PYRROLIDINE DITHIOCARBAMATE INHIBITS NF-κB ACTIVATION AND ENHANCES TNF-INDUCED APOPTOSIS IN HUMAN BREAST CANCER CELLS

TU Gang 涂刚*, YAO Zhen-xiang 姚榛祥

Department of Surgery, First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016

CLC number: Q255; R737.9 Document code: A Article ID: 1000-9604(2004)-02-0099-06

ABSTRACT

Objective: To determine whether pyrrolidine dithiocarbamate(PDTC) enhances TNFα-induced apoptosis in cultured breast cancer cells and explore the role of NF-κB in TNFα-induced apoptosis. Methods: Human breast cancer cell lines MCF-7 and MDA-MB-435s were treated with TNFα, PDTC and combination therapy. Cell survivals were determined by MTT assay. Apoptosis was detected by TUNEL and flow cytometry. NF-κ B DNA binding activity was detected using electrophoresis mobility shift assay (EMSA). Western blots were performed to demonstrate IκBα(Inhibitor protein of nuclear factorκB) phosphorylation and degradation. Results: Cell growth was not suppressed by either TNFα(2000 U/ml or less) or PDTC alone. Both cell lines treated with TNFα (2000 U/ml) combined with PDTC(50 μmol/L) showed significant growth inhibition. PDTC inhibited TNFα-induced IκBα phosphorylation and degradation in both cell lines. EMSA showed that PDTC continuously inhibited TNFα induced NF-κ B DNA binding activity. TNFα induced apoptosis (TUNEL) was increased significantly when both cells were pretreated with PDTC, and this was confirmed by Flow cytometry. Conclusion: PDTC enhances TNFα-induced apoptosis via inhibiting IκBα phosphorylation and degradation in human breast cancer cells. NF-κ B protects against TNFα-induced apoptosis.

Key words: Breast cancer; Apoptosis; Nuclear factor kappa B; Tumor necrosis factor α.

Tumor necrosis factor α (TNFα) deactivates cancer cells. both *in vivo* and *in vitro* mainly via triggering apoptosis[1]. However, several cancer cells are not sensitive or are minimally sensitive to TNFα even though they have appreciable TNFα receptor on their surface. The mechanism remains unclear. Recent findings have demonstrated that nuclear factor kappa B (NF-κ B) protects against TNFα-induced apoptosis[2].

NF-κ B is a transcription factor, which plays an important role in cell proliferation and malignant transformation[3,4]. NF-κ B is well established as a regulator of genes encoding cytokine, cytokines receptors, and cell adhesion molecules that drive immune and inflammatory responses. More recently, NF-κ B activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis , the cell cycle, differentiation and cell migration[5]. Additionally, activation of NF-κ B in cancer cells by chemotherapy and by biotherapy can blunt the ability of the cancer therapy to induce cell death. NF-κ B perhaps plays a role in the resistance to TNF and chemotherapy and escaping from apoptosis[6,7]. The activation or expression of NF-κ B is evident in human cancer, including breast cancer, non-small cell lung carcinoma, thyroid cancer, and several T-or B-cell lymphocyte leukemia[8]. It was demonstrated that the level of NF-κ B P65 protein and NF-κ B DNA binding activity in breast tumor tissue were increased compared with those in adjacent normal tissues in our previous investigation[9]. These findings make us believe that NF-κ B could protect TNFα-induced apoptosis in breast cancer cells. Using PDTC (an antioxidant) to inhibit NF-κ B activation, this study examined whether blockade of NF-κ B activation by PDTC potentiates TNFα-induced apoptosis in human breast cancer cells, thus provides a potential biological strategy to enhance tumor cell killing by radiotherapy, chemotherapy or biotherapy.

MATERIALS AND METHODS

Cell Culture and Treatment

Received date: Dec. 4, 2003; **Accepted date:** Mar. 19, 2004.
***Author** to whom correspondence should be addressed.
Phone: (0086-23)-89011016; Fax: (0086-23)-68812985;
E-mail: tugang68@126.com
Biography: TU Gang(1968-), male, doctor of medicine, attending physician, Chongqing University of Medical Science Sciences, majors in surgery.

