Imatinib enhances docetaxel-induced apoptosis through inhibition of nuclear factor-κB activation in anaplastic thyroid carcinoma cells

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Running title: Combination of Imatinib and Docetaxel in ATC cells

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Abstract

Background: We previously reported the partial effectiveness of imatinib (also known as STI571, Glivec, or Gleevec) on anaplastic thyroid cancer (ATC) cells. Imatinib is a selective tyrosine kinase inhibitor which has been used for various types of cancer treatments. Recently, several reports have demonstrated that imatinib enhanced the sensitivity of cancer cells to other anti-cancer drugs. In this study, therefore, we investigated whether imatinib enhances anti-tumor activity of docetaxel in ATC cells.

Methods: Two ATC cell lines, FRO and KTC-2 were treated with imatinib and/or docetaxel. Cell survival assay and flow cytometry for annexin V were used to assess the induction of apoptosis. Changes of pro- and anti-apoptotic factors were determined by Western blot. NF-κB activity was measured by DNA-binding assay. Tumor growth was also investigated *in vivo*.

Results: The combined treatment significantly enhanced apoptosis compared to single treatment. ATC cells themselves expressed high levels of anti-apoptotic factors, XIAP and survivin. The treatment with docetaxel alone further increased their expressions; however, the combined treatment blocked the inductions. Although imatinib alone had no effect on NF-κB background levels, combined treatment significantly suppressed the docetaxel-induced NF-κB activation. Furthermore, the combined administration of the drugs also showed significantly greater inhibitory effect on tumor growth in mice xenograft model.

Conclusions: Imatinib enhanced anti-tumor activity of docetaxel in ATC cells. Docetaxel seemed to induce both pro- and anti-apoptotic signaling pathways in ATC cells, and imatinib blocked the anti-apoptotic signal. Thus, docetaxel combined with imatinib emerges as an attractive strategy for ATC treatment.

Introduction

Anaplastic thyroid cancer (ATC) is one of the most aggressive human malignant tumors. Multi-disciplinary treatments including radio- and chemotherapy still poorly control the progression of this disease, and its mean survival is less than one year after initial diagnosis (1). Therefore, there is a critical need to develop a novel approach to treat patients with ATC.

Docetaxel is an anticancer microtubule-stabilizing agent which induces apoptosis by suppressing the microtubule dynamics of mitotic apparatus. Docetaxel also arrests cells in G2/M phase, leading to increase of sensitivity of cancer cells to radio- and chemotherapy (2-4). Although the effect of the drug alone was only modest against ATC in a clinical trial (5), combined therapy with radiation and/or another type of drug may be effective (6-8).

Imatinib (also known as STI571, Glivec, or Gleevec) is a selective tyrosine kinase inhibitor and was originally developed to inhibit BCR/ABL fusion oncoprotein expressed in chronic myelogenous leukemia (CML). It also cross-reacts with other tyrosine kinases such as c-ABL, c-KIT, and platelet-derived growth factor receptors (PDGFRs) and has been used for the treatment of gastrointestinal stromal tumor, small cell lung cancer, ovarian cancer, and colorectal carcinoma (9-13). In preclinical cancer models, we have already reported the partial effectiveness of imatinib on ATC cells (14). We have also demonstrated that imatinib combined with ionizing radiation enhanced senescence-like growth arrest (SLGA) (15). Very recently, a clinical trial using imatinib as a single agent for the treatment of ATC has shown weak responses: no complete response, 25% partial response and 50% stable disease at 8 weeks but the rate of 6-month survival was only 45% (16).

Several reports have demonstrated that imatinib enhanced the sensitivity of various cancer cells to anti-cancer drugs (9, 17). A few studies have investigated the anti-cancer efficacy of imatinib/docetaxel combination in preclinical models. It was shown that the combination of imatinib and docetaxel was significantly more effective than either agent alone in the non-small cell lung carcinoma xenograft model. In this model,

imatinib, as an inhibitor of PDGF-Rβ, decreased microvessel density and interstitial fluid pressure, and thereby improved subsequent delivery of docetaxel (18). Another report showed that in human CML cells, the imatinib/docetaxel combination induced apoptosis through decreasing mitochondrial membrane potential and increasing caspase-3 enzyme activity (19). Kinsella *et al.* reported that imatinib combined with docetaxel strongly inhibited both proliferation and invasion, and had a pro-apoptotic effect in glioma cells (20). So far, there has been no report studying the therapeutic efficacy of the imatinib/docetaxel combination in ATC.

In this study, we demonstrate that imatinib enhanced anti-tumor activity of docetaxel in ATC cells. Docetaxel seemed to induce both pro-apoptotic and anti-apoptotic signaling pathways, and imatinib blocked the anti-apoptotic signal through inhibition of docetaxel-induced NF-κB activation.

Materials and Methods

Reagents

Imatinib (Novartis, Basel, Switzerland) was dissolved in dimethylsulfoxide (DMSO) at stock concentration of 7 mM for *in vitro* experiments, and for *in vivo* experiments, imatinib tablets were dissolved in distilled water, and insoluble material was removed by repeated centrifugation at 2,500 g as described previously (21). Docetaxel (Wako Chemicals, Osaka, Japan) was dissolved in DMSO at a stock concentration of 1 mM. The antibodies to p65, survivin, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the antibodies to X-linked inhibitor of apoptosis (XIAP), cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), IκBα, and anti-rabbit and anti-mouse HRP-conjugated antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human ATC cell line, FRO was originally provided by Dr James A. Fagin (University of Cincinnati College of Medicine, Cincinnati, OH, USA; currently Memorial

Sloan-Kettering Cancer Center, New York, NY, USA), and KTC-2 was from Dr. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan) (22). All cells were grown in RPMI 1640 (Wako Chemicals) supplemented with 5% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin (Wako Chemicals).

