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Original Article

Biological effects of novel "combi-targeting" molecule and its effect on DNA repair pathway in hormone-refractory prostate cancer

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Abstract: Objective: This study aimed to investigate the biological effects of "combi-targeting" JDF12 and its effect on the DNA repair pathway in hormone-refractory prostate cancer (HRPC). Methods: HRPC cell lines (PC3 cells and VCap cells) were treated with JDF12 at different concentrations, and SRB method was employed to detect the proliferation of HRPC cells; Annexin V-FITC kit was used to detect the apoptosis of PC3 cells; Alkaline comet assay was performed to detect DNA damage; Western blot assay was done to detect the expressions of autophosphorylated EGFR, XRCC1 and ERCC1 (later two are proteins in DNA repair pathway); the anti-tumor effect was evaluated in nude mice inoculated with PC3 cells. Results: JDF12 could inhibit the proliferation of PC3 cells and VCap cells in a concentration dependent manner (IC_{50}: 14.04 ± 1.22 for PC3 and 15.57 ± 1.13 for VCap) and significantly increase the apoptotic cells as compared to those treated with mitozolomide or iressa alone. In PC3 cells, JDF12 induced DNA damage and also inhibited the expressions of phosphorylated EGFR, XRCC1 and ERCC1 in a concentration dependent manner. Moreover, JDF12 markedly inhibited tumor growth in nude mice. Conclusion: The novel "combi-targeting" JDF12 may exert more potent anti-proliferative effect as compared to mitozolomide or iressa alone, and the inhibitory effect on the EGFR signaling pathway and down-regulated XRCC1 and ERCC1 expressions may be ascribed to the JDF12 induced DNA damage.

Keywords: Combi-targeting molecule, JDF12, hormone-refractory prostate cancer, DNA repair pathway

Introduction

Prostate cancer (PCa) is a malignancy with the highest incidence in males of western countries and has been the second cause of death following lung cancer [1]. In recent years, with the development of liver standard, change in dietary structure and population aging, the incidence of PCa is increasing in China. In 2012, the prevalence of PCa in registered regions is about 9.92 cases per 100000 [2, 3]. Currently, therapeutic strategies have limited effectiveness for the advanced PCa with metastasis which thus may finally progress into hormone refractory prostate cancer (HRPC) [4]. The therapy of HRPC is still a challenge in clinical practice and has been the major cause of death in PCa patients.

Drug resistance and lack of target are the major reasons for therapeutic failure in HRPC patients. Traditional chemotherapy lacks favorable specificity and usually causes severe adverse effects although effectiveness is achieved to a certain extent. Significantly increased epidermal growth factor receptor (EGFR) is one of important characteristics of PCa. The expression of EGFR and its homologous ligands may cause an autocrine stimulation cycle and activate receptors to promote growth, proliferation and invasion of cancer cells, affecting the prognosis of PCa [5, 6]. Thus, to develop new drugs targeting EGFR may bring promises for the clinical therapy of HRPC.

In our previous study, a novel "combi-targeting" chemical compound JDF12 was synthesized