Human breast cancer cell lines, MCF-7 (estrogen receptor-positive) and MDA-MB-435s(estrogen receptor -negative), were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). After serum starvation for 12h, cells were treated with PDTC(ranged from 0 to 200 μmol/L, Sigma Co.) or TNFα(ranged from 0 to 10000 U) for 48h in cell growth assay, depending upon the experiment. All cells, with the exception of negative controls, were pretreated for 1 h by PDTC(50 μmol/L), and then exposed to human recombinant TNFα(Bangding Co., Beijing) at 2000 U/ml (specific activity 6×10^4 U/mg) for assay combined effect. PDTC exposure was maintained at the desired concentration throughout TNFα stimulation.

Cell Growth Assay

For survival assays, experiments were performed in poly-D-lysine-coated 96-well plates seeded at a density of 1×10^3 cells/well/200 μ l. At the end of the experiment, twenty μl MTT (5mg/ml, Sigma Co., USA) was added into each well and incubated for 3 hours at 37℃, then 200 μl dimethylsulfoxide was added to solve formazan produced. Optical intensity (absorbance) was determined with microplate reader at a 570-nm wavelength. Cell survival was calculated as following formula: survival ratio = (OD in experiment / OD in control) × 100%. Each experiment was performed in triplicate.

Western Blots

Cellular proteins were extracted by adding prechilled RIPA lysis buffer, and then incubated 30 min on ice. The lysate was centrifuged 12000g for 10 min at 4°C and the supernatant was prepared as the cytoplasmic extract. SDS-PAGE electrophoresis was performed. Samples were loaded at 60 μg per well, and then electrophoresed at 200V for 4h. The gel was transferred to nitrocellulose membrane at 100V for 2 h. The membrane was treated with Iκ Bα antibody and horseradish-peroxidase- couple goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology Co. USA). Then the membrane was developed using enhanced chemiluminescence and exposed to film.

Electrophoretic Mobility Shift Assay(EMSA)

Nuclear proteins were prepared as described[10]. NF-κ B double-stranded DNA oligonucleotide 5'-AGTT-GAGGGGACTTTCCCAGGC-3', 3'-TCAACTCCCCT-GAAAGGGTCCG-5'(Promega Co. USA) was labeled with^{32}P- γ dATP by standard protocols employing T4 kinase. Nuclear proteins (4μg) and ^{32}P-labeled oligonucleotide probes (1.75pmol) were added to the DNA binding buffer .The DNA-protein complexes were resolved in a 7% nondenaturing acrylamide gel and electrophoresed for 2h. The gel was dried and exposed with Kodak film at -70°C for 48h to 72 h.

Apoptosis Determination by Flow Cytometry

Cells were plated in six-well plates at a concentration of 5×10^5 cells per well. Twenty-four hours following treatment with TNFα (2000 U/ml) and/or pretreatment with PDTC(50 μmol/L)1h, cells were harvested and stained with propidium iodide and evaluated by flow cytometry. Apoptosis ratio was determined using software ModFit. Apoptosis cells were defined as cells containing less DNA than the G_0/G_1 peak.

Apoptosis Analysis by TUNEL Stain

Breast cancer cells treated with TNFα, PDTC and combination of the both, were plated onto poly-D-lysine-coated glass coverslips in 24-well plates and incubated for 24 h. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and TUNEL was performed as the instructional manual. Nuclei of apoptotic cells were in brown-yellow color.

Statistical Analysis

Unpaired *t* test was adopted and the difference was significant if *P* value was less than 0.05.

RESULTS

Effect of TNFα or PDTC on the Growth of Breast Cancer Cells

After 48 hours incubation, TNFα did not result in significant growth inhibition on MCF-7 cells with concentration less then 4000 units/ml. However, growth inhibition was detected when the concentrations reached 5000units/ml (controls OD: 0.86±0.03, experiment OD: 0.61±0.02, $P<0.05$). TNFα at a concentration of 10000 units/ml induced significant growth inhibition in MDA-MB-435s cells (controls OD:0.78±0.06, experiments OD:0.53±0.01, $P<0.05$). PDTC did not lead to growth inhibition on both breast cancer cells when the concentration was less then 200 μmol/L (controls OD:0.95±0.03, experiment OD: 0.93±0.01 in MCF-7, $P>0.05$; controls OD:0.88±0.05, experiment OD: 0.87±0.04 in MDA-MB-435s, $P>0.05$) (Figure 1).