Cell growth assay

Cells were seeded onto each well of 24-well plate (500 μL, 15 x 10^3 cells per well) and incubated for 24 h before treatment. Solutions containing various concentrations of docetaxel and/or imatinib were added to each well in 55 μL medium, with three wells used for each concentration. In the control wells, DMSO was added, and the final concentration of DMSO in any well did not exceed 0.2% (v/v). After incubations, the number of cells were counted with a Coulter counter (Beckman Coulter, Fuller, CA, USA).

Flow cytometry analysis with the annexin V/PI staining

Detection of apoptotic cells was performed with an Annexin V-PI apoptosis detection kit (Wako Chemicals) according to the manufacturer's instructions. In brief, 4×10^5 cells were double stained with FITC-conjugated Annexin V and propidium iodide (PI) for 15 min at room temperature in a Ca^{2+}-enriched binding buffer and then analyzed on a FACS Vantage SE (BD Biosciences, San Jose, CA, USA). FITC and PI emissions were detected in the FL-1 and FL-3 channels, respectively. Analysis was done with Cell Quest software (BD Biosciences).

Senescence-Associated β-Galactosidase (SA-β-gal) staining

The SA-β-gal staining was performed as described elsewhere (23). Briefly, after experimental treatment, cells on plates were fixed with 2% (v/v) formaldehyde/0.2% (v/v) glutaraldehyde, washed with PBS and assayed for SA-β-gal activity using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) at pH 6.0. SA-β-gal^+ cells were detected by bright-field microscopy.

Western blotting

Forty micrograms of protein were separated with SDS-PAGE and transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA). After incubation with an appropriate primary antibody, the antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and a chemiluminescence system (Nacalai Tesque, Kyoto, Japan) Detection was performed using a LAS3000 imaging system (FUJIFILM, Tokyo, Japan).

DNA-binding assay

The multiwell colorimetric assay for active NF-κB was performed as described previously (24). Briefly, equal amount of nuclear extracts were incubated in 96-well plate coated with immobilized oligonucleotide containing a NF-κB consensus binding site. NF-κB binding to the target oligonucleotide was detected with primary antibody specific for p65 subunit and HRP-conjugated secondary antibody. For quantification of activity, ODs were measured at 450 nm using a microplate reader 2030 ARVO X (PerkinElmer Inc., Waltham, MA, USA).

***In vivo* xenograft model**

All mice were maintained at Nagasaki University animal facility, and all animal experiments described in this study were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals of Nagasaki University. FRO cells (3×10^6) resuspended in RPMI 1640 were injected *s.c.* into both flanks of 6-week-old male BALB/c *nu/nu* mice (CLEA Japan, Tokyo, Japan), five animals per group. Then they were randomly assigned into four groups. The tumor sizes were measured every three days with calipers, and tumor volumes were calculated according to the formula: $a^2\times b\times 0.4$, where a is the smallest tumor diameter and b is the diameter perpendicular to a. Imatinib solution in sterile water/PBS (ratio 1:1) was injected *i.p.* daily for 2 weeks at a dose of 50 mg/kg beginning from day 9 after tumor implantation. Docetaxel, diluted in PBS/DMSO (ratio 1:1), was injected *i.p.* at a dose of 5 mg/kg on day 9 and 16. Combined treatment mice were given both drugs. Control group mice received vehicle injections only. For 27 days, tumor size was monitored, and body weight, feeding behavior, and motor activity of each animal were monitored

as indicators of general health.

Statistical Analysis

All data were expressed as the mean ± SD. Differences between groups were examined for statistical significance with ANOVA followed by Tukey's post test. A p value not exceeding 0.05 was considered statistically significant.

Results

Inhibition of cell growth by docetaxel and/or imatinib

To investigate the effect of docetaxel and/or imatinib, cell growth assay was done. Our previous experiments (7, 14); (unpublished data) demonstrated that moderate cell growth inhibition by imatinib and docetaxel was observed at concentrations of 7 μM and 1~2 nM in FRO cells, respectively. Thus, we conducted the experiments using 7 μM of imatinib and 1 nM of docetaxel. As shown in Fig. 1, both docetaxel and imatinib had moderate inhibitory effect on the growth of FRO cells, and combined treatment significantly reduced the cell number compared to single treatment (Fig. 1). We also used another ATC cell line, KTC-2 cells, in which the growth inhibition by imatinib was less effective compared to FRO cells, and the potency of docetaxel was more pronounced. The combined treatment similarly inhibited the cell growth, almost completely (Fig. 1). The rates of growth reduction at 4 days in docetaxel, imatinib, and combined treatment were 47.3%, 68.5%, and 96.0% in FRO cells and 76.1%, 29.6%, and 99.5% in KTC-2 cells, respectively (Fig. 1).

Apoptotic changes in cells treated with the drugs

Although the degree of cell detachment was more notable in the combined treatment than in single treatment group, we first explored the possibility of involvement of senescence-like terminal growth arrest (SLGA) because we previously demonstrated

that imatinib plus radiation therapy enhanced SLGA in ATC cells (15). Neither single treatment nor combined treatment did induce SA-β-gal activity in both FRO and KTC-2 cell lines, suggesting that the growth inhibitory effect was not due to SLGA (data not shown). Next, we examined whether the effect was associated with apoptosis. Cells were treated with the drugs for 16 hours and then double stained with FITC-conjugated annexin V and PI to look at early apoptotic response (right lower quadrant in each dot plot in Fig. 2) and subsequent cell death (right upper quadrant). Imatinib barely caused apoptosis, and docetaxel moderately induced it. On the other hand, the combined treatment further increased apoptosis (Fig. 2).

