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Cathepsin E enhances anticancer activity of doxorubicin on human prostate cancer cells showing resistance to TRAIL-mediated apoptosis

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Abstract

We previously described that cathepsin E specifically induces growth arrest and apoptosis in several human prostate cancer cell lines *in vitro* by catalyzing the proteolytic release of soluble tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) from the tumor cell surface. It also prevents tumor growth and metastasis *in vivo* through multiple mechanisms, including induction of apoptosis, angiogenesis inhibition and enhanced immune responses. Using the prostate cancer cell line PPC-1, which is relatively resistant to cell death by doxorubicin (40–50% cytotoxicity), we first report that a combination treatment with cathepsin E can overcome resistance of the cells to this agent. *In vitro* studies showed that combined treatment of PPC-1 cells with the two agents synergistically induces viability loss, mainly owing to down-regulation of a short form of the FLICE inhibitory protein FLIP. The enhanced antitumor activity was corroborated by *in vivo* studies with athymic mice bearing PPC-1 xenografts. Intratumoral application of cathepsin E in doxorubicin-treated mice results in tumor cell apoptosis and tumor regression in xenografts by enhanced TRAIL-induced apoptosis through doxorubicin-induced c-FLIP down-regulation and by a decrease in tumor cell proliferation. These results indicate that combination of cathepsin E and doxorubicin is sufficient to overcome resistance to TRAIL-mediated apoptosis in chemoresistant prostate cancer PPC-1 cells, thus indicating therapeutic potential for clinical use.

Keywords: aspartic proteinase; cathepsin E; doxorubicin; prostate cancer; tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

Introduction

Prostate cancer is one of the most common malignancies and one of the leading causes of cancer death in Western countries (Jemal et al., 2009). Unfortunately, a large percentage of prostate cancer patients are in an advanced stage of disease and eventually develop to an androgen-independent phenotype with incurable disease. Moreover, resistance to conventional anticancer chemotherapeutic agents becomes another problem in the treatment of prostate cancer. Thus, considerable efforts have been made to search for novel, effective agents for treating hormonal ablation-ineffective and chemoresistant prostate cancer patients.

It is generally believed that chemotherapy kills cancer cells by induction of a final common pathway that leads to cell death. However, a common hurdle that most of the anticancer agents have not overcome is their severe side effects induced by damaging various cellular components. Under these circumstances, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has attracted intense interest in cancer therapy, because it can induce apoptosis in a variety of cancer cells without harming normal cells (Hall and Cleveland, 2007; Kruyt, 2008; Yang, 2008). Indeed, TRAIL can induce apoptosis in both hormone-sensitive and -insensitive prostate cancer cells (Holen et al., 2002; Kawakubo et al., 2007). However, several prostate cancer cell lines still remain resistant to TRAIL-mediated apoptosis (Bonavida et al., 1999; Van Ophoven et al., 1999; Nagane et al., 2001; Nimmanapalli et al., 2001; Kelly et al., 2002; Voelkel-Johnson et al., 2002). An additional problem with *in vivo* use of TRAIL is that a high concentration of this molecule is required to obtain definite therapeutic efficacy, probably owing to the short half-life of soluble TRAIL in plasma (Walczak et al., 1999; Kelley et al., 2001). Therefore, there is a need to search for new regimens to enhance sensitization of prostate cancer cells to TRAIL-induced apoptosis. Recently, higher expression of cellular FLICE-inhibitory protein (c-FLIP), a potent inhibitor of death receptor signals that exists in two forms of the spliced transcripts, termed long (c-$FLIP_L$) and short (c-$FLIP_S$), has been shown to correlate with TRAIL-resistant apoptosis in various malignant cells (Griffith et al., 1998; Hao et al., 2001; Voelkel-Johnson et al., 2002). In this context, down-regulation of c-$FLIP_L$ can lead to the induction of a caspase-dependent mitochondrial apoptosis pathway in TRAIL-resistant ovarian cancer SKOV3 cells (Park et al., 2009), indicating that c-FLIP is an important mediator of anti-apoptotic response. Additionally, high expression levels of anti-apoptotic molecules such as TRAIL decoy receptors and the BcL-2 family members Bcl-2 and Bcl-X_L are known to inhibit or delay TRAIL-induced apoptosis in various cancer cell lines, including pancreatic and prostate cancer cell lines (Hinz et al., 2000; Walczak et al., 2000; Munshi et al., 2001; Holen et al., 2002; Park et al., 2009). Thus, cellular expression levels of these anti-apoptotic

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molecules are most likely to affect TRAIL-induced apoptosis in cancer cells and thus they might be promising targets in the treatment of several human cancers. Meanwhile, to overcome resistance to apoptosis in prostate cancer cells by chemotherapeutic agents, their combination with TRAIL has been performed (Keogh et al., 2000; Chen et al., 2001; Kelly et al., 2002; Voelkel-Johnson et al., 2002; Kang et al., 2005; Shankar et al., 2005).

Cathepsin E is an intracellular aspartic proteinase of the pepsin superfamily, which is predominantly expressed in cells of the immune system and is highly secreted as the catalytically active enzyme by activated phagocytes (Sakai et al., 1989; Sastradipura et al., 1998; Nishioku et al., 2002; Yanagawa et al., 2006). The increased expression of cathepsin E was detected in antigen-presenting cells in response to interferon-γ and lipopolysaccharide (Yanagawa et al., 2006) and inflammatory cells within and nearby carcinoma (Matsuo et al., 1996). In this regard, recent gene or protein expression profiles have demonstrated that increased expression of cathepsin E is associated with survival of lung and gastric cancer patients (Ullmann et al., 2004; Sakakura et al., 2005). Moreover, we have recently shown that cathepsin E specifically induces apoptosis in various prostate cancer cell lines *in vitro* by catalyzing the proteolytic release of soluble TRAIL from the tumor cell surface and that exogenously administered mature cathepsin E in athymic nude mice bearing tumors plays a substantial role in host defense against the cancer cells by multiple mechanisms, including the enhancement in cancer cell apoptosis, tumor-associated macrophage-mediated cytotoxicity, tumor angiogenesis inhibition and immune responses (Kawakubo et al., 2007; Shin et al., 2007). Thus, cathepsin E is likely to have significant advantages over TRAIL in inducing effective cancer cell apoptosis and overcoming the resistance of prostate cancer cells.

