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Low-dose Docetaxel Enhanced the Anticancer Effect of Temsirolimus by Overcoming Autophagy in Prostate Cancer Cells

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Abstract. *Background/Aim: Chemotherapy with docetaxel (DTX) is used for castration-resistant prostate cancer (CRPC), but it is inadequate. Materials and Methods: We evaluated the effect of the combination treatment DTX and the mTOR inhibitor temsirolimus (TEM) in the PC3 prostate cancer cell line, by focusing on the induction of autophagy and apoptosis. Results: TEM induced autophagy but not apoptosis even at a high dose, whereas DTX induced apoptosis. The combination of low-dose DTX and TEM caused a 34% suppression in cell proliferation compared to monotherapy with a higher dose of DTX. The induction of apoptosis was increased by their combination. The combination with DTX overcame the induction of autophagy by TEM. The combination treatment suppressed tumor growth 72% less than the control group after 14 days of treatment in vivo. Conclusion: The combination of TEM and DTX induced apoptosis by overcoming autophagy and enhanced the anticancer effect compared to monotherapy.*

Androgen deprivation therapy (ADT) is the standard therapy for metastatic prostate cancers. Most patients with ADT will experience disease progression (1) to castration-resistant prostate cancer (CRPC), which carries a much poorer prognosis (2). Today, chemotherapy with docetaxel (DTX) is widely used for the treatment of CRPC, but the effect of DTX in CRPC is not satisfactory. New agents for CRPC include the cytochrome *P17* inhibitor abiraterone acetate, the new anti-androgen enzalutamide and the new taxane cabazitaxel (3-6). Although these new agents have shown promise for prolonging survival, the effects of these new androgen receptor target drugs and chemotherapy are limited.

There is a report that mammalian target of rapamycin (mTOR) inhibitors suppress the progression of CRPC (7) and Morikawa *et al.* revealed that the mTOR inhibitor rapamycin enhanced the cytotoxicity of DTX in an androgen-independent prostate cancer xenograft model (8). These reports indicate that an mTOR inhibitor could be a key therapy for CRPC and could have a synergistic effect with DTX. mTOR has various functions in the human body, and one of its key roles is regulation of autophagy. Autophagy is a response to stressors such as nutrient deprivation and bioenergetic stress (9-11). Thus, cells can survive under various types of stress by inducing autophagy. However, excessive autophagy may induce cell death (10).

This dual role of autophagy is supported by a variety of cancer treatments. For example, autophagy is protective in response to radiation therapy in breast cancer (12), but toxic in response to anti-estrogen treatment in human mammary carcinoma cells (13). Two studies revealed that autophagy inhibitors enhanced the cytotoxicity of an mTOR inhibitor in prostate cancer cells (14, 15). Thus, the suppression of autophagy in treatment using mTOR inhibitors could be effective against prostate cancer cells.

The disadvantage of DTX treatment is its adverse events (AEs). We hypothesized that combination therapy with a low-dose DTX and an mTOR inhibitor would have an effect equivalent to that of standard-dose DTX therapy with a reduced rate of AEs.

The present study was conducted to evaluate the effect of combination therapy with TEM + DTX, focusing on the induction of autophagy and apoptosis.

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Key Words: CRPC, temsirolimus, docetaxel, autophagy, apoptosis.

Materials and Methods

Cell lines. Two human prostate cancer cell lines, PC3 and LNCaP were maintained in RPMI with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 incubator. The cells were treated with DTX (1, 5, 10, 50, or 100 nM), TEM (1, 10, 100, 500, or 1 μM), or the combination of 10 nM DTX + TEM (10, 50, or 100 nM) for 48 h. For the evaluation of autophagy induced by TEM, PC3 cells were treated with TEM and the autophagy inhibitor chloroquine (CQ) or 3-methyladenine (3-MA) for 48 h.

Drugs. TEM, DTX, and the autophagy inhibitors CQ and 3-MA were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation assay. Cells were seeded in 96-well plates at the density of 1×10^4 cells/well and incubated in a CO_2 incubator at 37˚C overnight. The next day, the drugs (TEM, DTX, or DTX + TEM) were added to each well at different concentrations and the cells were further cultured for 48 h. Cell proliferation was measured by the CellQuanti-MTT Cell Viability Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

Antibodies. Anti-LC3 (microtubule-associated protein 1A/1B-light chain 3) was obtained from MBL (Nagoya, Japan). Anti-caspase 3, anti-cleaved caspase 3, anti-phospho-mTOR, anti-PI3K class III, anti-p62, anti-Atg 5, anti-Akt, anti-phospho-Akt and anti-beta-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako (Carpinteria, CA, USA).