Effects of docetaxel and imatinib on pro-apoptotic and anti-apoptotic factors

We next examined the status of several key proteins involved in apoptosis by Western blotting. After ATC cells were treated with docetaxel, the cleavages of caspase-3 (19 kDa and 17 kDa) and PARP (89 kDa) levels were induced (Fig. 3). The cleaved PARP and caspase-3 were further increased by the combined treatment, suggesting stronger apoptotic change (Fig. 3). XIAP and survivin belong to the human inhibitors of apoptosis (IAP) family, and their overexpression in cancer cells suggests an important role for these proteins in cancer progression. We tested whether docetaxel and/or imatinib modulate the expression of these anti-apoptotic gene products by Western blotting. As shown in Fig. 3, although ATC cells themselves expressed high levels of XIAP and survivin, docetaxel further increased the levels (Fig. 3). Single treatment of imatinib did not change their background levels; however, the increased docetaxel-induced XIAP and survivin expression was suppressed in the combination treatment (Fig. 3).

Docetaxel induces NF-κB activation, and imatinib inhibits the effect

Since we already reported that the expression of those anti-apoptotic factors was regulated through NF-κB signaling pathway in ATC cells (7), we next performed DNA-binding assay using nuclear extracts to assess NF-κB activity. In both FRO and KTC-2 cell lines, the binding activity of nuclear p65 was increased by docetaxel treatment. Although imatinib alone had no effect on NF-κB background levels, in the combined treatment, imatinib significantly suppressed the docetaxel-induced NF-κB activation (Fig. 4A). A similar trend was observed in the amount of nuclear p65 (Fig. 4B). We also checked IκBα expression. As shown in Fig. 4B, the combined treatment reduced IκBα protein level, consistent with our previous studies (7, 25). NF-κB is known to bind the IκBα promoter and activate its synthesis, and therefore the inhibition of NF-κB probably suppressed *de novo* synthesis of IκBα. Presumably, for the same reason, IκBα expression after docetaxel treatment was not changed (Fig. 4B).

In vivo effects of the combined treatment with docetaxel and imatinib

To explore the effects of the combined treatment *in vivo*, we used an animal xenograft model inoculated with FRO cells, and the treatments were done as described in Materials and Methods section. As shown in Fig. 5, the mean tumor size of imatinib-treated group was smaller than that of control, but there was no statistical difference. Although the docetaxel treatment alone was able to delay tumor growth moderately, the effect of the combined treatment with docetaxel and imatinib was far greater. The body weight and physical activity of mice exposed to drugs were not significantly affected.

Discussion

We and other groups have reported that taxanes (including paclitaxel and docetaxel) induce both pro- and anti-apoptotic signaling pathways, and the anti-apoptotic factors

are induced through NF-κB activation (7, 26, 27). NF-κB is a transcription factor that regulates genes involved in cellular proliferation and survival (28). In most cases, NF-κB suppresses apoptosis by up-regulation of anti-apoptotic proteins, including inhibitor of apoptosis (IAP), such as c-IAP, BCL-xL, XIAP, and survivin (29, 30). In addition, basal NF-κB activity is often increased in various types of human cancers, which causes chemotherapy resistance (31). Under certain conditions, cytotoxic drugs such as taxanes induce NF-κB activation in different types of malignant cells (26, 32-36). The present data also showed that docetaxel induced NF-κB activation and then up-regulated anti-apoptotic factors in two ATC cell lines, FRO and KTC-2.

In our experiments, imatinib seemed to enhance apoptosis presumably through the inhibition of docetaxel-induced NF-κB activation. There are several reports proposing the mechanism by which imatinib inhibits the docetaxel-induced NF-κB activation. The PI3K/AKT pathway has been associated with cancer cell resistance to chemotherapeutic drugs including paclitaxel (34, 37). Possible mechanisms of such resistance may be due to the activation of the NF-κB pathway via PI3K/AKT activation (38). Qian *et al.* compared the gene expression profiles in individual human prostate cancer specimens before and after chemotherapy and showed that docetaxel treatment increased *CCL2* expression (39). They also showed that up-regulation of *CCL2* contributed to chemotherapy resistance through stimulating MAP kinase and PI3K/AKT signaling pathways. Several other reports have demonstrated that imatinib inhibited the PI3K/AKT pathway, resulting in the inhibition of NF-κB activation in cancer cells. Fang *et al.* reported that imatinib induced apoptosis in BCR-ABL-positive human leukemia cells in association with the down-regulation of anti-apoptotic factors such as XIAP through the inhibition of AKT and NF-κB activities (40). Xu *et al.* reported that imatinib inhibited IR-induced RelB nuclear translocation by decreasing the phosphorylation levels of PI3K (Tyr458) and AKT (Ser473) in androgen-independent

prostate cancer cells (41). They showed that imatinib inhibited PI3K tyrosine phosphorylation, leading to the down-regulation of the AKT/IKKα-activated NF-κB pathway. We examined whether docetaxel and/or imatinib modulate the phosphorylation level of AKT. However, we could detect neither the activation of AKT by docetaxel treatment nor the inhibition of AKT by imatinib (data not shown). Further experiments are necessary to clarify the exact mechanism by which imatinib inhibits the docetaxel-induced NF-κB activation in ATC cells.