Doxorubicin is an anticancer agent having broader anticancer activity against human neoplasms, including a variety of solid tumors and is known to affect many functions of DNA, including DNA and RNA synthesis. It is also known that this agent induces breakdown of DNA by the action of topoisomerase II or by the generation of free radicals. It has also been demonstrated that doxorubicin induces both apoptosis and necrosis in cancer cells, depending on its concentrations, respectively (Carter et al., 2003; Mansilla et al., 2006; Ongkeko et al., 2006; Clyburn et al., 2010). In addition, this agent alters functions of the cell membranes through the increase in the TRAIL death receptors DR4 and DR5 expression (Kang et al., 2005; Shankar et al., 2005; Wu et al., 2007) or the decrease in the c-FLIP expression (Kelly et al., 2002; Watanabe et al., 2005).

Recent studies have shown that doxorubicin has limited cytotoxicity in various human prostate cancer cell lines, including PPC-1 cells, at a dose of 1 μg/ml (1.84 μM), which is clinically achievable (Voelkel-Johnson et al., 2002). PPC-1 cells were the most resistant (90%) to doxorubicin among the prostate cancer cell lines examined (Voelkel-Johnson et al., 2002). PPC-1 cells were also slightly susceptible to soluble TRAIL at a dose of 100 ng/ml (approx. 20% cytotoxicity), but the combination of doxorubicin and TRAIL resulted in approximately 75% killing in these cells (Voelkel-Johnson et al., 2002). We thus assumed that the combination treatment of cathepsin E with doxorubicin might synergistically induce TRAIL-mediated apoptosis in PPC-1 cells and thereby could be able to minimize the deleterious side effects of the drug by reducing its dosage. In this study, we first demonstrate that a combination of cathepsin E and doxorubicin can overcome resistance to TRAIL-induced apoptosis in PPC-1 cells, which are relatively resistant to either of the agents *in vitro* and *in vivo*. The present data provide a mechanistic basis for the enhanced anticancer activity of the combination of cathepsin E with commonly used chemotherapeutic agents in the treatment of prostate cancer cells.

Results

Synergistic interaction of cathepsin E and doxorubicin on apoptosis in prostate cancer PPC-1 cells *in vitro*

We have previously shown that cathepsin E can induce TRAIL-mediated apoptosis of all the prostate cancer cell lines tested without harming the viability of normal prostate epithelial cells (Kawakubo et al., 2007). However, the extent of apoptosis by cathepsin E varied among the cell lines. Among these cell lines, PPC-1 cells showed the most resistance to cathepsin E-induced apoptosis, which was explained in part by the expression of the soluble decoy receptor osteoprotegerin or by the efficiency of cathepsin E-mediated cleavage of TRAIL at the cell surface. It has also been shown that PPC-1 cells, as with PC-3, Du145 and LNCap, were resistant to doxorubicin or TRAIL (Kelly et al., 2002; Voelkel-Johnson et al., 2002; Shankar et al., 2005). However, the resistance of the prostate cancer cell lines was demonstrated to be overcome when they were treated with a combination of these two agents (Kelly et al., 2002; Voelkel-Johnson et al., 2002; El-Zawahry et al., 2005; Shankar et al., 2005). Hence, to determine whether the combination treatment of doxorubicin and cathepsin E is useful in killing PPC-1 cells resistant to either of the agents, we first analyzed the susceptibility of PPC-1 cells to apoptosis by cathepsin E alone or doxorubicin alone. The cultured PPC-1 cells were incubated for 20 h with varying concentrations of either of the agents and then cell viability was measured with a colorimetric assay. The viability of the cells was significantly decreased and plateau values were attained by treatment of either of the agents (maximally approx. 10% and 50% cytotoxicity for cathepsin E and doxorubicin, respectively) (Figure 1A,B), confirming that the cells are relatively resistant to apoptosis by each of the agents.

To examine whether resistance of PPC-1 cells to apoptosis by either of the agents can be overcome by co-treatment with both agents, the cytotoxic effect of a combination of a fixed concentration of doxorubicin with increasing concentrations of cathepsin E was assessed using a cell viability assay. Based on their dose-dependent curves, we reasoned that a dose of doxorubicin suitable for combination with cathepsin E might be 1 μg/ml (1.84 μM), because this dose is clini-

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cally achievable and leads to a plateau value in the cell viability loss. The combination with cathepsin E resulted in 55–70% killing in the cells in a dose-dependent manner (Figure 2A). The enhanced cytotoxicity by the combination treatment with the two agents was shown to be synergistic by isobolographic analysis (Berenbaum, 1977, 1978) (Figure 2B). The synergy was also substantiated by calculating the combination index (CI; Chou, 2006). The CI values for combinations at all the doses tested were shown to be $CI<1$ (Table 1), indicating that the enhanced cytotoxicity by this combination achieved synergism. At the microscopic level, the cells treated with 0.001% DMSO and 0.74% glycerol as vehicle for doxorubicin and cathepsin E, respectively, exhibited a morphology characteristic of growth phase, whereas the cells treated with doxorubicin alone (1 μg/ml) or cathepsin E alone (25 μg/ml) manifested morphologic hallmarks of apoptosis (e.g., cell shrinkage, nuclear fragmentation and apoptotic body-like structure formation) and/or necrosis (e.g., cell swelling and disintegration of cellular and nuclear structures; Figure 2C). The cells treated by a combination of both agents showed more profound cell damage compared with those treated by either of the agents alone. To confirm whether morphological changes with cathepsin E alone and doxorubicin alone were apoptosis and/or necrosis, the cells were analyzed by staining with annexin V and propidium iodide (PI; Figure 1C). It was found that the cells treated with cathepsin E were positive only for annexin V staining, whereas those treated with doxorubicin were positive for both types of staining, indicating that cathepsin E exclusively induced apoptosis and doxorubicin resulted in both apoptosis and necrosis under these conditions.

We next examined whether sequential treatment of the two agents was also effective. The cells were preincubated with doxorubicin (1 μg/ml) alone or cathepsin E (25 μg/ml) alone for 16 h and then incubated for an additional 4 h with cathepsin E (25 μg/ml) or doxorubicin (1 μg/ml), respectively. Pretreatment of the cells with either of the agents had no substantial effect on the viability loss by the other agent (Table 2). Thus, the enhanced antitumor effect of the combination of cathepsin E and doxorubicin on the cells is probably based on their immediate interaction in TRAIL-mediated apoptosis pathways.

