition, the bicombination of docetaxel, gefitinib, and cyclopamine was more cytotoxic in all tested prostate cancer cells than agents alone, and the combination of the three drugs caused the death of the majority of cells. Additional evidence that these cytotoxic agents induce apoptosis in PC3 cells is also supported by the increase in DNA fragmentation observed in the presence of these drugs compared with untreated cells (data not shown). Comparable results were also obtained from the trypan blue exclusion analyses of PC3 cell viability after 4 days (Table 1).

**Estimation of the Role of the Caspase and Ceramide Pathways in the Apoptotic Effect Induced by Docetaxel, Gefitinib, and Cyclopamine in PC3 Cells**

To assess whether the molecular mechanisms whereby drugs induce cell death involve the caspase and proapoptotic lipid ceramide pathways, the effect of the drug treatment on MMP, cytosolic cytochrome c release, and apoptotic death was estimated in the absence and presence...
of different inhibitors of caspase activation and ceramide metabolism on EGF plus SHH–stimulated PC3 cells. As shown in Fig. 6A and B, the continuous treatment of PC3 cells for 4 d with 2 nmol/L docetaxel, 1 μmol/L gefitinib, or 2 μmol/L cyclopamine alone caused only a slight decrease of MMP as indicated by the weak shoulder of the peak compared with the stained PC3 cells untreated (control). On the other hand, the treatment of PC3 cells with 5 nmol/L docetaxel or 2 nmol/L docetaxel plus 1 μmol/L gefitinib or 2 μmol/L cyclopamine was accompanied by a marked decrease of MMP. Furthermore, the addition of 2 nmol/L docetaxel, 1 μmol/L gefitinib, plus 2 μmol/L cyclopamine induced a higher mitochondrial membrane–depolarizing effect and cytochrome c amount released in the cytosol compared with the treatment of PC3 cells with individual drugs and bicomination treatment. The broad-spectrum caspase inhibitor Z-VAD-fmk at 50 μmol/L also abrogates the mitochondrial transmembrane–depolarizing effect and apoptotic death induced by these agents in PC3 cells (Fig. 6; Table 1). In addition, the results indicated that the inhibition of acid ceramidase, whose enzyme is involved in ceramide degradation and overexpressed in prostate cancers (36), by using 10 μmol/L N-oleoyl ethanolamine, enhanced the apoptotic effect induced by 2 nmol/L docetaxel on PC3 cells, whereas N-oleoyl ethanolamine alone had no significant cytotoxic effect at this concentration (Table 1). Moreover, the inhibition of cellular ceramide production from de novo synthesis or acidic sphingomyelinase pathways by using a specific ceramidase synthase inhibitor, fumonisin B1, or aSMase inhibitor, desipramine, also attenuated the rate of apoptotic death induced by 2 nmol/L docetaxel, 1 μmol/L gefitinib, plus 2 μmol/L cyclopamine in PC3 cells (Table 1). A significant decrease of the apoptotic death of PC3 cells induced by this triple combination of drugs was also observed by using a scavenger of hydrogen peroxide (H2O2) catalase, which inhibits H2O2 accumulation.

Estimation of the Inhibitory Effect Induced by Docetaxel, Gefitinib, and Cyclopamine on the Invasive Ability of PC3 Cells

To estimate the inhibitory effect of the docetaxel, gefitinib, and cyclopamine, alone or in combination, on the invasiveness of the EGF plus SHHNp–stimulated prostate cancer cells, the in vitro invasive potential of untreated or drug-treated PC3 cells was evaluated by their ability to penetrate a Matrigel invasion chamber. As shown in Fig. 7, the treatment of PC3 cells with 2 nmol/L docetaxel, 0.1 μmol/L gefitinib, or 1 μmol/L cyclopamine significantly inhibited their invasion ability compared with untreated cells (control). In addition, the combination of 2 nmol/L docetaxel plus 0.1 μmol/L gefitinib, or 1 μmol/L cyclopamine also induced a more marked reduction of the percentage of invasive PC3 cells compared with individual agents and the inhibitory effect induced by the three drugs was also greater compared with the bicomination of these agents at the same concentrations (Fig. 7).