Combined Effect of TNFα and PDTC on the Growth of Breast Cancer Cells

For MCF-7 cells, the cell survival ratios of TNFα(2000 U/ml) and TNFα(2000 U/ml) with PDTC(50 μmol/L) were 0.98±0.08 and 0.88±0.06 (P<0.05), respectively 24 hours later, and 0.98±0.06 and 0.75±0.07(P<0.01), respectively 48 h later. In MDA-MB-435s cells, the cell survival ratio of TNFα (2000 U/ml) and TNFα (2000 U/ml) with PDTC (50 μmol) were 1.01±0.08 and 0.79±0.06 (P<0.01) respectively 24 hours later. Both MCF-7 and MDA-MB- 435s treated with TNFα plus PDTC showed significant and dramatic growth inhibition compared with those treated with TNFα 24 h later (Figure 2).

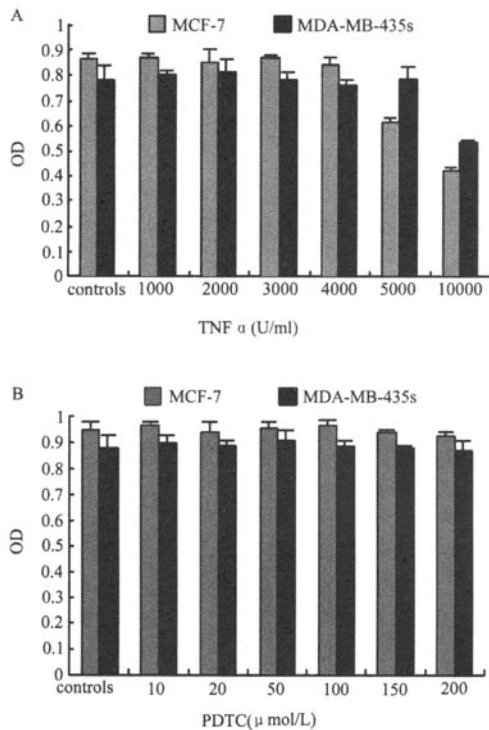


Fig. 1. Effect of TNF α or PDTC on the growth of breast cancer cells.

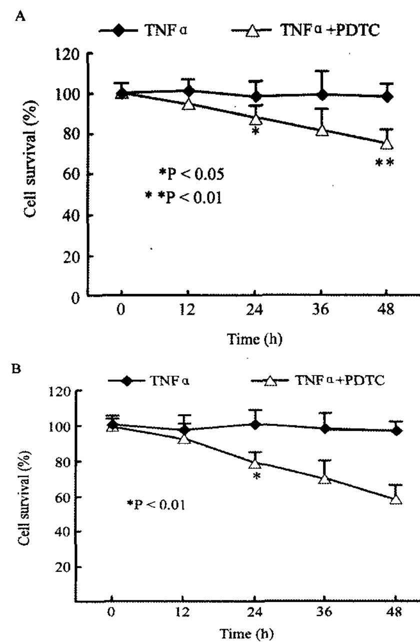


Fig.2. Combined effecdt of TNF α and PDTC on the growth of breast cancer cells.

PDTC Inhibit TNFα Induced Iκ B α Phosphorylation and Degradation in Culture Human Breast Cancer Cell Lines

In Both MCF-7 and MDA-MB-435s cell lines, TNFα treatment (2000 U/ml) for 30 min induced brisk degradation of IκBα (68.04±4.43 and 16.47±2.5), and there was significant difference compared with the control group (285.41±11.87 and 183.45±7.9, P<0.01) Pretreatment of both cell lines with PDTC (50 mol) for 1 h markedly inhibited TNFα-induced IκBα degradation compared with TNFα treated alone in both breast cancer cells (172.24±6.92 and 177.88±10.43, P<0.01) (Figure 3) .