Expression of TRAIL receptors DR4 and DR5 and c-FLIP at mRNA and protein levels in PPC-1 cells treated with doxorubicin

We have previously shown that treatment of all of the prostate cancer cell lines tested with cathepsin E (1 μM) has little or no effect on the expression of TRAIL and its membrane-associated receptors (DR4, DR5, DcR1, DcR2) (Kawakubo et al., 2007). Only the soluble decoy receptor osteoprotegerin

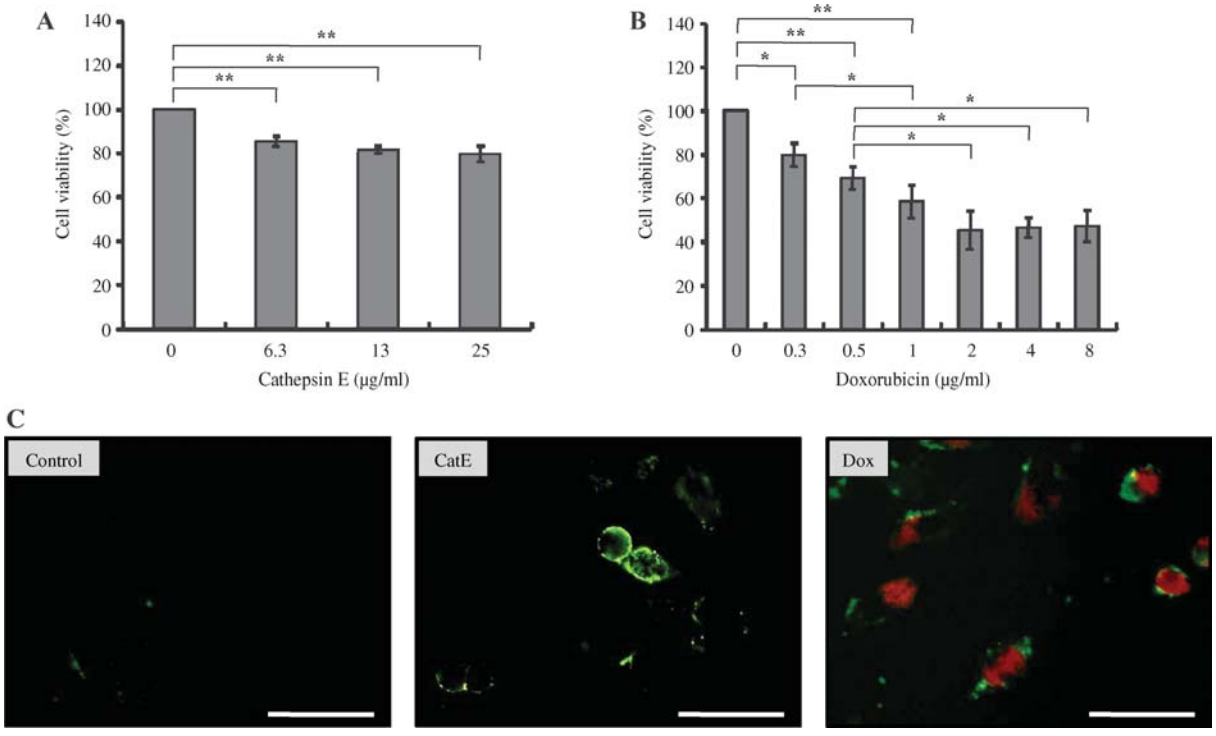

Figure 1 *In vitro* effects of doxorubicin and cathepsin E on the viability of human prostate carcinoma PPC-1 cells.
(A,B) The cells were treated with various concentrations of cathepsin E or doxorubicin for 20 h. The cell viability was determined by a colorimetric assay using a Cell Counting Kit-8. Data are expressed relative to the value of cells treated with vehicle (0.75% glycerol for cathepsin E and 0.030% DMSO for doxorubicin) and are the means±SD of values from four independent experiments. *$p<0.05$, **$p<0.01$, versus the values for cell viability of the corresponding cells treated with vehicle or each agent. (C) Cells treated with cathepsin E or doxorubicin were stained with annexin V and PI for 4 h, Annexin V, green; PI, red. Scale bars, 50 μm.

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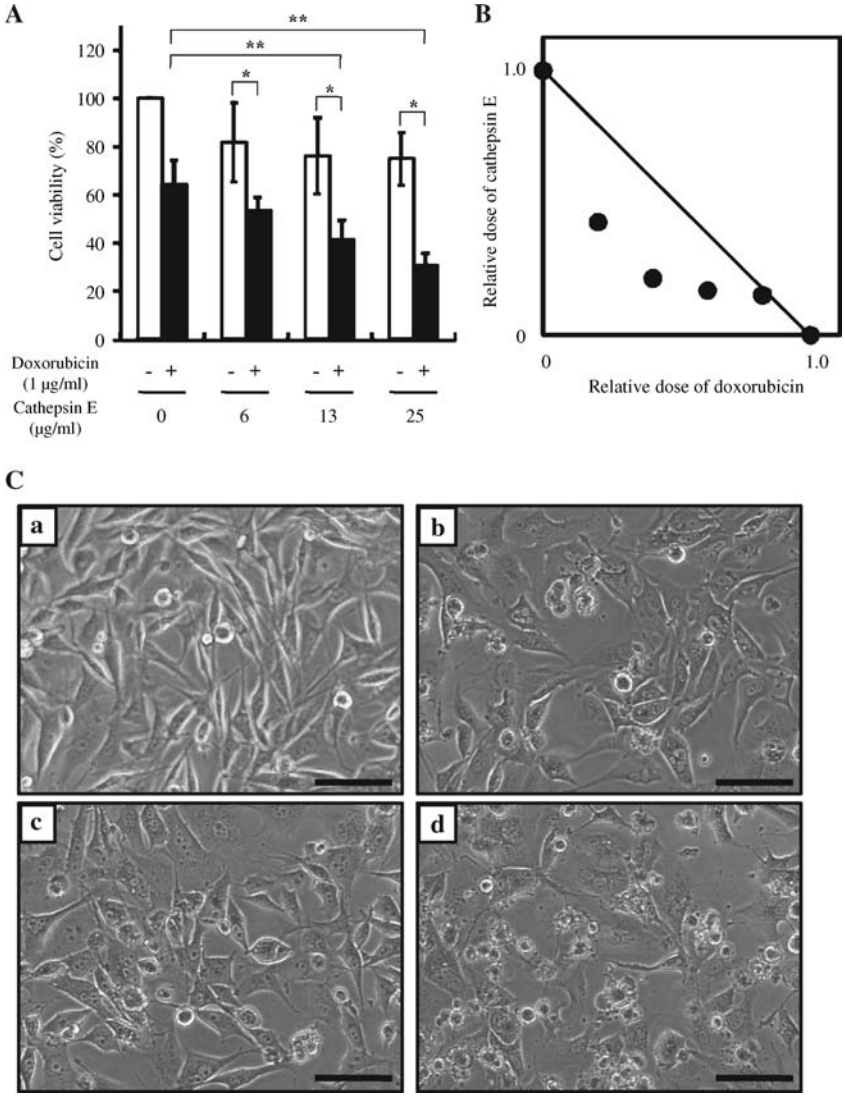

