Effects of ulinastatin and docataxel on breast tumor growth and expression of IL-6, IL-8, and TNF-α

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Abstract

Objective: This study investigated the effects of Ulinastatin (UTI) and docataxel (Taxotere, TAX) on tumor growth and expression of interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) in breast cancer.

Methods: MDA-MB-231 human breast carcinoma cells were cultured in vitro and injected into nude mice to establish breast tumor xenografts in vivo. Cultured cells and mice with tumors were randomly divided into four groups for treatment with TAX, UTI, and TAX+UTI. The effects of these drug treatments on cell proliferation and apoptosis was measured using the MTT assay and the Annexin V/propidium iodide (PI) double-staining method, respectively. IL-6, IL-8, and TNF-α expression levels were determined by measuring mRNA transcripts in cultured cells by RT-PCR and cytokine proteins in solid tumors using immunohistochemistry.

Results: UTI, TAX, and UTI+TAX inhibited the growth of MDA-MB-231 cells in vitro and tumors in vivo. These two drugs, particularly when used in combination, promote tumor cell apoptosis and down-regulate the expression IL-6, IL-8, and TNF-α cytokines.

Conclusion: Both UTI and TAX inhibited the growth of MDA-MB-231 breast carcinoma cells. UTI enhanced the inhibitory effect of TAX by a mechanism consistent with the down-regulated expression of IL-6, IL-8, and TNF-α.

Background

Along with the increasing incidence of breast cancer tumors, which now account for 18% of all female tumors, 1.2 million women suffer from breast cancer worldwide. Many important problems pertaining to the oncological details of invasion and metastasis pose significant challenges to scientists.

With the development of new techniques in molecular biology, further exploration into the mechanisms related to the occurrence of breast cancer have become a hotspot in the field of cancer research. The cytokines, which play regulatory roles in disease development have become an important topic for many researchers. IL-6, IL-8, and TNF-α are one group of cytokines produced by mononuclear macrophages and endotheliocytes involved in activating and inducing T cells, B cells, and natural killer cells to target and phagocytosize pathogenic cells. Additionally, these cytokines are important factors in inflammation and pathophysiology.

In this study, we monitored the effects of UTI and TAX, individually and in combination, on the growth of the negative estrogen receptor (ER-) human breast carcinoma cell line, MDA-MB-231. Using both cultured cells in vitro and xenografted tumors in vivo, we also examined the effects of UTI and TAX on apoptosis and the expression levels of IL-6, IL-8, and TNF-α cytokines.

Materials and methods

1.1 Cell lines and animals

The human breast cancer cell line MDA-MB-231(ER-) was a generous gift from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (CAS). Fifty female BALB/c-nc/nu nude mice, 5 weeks old and weighing 17-21 g, were purchased from the Beijing Institute of Experimental Zoology, CAS, and maintained in the Chongqing Medical University Animal Research Center (production license No. SCXK (Jing), 2005-0014, usage permit No. (Yu), 2007-0001).
1.2 Reagents
UTI was kindly provided by Techpool Bio-Pharma Co., Ltd. TAX was a generous gift from Sanofi-aventis Pharma Co., Ltd. Maxima™ SYBR Green/ROX qPCR Master Mix (2X) and RevertAid™ First Strand cDNA Synthesis Kits was purchased from Fermentas Co. Ltd., Canada; Trizol kit was purchased from Invitrogen Co., Ltd; RT-PCR kit was purchased from Nanjing KeyGen Biotech Co, Ltd. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), propidium iodide(PI), and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co., Ltd; RPMI-1640 was purchased from Gibco Co., Ltd; AMV reverse transcriptase was purchased from Promega Co, Ltd; RT-PCR kit was purchased from Nanjing KeyGen Biotech Co, Ltd; RPMI-1640 was purchased from Gibco Co., USA. The secondary antibody kit and diaminobenzidine (DAB) chromogenic substrate were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. Vascular endothelial growth factor-C (VEGF-C), basic fibroblast growth factor (bFGF), and nerve growth factor (NGF) primary antibodies were purchased from Abcam Co., Ltd., UK.