Discussion

The results from the present study indicated that the enhanced expression and activation of EGF-EGFR and...
hedgehog pathways may contribute to the sustained growth and survival of prostate cancer cells during the progression from localized invasive prostate cancers to metastatic disease. The immunohistochemical analyses of SHH revealed that its expression is enhanced in 39% of patients with the primary prostatic adenocarcinomas analyzed (Gleason scores 4–10) relative to normal prostate tissues (Fig. 1). SHH was focally expressed in the cytoplasm of some basal cells and weakly in luminal epithelial cells in normal prostate tissues, whereas a higher expression was detected in the cytoplasm and at or near the cell surface in the luminal tumor cells (Fig. 1). As shown in Fig. 2, the confocal immunostaining analysis has also revealed the higher levels of cytoplasmic staining for SHH in metastatic and androgen-independent LNCaP-C81, DU145, and PC3 cells compared with androgen-responsive LNCaP-C31 cells. Furthermore, the significant expression levels of the SHH precursor protein and its NH$_2$-terminal autocleavage product, SHH-N, were also detected by Western blot in cell lysates and conditioned medium for LNCaP-C81, DU145, and PC3 cells. In contrast, their expression was weaker for LNCaP-C33 cells and very low to undetectable for immortalized PZ-HPV-7 prostate epithelial cells (Fig. 2B). Because we have observed a significant SHH immunostaining at the prostate cancer cell surface in luminal compartment in malignant prostate tissues as well as the presence of NH$_2$-terminal fragment SHH-N in conditioned medium and lysate samples prepared from prostate cancer cells (Figs. 1 and 2), it seems likely that certain SHH-N ligand molecules may be retained near or at the prostate cancer cell surface (37–40). In fact, as noticed previously for several types of diffusible ligands, the localization of SHH-N ligand molecules near the cell surface, and more particularly within the lipid rafts, would permit ligand molecules in these microdomains to reach a higher local concentration necessary for a more rapid transduction of signals (37–40). Hence, these findings indicate that the up-regulated expression of the SHH-N ligand in prostate cancer cells might be an important factor associated with the most aggressive and metastatic phenotypes of the tumor cells. In support of this, it has been observed that the specimens

Figure 4. FACS analyses of the inhibitory effect induced by mixed docetaxel, gefitinib, and cyclopamine on EGF plus SHHNp–stimulated PC3 cells. The inhibitory effect induced by drugs on the progression of EGF plus SHHNp–stimulated PC3 cells in the cellular cycle was investigated by flow cytometric analysis. The stimulated PC3 cells were untreated or treated with the indicated concentrations of 1 or 2 nmol/L docetaxel, 0.1 μmol/L gefitinib, and 1 μmol/L cyclopamine, alone or in combination, for 2 d. At the end of incubation time, the cells were prepared as described in Materials and Methods and the cell cycle distributions were assessed by FACS analyses. Representative results obtained from three separate experiments.

Figure 5. FACS analyses of the apoptotic effect induced by docetaxel, gefitinib, and cyclopamine on EGF plus SHHNp–stimulated prostate cancer cells. The prostate cancer cell lines LNCaP-C33, LNCaP-C81, DU145, and PC3 cells were untreated (control) or treated with indicated concentrations of docetaxel, gefitinib, and cyclopamine, alone or in combination, for 4 d. Then, the cell nuclei were stained with propidium iodide and the DNA content was analyzed by flow cytometry.
from human nonlocalized or metastatic prostate tumors expressed higher levels of SHH, PATCHED-1 receptor, and GLI-1 transcription factor compared with samples from localized prostate cancers and normal tissues or benign prostate epithelial cells (41–44). Moreover, our recent work combined with several other studies has also revealed the significant expression levels of hedgehog signaling components (SHH, PATCHED-1, and GLI-1) in metastatic prostate cancer cell lines, including CWR22RV1, LNCaP, DU145, and PC3 cells (22, 24, 42, 43). Similarly, the immunohistochemical analyses of the EGFR and its active phosphorylated form, p-Tyr \(^{1173}\)-EGFR, have also indicated more elevated expression levels of this receptor in metastatic and androgen-independent LNCaP-C81, DU145, and PC3 cells as well as in androgen-responsive LNCaP-C33 cells (Fig. 2A). This supports the data from several prior immunostaining analyses, which indicated that the EGFR expression was enhanced in human prostate cancer cells in approximately 38% to 41% of specimens from patients with primary adenocarcinomas, although its expression was detected in up to 89% of patients with metastatic and androgen-independent tumors (31, 45–47). Moreover, the higher levels of EGFR expression were also detected by reverse transcription-PCR, ELISA assays, and Western blot in metastatic and androgen-independent LNCaP-C81, DU145, and PC3 cells than in androgen-responsive LNCaP-C33 cells and nonmalignant prostate cell line (22, 23, 28, 31, 48). Altogether, these observations suggest that the up-regulated expression of the SHH-N ligand and EGFR in androgen-independent prostate cancer cells might contribute to their acquisition of more malignant phenotypes during the transition from localized prostate cancers into aggressive and metastatic high-stage diseases.