Figure 2 Cooperation of doxorubicin and cathepsin E in induction of viability loss in PPC-1 cells *in vitro*.
(A) The cells were treated with various concentrations of cathepsin E in the presence of doxorubicin (1 μg/ml) (■) or vehicle (□) and then incubated under the same conditions as described in Figure 1. Data are expressed relative to the value of vehicle-treated cells and are the means±SD of values from four independent experiments. $^{**}p<0.01$, versus the corresponding value for cell viability of cells treated with vehicle or each agent. (B) The combined effect of cathepsin E and doxorubicin on cell viability was evaluated by the isobolographic analysis method. Data are expressed as experimental isoeffective points at 80% cell viability. The oblique line indicates the alignment of theoretical values of an additive effect between the two agents. Values above the oblique line indicate an antagonistic effect, and values below the line indicate synergistic effect. (C) Microscopic images of cells treated for 20 h with vehicle (a), doxorubicin (1 μg/ml) (b), cathepsin E (25 μg/ml) (c) or both agents (d). Scale bars, 100 μm.

was significantly increased in the culture medium of PPC-1 cells but not the other cell lines, suggesting that resistance of PPC-1 cells to cathepsin E-induced apoptosis is at least in part owing to higher expression of osteoprotegerin in the cells. By contrast, it has been reported that doxorubicin up-regulates DR4 and DR5 and down-regulates c-FLIP but has no effect on expression levels of Bcl-2 family members, including Bcl-2, Bcl-X_L and Bax, and XIAP in various human prostate cancer cell lines such as PPC-1 (Kelly et al., 2002; Shankar et al., 2005) and LNCap (Kang et al., 2005). The effect of doxorubicin, however, is complicated and probably varies with individual cases. We thus attempted to elucidate the mechanism of synergistic interaction of cathepsin E and doxorubicin on apoptosis in PPC-1 cells. The cells were treated with doxorubicin (1 μg/ml) or vehicle for 20 h and then the cell extract were analyzed by Western blotting with antibodies specific DR4, DR5, and c-FLIP. Expression of c-$FLIP_S$ but not c-$FLIP_L$ was most clearly reduced, whereas the expression levels of DR4 and DR5 were not significantly changed under the same conditions (Figure 3A). Unexpectedly, mRNA levels of DR4 and c-$FLIP_L$, as well as c-$FLIP_S$, were significantly decreased by doxorubicin (Figure 3B), indicating that the changes in these molecules were not reflected on the protein levels. Given that cathepsin E alone

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Table 1 Analysis of combination effect with the combination index method.

Dose of CatE (μg/ml) ($[D]_1$)	Dose of Dox (μg/ml) ($[D]_2$)	Relative dose of CatE ($[D]_1/[D_{20}]_1$)	Relative dose of Dox ($[D]_2/[D_{20}]_2$)	Combination index (CI) ($(D)_1/[D_{20}]_1+[D]_2/[D_{20}]_2$)
86.3 (=$[D_{20}]_1$)	0	1	0	1
36.8	0.05	0.426	0.205	0.631
18.5	0.10	0.214	0.411	0.625
14.5	0.15	0.168	0.616	0.784
13.0	0.20	0.151	0.822	0.973
0	0.24 (=$[D_{20}]_2$)	0	1	1

The combined effect of cathepsin E and doxorubicin on the cell viability was evaluated by the combination index (CI). $CI=[D]_1/[D_{20}]_1+[D]_2/[D_{20}]_2$, where CI is the value for 20% cytotoxicity, $[D_{20}]_1$ and $[D_{20}]_2$ are the doses of cathepsin E and doxorubicin required to exert 20% effect alone, whereas $[D]_1$ and $[D]_2$ are the doses of cathepsin E and doxorubicin that exert the same 20% effect in combination with the other agent, respectively. CI=1 indicates additivity, CI<1 synergism, and CI>1 antagonism.

did not affect levels of DR4 and c-FLIP$_S$ in the cells at mRNA and protein levels (data not shown), our results indicate that synergistic interaction of cathepsin E with doxorubicin is induced by an increase in the efficacy of the cathepsin E-mediated, TRAIL-induced apoptosis, at least in part, via down-regulation of c-FLIP, in particular c-FLIP$_S$, in the cells treated with doxorubicin.

Enhanced anticancer activity cathepsin E and doxorubicin combination treatment in xenograft PPC-1 tumor models

To further investigate whether the enhanced TRAIL-induced apoptosis by combination of cathepsin E and doxorubicin was also apparent *in vivo*, PPC-1 cells were implanted subcutaneously into athymic nude mice. When the size of xenografts had reached approximately 200 mm^3 (approx. 25 days post-inoculation), the animals were injected once with phosphate-buffered saline (PBS) or doxorubicin (8 mg/kg) by intraperitoneal injection and daily with or without cathepsin E (5 nM/kg) by intratumoral injection into the center of xenografts for 13 days. The size of xenografts treated with PBS was time-dependently increased, whereas a single injection of doxorubicin followed by daily vehicle injection resulted in significant inhibition of tumor growth (Figure 4A). Interestingly, the growth of xenografts from animals treated with both agents followed by cathepsin E was more profoundly inhibited compared with that treated with vehicle. Experiments with the animals bearing PPC-1 xenografts without pretreatment with doxorubicin revealed that daily injection of cathepsin E alone resulted in significant inhibition of tumor growth, but the extent of the inhibition was lower, but not significant, than that pretreated with doxorubicin. Thirteen days after doxorubicin injection, tumors were harvested and analyzed for apoptosis by TUNEL staining (Figure 4B). There were significant differences in the number of TUNEL-positive cells in xenografts among the four groups, increasing according to the rank order vehicle alone < doxorubicin alone < cathepsin E alone without pretreatment of doxorubicin < doxorubicin/cathepsin E combination followed by cathepsin E.

Mechanisms of tumor regression in combination of cathepsin E and doxorubicin

To investigate the mechanisms by which the combination of cathepsin E and doxorubicin was most effective in reducing tumor growth and increasing apoptosis in xenografts, the

Table 2 Cytotoxic effects of sequential and co-treatments of PPC-1 cells with cathepsin E and/or doxorubicin.