Protein extraction and immunoblotting. Cultured cells and their supernatant were prepared in CelLytic MT Mammalian Tissue Lysis/Extraction Reagent from Sigma-Aldrich (St. Louis, MO, USA). After determination of the protein content, samples were separated using 4%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Each membrane was incubated with the primary antibodies described above. Blots were developed with HRP-conjugated secondary antibodies. Proteins were visualized using ECL Prime Western Blotting Detection Reagent and captured by an ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK). The LC3-I and LC3-II levels were detected by the ImageQuant TL Analysis toolbox (GE Healthcare).

Animal studies. Athymic nude mice (BALB/c-nu/nu, aged 4-5 wks old) were purchased from Charles River Laboratory (Worcester, MA, USA) and acclimatized in the animal colony for 1 week before the experiments. The mice were housed in microisolator cages, five per cage, in a 12 h light/dark cycle. The mice received filter-sterilized water and food. A total of 24 mice were injected subcutaneously in the right flank with 2.0×10^6 PC3 cells in serum-free medium and 0.1 ml Matrigel (BD Bioscience-Discovery Labware, Franklin Lakes, NJ, USA). We randomly divided the mice into the following four treatment groups of six mice each: 1) DTX alone [10 mg/kg on days 11 and 18, intraperitoneally (i.p.)], saline 0.2 ml on days 12, 13, 19, 20,); 2) TEM alone (4 mg/kg on days 11, 12, 13, 18, 19, 20, i.p.); 3) TEM (4 mg/kg on days 11, 12, 13, 18, 19, 20, i.p) with DTX (10 mg/kg/days 11 and 18, i.p.); and 4) a control group that was treated with 0.2 mL saline (days 11, 12, 13, 18, 19, 20, i.p) (Figure 1).

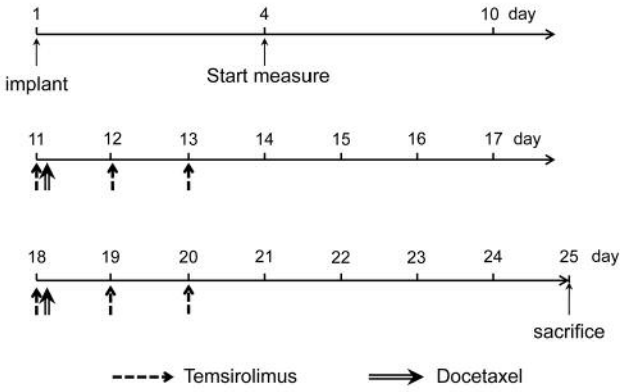

Figure 1. *Treatment schedule. Sequence of DTX and TEM treatment in vivo (n=6 per group). Xenografts were obtained when the mice were sacrificed on day 25.*

Measurements of tumor sizes began on day 4. The tumor sizes were measured with a caliper every day, and the volume was calculated with the following formula: $A \times B^2 \times \pi/6$, where A is the length of the longest aspect of the tumor, and B is the length of the tumor perpendicular to A. The tumor size on day 4 was used as a benchmark of tumor size and was set as 1. The body weight of each mouse was measured every day. All mice were euthanized on day 25.

Statistical analysis. All analyses were performed using the SPSS statistics ver. 20 software (IBM, Armonk, NY, USA). Data are expressed as mean±standard deviation (SD). We used a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* analysis to compare the differences between treatment groups in the *in vitro* experiments. A two-way ANOVA with Tukey's *post-hoc* analysis was used to compare tumor sizes and body weights in the mice. Differences were considered significant when the *p*-value was <0.05.

Results

The anticancer effect of the combination treatment of DTX + TEM. The growth inhibition of the PC3 and LNCaP cell lines by the various treatments is shown in Figure 2A. The growth of both PC3 and LNCaP cells was inhibited by TEM and by DTX in a dose-dependent manner. The viability of both cell lines treated with either DTX or TEM at 100 nM decreased to approximately 60% compared to their respective control groups. The growth suppression effect of both agents did not depend on the cell's androgen sensitivity. Hereafter, we used PC3 cells to reveal the treatment effect on CRPC.