Several recent lines of evidence have indicated that the accumulation of genomic alterations leading to the sustained activation of distinct developmental signaling in multi-
combined use of docetaxel, gefitinib, and cyclopamine induced an arrest of the growth and an inhibition of invasion of metastatic prostate cancer cells (Figs. 3, 4, and 7), it seems that this novel combination of drugs could be effective to prevent tumor growth and distant metastases. Importantly, we have also observed that the combined docetaxel, gefitinib, and cyclopamine also induced a massive rate of apoptotic death in the metastatic androgen receptor–positive LNCaP cell line, which possesses a luminal phenotype (CK5+, CD44+, and CK8/CK18+), as well as in androgen-independent and highly tumorigenic PC3 and DU145 cell lines characterized by an intermediate phenotype (CK5/CK18+, CD44+, and low or undetectable androgen receptor; refs. 2, 56–59). This then suggests that this triple combination of cytotoxic drugs could also be effective to eliminate the entire tumor masses of intermediate and luminal prostate cancer cells in primary and secondary neoplasms and thereby prevent disease relapse. In support of this, the results from a recent study have revealed that docetaxel may reduce the tumor growth and the occurrence of metastases of the intraprostatic orthotopically implanted PC3 cell model established in nude mice in vivo (26). Moreover, it has been observed that the blockade of the EGFR and/or hedgehog signaling pathways by using gefitinib and cyclopamine resulted in a growth arrest, apoptosis, and an inhibition of invasion.

**Figure 6.** Stimulatory effect induced by docetaxel, gefitinib, and cyclopamine on MMP (A) and (B) cytosolic cytochrome c level. The PC3 cells were untreated (control) or treated with 2 or 5 nmol/L docetaxel, 1 μmol/L gefitinib, and 2 μmol/L cyclopamine, alone or in combination, in the absence and presence of 50 μmol/L Z-VAD-fmk for 4 d. After the treatment, the cells were prepared by staining with 40 nmol/L 3,3'-dihexyloxacarbocyanine iodide (DIOC6(3)) for analyses of mitochondrial potential by flow cytometry. Moreover, the amount of cytochrome c released into the cytosol was estimated by ELISA assays as described in Materials and Methods. Representative data (A) and results (B) obtained from two to three separate experiments. *, P < 0.05, significant difference of either the stimulatory effects on the cytosolic cytochrome c release induced by the docetaxel, gefitinib, and cyclopamine alone compared with two combined drugs or the stimulatory effects induced by a combination of two drugs compared with the three combined drugs at the same concentrations.
accumulation of EGFR-positive tumor cells expressing and thereby result in disease relapse and the death of patients. More specifically, it has been reported that an androgen-independent prostate cancers initially respond to androgenic therapies, radiotherapy and chemotherapeutic agents, such as bicalutamide, on the androgen-sensitive and androgen-independent prostate cancer cells in vitro and in vivo (2, 18, 20, 22–24, 31, 41–43). Particularly, gefitinib has been observed to inhibit tumor growth and bone metastasis of PC3 cells and its high metastatic subline PCb2 in vitro (23). Importantly, it has also been reported that the continued cyclopamine treatment of PC3 and CWR22RV1 cell xenografts established in nude mice in vivo was accompanied by apoptotic cell death and tumor regression, and no sign of the tumor recurrence was detected 72 and 148 days after the cessation of treatment, respectively (24). Altogether, these observations support the potential benefit to adding the EGFR and hedgehog signaling inhibitors, such as gefitinib and cyclopamine, to docetaxel-based regimens for a more effective treatment of advanced and metastatic HRPCs in the clinics.