Data obtained in our experiments showed that combination of docetaxel and imatinib effectively killed ATC cells, both *in vitro* and *in vivo*. Based on our and other's findings, we propose a mechanistic scheme in Fig. 6. As previously shown, docetaxel activates both pro-apoptotic and anti-apoptotic signals. In pro-apoptotic pathway, docetaxel binds to microtubules, impairs mitosis, and induces apoptosis. In anti-apoptotic pathway, docetaxel also induces NF-κB activation and in turn increases the expression of anti-apoptotic molecules. Imatinib inhibits the docetaxel-induced NF-κB activation (but does not reduce the basal NF-κB level, even though it is high). By this mechanism, imatinib presumably modulates the balance between pro- and anti-apoptotic signals and enhances docetaxel-induced apoptosis.

In conclusion, our present study demonstrates that imatinib enhanced anti-tumor activity of docetaxel in ATC cells, suggesting that this combination may be a promising approach for the treatment of ATCs. Since docetaxel and imatinib have already been approved and currently being used for other type of cancers, this combination strategy can be rapidly applied to clinical trial.

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Author Disclosure Statement

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Cytotoxic effect of docetaxel and/or imatinib on anaplastic thyroid cancer cells

Growth of FRO and KTC-2 cells treated with 1 nM of docetaxel and/or 7 μM of imatinib for 2 and 4 days were determined by cell count assay. Each point represents mean ± SD. *p<0.01 *vs.* any other group. Similar results were obtained in three independent experiments.

Fig. 2. Apoptotic changes in cells treated with drugs

FRO cells were treated with the drugs (1 nM docetaxel and/or 7 μM imatinib) for 16 h and then double stained with FITC-conjugated annexin V and PI, then analyzed on a FACS Vantage SE. FITC and PI emissions were detected in the FL-1 and FL-3 channels, respectively. The cells in left lower, right lower, and right upper quadrant represent viable cells, early apoptotic cells, and terminal stage of apoptotic or necrotic cells, respectively. Data are representative of two independent experiments.

Fig. 3. Effects of docetaxel and imatinib on apoptotic factors

Cells were treated with the drugs (4 nM docetaxel and/or 7 μM imatinib) for 16 hours (XIAP, survivin) or 24 hours (caspase 3, PARP), and whole-cell lysates were examined by Western blotting. β-actin was used as a loading control. Data are representative of at least two independent experiments.

Fig. 4. Docetaxel induces NF-κB activation, and imatinib inhibits the effect

A, Cells were treated with 4 nM docetaxel and/or 7 μM imatinib for 24 (FRO) and 48 hours (KTC-2). Nuclear extracts were prepared and subjected to DNA-binding assays. Bars represent mean ± SD of three wells. *p<0.001. B, FRO cells were treated with 4 nM docetaxel and/or 7 μM imatinib for 16 hours, and then nuclear extracts (for nuclear p65) or total cell lysates (for IκBα) were examined by Western blotting. β-actin was used as a loading control. Similar results were obtained in at least two independent experiments.

Fig.5. Effect of docetaxel and imatinib in FRO tumor xenograft model

FRO cells were implanted and imatinib was injected *i.p.* at a dose of 50 mg/kg/day for 14 days, beginning on day 9 after tumor implantation. Docetaxel was injected *i.p.* at a dose of 5 mg/kg on day 9 and 16. Combined treatment mice were given both drugs. Control group mice received vehicle injection only. Each point represents mean ± SD of 10 tumors (in five mice). *$p<0.01$ *vs.* any other group.

Fig.6. A proposed mechanistic model of enhancement of docetaxel-induced apoptosis by imatinib