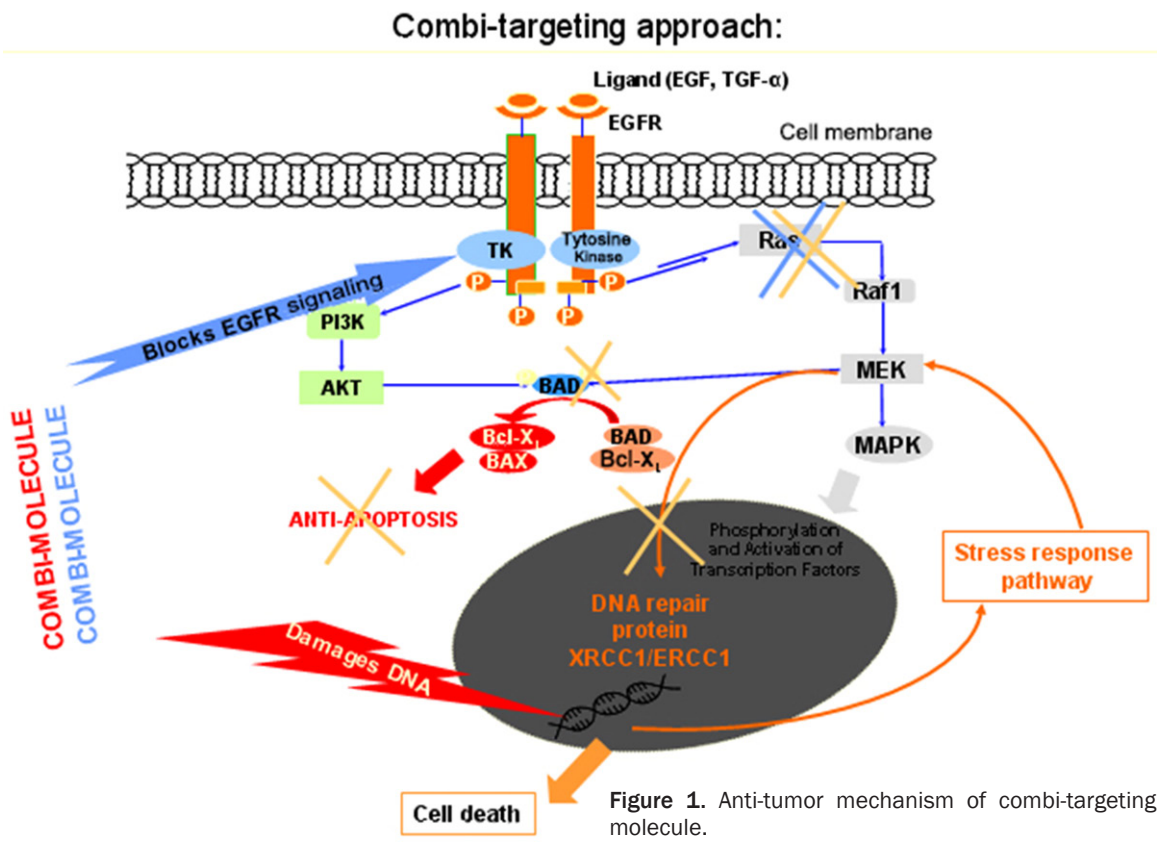

Figure 1. Anti-tumor mechanism of combi-targeting molecule.

[7]. This drug can specifically bind to EGFR over-expressed in PCa, which then blocks the EGFR signaling pathway. This drug may be further hydrolyzed into an inhibitory molecule of EGFR tyrosine kinase (TK) and DNA alkylating molecule. This process may result in a tadem-like release of anti-tumor molecules, exerting potent and sustained anti-tumor effect on HRPC [7]. Theoretically, EGFR TK inhibitor may block the EGFR signaling pathway and also block or significantly inhibit the activation of DNA repair signaling pathway due to the hydrolyzed DNA alkylating agent, which may be more effective to damage DNA in cancer cells. Furthermore, DNA alkylating agent induced DNA damage may reduce risk for gene mutation and subsequent resistance of cancer cells due to treatment with one EGFR TK inhibitor (**Figure 1**) [8, 9].

In our previous study, results confirmed that the anti-proliferative effect of JDF12 in PCa cell lines (LNCap cells and DU145 cells) was more than 10 times that of MTZ or JDF04R alone (two active components of JDF12) and more than 2 times that of combined therapy at the same or equivalent doses. The combination index (CI50) was < 1, suggesting the synergistic anti-tumor effect of both active components on PCa [7]. To further detect the anti-tumor effect of JDF12 on HRPC and to elucidate the potential mechanisms underlying the synergistic anti-tumor effect of both active components, this study was undertaken to detect the biological effect of JDF12 on PC3 cells and VCap cells, and the influence of JDF12 on the key proteins in the DNA repair signaling pathway was also evaluated.

Materials and methods

Preparation of drugs

JDF12 was synthesized by Prof Bertrand Jeanlaude in the Cancer Drug Research Laboratory Department of Medicine, McGill University Health Centre. Iressa was purchased from AstraZeneca (UK), and mitozolomide (MTZ) from Beijing Huamei Huli Biological Chemical Company. Antibodies against EGFR, ERCC1, XRCC1 and other proteins were purchased from Santa Cruz Biotechnology, INC (California, USA). Annexin V-FITC apoptosis assay kit (BD), Single Cell Gel Electrophoresis Assay kit (SYBR Gold) and other chemicals were purchased from Sigma (USA). Drugs were dissolved in DMSO