	Cell viability (%)
Vehicle	100
Doxorubicin (1.0 μg/ml), 20 h	58.3±7.9*
Cathepsin E (25 μg/ml), 20 h	88.1±3.8*
Doxorubicin (1.0 μg/ml)+cathepsin E (25 μg/ml), 20 h	30.9±4.7*
Doxorubicin (1.0 μg/ml), 16 h → cathepsin E (25 μg/ml), 4 h	61.6±10.5*
Cathepsin E (25 μg/ml), 16 h → doxorubicin (1.0 μg/ml), 4 h	65.5±11.7*

The cells were treated with vehicle, cathepsin E (25 μg/ml) alone, doxorubicin (1.0 μg/ml) alone or with a combination of the two agents for 20 h. The cells were also pretreated with doxorubicin (1.0 μg/ml) alone or cathepsin E (25 μg/ml) alone for 16 h and then incubated for an additional 4 h with cathepsin E (25 μg/ml) or doxorubicin (1.0 μg/ml), respectively. At the end of incubation period, cell viability was determined by using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). *$p<0.05$, versus the corresponding value for the cells treated with vehicle.

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status of c-FLIP that are well known to interfere with the initial stages of caspase activation in the death receptor-mediated signaling pathway (Safa et al., 2008) was analyzed by immunohistochemical staining in xenograft sections from each animal group (Figure 5). Administration of doxorubicin alone followed by vehicle significantly decreased the expression level of c-FLIP$_{L/S}$ proteins, whereas administration of cathepsin E alone had little effect on c-FLIP$_{L/S}$ expression. Down-regulation of c-FLIP$_{L/S}$ proteins in xenografts of animals treated with a combination of cathepsin E and doxorubicin followed by cathepsin E was comparable with that treated with doxorubicin followed by vehicle. To further investigate whether the observed effect of the combined agents is the end result of growth arrest followed by apoptosis of tumor cells, the number of proliferating cells in xenografts from each animal group at 13 days post-injection with both agents was assessed by immunohistochemical staining with the monoclonal antibody Ki-67, which reacts with a nuclear antigen present only in proliferating cells (Gerdes et al., 1984). The number of Ki-67 positive cells was markedly decreased in xenografts treated with doxorubicin or cathepsin E after a combination of both agents, whereas xenografts treated with PBS alone showed high proliferative activity (Figure 6). A moderate, but significant, decrease of Ki-67-positive cells was also observed in xenografts treated with cathepsin E alone. The present results suggest that both induction of TRAIL-mediated apoptosis via c-FLIP down-regulation and cell cycle arrest might be underlying mechanisms of enhanced anticancer activity of combination treatment of both agents followed by cathepsin E in PPC-1 xenografts.

Discussion

We have previously shown that cathepsin E can induce TRAIL-mediated apoptosis in all human prostate cancer cell lines tested without regard to site of origin (ALVA-41, ALVA-101, and PC3, from bone metastasis; DU145, from brain metastasis; PPC-1, from primary prostate carcinoma), androgen dependency (dependent, ALVA-41; moderately dependent, ALVA-101; independent, PPC-1, PC3, and DU145), and extent of differentiation (well, ALVA-41; moderately, DU145; poorly, PPC-1 and PC3), without harming the via-

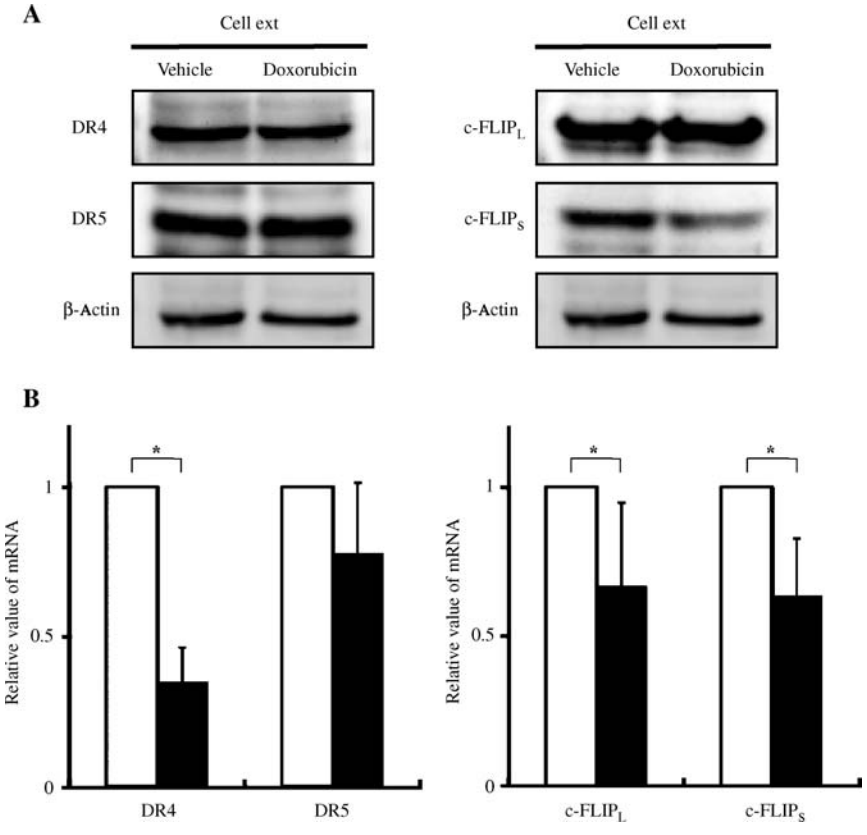

Figure 3 Expression levels of the death receptors DR4 and DR5 and c-FLIP$_{S/L}$ in PPC-1 cells treated with vehicle or doxorubicin *in vitro*. (A) Whole cell lysates were prepared from the cells treated with doxorubicin (1 μg/ml) or vehicle for 20 h and then analyzed by Western blotting with antibodies specific DR4, DR5, c-FLIP$_L$ and cFLIP$_S$. β-Actin is provided as a loading control. (B) Induction of the death receptors and c-FLIP mRNAs in the cells treated by vehicle (□) and doxorubicin (■) was analyzed by real-time RT-PCR, as described in the Materials and methods section. The relative values were defined as the ratio of mRNA level of each protein in doxorubicin-treated cells to that in vehicle-treated cells. The data are the mean±SD of values from five independent experiments and expressed as the value of doxorubicin-treated cells relative to that of vehicle-treated cells. *$p<0.05$: versus the corresponding value for levels of cells treated with vehicle.