For the determination of the effect of the combination of TEM and DTX, we exposed PC3 cells to low-dose (10 nM)

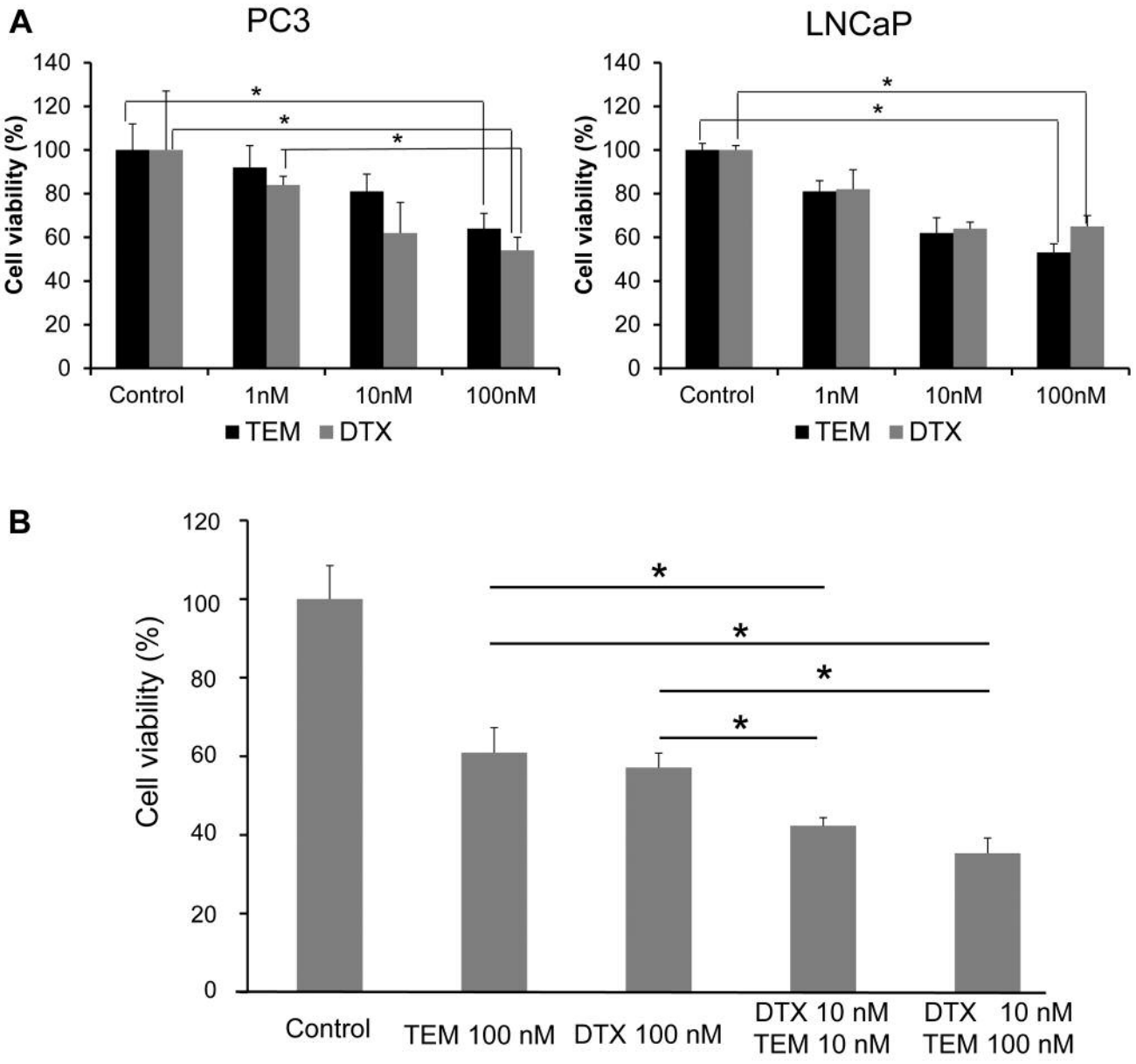

Figure 2. *Antitumor effect of TEM and DTX. A: The effects of DTX and TEM monotherapies on cell lines PC3 and LNCaP. Each drug inhibited the prostate cancer cell growth in a dose-dependent manner. One-way layout ANOVA. *p<0.05. B: Comparison between the monotherapy with TEM or DTX and the combination therapy of TEM + DTX. The combination was significantly more effective than the monotherapy with TEM or DTX (One-way layout ANOVA. p<0.001, respectively).*

DTX with different concentrations of TEM (10 or 100 nM) for 48 h. The results revealed that the combination of 10 nM TEM with 10 nM DTX was more effective than the 100 nM TEM or 100 nM DTX monotherapy (Figure 2B). There were significant differences in cell viability between the combination group and the single-agent groups ($p<0.001$, respectively).