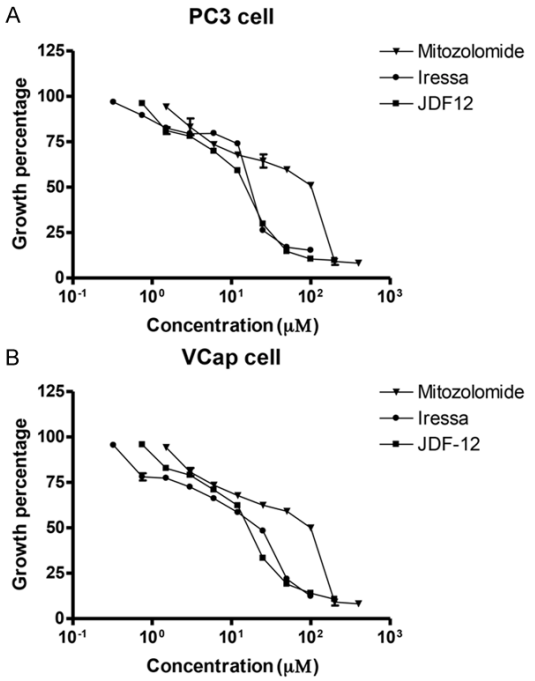

Figure 2. Antiproliferative effects of JDF12, Iressa, and MTZ against PC3 cells (A), VCap cells (B). Cells were treated with JDF12, Iressa, and MTZ over a period of 6 days. Growth inhibition was measured using the SRB assay. Each point represents at least two independent experiments run in triplicate.

and diluted with sterilized RPMI 1640 containing 10% fetal bovine serum (FBS) before use. The final DMSO concentration was lower than 0.2%.

Cell culture

Human PCa cell lines (PC3 cells and VCap cells) were purchased from ATCC. Cells were maintained in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin in a humidified environment of 5% CO_2 at 37°C. Cells in logarithmic growth phase were used in following experiments. In all assays, the cells were plated for 24-48 h before drug administration.

Detection of cell proliferation with sulforhodamine B (SRB)

Cells were seeded into 96-well plates at a density of 1×10^4 and incubated at 37°C for 24 h followed by treatment of drugs at different concentrations. There were 3 wells in each group. After drug treatment for 6 d, 50-μL of pre-cold trichloroacetic acid (50%) was added, followed by fixation at 4°C for 1 h. After washing with deionized water four times, cells were air-dried. Then, 50 μl of 0.4% SRB solution was added into each well, followed by incubation for 30 min. Cells were washed in 1% acetic acid to remove background staining and dried at room temperature. Cells were subsequently incubated with 200 μl of 10 mM Tris-base to resolve the dye with constant shaking for 15 min. Absorbance of each well was measured at 492 nm.

Detection of apoptosis by Annexin V-FIT apoptosis assay

After drug treatment for 2 d, cells and culture medium were collected and centrifuged, and cells were harvested and re-suspended in PBS for further cell counting. Then, 5×10^5 cells were centrifuged at 1000 g for 5 min, the supernatant was removed and 195 μl of binding solution was added, followed by addition of 5 μl of Annexin V-FITC and 10 μl of propidium iodide. Incubation was done at room temperature in dark for 15 min and then cells were placed on ice, followed by flow cytometry.

Western blot assay

Western blot assay was performed according to previously described [10]. After drug treatment for 2 d, total protein was extracted and protein concentration was determined. Equal amounts of protein (100 μg) were subjected to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 70 V for 20 min in separating gel and 110 V for 60-80 min in stacking gel, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) at 300 mA for 60 min. The membrane was incubated with 5% non-fat milk for 2 h with constant shaking and then treated with primary antibody (1:1000) at 4°C overnight. Following washing in PBST thrice (10 min for each), the membrane was treated with secondary antibody (1:10000) at room temperature for 1 h in dark. After washing in PBST thrice (10 min for each), the proteins on the membrane was visualized under an Odyssey infrared fluorescence imaging system.

Alkaline comet assay for quantitative analysis of DNA damage

Alkaline Comet Assay was performed according to previous described [11]. In brief, 100 μl of 1% agarose with a normal melting point at 45°C was dropped onto frosted slides, followed consolidation at 4°C for 10 min. Then, the coverslip was removed, and 75 μl of 0.7% agarose with a low melting point at 37°C and 10 μl of