1.3 Cell cultures and nude mice
MDA-MB-231 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin at 37°C in a 5% CO2 atmosphere. Following propagation for 2-3 days, cells in logarithmic growth phase were digested with 1.0 mL of 0.25% trypsin for 2-3 min, separated from trypsin, and incubated with double antibody solution in RPMI-1640 medium containing 10% FBS. Nude mice were housed in a specific pathogen free (SPF) environment at 22-25°C and 50-65% relative humidity with sterile drinking water, food, and experimental equipment.

1.4 Experimental groups and drug treatments
Cultured MDA-MB-231 cells were divided into four random groups: Control (RPMI-1640 medium alone), UTI (8000 U/mL), TAX (3.7 ug/mL; 5 × 10^-6 M), and UTI+TAX. MDA-MB-231 cells were harvested, rinsed twice in PBS, resuspended in serum-free RPMI-1640 medium at a density of 2.5 × 10^5 cells/L, and inoculated into the right axillary breast tissue of nude mice (0.2 mL/mouse × 50 mice). At 21 days post-inoculation, 29 mice with tumors ≥ 500 mm^3 were divided into four experimental groups: 1) Control (8 mice injected with PBS); 2) UTI (7 mice injected with 8000 U/mL UTI); 3) TAX (7 mice injected with 20 mg/kg TAX); and 4) UTI+TAX (7 mice injected with both UTI and TAX as in groups 2 and 3). All inoculations were i.p. For groups 1 and 2, 0.2 mL was injected per mouse every day for 20 days. For groups 3 and 4, 20 mg/kg was injected on days 1, 7, and 14. After 21 days, the mice were sacrificed for sample preparation. The maximum length (L) and the minimum diameter (D) of each tumor was measured using vernier calipers to calculate the tumor volume (cm^3). Tumor growth curves were constructed and tumor growth rates were calculated for each experimental group. We validated the synergistic or antagonistic effects of the drugs by calculating the q value using King’s formula. Synergistic, additive, or antagonistic effects were determined by q > 1.15, 1.15 > q > 0.85, q < 0.85, respectively. The formulas used were: tumor volume (cm^3) = (L2 × D)/2; tumor growth inhibition rate(%) = [1-(V1-V2)/(V3-V4)] × 100%, where V1 and V2 are the respective starting and ending average tumor volumes in the drug-treated groups and V3 and V4 are the respective starting and ending tumor volumes in the control group; and q = Ea+b/[(Ea+Eb)-Ea × Eb], where Ea, Eb, (Ea+Eb) represent the inhibitory rates of UTI, TAX, and UTI+TAX, respectively (King’s formula).

1.5 Quantitation of cell proliferation using the MTT assay
Cells were seeded into 96-well plates at a density of 4 × 10^3 cells per 200 µL per well. The cells were divided into four experimental groups (6 wells/group) as described in 1.4.1 and cultured in RPMI-1640 + 10% FBS. After 24, 48, and 72 h, 20 µL of 5 mg/ml MTT was added to each well for 4 h. Then 150 µL of DMSO was added to each well with shaking for 10 min. The absorbance (A) at 570 nm was measured using an enzyme-linked immunosorbant assay (ELISA) plate reader to quantitate the inhibitory rate. The experiment was repeated three times. Inhibitory rate (%) = (1-experimental group A570/control group A570) × 100%

1.6 MDA-MB-231 cell apoptosis
Adherent MDA-MB-231 cells were detached from their substrates by digestion with 0.125% EDTA-free trypsin, centrifuged for 5 min, resuspended, and rinsed by centrifugation in PBS at 4°C. The cell pellet was resuspended in 490 µL PBS containing 5 µL of FITC-Annexin and 5 µL of 250 µg/mL PI and incubated on ice for 10 min. After two rinses, the cells were analyzed by flow cytometry using a FACS Vantage SE from Becton-Dickinson, USA.