Fig. 1

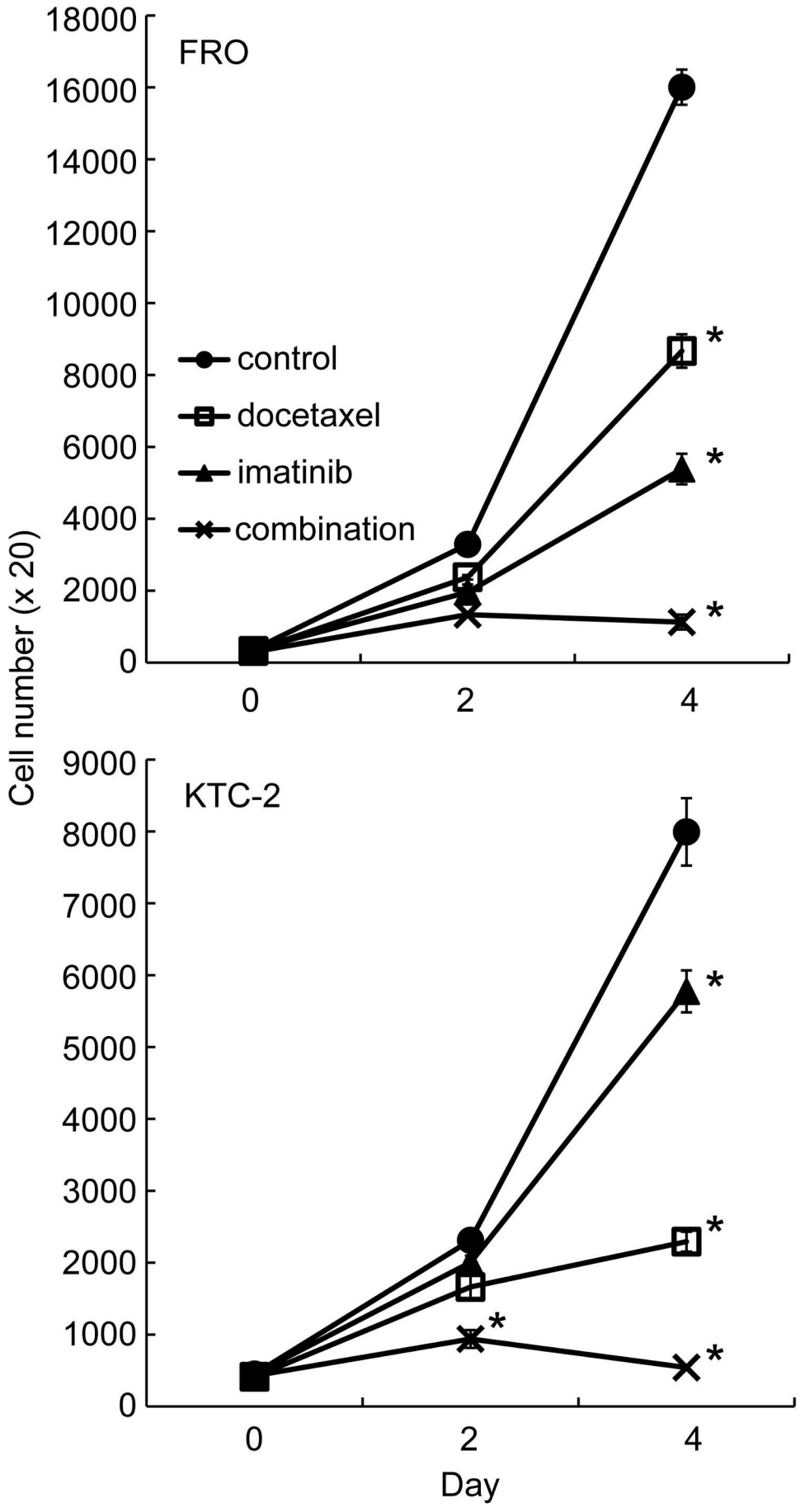

FRO
KTC-2
control
docetaxel
imatinib
combination
Cell number (x 20)
Day
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
9000
7000
5000
3000
1000
0
2
4
*

Fig. 2

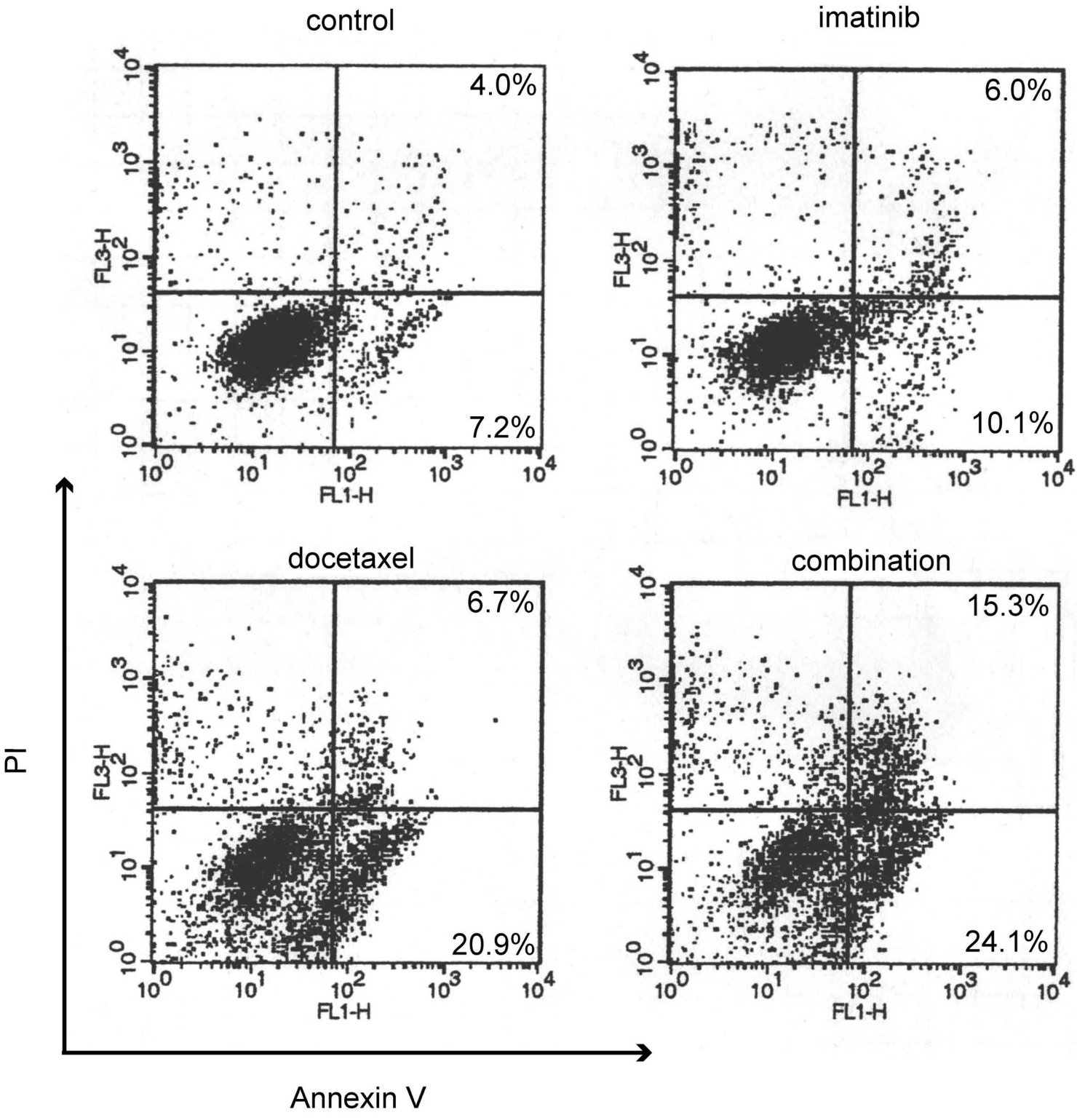

control
4.0%
7.2%
imatinib
6.0%
10.1%
docetaxel
6.7%
20.9%
combination
15.3%
24.1%
FL3-H
FL1-H
PI
Annexin V