TEM-induced autophagy and the effect of an autophagy inhibitor on the cytotoxicity of TEM. Elevated expression levels of the LC3-phospholipid conjugate LC3-II and its ratio indicate the induction of autophagy. Here, expression of LC3-II was induced by TEM treatment in a dose-dependent manner (Figure 3A). DTX treatment did not increase LC-II expression (Figure 3A). The growth suppression effect of TEM was

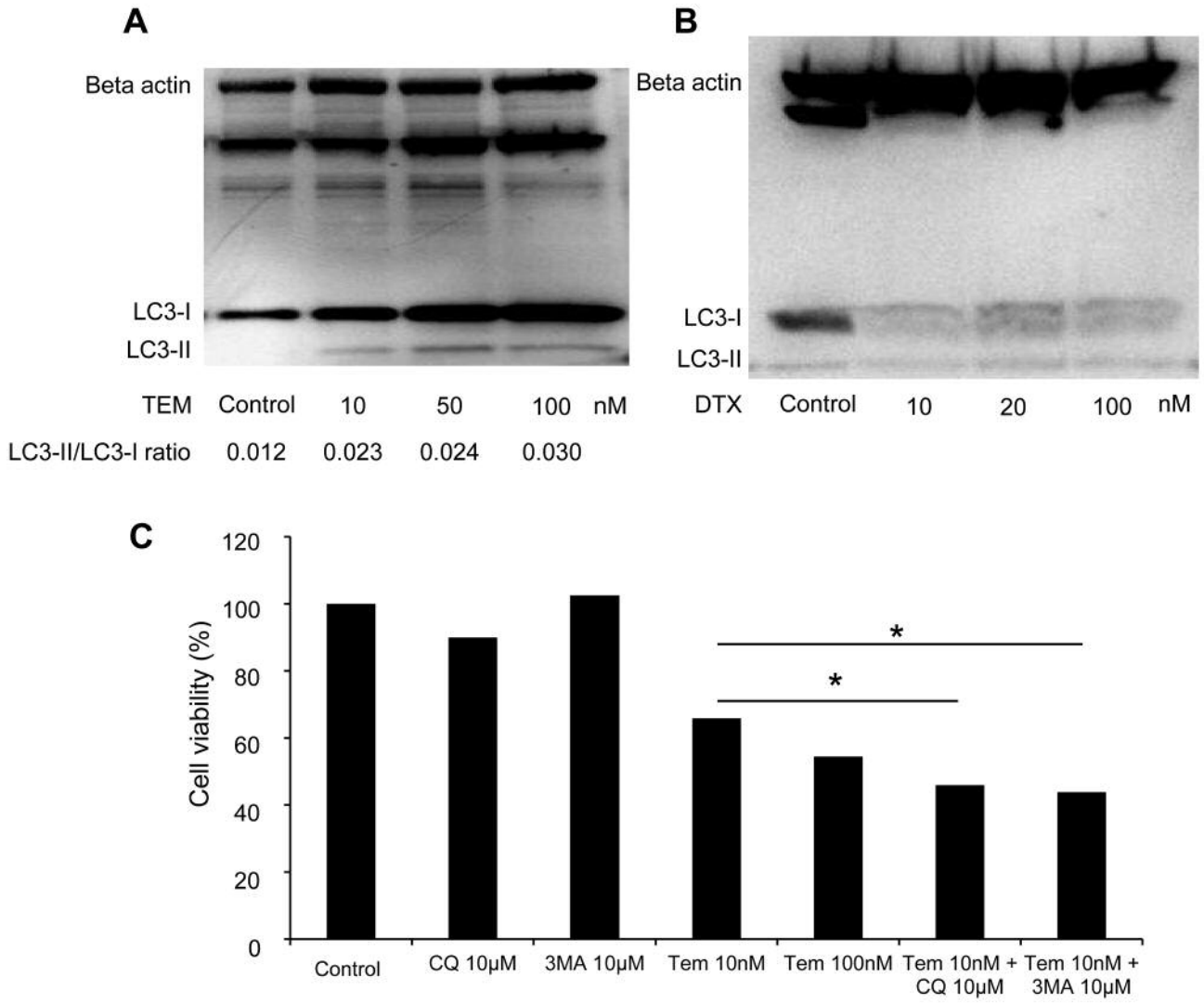

Figure 3. *Induction of autophagy and the effect of autophagy inhibitors. A: TEM induced autophagy in a dose-dependent manner. B: DTX suppressed autophagy. C: The growth suppression effect of TEM was increased by co-exposure with autophagy inhibitors (10 μM CQ, p=0.02; 10 μM 3-MA, p<0.001). n=4. One-way layout ANOVA. *p<0.05.*

increased by co-exposure with an autophagy inhibitor, *i.e.*, CQ or 3-MA (Figure 3B, 10 nM TEM with 10 μM CQ; p=0.02, 10 nM TEM with 10 μM 3-MA; p<0.01) indicating that the anticancer effect of TEM is increased by suppressing autophagy induction in PC3 cells.