1.7 Detection of IL-6, IL-8, and TNF-α mRNA transcripts by RT-PCR
Based on the complete nucleotide sequences of IL-6, IL-8, TNF-α, and control gene β-actin supplied by GenBank, Primer 5.0 software was used by Nanjing Keygen Biotech Co. Ltd. to design and synthesize primers for reverse transcriptase-polymerase chain reaction (RT-PCR). The product lengths for IL-6, IL-8, TNF-α, and β-actin were 84, 160, 108, and 136 base pairs, respectively. The primer pairs used were:
IL-6 sense: 5’ AAATTCCGTACATCCTCAGAC 3’,
IL-6 anti-sense: 5’ CCTCTTTTGCCTTTCACAC 3’,
IL-8 sense: 5’ TACTTCAACCTTTCCAGCC 3’, IL-8 anti-sense: 5’ AAAACTTCTCCACACCTCCT 3’,
TNF-α sense: 5’ GCCCTGCCTCCTTGGAGTG 3’,
TNF-α anti-sense: 5’ TCGGGGTTCGAGATGTAT 3’,
β-actin sense: 5’ GCAGAAAGGATACACAGCCCT 3’, and β-actin anti-sense: 5’ GCTGATCCACATCTGC 3’.

The SYBR Green/ROX qPCR master mix was used with initial denaturation at 95°C for 5 min followed by:
45 cycles of denaturation at 94°C for 15 s; annealing at
60°C for 30 s; and extension at 55°C for 1 min, and
1 min extension at 95°C. The luminescence signal was measured during the extension process. The transcrirical
cycle (Ct) was analyzed using the PCR apparatus pro-
dure and copy numbers were calculated from 2^-ΔΔCt, the
copy number ratio of expanding target genes and the
internal control gene (β-actin) to determine the mRNA
expression levels of the target genes.

1.8 Detection of IL-6, IL-8, and TNF-α cytokines in
xenografted tumors by immunohistochemistry
Carcinoma tissues were dehydrated using a graded series
from 75, through 80 and 95, to 100% ethanol. Dehy-
drated samples were completely immersed in wax, cut
into 5 μm sections, and mounted on 3-triethoxysilylpro-
pylamine (APES)-treated glass. Sections were treated with
50 μL non-immune animal serum plus 50 μL of a
1:50 dilution of anti-IL-6, IL-8, and TNF-α antibodies
for 10 min. PBS was used as a negative control. Primary
antibody incubations were followed by 50 μL of biotin-
labeled secondary antibody and 50 μL of streptavidin-
peroxidase (SP) solution for 10 min. The sections were
rinsed with PBS three times for 3 min and 100 μL of
fresh DAB chromogenic substrate solution was added.
Sections were examined microscopically for color devel-
opment for 5-10 min, redyed with hematoxylin (HE), re-
blued with saturated lithium carbonate, dehydrated with
the graded ethanol series (as above), and sealed in neu-
tral gum.

Imaging of all immunohistochemical sections was per-
formed using a Leica microscope electronic imager. The
appearance of tan color or tan particles indicated a posi-
tive reaction in the cells. We performed IOD analysis on
the sections in each group using Image Pro-plus v6.0
software to compare the differences between the group.

1.9 Statistical analysis
All data were analyzed using PASW 18.0 software and
represented as X ± s. The variance analysis was adopted for
comparisons between groups. P < 0.05 was considered
to be statistically significant.

Results
2.1 Effects of UTI and TAX on MDA-MB-231 cell
proliferation
Relative to the control group, the growth of MDA-MB-
231 cells treated with UTI, TAX, and UTI+TAX for
24 h was significantly inhibited (P < 0.05; Table 1). The
inhibitory effect increased in a time-dependent manner
when the cells were treated for 48 and 72 h (P < 0.01;
Table 1). The strongest inhibitory effect was produced
by co-treatment with both drugs and the weakest effect
occurred with UTI alone (UTI+TAX > TAX > UTI).
The differences were statistically significant (P < 0.01;
Table 1).

2.2 Effects of UTI and TAX on MDA-MB-231 cell apoptosis
Compared to the control group (1.00), the level of
apoptosis increased to 1.84 for the UTI group, 3.90 for
the TAX group, and 6.79 for the UTI+TAX group
(Table 2).

2.3 Expression of IL-6, IL-8, and TNF-α mRNA in
MDA-MB-231
Treatment of MDA-MB-231 cells with both UTI and TAX
down-regulated the expression of IL-6, IL-8, and TNF-α
transcripts greater than treatment with either UTI or TAX
alone (P < 0.05; Figure 1, Figure 2, Figure 3).