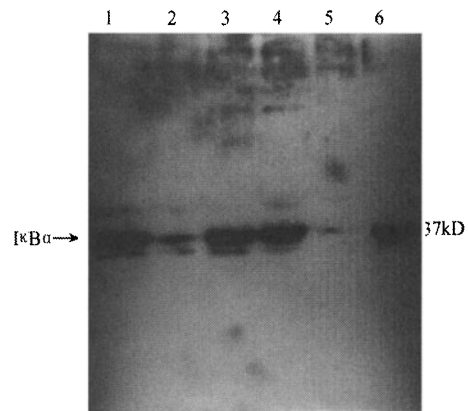


Fig. 3. Western blot analysis of I κ B α protein in breast cancer cells lane 1 and 4: control lane 2 and 5: TNF α lane 3 and 6: TNF α +PDTC (lane 1－3; MCF-7 cell line; lane 4－6

PDTC Inhibited NF-κ B DNA Binding Activity

In both MCF-7 and MBA-MB-435s cell lines, after TNFα treatment (2000 U/ml) at 4 h, NF-κ B DNA binding activity was markedly increased (64.29±2.84 and 133.28±5.6) compared to that of untreated cells (29.62 ± 1.23 and 83.79 ± 4.3, P<0.05). Pretreatment with PDTC (50 μmol) for 1 h markedly suppressed TNFα-induced NF-κ B DNA binding activity compared with TNFα treated alone in both breast cancer cells (34.56±3.2 and 31.62±2.52, P<0.05)(Figure 4).

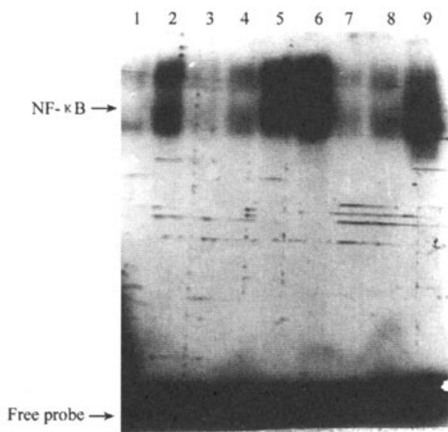
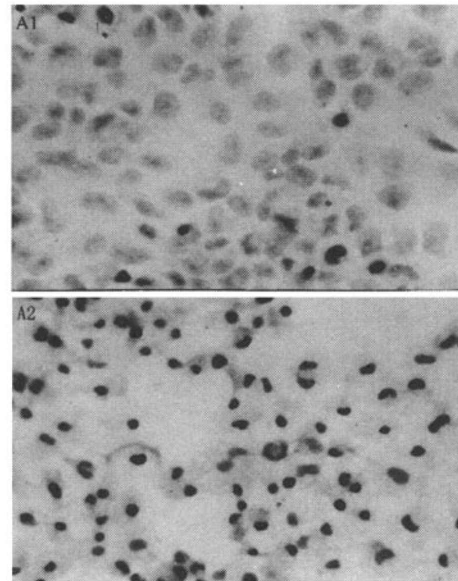


Fig. 4. Detection of NF-κ B DNA binding activity by EMSA in breast cancer cells lane 1 and 5: Control lane 2 and 6: TNFα Lane 3 and 7: NF-κ B decoy lane 4 and 8: TNF α +NF-κ B decoy lane 9: Hela nuclear extract used as the positive control (lane 1—4: MCF-7 cell line; lane 5—8: MDA-MB-453s cell line)



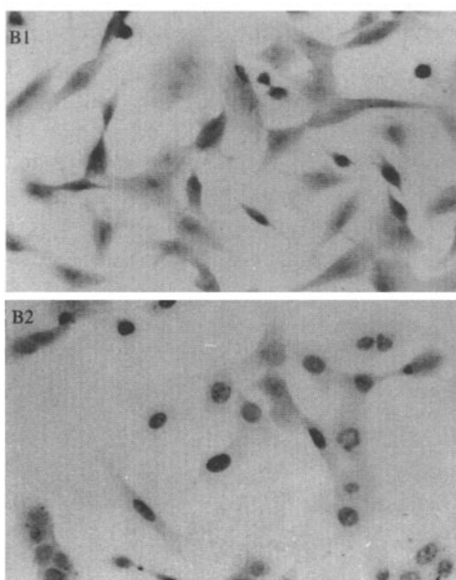
B1: TNFα B: TNFα+PDTC

Fig. 5. Apoptosis analysis by TUNEL in breast cancer cells [MCF-7(A), MDA-MB-435s(B)]