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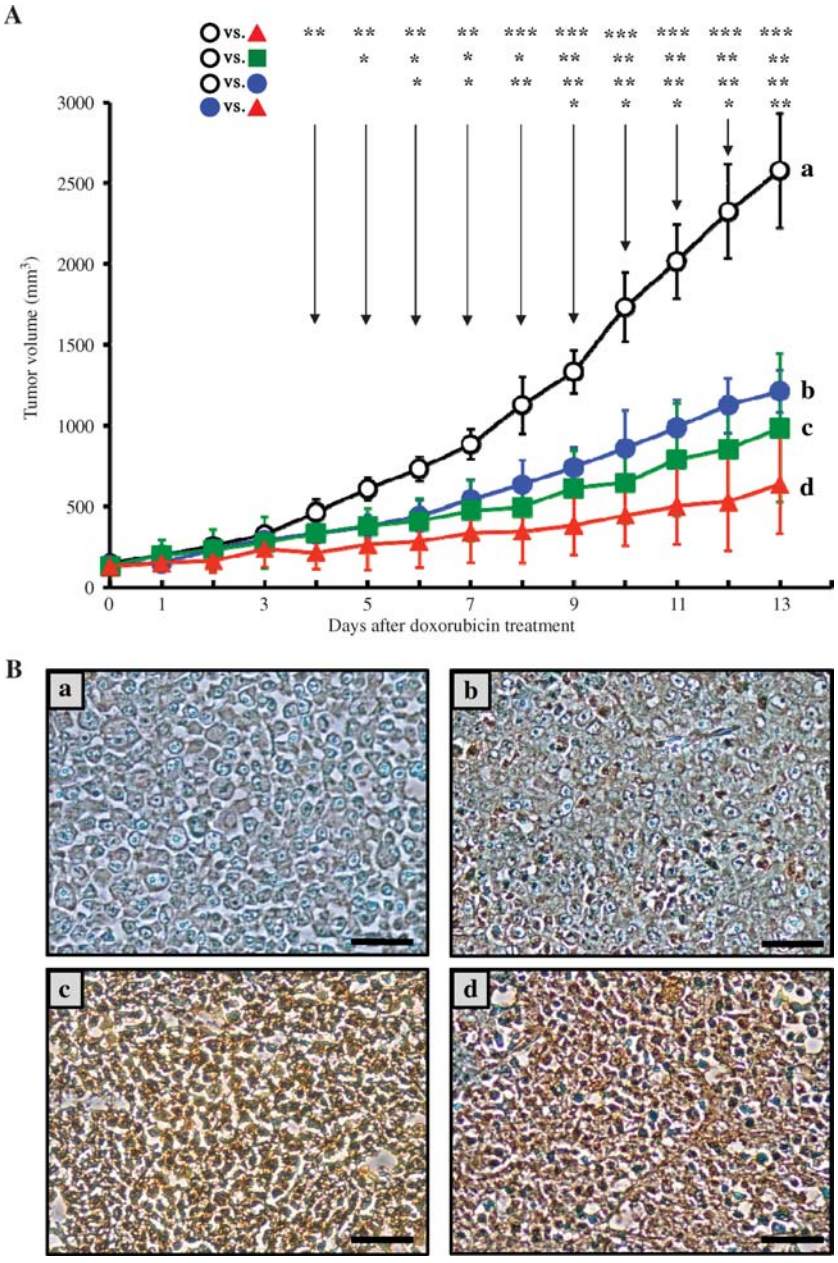

Figure 4 Effect of administration of cathepsin E on the growth of tumors formed by PPC-1 cells and the tumor cell apoptosis into nude mice treated with the two agents.

(A) Tumor size of xenografts treated with PBS alone (a), doxorubicin followed by vehicle (b), cathepsin E alone (c) or doxorubicin/cathepsin E followed by cathepsin E (d). Data are means±SD of values from five mice per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus the corresponding value for the volume of PBS-treated xenografts (a) or that treated with doxorubicin followed by vehicle (b) at each time point. (B) TUNEL staining of xenografts treated with PBS alone (a), doxorubicin followed by vehicle (b), cathepsin E alone (c) or doxorubicin/cathepsin E followed by cathepsin E (d) at day 13. Data are representative of results obtained with five mice per each group. Scale bars, 50 μm.

bility of normal prostate epithelial cells (Kawakubo et al., 2007). The growth arrest of these cell lines by cathepsin E was induced *in vitro* exclusively by proteolytic release of soluble TRAIL from the tumor cell surface and *in vivo* by multiple modes including angiogenesis inhibition (Shin et al., 2007), tumor-associated macrophage cytotoxicity (Kawakubo et al., 2007) and enhanced immune responses (Shin et al., 2007), in addition to soluble TRAIL release (Kawakubo et al., 2007). However, the extent of apoptosis induced by cathepsin E varied among the cell lines, increasing according to the rank order PPC-1 < DU145 ≤ ALVA-101 < ALVA-41 < PC3. Resistance of the most insensitive cell line PPC-1 to cathepsin E-induced apoptosis was approximately 20 times that apparent for the most sensitive cell line PC-3 and

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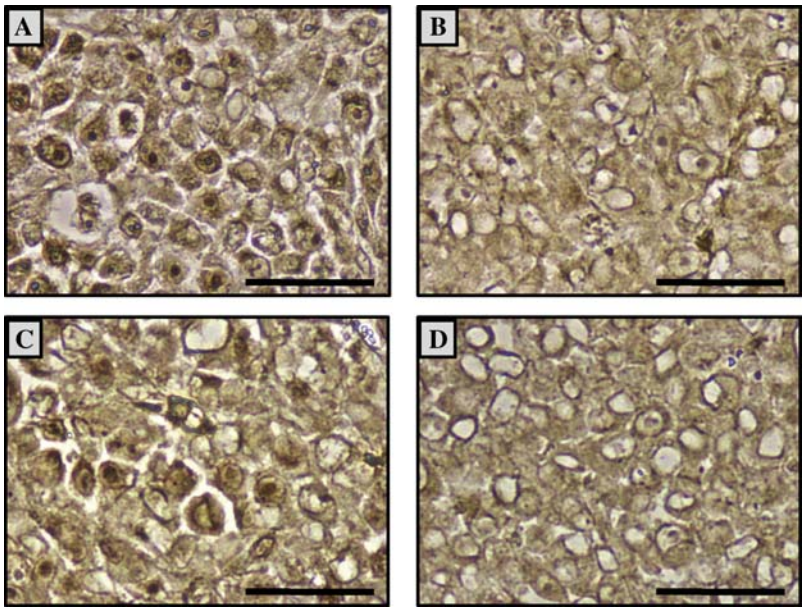

Figure 5 Immunohistochemical staining of xenografts with anti-cFLIP$_{S/L}$ antibody.
cFLIP$_{S/L}$ staining of xenografts treated with PBS alone (A), doxorubicin followed by vehicle (B), cathepsin E alone (C) or doxorubicin/cathepsin E followed by cathepsin E (D) at day 13. Data are obtained with three mice of each group. Scale bars, 100 μm.

explained in part by the expression of the soluble decoy receptor osteoprotegerin or by the efficiency of cathepsin E-mediated cleavage of TRAIL at the cell surface. Several studies have shown that most of the prostate cancer cell lines, including PPC-1, PC-3, DU-145 and LNCaP, are resistant to apoptosis by doxorubicin or TRAIL but are sensitized to TRAIL-induced apoptosis by a combination of the two agents (Kelly et al., 2002; Voelkel-Johnson et al., 2002; El-Zawahry et al., 2005; Shankar et al., 2005).