2.4 Effects of UTI and TAX on the growth of ed breast
tumor xenografts
One mouse in the control group died on day 13 and
one mouse in the UTI group died on day 18 due to con-
sumption and cachexia. The 7 tumors in the control
group enlarged in a time-dependent manner, with no
spontaneous tumor deflation or regression. For the
6 mice in the UTI group, the volume of their xeno-
grafted tumors gradually increased at a rate less than
that of the mice in the control group (P < 0.05). For the
7 mice in the TAX group, the volume of their xeno-
grafted tumors gradually increased at a rate less than
that of the mice in the control group (P < 0.05). For the
6 mice in the UTI group, the volume of their xeno-
grafted tumors decreased with the greatest rate
and extent over time (P < 0.05; Table 3; Figure 4).

2.5 Effects of UTI and TAX on the expression of IL-6, IL-8,
and TNF-α proteins in breast tumor xenografts
Relative to untreated MDA-MB-231 tumor xenografts,
the xenografts from mice treated with UTI, TAX, and
UTI+TAX showed decreased expression of IL-6 (Figure 5, Figure 6), IL-8 (Figure 7, Figure 8), and
TNF-α (Figure 9 Figure 10) proteins. Treatment with
UTI+TAX decreased cytokine expression greater than
treatment with either UTI or TAX alone (P < 0.01;
Figures. 5,6,7,8,9,10).
Discussion

Ulinastatin (UTI) is a serine protease inhibitor (SPI) with extensive inhibitory effects on cell proliferation and extracellular matrix degradation. Consequently, the protection of patients in radiotherapy and chemotherapy becomes an important consideration for researchers. The experiment of Kobayashi [1] showed that UTI inhibited human ovarian cancer and the effect could be related to UTI down-regulation of protein kinase C (PKC), which regulates the methionine/extracellular-signal of the MEK/ERK/c-Jun-dependent signal pathway to collaboratively down-regulate the plasminogen activator urokinase. The application of UTI and etoposide can enhance the inhibition of metastasis in Lewis lung carcinoma (3LL) [2]. Our experiments show that UTI can inhibit the growth of xenografted breast carcinoma tumors with the co-application of both UTI and TAX being most effective.

As one of the core cytokines, interleukin-6 (IL-6), is produced by lymphocytes, mononuclear cells, fibroblasts, vascular endothelial cells, and some cancer cells, primarily in autocrine and paracrine secretions. After secretion, IL-6 combines with the α-subunit of the membrane-bound IL-6 receptor (IL-6R) and the β-subunit of glycoprotein 130 (gp130) for cell signaling. Goswami [3] used an anti-IL-6 primary antibody to inhibit the proliferation of human glioblastoma multiforme cells, demonstrating that IL-6 has some effect on promoting tumor cell proliferation. Burger [4] also reported that cancer cells and tumor-related macrophages can release high concentrations of IL-6. Hussein [5] showed that high-levels of IL-6 indicate poor prognosis and the concentration of IL-6 in the serum of breast cancer patients is not only elevated, but increases with the clinical stage of breast cancer. Sasser [6] found that the growth rate of MCF-7 estrogen-receptor-positive (ER+) breast carcinoma cells doubled in vitro and increased even more in vivo following treatment with recombinant human IL-6. Our results show that UTI inhibits the expression of IL-6.

Interleukin-8 (IL-8) is produced by monocytes, macrophages, T cells, and vascular endothelial cells. UTI enables neutrophil chemotaxis, defluvium, and lyase release. Additionally, UTI can protect against inflammation, promote T cell chemotaxis, and reinforce the immune response. Heideman [7] suggested that IL-8 promotes leukin chemotaxis into tumors, leading to tumor neovascularization and the acceleration of tumor growth and metastasis. IL-8 enters cells by combining with the chemokine receptor CXCR1, to activate the extracellular ERK2/1 signaling pathway and promote the formation of new microvessels. It has been reported that the expression of IL-8 in breast carcinoma cells is inversely proportional to the level of estrogen receptors (ER). Based on this relationship, decreased expression of ER increases the expression of IL-8, leading to increased tumor deterioration [8]. Our prophase experiment showed that UTI can inhibit the expression of CXCR4 [9], which is produced by stroma derived factor-1. In the present study, UTI and TAX inhibited the expression of IL-8 in xenografted breast tumors in nude mice.