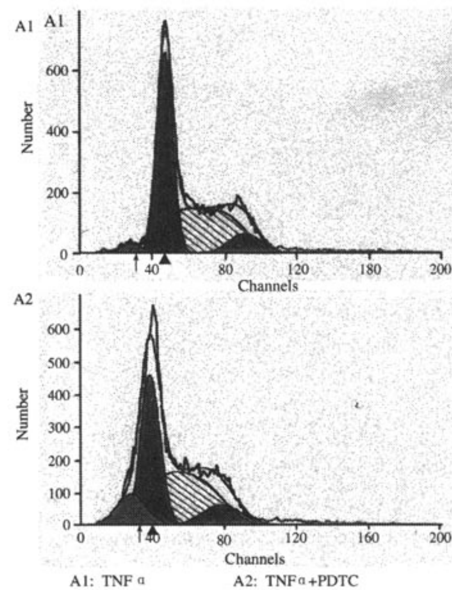
PDTC Enhanced TNFα induced Apoptosis in Both Breast Cancer Cell Lines

In both MCF-7 and MBA-MB-435s cell lines, treatment with neither TNFα (2000 U/ml) nor PDTC(50 μmol) alone for 24 h produced statistically significant changes in TUNEL stain compared to untreated cells. However, the synergistic effect of these same doses (50 μmol PDTC as pretreatment to 2000 U/ml TNFα 24h) produced a great deal apoptosis cells (Figure 5, cells with brown-yellow nucleus indicated by arrows).

These results were confirmed by flow cytometry. In both MCF-7 and MBA-MB-435s cell lines, the apoptotic fraction accounted for approximately 2.5% and 0% treated with TNFα (2000 U/ml) alone for 24 h. With PDTC(50 μmol) treatment alone for 24 h, the apoptotic fraction accounted were 0% for both cell lines. Again, TNFα and PDTC had a synergistic effect, increasing the apoptotic fraction to 11.14% and 22.5% (Figure 6, the apoptotic fraction indicated by arrow).



A1: TNFα A2: TNFα+PDT



A1: TNFα A2: TNFα+PDTC

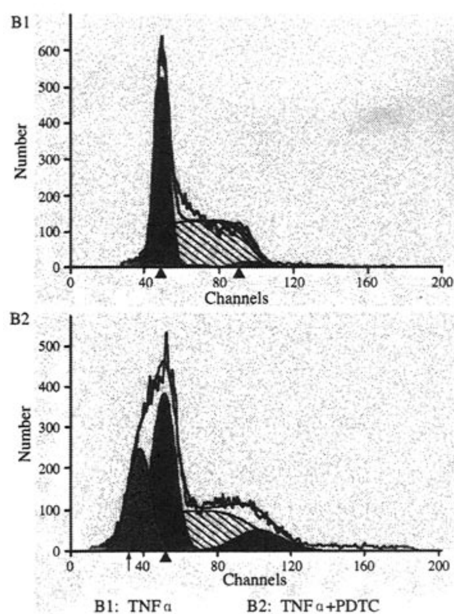


Fig. 6. Apoptosis analysis by flow cytometry in breast cancer cells [MCF-7(A), MDA-MB-435s(B)]

DISCUSSION

In an *in vitro* model of human breast cancer, we have demonstrated that TNFα-induced phosphorylation and degradation of I κ Bα, the cytoplasmic NF-κ B repressor molecule. I κ B α degradation is necessary for the NF-κ B translocation to the nucleus, which correlates with decreased activation of the transcription factor[2]. In both treated cell lines, PDTC inhibited activation of the NF-κ B pathway. PDTC also dramatically enhanced TNFα induced apoptosis. When used separately, the effects of TNFα and PDTC are unimpressive. When used in combination, however, their effects are synergistic, being much greater than simply additive.

We demonstrated that NF-κ B inhibited TNFα-induced cell killing in both MCF-7 and MDA-MB-435s. Recently, several investigations showed that NF-κ Bα could counteract the cytotoxicity of TNFα and chemotherapy agents [11,12]. Such a role may be mediated via regulating antiapoptotic oncogenic proteins. Cheng et al[13] believed that TNFα and several commonly used chemotherapeutic agents upregulated the expression of Bcl-x and Bfl-/A1 through an NF-κ B-dependent pathway. While cancer cells were resistant to the cytotoxic effects of both TNFα and chemotherapy agents. These finding suggested that NF-κ B suppresses apoptosis through activation of various oncogenic proteins, although further investigation will be needed to clarify the signal transduction pathway connecting NF-κ B with these oncogenic proteins.