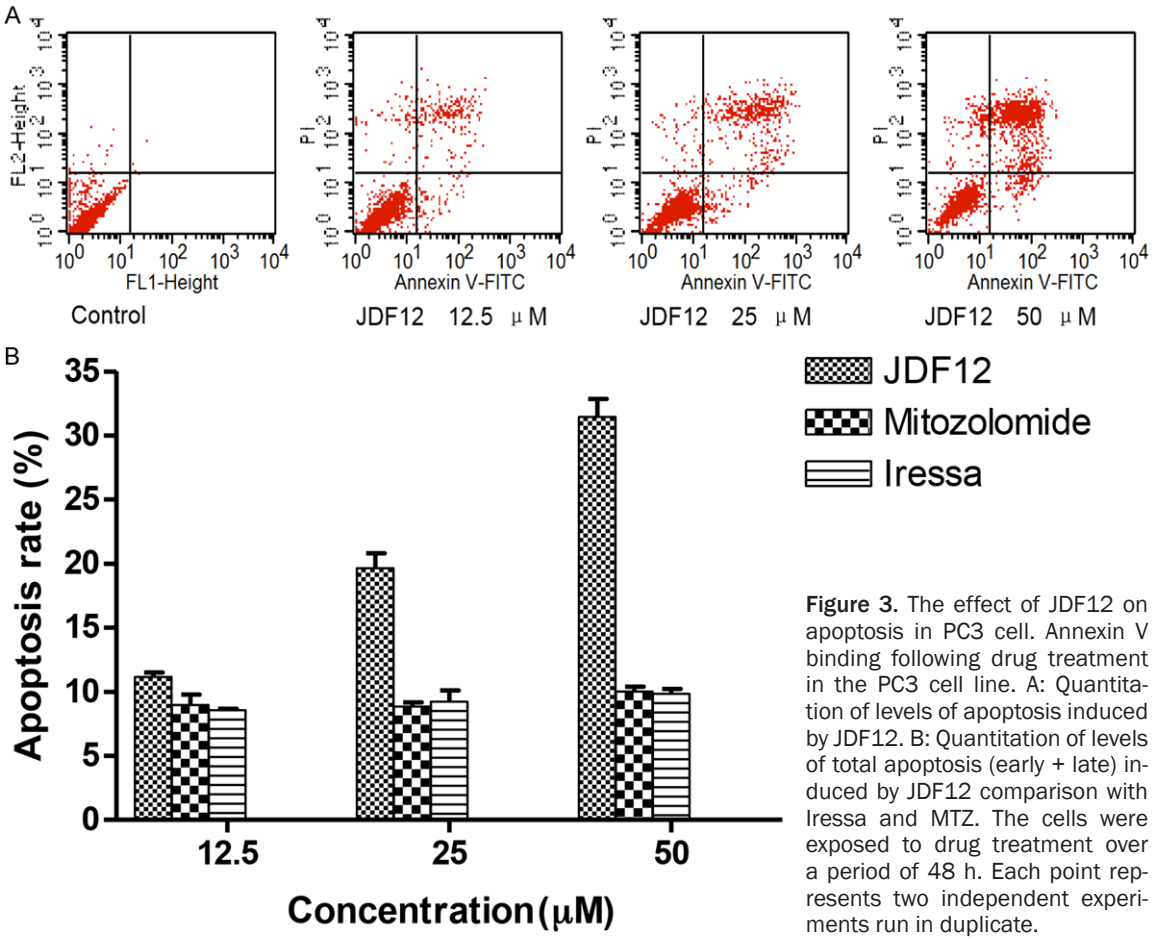

Figure 3. The effect of JDF12 on apoptosis in PC3 cell. Annexin V binding following drug treatment in the PC3 cell line. A: Quantitation of levels of apoptosis induced by JDF12. B: Quantitation of levels of total apoptosis (early + late) induced by JDF12 comparison with Iressa and MTZ. The cells were exposed to drug treatment over a period of 48 h. Each point represents two independent experiments run in duplicate.

cell suspension were rapidly dropped into slides, followed by consolidation at 4°C for 10 min. Electrophoresis was performed after cell lysis and DNA unwinding, followed by SYBR Gold staining. The resultant products were stored at 4°C in a humidified environment in dark for 24 h and observed under a fluorescence microscope at 330 × magnification. Single cell Comet Assay IV v4.2 image analysis software was used to analyze the proportion of cell comets and calculate the DNA migration distance. DNA damage was assessed with tail length and tail moment (the distance between the barycenters of the head and the tail of the comet multiplied by the percentage DNA in the tail region).

In vivo tumorigenicity

In brief, 3×10^6 cells were re-suspended in 100 μl of PBS, which was then subcutaneously inoculated into 4-week-old CD-1 nude mice. Ten days later, rice-sized nodules were observed under the skin, suggesting successful tumorigenicity. Mice were randomly assigned into control group, iressa group and JDF12 group (n = 5 per group), and drugs were injected intraperitoneally at 100 mg/kg. In control group, 400 μl of vehicle was injected. Injection was done once every 2 days for consecutive 7 times. The tumor width and tumor length were measured with a vernier caliper once every 3 days after treatment initiation, and tumor volume was calculated as follow: tumour volume (TV, mm^2) = $[(\text{tumor width} + \text{tumor length})/4]^3 \times 4/3 \times \pi$.