Given the manifold functions of cathepsin E *in vivo* (Kawakubo et al., 2007; Shin et al., 2007), the enzyme is likely to have significant advantages over TRAIL for inducing tumor cell apoptosis and tumor growth inhibition. Additionally, one of the practical problems with TRAIL is that its *in vivo* use requires high concentrations to obtain definite therapeutic efficacy, probably owing to its short half-life in plasma (Walczak et al., 1999; Kelley et al., 2001). Thus, we reasoned that the combination of doxorubicin with cathepsin E was more effective than that with TRAIL in overcoming resistance of prostate cancer cell lines to the drug. We first confirmed that PPC-1 cells were relatively resistant to cytotoxicity by either cathepsin E or doxorubicin and then extended this study to provide evidence that a combination of these agents could synergistically increase anticancer activity in the cells *in vitro* and *in vivo*. A previous study showed that simultaneous or sequential treatments with doxorubicin and TRAIL were sufficient to sensitize prostate cancer cells to TRAIL-induced apoptosis through down-regulation of c-FLIP$_L$ *in vitro* (Kelly et al., 2002) or up-regulation of DR4, DR5, Bax and Bak *in vivo* (Shankar et al., 2005). Our *in vitro* studies showed that doxorubicin induced down-regulation of c-FLIP$_S$, c-FLIP$_L$ and DR4 at the mRNA level but such changes of these molecules, except for c-FLIP$_S$, were not reflected at the protein level. Because the expression levels of Bcl-2 family members Bax, Bcl-2, and Bcl-X$_L$ in PPC-1 are known to be unaffected by doxorubicin (Kelly et al., 2002) and because cathepsin E has no substantial effect on the expression of these molecules including c-FLIP (data not shown), the observed effect *in vitro* in combination treatment of the two agents is more likely to be induced through down-regulation of c-FLIP$_S$ by doxorubicin.

The enhanced anticancer activity by combination treatment of cathepsin E and doxorubicin observed *in vitro* was also corroborated by *in vivo* studies with nude mice bearing PPC-1 xenografts. When the animals bearing the xenograft which had been treated with doxorubicin and cathepsin E were treated daily with cathepsin E, tumor growth arrest and apoptosis in tumor cells were more profoundly induced as compared with those treated with either of the agents and PBS (Figure 4). Importantly, the anticancer potential by combination of cathepsin E and doxorubicin *in vivo* was induced more profoundly than that *in vitro*, because, differing from *in vitro*, cathepsin E is likely to exert anti-cancer activity via manifold functions *in vivo* (Kawakubo et al., 2007; Shin et al., 2007). In addition, several tumor effector cells such as activated T cells, B cells, natural killer cells, dendritic cells and monocytes are known to express TRAIL on their cell surface (Mariani and Krammer, 1998; Fanger et al., 1999; Griffith et al., 1999; Kashii et al., 1999; Kayagaki et al., 1999). Thus, cathepsin E *in vivo* might exert its anticancer activity via the proteolytic release of soluble TRAIL from not only cancer cells but also these immune system cells. The present study also showed that xenografts treated with a combination of both agents contained fewer c-FLIP-positive cells than those with either of the agents alone. These

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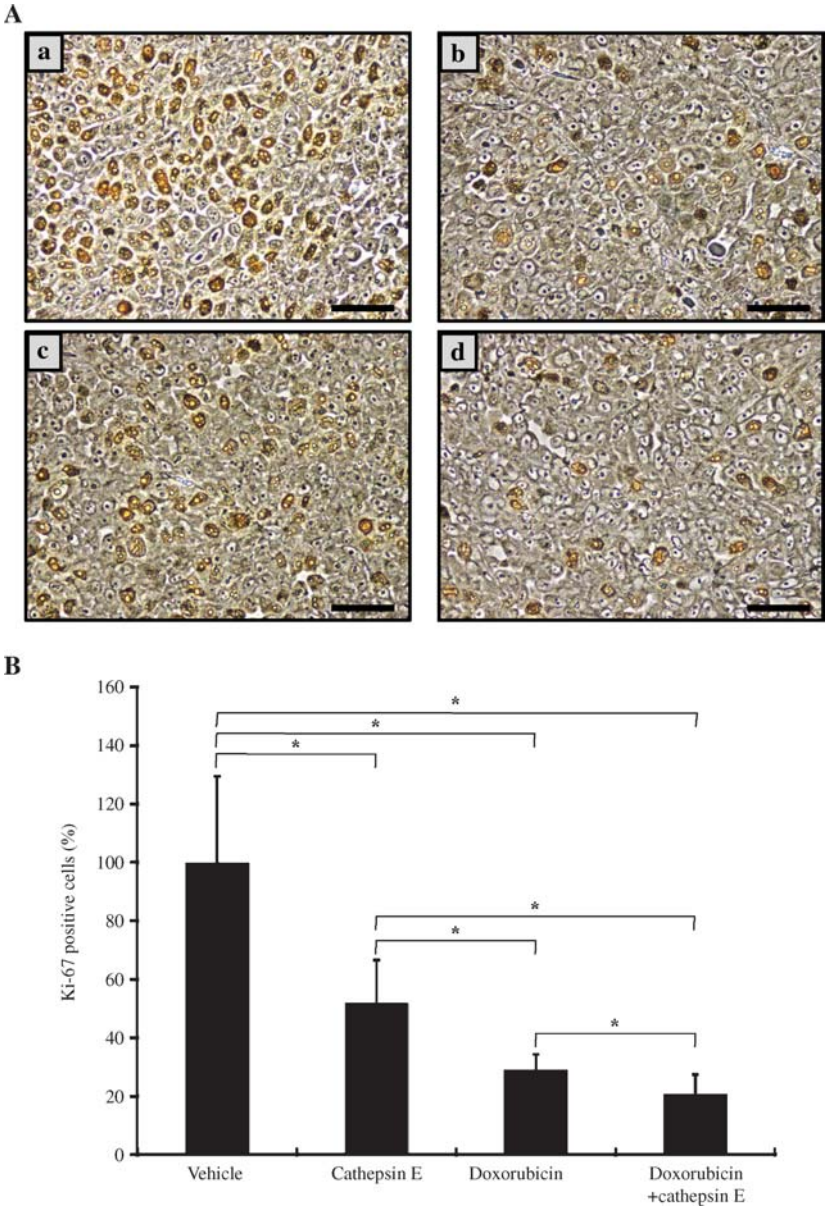