TNF-α is a peptide hormone that affects tumor cell necrosis, inflammation, and the immune response. The effects of TNF-α are widespread and mediated through nearly all of the TNF-α receptors on tumor cells and many other cells. Gong [10] demonstrated that increased TNF-α promotes invasion and metastasis in ductal carcinomas in a scalar fashion. The TNF secreted by tumor-related macrophages can enhance the invasion of tumors by increasing the expression of matrix metalloproteases (MMPs) in breast carcinoma and vascular endothelial growth factor (VEGF) in the c-Jun N-terminal kinase (JNK) and the NF-KB signaling pathways [11]. Also, the inflammatory cells of the tumor microenvironment, consisting primarily of tumor-related macrophages, can secrete TNF-α continuously to promote tumor formation, invasion, and metastasis via activation of protein-1 (AP-1) and the NF-KB pathway [12].

### Table 1 Effects of UTI and TAX on the proliferation of human breast cancer MDA-MB-231 cells in vitro (A570, x ± s)

| Groups     | 24 h         | 48 h         | 72 h         |
|------------|--------------|--------------|--------------|
|            | A value (x ± s) | Inhibition rate (%) | A value (x ± s) | Inhibition rate (%) | A value (x ± s) | Inhibition rate (%) |
| Control    | 1.086 ± 0.082 | 0            | 1.366 ± 0.042 | 0                  | 1.881 ± 0.106 | 0                  |
| UTI        | 1.000 ± 0.067 | 7.919        | 0.867 ± 0.102 | 36.530             | 0.631 ± 0.067 | 66.454             |
| TAX        | 0.853 ± 0.051 | 21.455       | 0.703 ± 0.043 | 48.536             | 0.440 ± 0.063 | 76.608             |
| UTI+TAX    | 0.773 ± 0.041 | 28.821       | 0.590 ± 0.059 | 56.808             | 0.315 ± 0.068 | 83.254             |

* P < 0.05 for all treatment groups versus control; b P < 0.01 for TXT and UTI+TAX groups versus UTI group; c P < 0.01 for UTI+TAX group versus TAX group.

### Table 2 Apoptosis of MDA-MB-231 cells treated with different drugs

| Treatment     | Apoptotic rate(%) | Fold increase |
|---------------|-------------------|---------------|
| Control       | 2.52 ± 0.53       | 0             |
| UTI           | 7.16 ± 1.59       | 1.84          |
| TAX           | 12.35 ± 1.88      | 3.90          |
| UTI+TAX       | 19.64 ± 2.26      | 6.79          |

Data expressed as mean ± sd. Note: p < 0.05 among different treatments.
Our *in vitro* experiments show that UTI can inhibit the proliferation and invasion of MCF-7 human breast carcinoma cells [9] and the growth of MDA-MB-231 (present study). Taken together, these effects could be related to the down-regulation of MMP-9 in breast carcinoma cells by UTI [13]. We show here that both UTI and TAX inhibit the expression of TNF-α.

Ulinastatin (UTI) and docataxel (Taxotere, TAX) inhibit the growth of MDA-MB-231 human breast cancer cells cultured *in vitro* and xenografted into nude mice.
Figure 5 Effects of UTI and TAX on IL-6 protein expression in human breast cancer xenografts in immunohistochemistry:
1. Control group SP × 400 2. UTI group SP × 400, 3 TAX group SP × 400 4. UTI+TAX group SP × 400.

Figure 7 Effects of UTI and TAX on IL-8 protein expression in human breast cancer xenografts in immunohistochemistry:
1. Control group SP × 400 2. UTI group SP × 400, 3 TAX group SP × 400 4. UTI+TAX group SP × 400.

Figure 6 Effects of UTI and TAX on IL-6 protein expression in human breast cancer xenografts in histogram.

Figure 8 Effects of UTI and TAX on IL-8 protein expression in human breast cancer xenografts in histogram.
in vivo. The combination of both drugs is stronger than either drug alone under the conditions tested. The growth inhibition of human breast carcinoma cells and tumors could be related to the concomitant down-regulation of IL-6, IL-8, and TNF-α in breast carcinoma cells by these drugs.

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Authors’ contributions
XZ did the MTT essay and immunohistochemistry, XS did the Cell-culturing, submitted paper and revised the paper, FG did the medical statistics, JL cultured the cell and did PCR, ZS designed this experiment and wrote this paper. All authors read and approved this final draft.

Competing interests
The authors declare that they have no competing interests.

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