Fig. 3

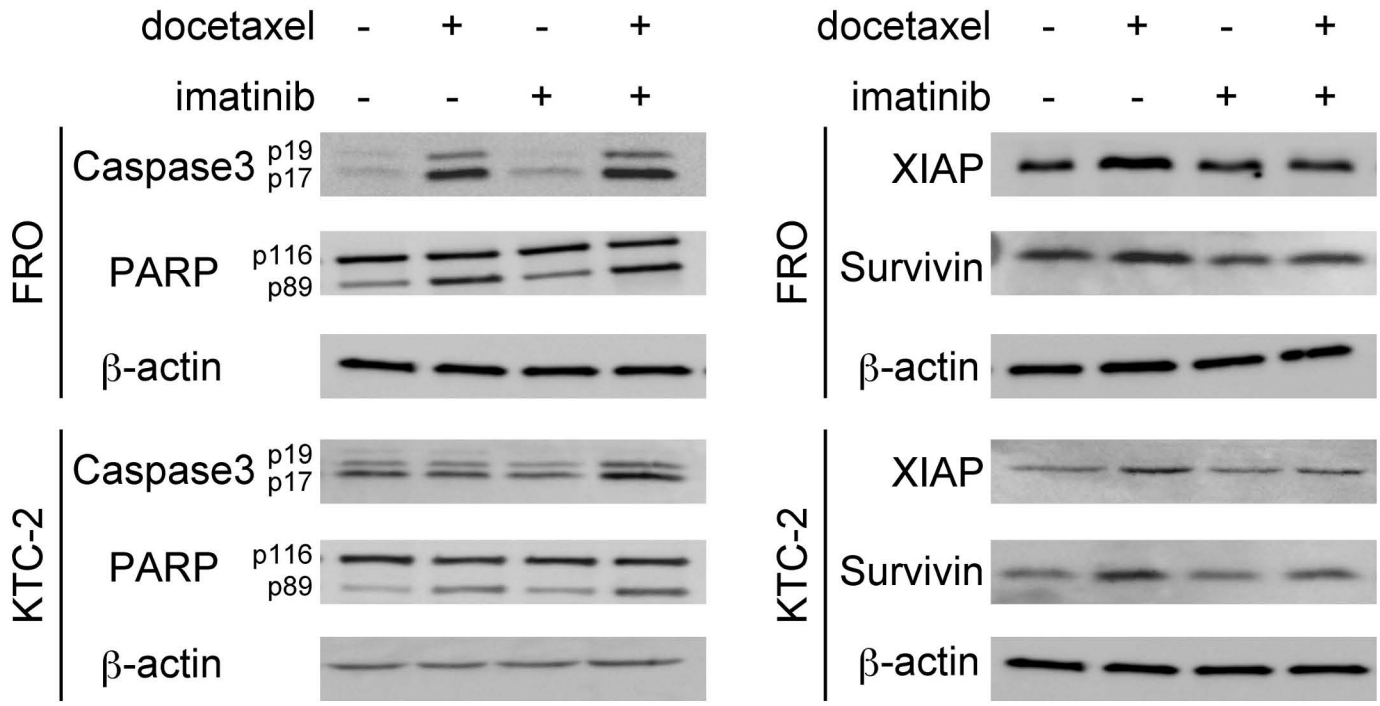
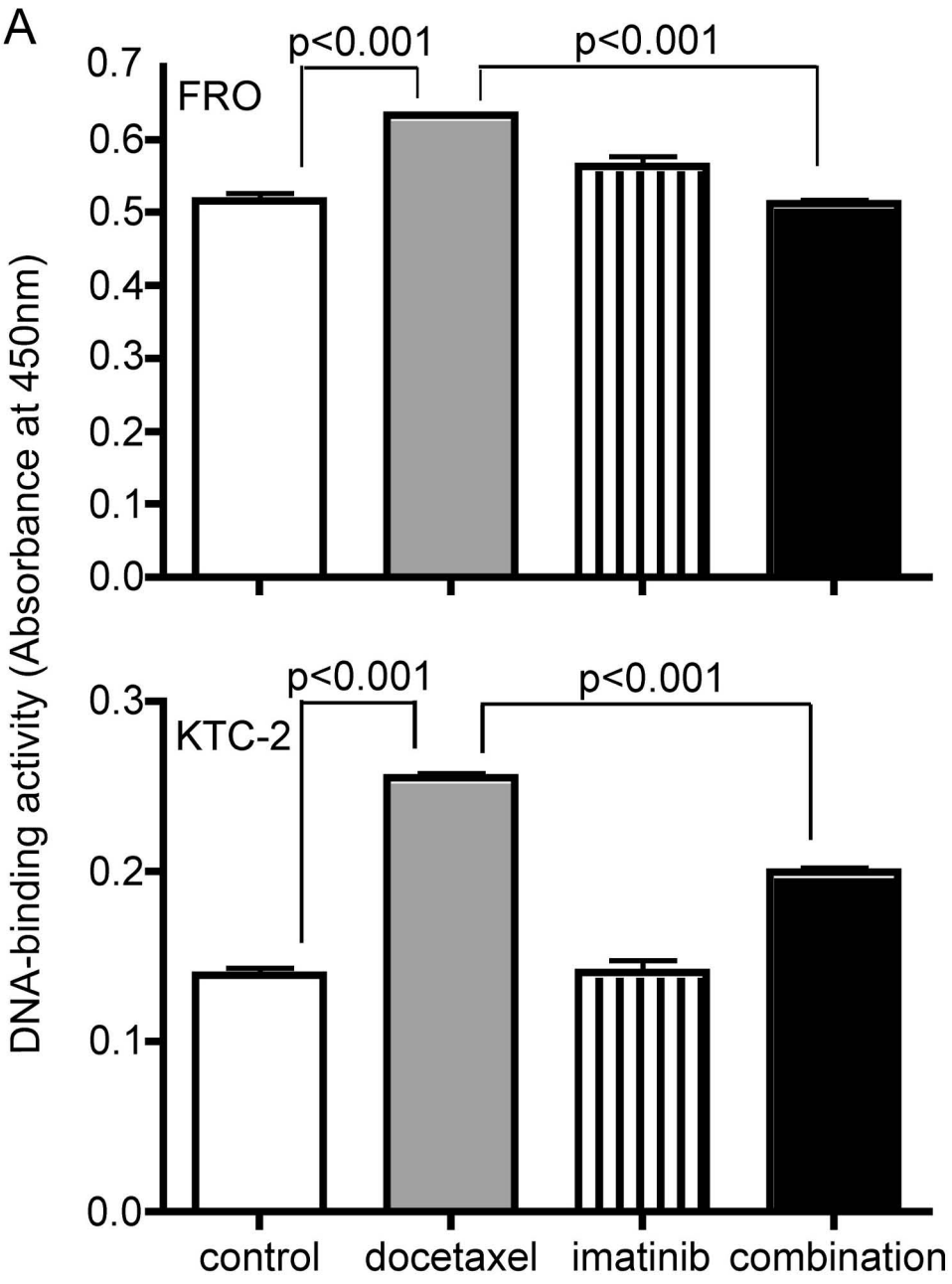