Statistical analysis

Statistical analysis was performed with SPSS version 16.0, and a value of $P < 0.05$ was considered statistically significant.

Results

JDF12 significantly inhibits the proliferation of PC3 cells and VCap cells

Detection with SRB method showed JDF12 treatment for 6 d significantly inhibited the proliferation of PC3 cells and VCap cells in a con-

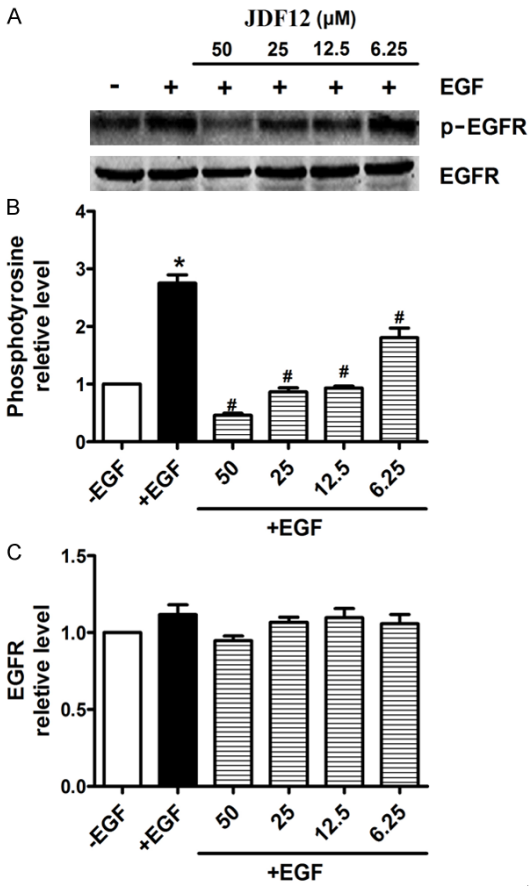

Figure 4. Inhibition of EGFR phosphorylation in PC3 cells. Serum-starved cells were preincubated for 2 h with the indicated concentrations of JDF12 (A). Phosphorylation level of EGFR by difference JDF12 concentrations (*$p < 0.05$ *vs* -EGF, #$p < 0.05$ *vs* +EGF) (B). EGFR expression level in indicated concentrations of JDF12 (C) before stimulation with EGF (50 ng/mL) for 15 min. Equal amounts of cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibodies. Membranes were stripped of anti-phosphotyrosine and reprobed with anti-EGFR antibodies as a loading control.

centration dependent manner, and the IC_{50} was 14.04 ± 1.22 for PC3 cells and 15.57 ± 1.13 for VCap cells. As compared to Iressa or MTZ alone, the IC_{50} of JDF12 markedly lowered, and JDF12 at a low concentration could achieve good inhibitory effect on the proliferation of PCa cells (**Figure 2**).

JDF12 significantly induces apoptosis of PC3 cells

Annexin V-FITC apoptosis assay was employed to detect the apoptosis of PC3 after JDF12 treatment. Results showed JDF12 treatment significantly increased the proportion of apoptotic cells in a concentration dependent manner. The apoptosis index was about 31.47% after treatment with 50 μM JDF12, but that of mitozolomide and iressa was 10.02% and 9.84%, respectively. When compared with mitozolomide and iressa, JDF12 was more potent to induce the apoptosis of PC3 cells (**Figure 3**).

JDF12 inhibits the EGFR phosphorylation in a concentration dependent manner

PC3 cells were incubated with JDF12 at 50, 25, 12.5 and 6.25 μM for 2 h and then with 50 ng/mL EGF for 15 min, followed by Western blot assay. Results showed the expression of phosphorylated EGFR increased markedly in cells treated with EGF alone. In the presence of EGF, JDF12 at different concentrations could inhibit the EGFR phosphorylation in a concentration dependent manner. Under different conditions, EGFR expression remained unchanged (**Figure 4**).

JDF12 induces DNA damage in PCa cells

Alkaline Comet Assay was employed to detect the DNA damage in PC3 cells after JDF12 treatment. Results showed JDF12 treatment for 24 h significantly caused DNA damage in a concentration dependent manner in PC3 cells. JDF12 at a high concentration was more potent to induce DNA damage as compared to mitozolomide, a DNA alkylating drug, but iressa failed to cause obvious DNA damage (**Figure 5**).