In the present study, we manifested that there were differences in levels of NF-κ B DNA binding activation and I κ Bα expression between MCF-7 and MDA-MB-435s cell lines. NF-κ B activation level in MDA -MB-435s cell line was higher than that in MCF-7 cells, but I κ Bα in MDA -MB-435s cell was lower than in MCF-7 cells. The molecular mechanism responsible for differential I κ Bα expression in different breast cancer cells is not known. The estrogen receptor expression level ultimately determines the ability of DNA binding competent NF-κ B to activate the target genes[14,15].

In order to investigate the role of NF-κ B in breast cancer cells, PDTC is used to inhibit NF-κ B function. PDTC was an antioxidant, which could scavenge free-radical species that are necessary for NF-κ B activation[16,17]. We utilized TNFα for the dual activation of apoptosis and the NF-κ B apoptotic-resistance pathway, and observed a powerful synergistic effect for induction of apoptosis when PDTC was used to block NF-κ B activation. Together, these data suggest that suppression of the NF-κ B activation pathway may increase the efficacy of therapies, such as ionizing radiation or chemotherapy, while minimizing dose-limiting toxicities and tumor resistance.

REFERENCES

[1] Vassalli P. The pathophysiology of tumor necrosis factors[J]. Ann Rev Immunol 1992; 10: 411.

[2] Vincent B, Mohamed B A, A C Hellin, et al. Nuclear factor-κ B, cancer, and apoptosis[J]. Biochem Pharmacol 2000; 60:1085－90.

[3] Baeuerle PA, Baltimore D. NF-κ B: Ten years after[J]. Cell 1996; 87:13－20.

[4] Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer[J]. Oncogene 1999; 18: 6938－47.

[5] Amit S, Ben-Neriah Y. NF-κ B activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach[J]. Semin Cancer Biol 2003; 13:15－28.

[6] Baidwin AS Jr. The transcription factor NF-κ B and human disease[J]. J Clin Invest 2001; 107:3－6.

[7] Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer[J]. J Clin Invest 2001; 107:135－42.

[8] Chen F, Castranova V, Shixianglin, et al. New insights into the role of nuclear factor-κ B, a ubiquitous transcription factor in the initiation of diseases[J]. Clin Chem 1999; 45:7－17.

[9] Tu Gang, Yao Zhenxiang, Dong Pujiang, et al. Expression of nuclear factor-κB in human breast cancer[J]. Chin J General Sug 2003; 12:348－50.

[10] Ye J, Young HA, Ortaldo JR, et al. Identification of a

DNA binding site for the nuclear factor YY1 in the human GM-CSF core promoter[J]. Nucleic Acids Res 1994; 5672－8.

[11] Englaro W, Bahadoran P, Bertolotto C, et al. Tumor necrosis factor alpha-mediated inhibition of melanogenesis is dependent on nuclear factor kappa B activation[J]. Oncogene 1999; 18:1553－9.

[12] Biawas DK, Martin KJ, McAlister C, et al. Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappa B activation[J]. Cancer Res 2003; 63:290－5.

[13] Cheng QW, Lee HH and Li Y, et al. Upregulation of Bcl-x and Bfl-1 as a potential mechanism of chemoresistance, which can be overcome by NF-κ B inhibition[J]. Oncogene 2000; 19:4936－40.

[14] Harikrishna N, Poornima B N, Daniel A M, et al. Constitutive activation of NF- κ B during progression of breast cancer to hormone-independent growth[J]. Mol Cell Biol 1997; 17:3629－39.

[15] Biswas DK, Dai SC, Cruz A, et al. The nuclear factor kappa B(NF- κ B): A potential therapeutic target for estrogen receptor negative breast cancers[J]. PNAS 2001; 98:10386－91.

[16] Gunawardena K, Murray DK and Swope RE, et al. Inhibition of nuclear factor kappaB induces apoptosis following treatment with tumor necrosis factor alpha and an antioxidant in human prostate cancer cells[J]. Cancer Detect Prev 2002; 26:229－37.

[17] Bach SP, Chinery R, O'Dwyer ST, et al. Pyrrolinedithiocarbamate increases the therapeutic index of %-fluorouracil in a mouse model[J]. Gastroenterology, 2000; 118:81－9.