docetaxel - + - +
imatinib - - + +
FRO
Caspase3
p19
p17
PARP
p116
p89
β-actin
KTC-2
Caspase3
p19
p17
PARP
p116
p89
β-actin
docetaxel - + - +
imatinib - - + +
FRO
XIAP
Survivin
β-actin
KTC-2
XIAP
Survivin
β-actin

Fig. 4


A
p<0.001
p<0.001
FRO
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DNA-binding activity (Absorbance at 450nm)
p<0.001
p<0.001
KTC-2
0.3
0.2
0.1
0.0
control
docetaxel
imatinib
combination

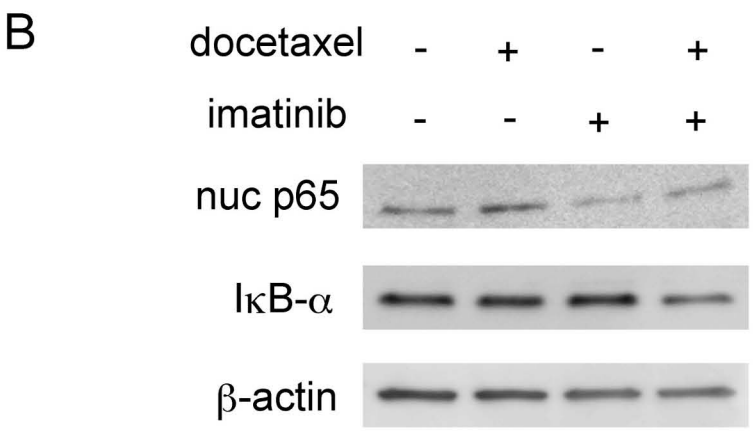

B
docetaxel - + - +