JDF12 alters the expressions of proteins related to DNA repair

XRCC1 and ERCC1 are two important proteins related to DNA repair. Western blot assay was employed to investigate the influence of JDF12 on the DNA repair signaling pathway. After EGF treatment, the expressions of XRCC1 and ERCC1 were significantly up-regulated, but JDF12 at different concentrations suppressed the expressions of XRCC1 and ERCC1 in a concentration dependent manner (**Figure 6**).

JDF12 inhibits tumor growth in vivo

The TV was measured once every 3 days (**Figure 7B**). At 33 days after inoculation of cancer cells, the tumors were collected, and TV was calculated (**Figure 7A**). The TV was 960.3 ± 245.0 in

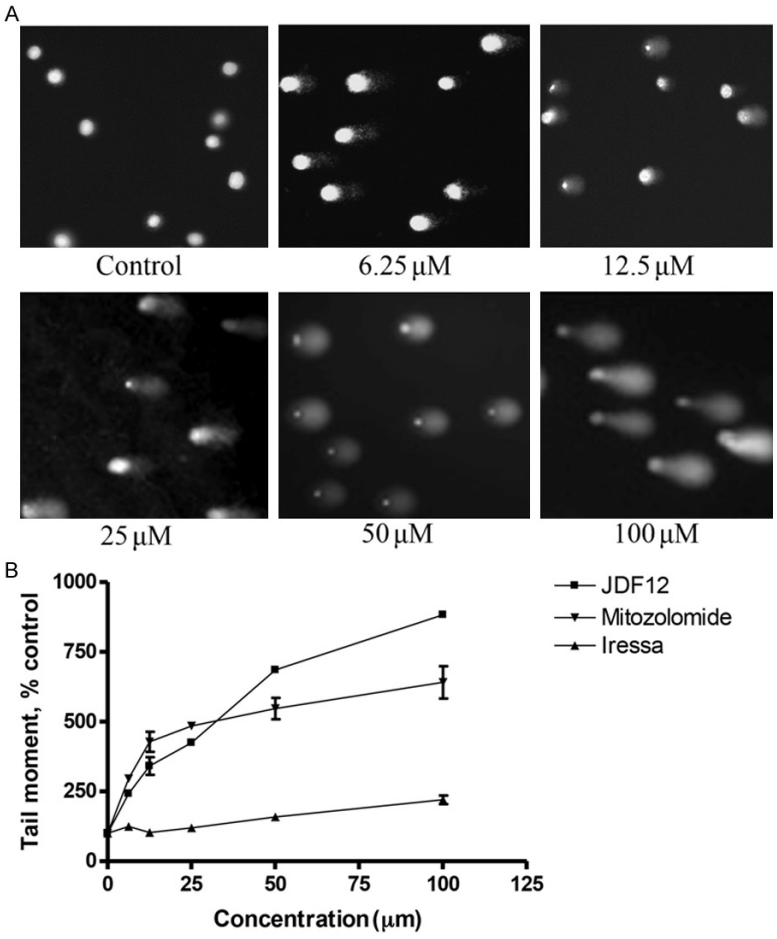

Figure 5. (Original magnification) (A) Typical comets formed by electrophoresis of PC3 cell nuclei after exposure to various concentrations of JDF12. Comets were visualized and examined using fluorescence microscopy at × 330 magnification; (B) Quantification of DNA damage in PC3 cells using the alkaline comet assay after exposure to JDF12, MTZ, and Iressa.

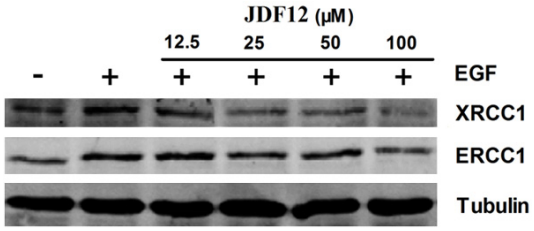

Figure 6. PC3 cells were starved overnight, treated with the indicated concentrations of JDF12 for 2 h, and thereafter stimulated or not stimulated with EGF (50 ng/mL) for 15 min. Afterwards, cellular proteins were analyzed by Western blot for the effect of JDF incubated with antibodies against ERCC1 and XRCC1 to detect DNA repair proteins, and anti-tubulin antibody was used to control for equal loading. Western blots were repeated twice, and similar results from two independent treatments were obtained.

control group, 570.6 ± 131.8 in iressa group and 220.4 ± 165.4 in JDF12 group, showing marked difference among them (Control *vs.* JDF12 P = 0.0008 and iressa *vs.* JDF12 P = 0.0066) (**Figure 7C**).