Low-dose DTX combined with TEM can induce apoptosis. As the combination of TEM + DTX was more effective than both monotherapies for cell growth suppression, we examined the induction of apoptosis by combination treatment. Cleaved caspase 3 was detected in DTX-treated cells (Figure 4A), which indicated that DTX induced apoptosis of PC3 cells. In contrast, the expression of cleaved caspase 3 was not detected in TEM-treated PC3 cells, even with a high-dose treatment (data not shown).

Although 10 nM DTX monotherapy and 10 nM TEM monotherapy did not induce apoptosis, the combination of 10 nM DTX with 50 or 100 nM TEM increased the cleaved caspase 3 expression (Figure 4B). Taken together, our findings demonstrate that the combination of low concentrations of DTX + TEM, at which the respective monotherapies did not induce apoptosis, can induce apoptosis of PC3 cells.

Combination treatment with TEM + DTX suppressed autophagy by interacting with the PI3K/Akt/mTOR pathway.

We examined the induction of autophagy by combination treatment with TEM + DTX in PC3 cells by analysing the expression of LC3-I and LC3-II using western blotting. The LC3-II/LC3-I ratio induced by the combination therapy with 10 nM DTX and 50 nM or 100 nM TEM was decreased to approximately one-third of that produced by 100 nM TEM monotherapy, indicating that DTX overcame the autophagy induction by TEM. There were significant differences in the LC3-II/LC3-I ratio between the combination-treated group and the monotherapy-treated groups ($p<0.01$) (Figure 4B).

p62 binds directly with LC3 and is decomposed selectively in autophagy. The expression level of p62 increases when autophagy is blocked. Here, the level of p62 was decreased in TEM treated cells compared to the control group and the DTX monotherapy group (Figure 4C), indicating that TEM induced autophagy. Interestingly, the expression levels of p62 in the combination treatment group were greater than those in the TEM-monotherapy group. These results indicate that TEM-induced autophagy was blocked by DTX.

We tested whether a combination treatment would block autophagosome membrane formation in PC3 cells. The first step in autophagosome formation requires the phosphatidyl inositol 3 kinase (PI3K) class III complex. TEM monotherapy increased the expression level of PI3K class III in PC3 cells compared to the control. The level of PI3K class III was decreased by the combination treatment with TEM + DTX compared to the same concentration of TEM alone (Figure 4C). This indicates that DTX inhibited the autophagosome formation by suppressing PI3K class III. DTX appears to have a role as an autophagy inhibitor in the combination therapy with TEM in PC3 cells via suppressing PI3K class III.

We examined the effect of TEM on the PI3K/Akt/mTOR pathway. TEM treatment decreased the expression levels of mTOR and p-Akt, whereas the expression levels were not changed by DTX treatment compared to the control group. These TEM-induced decreased expression levels were not changed by adding DTX. Taken together, these results indicate that DTX has no interaction with the PI3K/Akt/mTOR pathway.

Combination treatment with TEM + DTX and the growth of PC3 cells in vivo. In a preclinical prostate cancer xenograft mouse model, the effect of TEM + DTX combination was most striking. There was a significant difference in the tumor volume between the combination treatment and control groups ($p=0.04$). Moreover, the tumor sizes of the combination treatment group on day 25 were even smaller than the initial tumor sizes (Figure 5). The body weight of the mice did not change in any group during the treatment (data not shown), indicating that the treatment doses used were not toxic in this respect.

Together these findings indicate that the combination treatment with the low-dose TEM + DTX is more effective than TEM or DTX monotherapy.

Discussion

The present results showed that the combination therapy of TEM + DTX was more effective in prostate cancer cells than monotherapy with TEM or DTX both *in vitro* and *in vivo*. Our findings also revealed that DTX inhibited autophagy induced by TEM. This outcome suggests that DTX not only suppressed the proliferation of prostate cancer cells by its own toxicity, but also enhanced TEM's toxicity by overcoming autophagy induction. DTX inhibits depolymerization by the stabilization of tubulin and arrests cycling cells (16). Eventually DTX activates an apoptotic cascade leading to caspase activation by Bcl-2 phosphorylation (17). Taxanes also have another effect on cancer cells. Veldhoen *et al*. showed that paclitaxel suppressed autophagy through the inhibition of PI3K class III (18). Our present data showed that DTX also functions as an autophagy inhibitor via the suppression of PI3K class III.