imatinib - - + +
nuc p65
IκB-α
β-actin

Fig. 5

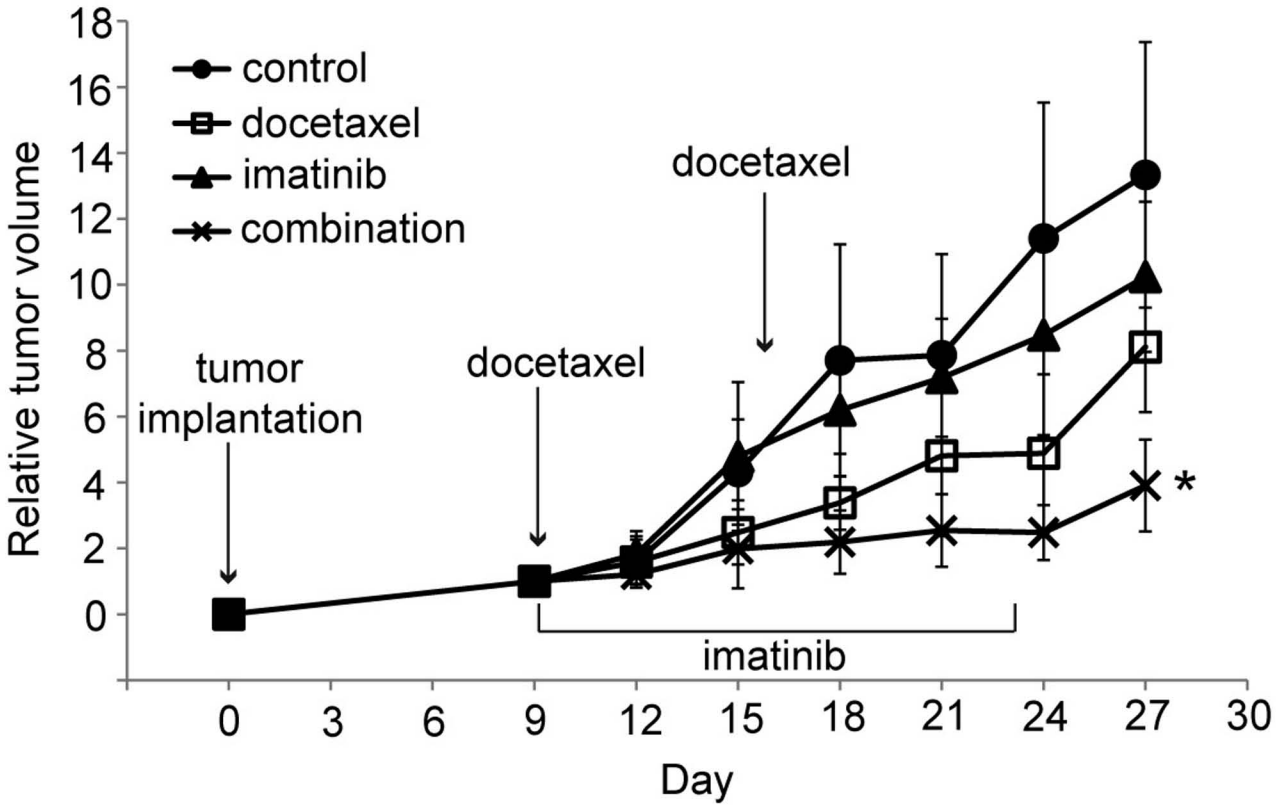
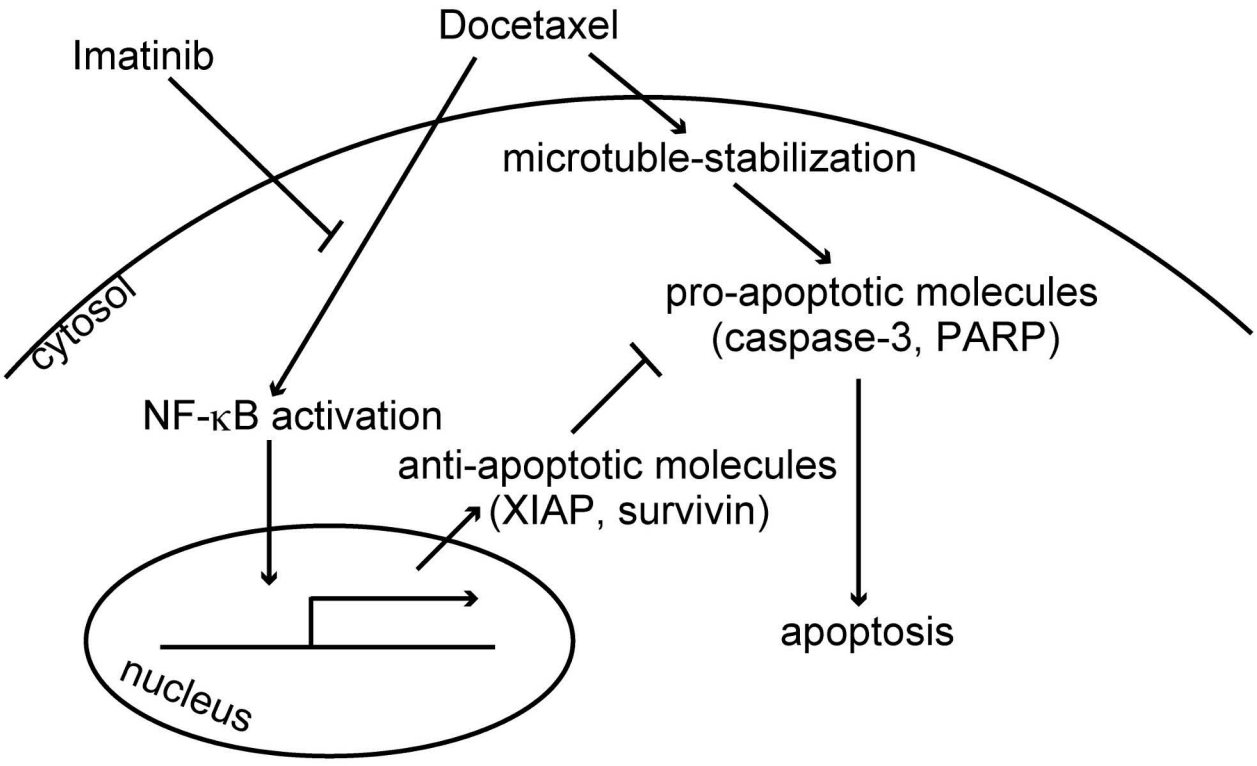

Relative tumor volume
18
16
14
12
10
8
6
4
2
0
control
docetaxel
imatinib
combination
tumor implantation
docetaxel
docetaxel
imatinib
*
0
3
6
9
12
15
18
21
24
27
30
Day

Fig. 6


Docetaxel
Imatinib
microtuble-stabilization
cytosol
pro-apoptotic molecules
(caspase-3, PARP)
NF-κB activation
anti-apoptotic molecules
(XIAP, survivin)
apoptosis
nucleus