Discussion

Development of “combi-targeting” drugs refers to synthesize a new compound with a chemical synthetic method. This compound is able to specifically bind to EGF receptor and inhibit its autophosphorylation to block the EGFR signaling pathway. Under physiological conditions, this compound may be further hydrolyzed into an EGFR TK inhibitor and DNA damaging molecule, which may exert sustained and potent anti-proliferative effect on the refractory and EGFR dependent cancer. This method is dependent on the stability of “combi-targeting” compound, the proportion of hydrolyzed anti-tumor molecule and the interaction among anti-tumor molecules under physiological conditions [12, 13].

On the basis of above design, we successfully synthesized anilinoquinazoline (able to competitively bind to the ATP of EGFR signaling pathway) and imidazotetrazinone (able to alkylate the guanine of DNA) into a chemical drug JDF12. It can specifically bind to EGF receptor and block the autophosphorylation of EGFR to interrupt the EGF signaling pathway [7]. Under physiological conditions, JDF12 may be further hydrolyzed into an EGFR TK inhibitor (JDF04R) and a DNA alkylating molecule (chloroethyl diazonium). Chloroethyl diazonium is a functional group of mitozolomide (a chemotherapeutic drug) and able to alkylate the DNA of cancer cells [7, 9, 14]. *In vitro* studies showed JDF12 was effective to inhibit the proliferation of LNCap cells and DU145 cells. Experiment in nude mice inoculated with DU145 cells also confirmed JDF12 had a favorable anti-tumor effect [7]. To further validate the biological effect of this “combi-targeting” chemothera-

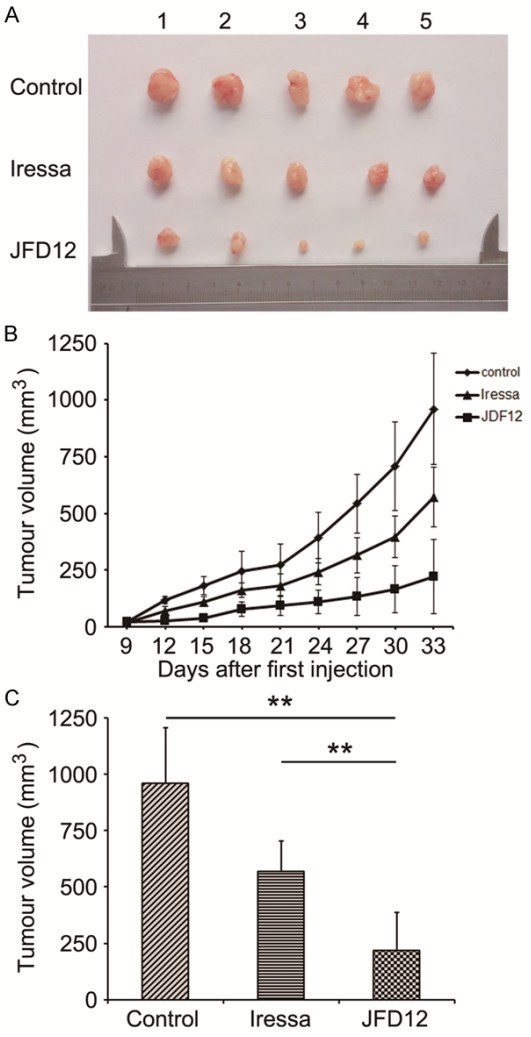

Figure 7. In vivo growth inhibition by JDF12. A: Tumour volume was measured by vernier caliper while mice were killed and tumour was anatomized 33 days post-treatment. B: Quantification of tumour volumes in PC3 cell line transplantation CD-1 mice after treatment with JDF12, Iressa, and vehicle (control). C: Comparison between the mean tumor volumes of groups of mice treated with JDF12 *vs* Iressa and JDF12 *vs* Control 33 day post-treatment. The mice were implanted with the PC3 cell line and were randomly divided into three groups. The first group was treated with vehicle (control), the second group was treated with Iressa, and the third group with JDF12. Twenty one days following administration, a significant difference in tumor volume could be observed between the group that received JDF12 and the group that received the control. Statistical analysis was carried out using Student’s t-test ($P < 0.05$ JDF12 *vs.* vehicle).

peutic drug on HRPC and investigate its influence on the DNA repair signaling pathway, PCa cell lines (PC3 cells and VCap cells) were employed and treated with JDF12. Our results may provide experimental evidence for the future studies on JDF12 and present theoretical evidence for the anti-tumor effect of JDF12. Our findings revealed that JDF12 was able to inhibit the proliferation of HRPC cells and increase their apoptosis index, which were JDF12-concentration dependent and better than that of mitozolomide or iressa alone. *In vivo* experiment in nude mice inoculated with PC3 cells also confirmed that JDF12 was able markedly inhibit the tumor growth, which was more potent than that of iressa. We not only confirmed JDF12 was potent to exert anti-tumor effect on HRPC but further investigated the potential mechanism related to the effect of JDF12 on the EGFR phosphorylation and DNA damage in PCa cells.