TEM is an ester analogue of the mTOR inhibitor rapamycin, and is currently being used for renal cancer treatment. The effect of TEM on CRPC has been reported in basic research, but the results of clinical studies on CRPC patients treated with mTOR inhibitors have been disappointing (19, 20). mTOR has critical roles in cell growth and cell maintenance. Among these effects, we focused on the induction of autophagy. Autophagy has dual roles as both a protective and toxic mechanism in cells (18). Autophagy induced by a cancer treatment may sometimes block the treatment's sufficient anticancer effect, and an autophagy inhibitor can sometimes enhance the treatment's effect. Xiaoqi *et al*. reported that combination treatment with TEM plus hydroxychloroquine, an autophagy inhibitor, suppressed melanoma growth (21). In the present study, the autophagy inhibitors CQ, 3MA, and DTX enhanced the toxicity of TEM. Similarly, DTX could enhance the tumor suppression effect of TEM by inhibiting autophagy.

The combination therapy of DTX with various agents has been tested in several laboratories (22, 23). Some reports have shown that the combination of DTX + TEM was more effective than the respective monotherapies in various types of cancer cells including prostate cancer cells (24, 25). Our data are consistent with these reports.

One of the problems of chemotherapies is their AEs. The reduction of AEs has been attempted by altering the administration schedule. For example, Kellokumpu-Lehtinen *et al*. reported the phase III trial of DTX treatment for CRPC administered once every 2 weeks *versus* once every 3 weeks, and found that the administration of DTX once every 2 weeks was well tolerated by patients with CRPC and could be a useful option when once every 3 weeks single-dose administration is unlikely to be tolerated (26). In the present *in vivo* investigation, we used reduced doses of