Although the patients with localized and androgen-dependent prostate cancers initially respond to androgen deprivation and radiotherapy, the up-regulation of numerous survival growth factors, such as EGFR and hedgehog, and deregulated expression and/or activity of apoptotic signaling elements, including endogenous lipid ceramide and caspases, may occur during the prostate cancer progression (2, 41–46, 60, 61). This may lead to the development of resistance to current clinical therapies and thereby result in disease relapse and the death of patients. More specifically, it has been reported that an accumulation of EGFR-positive tumor cells expressing intermediate markers CK5/CK18 occurred in a subset of patients with regressed and hormone-escaped prostate cancers (56, 62). Thus, the molecular targeting of EGFR and hedgehog cascades and the activation of ceramide and caspase-induced apoptotic death in prostate cancer cells may represent promising approaches to improve the current antiandrogenic therapies, radiotherapy and chemotherapy, in the clinics. In this matter, the data from the present investigation have indicated that the cytotoxic effects induced by combined docetaxel, gefitinib, and cyclopamine on EGF plus SHH–stimulated metastatic and androgen-independent PC3 cells may be mediated at least in part via the cellular ceramide accumulation and caspase activation through a mitochondrial pathway. More specifically, because we have observed that acid ceramidase inhibitor N-oleoyl ethanolamine enhanced the apoptotic effect induced by docetaxel on PC3 cells, it seems that this chemotherapeutic drug may mediate its cytotoxic effect through a cellular ceramide accumulation in vivo (26). This led to an elevation of ceramide/sphingosine 1-phosphate ratio in PC3 cells concomitant with the apoptotic cell death in vitro (26). Importantly, it has also been observed that the inhibitory effect induced by docetaxel on the metastatic properties of PC3 cells in vivo could be counteracted by overexpressing sphingosine kinase-1 in these cells. Furthermore, our recent works revealed that the selective inhibitors of EGFR and hedgehog signaling, gefitinib and cyclopamine, alone or in combination, at lower doses with the activator of cellular ceramide accumulation, such as tamoxifen and etoposide, also induced the apoptotic death of metastatic androgen-responsive LNCaP-C33 and androgen-independent LNCaP-C81, DU145, and PC3 cells in vitro (22, 31). The results from clinical trials have also revealed the potential benefit of docetaxel treatment as a neoadjuvant therapy or adjuvant therapy after surgery, alone or in combination with antiandrogenic therapy, as well as a radiosensitizing agent in patients with locally advanced prostate cancers at high risk of disease relapse (63–66). Particularly, a recent study indicated that the neoadjuvant treatment with docetaxel before radical prostatectomy was often accompanied by a significant decrease (>50%) of the prostate-specific antigen levels associated with tumor regression in patients with high-risk localized prostate cancer (63). Similarly, it has been reported that gefitinib might inhibit the activity of androgen receptor and enhance the antiproliferative effect induced by the antiandrogenic agents, such as bicalutamide, on the androgen-sensitive cell lines in vitro and in vivo (15, 67, 68). Moreover, gefitinib has been observed to enhance the antitumoral effects induced by radiation and various chemotherapeutic agents, such as cisplatin and carboplatinum, and paclitaxel on PC3 prostate cancer cell xenografts established in nude mice (69). Importantly, the clinical trials with a low dose of oral

Figure 7. Inhibitory effect induced by docetaxel, gefitinib, and cyclopamine on the invasive potential of PC3 cells. The PC3 cells were untreated (control) or pretreated with 2 nmol/L docetaxel, 0.1 μmol/L gefitinib, and 1 μmol/L cyclopamine, alone or in combination, for 24 h and during in vitro invasion assays, which were done for an additional 24 h using BD BioCoat cell invasion chambers as described in Materials and Methods. At the end of the assays, the invasive PC3 cells were stained and counted using a hemocytometer by phase-contrast microscopy. Data as the percentage of invasive cells. Columns, mean of three different experiments; bars, SE. *, P < 0.05, significant difference of either the inhibitory effects on the invasive potential of PC3 induced by the docetaxel, gefitinib, and cyclopamine alone compared with two combined drugs or the inhibitory effects induced by a combination of two drugs compared with the three combined drugs at the same concentrations.
active gefitinib have also indicated a potential benefit of using this type of agent, which generally shows a good bioavailability and little side effects, alone and in combination with antihormonal agents, radiation, and/or other chemotherapeutic drugs. This has been found in numerous cancer types overexpressing EGFR, including prostate, breast, brain, lung, and gastrointestinal cancers (70). Hence, based on these observations, it seems that the combined use of docetaxel, gefitinib, and/or cyclopamine, which induce a simultaneous activation of ceramide- and caspase-induced apoptotic responses, could represent a potential adjuvant therapeutic approach to improve the current clinical treatments, such as radical prostatectomy, androgenic agent treatment, and radiotherapy, for the patients with locally advanced prostate cancers at high risk of disease relapse and progression.

Conclusions

Altogether, the results suggest that EGFR-EGFR and SHH signaling cascades may play an important role for the sustained growth, survival, and invasion of prostate cancer cells during the progression from locally advanced prostate cancers into metastatic and recurrent disease states. Therefore, the combined use of docetaxel with both the selective inhibitors of EGFR and hedgehog signaling cascades, such as gefitinib and cyclopamine, may represent a promising strategy for improving the efficacy of current standard antihormonal and radiotherapeutic treatments used in the early stages against localized prostate cancers. Moreover, this triple combination could also be beneficial in late stages of advanced prostate cancer to eliminate the total population of metastatic HRPC cells in primary and secondary neoplasms and thereby prevent disease relapse. Hence, this novel combination of drugs offers a new alternative to current docetaxel-based regimens and may constitute a rational basis for future clinical trials to estimate its efficacy in patients with highly aggressive prostate cancers, which yet remain incurable in the clinics.

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References

11. Mimeault M, Batra SK. Recent advances on the significance of stem cells in tissue regeneration and cancer therapies. Stem Cells 2006;24:2319–45.
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Molecular Cancer Therapeutics

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