Clinical studies have found that 50-90% of HRPC cells have EGFR over-expression [15]. EGFR is able to phosphorylate EGFR TK to abnormally activate the downstream survival related signaling pathway and activate the anti-apoptosis signaling pathway, which are helpful to promote the cancer cell proliferation [16, 17]. Thus, in the present study, EGFR was employed as a target to validate whether the new combi-targeting chemotherapeutic drug JDF12 was able to block EGFR signaling pathway to exert anti-tumor effect. Moreover, results also revealed that additional EGF could significantly induce EGFR phosphorylation, but JDF12 could inhibit this EGF-induced EGFR phosphorylation in a concentration dependent manner, which were consistent with above hypothesis.

DNA alkylating agents are cytotoxic drugs. The active group of alkylating agents may covalently bind to DNA in cells, resulting in DNA inactivation or DNA fracture, which is also known as DNA alkylation damage [18]. DNA has the ability to repair itself in which DNA hydrocarbyl transferase is responsible for the de-alkylation (including demethylation). In addition, the entry of methylated DNA into subsequent biological processes is dependent on the repair capability of DNA mismatch. Once both steps are completed, methylated DNA may damage the DNA repair, exerting cytotoxic effect. Thus, an alkylating agent is only effective in some cancer cells with weak or no DNA repair. In the programmed administration of drugs, the first drug may inhibit the enzyme and consume DNA repair enzymes, which is helpful for the action

of following drugs. In the present study, comet assay was performed to detect DNA damage. Results showed JDF12 was able to increase DNA damage in a concentration dependent manner. JDF12 at a high concentration was more potent to cause DNA damage as compared to mitozolomide alone. This suggests that, when the hydrolyzed molecules (EGFR TK inhibitor and DNA alkylating molecule) of JDF12 reach a certain level, the ability of JDF12 to cause DNA damage increases significantly, and EGFR TK inhibitor and DNA alkylating molecule may synergistically exert anti-tumor effect, suggesting that to block EGFR signaling pathway significantly deteriorates DNA damage due to DNA alkylation. In our previous study, we compared the anti-proliferative effect of JDF12 with that of both mitozolomide and iressa at the same or equivalent doses. Results showed the anti-proliferative effect of JDF12 was 2 times that of mitozolomide and iressa, implying that the hydrolyzed molecules of JDF12 may exert synergistic anti-tumor effect [7]. There is evidence showing that DNA repair may remove DNA damage before the presence of adverse consequence of DNA damage, which reduces the cytotoxicity of DNA damage inducing agents [19]. Thus, the DNA repair mechanism may become a new target in the anti-tumor therapy. The known DNA repair mechanism includes nucleotide excision repair (NER), base excision repair, mismatch repair (MMR) and others, of which NER and BER are the most important mechanisms [20]. NER is a rate-limiting step in the recognition and excision of damaged DNA during DNA repair, in which excision repair cross-completion1 (ERCC1) plays a crucial role. The ERCC1 activity reflects the whole NER activity. X-ray repair cross-complementing group 1 (XRCC1) mainly acts in the BER and may interact with DNA ligase III, DNA polymerase β and ADP-ribose polymerase to repair DNA damage of any cause [20, 21]. In the present study, we also found EGF was able to increase XRCC1 and ERCC1 expressions, which, however, were down-regulated significantly by JDF12. This suggests that JDF12 may block the EGFR signaling pathway and reduce the expressions of XRCC1 and ERCC1 (two proteins in the DNA repair) in PCa cells, which increase the alkylating DNA damage and the subsequent anti-tumor effect on HRPC.

This study investigates the effects of JDF12 as a pro-drug on the EGFR and DNA and their role in the anti-tumor effect of JDF12 on HRPC. Furthermore, we confirm that the DNA damage inducing effect of JDF12 is related to the blocking of EGFR signaling pathway and down-regulated expressions of XRCC1 and ERCC1 in the DNA repair signaling pathway. Our findings provide new theoretical evidence for the use of combi-targeting drugs.

Acknowledgements

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Disclosure of conflict of interest

None.

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