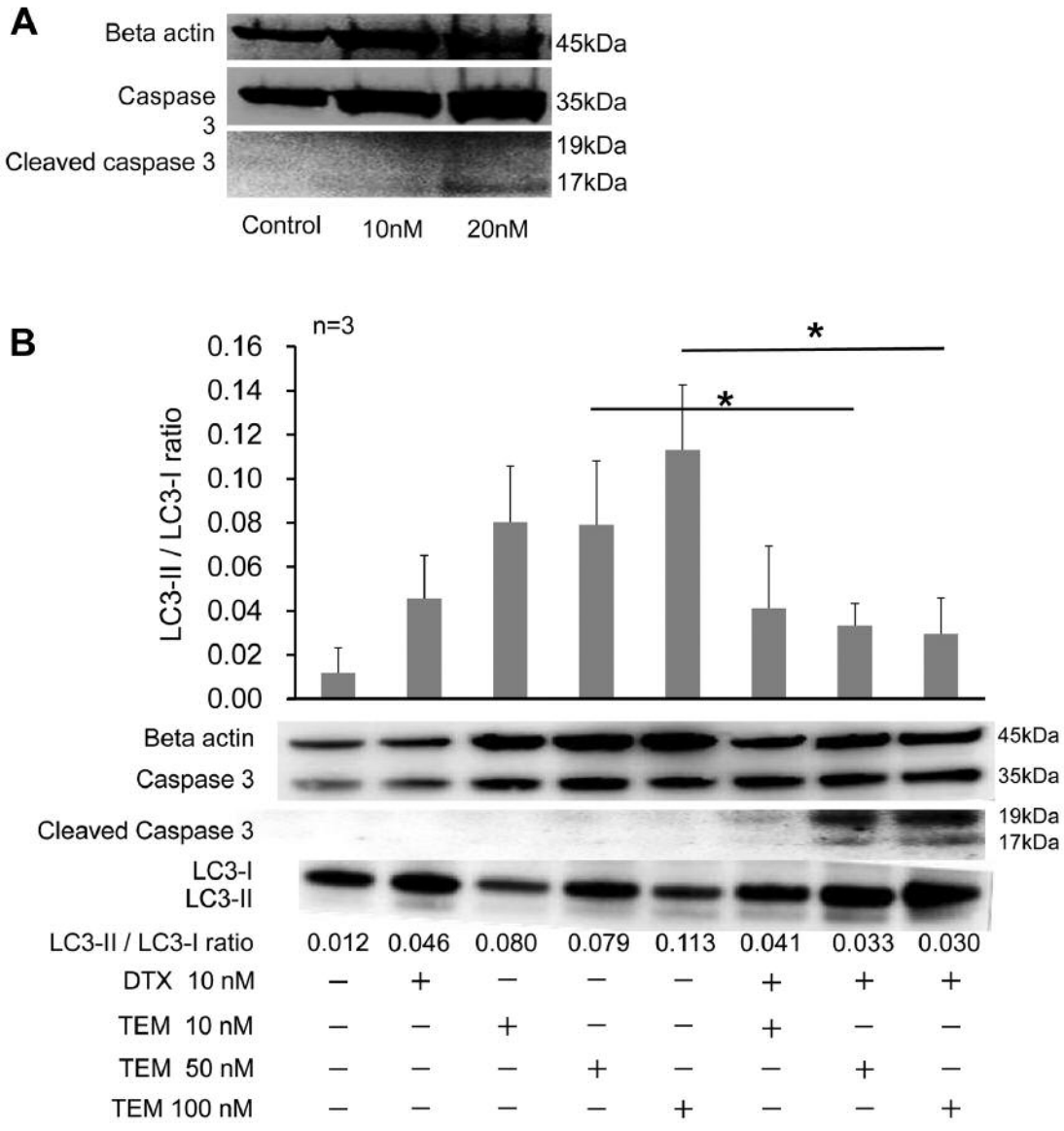

Figure 4. *Continued*

each chemotherapeutic agent to minimize AEs. The results showed no loss of body weight of mice in each treatment group, indicating that the combination of low-dose TEM + DTX can be administered safely.

The results of our experiments demonstrated that the combination of TEM + DTX was more effective than TEM or DTX monotherapy both *in vitro* and *in vivo*. DTX acted as an autophagy inhibitor in this therapy. DTX inhibited PI3K class III complex, which regulates the first step in autophagosome formation. The combination of TEM + DTX could be a new therapeutic option for the treatment of CRPC, but this hypothesis should be tested in prospective clinical trials.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Conception and design: Hideaki Ito; Acquisition of data: So Inamura. Analysis and interpretation of data: So Inamura; Drafting of the manuscript: So Inamura; Critical revision of the manuscript for important intellectual content: Hideaki Ito, Motohiro Kobayashi; Statistical analysis: Katsuki Tsuchiyama; Administrative, technical or material support: Minekatsu Taga, Hitomi Hoshino; Supervision: Osamu Yokoyama; Approval of the version of the manuscript to be

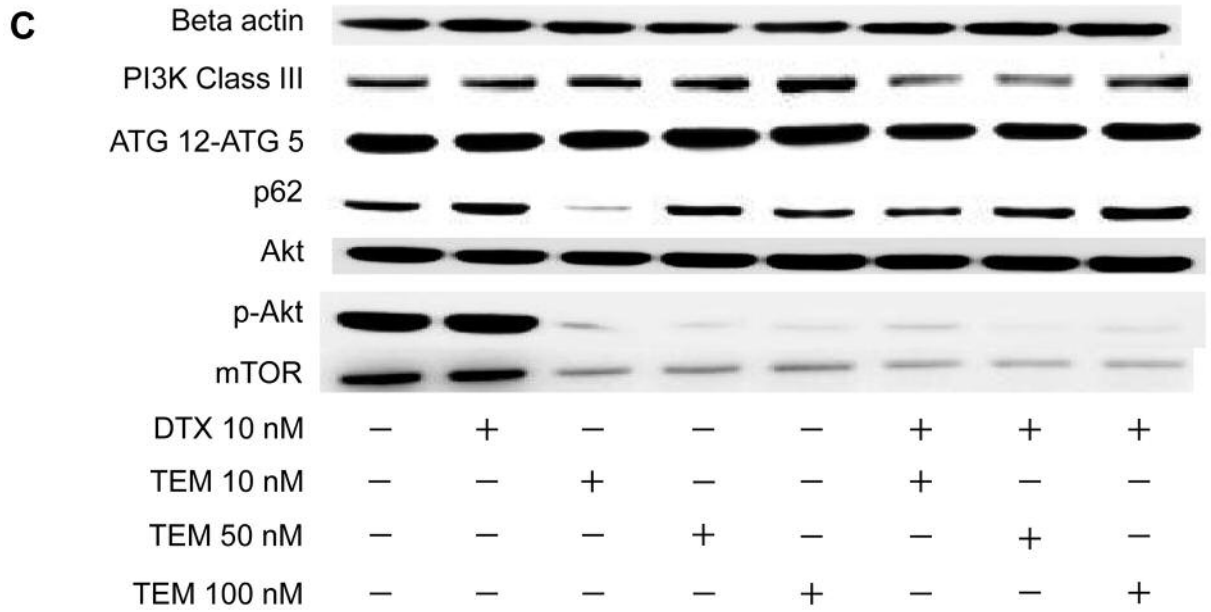

Figure 4. *Induction of apoptosis by the combination treatment of DTX + TEM. A: Western blotting showed that DTX induced cleaved caspase 3 expression levels in PC3 cells. B: Western blot showing that the cleaved caspase 3 level was enhanced in the group treated with the combination of TEM + DTX. The LC3-II/LC3-I ratio was decreased in the TEM + DTX group. One-way layout ANOVA. *p<0.05. C: Western blot showing that the combination treatment resulted in decreased expression of PI3K Class III and increased p62 expression on PC3. TEM suppressed the level of phospho-Akt and mTOR as shown in the western blot. DTX did not interact with the PI3K/Akt/mTOR pathway.*

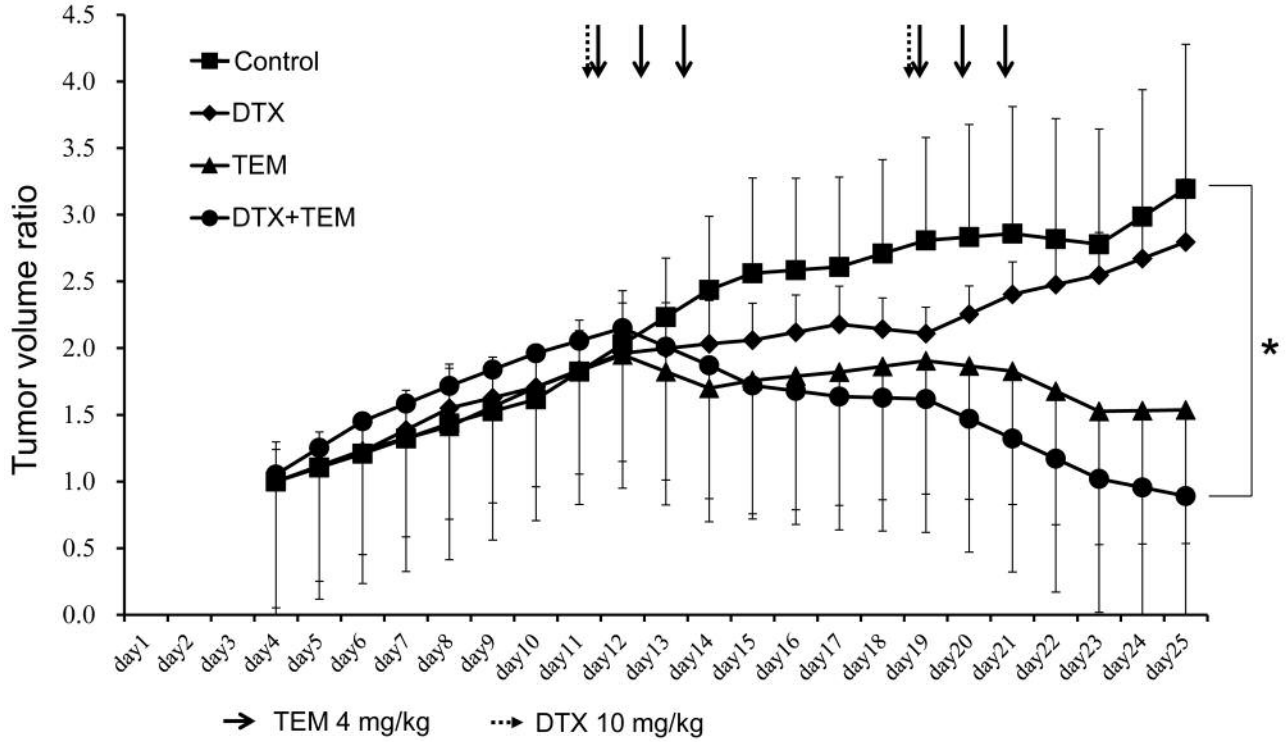

Figure 5. *The effects of monotherapy and combination treatments on the growth of PC3 xenografts. The combination of TEM + DTX was more effective than each monotherapy. There was a significant difference in tumor size between the control and combination groups (p=0.04). Two-way layout ANOVA. *p<0.05.*

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