Figure 6 Immunohistochemical staining of xenografts with anti-Ki-67 antibody.
(A) Expression of the growth-phase specific antigen Ki-67 in xenografts treated with PBS alone (a), doxorubicin followed by vehicle (b), cathepsin E alone (c) or doxorubicin/cathepsin E followed by cathepsin E (d) at day 13. Scale bars, 50 μm. (B) Number of Ki-67-positive cells determined in six microscopic fields of each xenograft. The data are the percentage of Ki-67 positive cells was measured in each four groups. Data are the mean±SD of values from five independent experiments and expressed relative to the value of vehicle-treated ones. *$p<0.01$ versus the values for the corresponding group.

results were consistent with those *in vitro*. Given that both c-FLIP$_L$ and c-FLIP$_S$ proteins confer resistance to the death receptor-mediated apoptosis and block caspase activation (Krueger et al., 2001; Bin et al., 2002; Kim et al., 2002; Yang 2008), the reduced expression of c-FLIP, in particular c-FLIP$_S$, is an important component to overcome resistance to TRAIL-mediated apoptosis in the cells by cathepsin E or doxorubicin. We also found that xenografts treated with both agents markedly decreased in the number of Ki-67 positive cells, the extent of which was significantly greater than that with doxorubicin alone. Interestingly, xenografts treated with cathepsin E alone also significantly decreased the Ki-67 positive cell number. Given that the Ki-67 nuclear antigen is present in all phases of the cell cycle, except G_0 (Gerdes et al., 1984), the reduction of Ki-67-positive cells in xenografts treated with cathepsin E suggest that this enzyme might regulate cellular proliferation rates, although it is not clear how cathepsin E regulates cell proliferation. Importantly, neither histological abnormalities nor metastasis were detected in tissues and cells, other than tumors, from PPC-1-bearing nude mice treated with doxorubicin and/or cathepsin E (data not shown). No significant difference in terminal body weight

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was also observed between the mice with and without either of the agents during experimental periods.

In conclusion, cathepsin E showed a synergistic cytotoxicity on PPC-1 cells in combination with doxorubicin, even though either of the agents by itself was unable to efficiently induce cell death in these cells. It also emphasizes that intratumoral injection of cathepsin E in athymic mice can induce apoptosis in cancer cells alone without affecting normal cells and that the mice overexpressing the protein have significant advantages over the wild type and cathepsin E-deficient mice in preventing tumor growth and metastasis and in enhancing immune responses and survival (Kawakubo et al., 2007). Although detailed mechanistic studies are needed to obtain more insight into the synergistically enhanced anticancer activity by the combination treatment of cathepsin E and doxorubicin in cancer cells, we can provide the hypothesis evolved from these data that cathepsin E-based cancer therapy could be an efficient strategy in the treatment of prostate cancer.

Materials and methods

Cell culture and reagents

Doxorubicin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The human prostate carcinoma cell line PPC-1 was kindly provided by Dr J.Y. Bahk (Gyeogsang National University, Korea). The cells were cultured as previously described (Kawakubo et al., 2007). Cathepsin E was purified from rat spleen as described previously (Yamamoto et al., 1978).

Animals

Balb/c athymic nude mice (8 weeks old) were purchased from KYUDO Co., Ltd. (Saga, Japan). The use and care of animals were reviewed and approved by the Animal Research Committee of the Graduate School of Pharmaceutical Science, Kyushu University, Japan. All animals were maintained according to the guidelines of the Japanese Pharmacological Society in a specific pathogen-free facility at the Kyushu University Station for Collaborative Research.

Measurement of cell viability

PPC-1 cells (1×10^4) were seeded in 96-well plates in a volume of 100 μl and cultured in DMEM at 37°C for 24 h in a 5% CO_2 incubator. Then the cells were incubated with various concentrations of cathepsin E and doxorubicin in serum-free Opti-MEM for 20 h. After the addition of the Cell Counting Kit-8 reagent (10 μl) (Dojindo Laboratories, Kumamoto, Japan) to each well, the cells were incubated for an additional 1 h. Then, the absorbance was determined on a microplate reader at the wavelength of 450 nm with a reference wavelength at 650 nm. Cell viability was also examined by TUNEL assay as described (Kawakubo et al., 2007).

Apoptosis assays

Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for apoptosis/necrosis detection. PPC-1 cells were seeded in 35 mm μ-dish (ibidi GmbH, Martinsried, Germany) and cultured. After reached at 80% confluence, the cells were incubated with cathepsin E or doxorubicin at 37°C under a humidified 5% CO_2 environment for 4 h. After the treatment, the medium was completely removed, and the cells were washed with PBS. The dishes were then covered with Annexin-V-FLUOS labeling solution in a HEPES buffer containing PI, following incubation for 10 min in the dark at room temperature. After several washes, the cells were immediately analyzed by fluorescence microscopy.

In vivo tumorigenicity assay

PPC-1 cells (5×10^6 cells; 0.2 ml in PBS) were injected subcutaneously in the right flank region of nude mice. At the point when tumors reached 200 mm^3 (approx. 25 days post-inoculation), the animals were randomly divided into following four groups. (i) PBS alone (control); (ii) doxorubicin alone (once intra-peritoneal injection at a dose of 8 mg/kg); (iii) cathepsin E alone (intratumoral injection at a dose of 5 nM/kg, every 24 h); (iv) one doxorubicin injection followed by daily cathepsin E injection. Tumor size was measured every day with digital calipers and the volume was calculated by the following formula: $ab^2/2$, where a and b are the largest and smallest central cross-sectional dimensions, respectively. Experiments were terminated when tumors reached approximately 2500 mm^3 in the control group.

mRNA extraction and quantitative real-time RT-PCR

Quantitative RT-PCR was performed using a Taqman real-time PCR system on a Light Cycler 480 system instrument (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA was purified with a High Pure RNA Isolation Kit (Roche), and cDNA was prepared from 1 μg total RNA in 20 μl reaction volume using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Primers to amplify transcripts are as follows: GAPDH: 5′-AGC CAC ATC GCT CAG ACA C-3′ (forward), 5′-GCC CAA TAC GAC CAA ATC C-3′ (reverse), DR4: 5′-GGA GGC ACA GTG TCT GCT G-3′ (forward), 5′-CAG CAC CAT TTG CTG GAA C-3′ (reverse), DR5: 5′-AGA CCC TTG TGC TCG TTG TC-3′ (forward), 5′-GGG TGA TCA GAG CAG ACT CAG-3′ (reverse), c-FLIP long form: 5′-GCT CAC CAT CCC TGT ACC TG-3′ (forward), 5′-CAG GAG TGG GCG TTT TCT T-3′ (reverse), c-FLIP short form: 5′-CAG GAA CCC TCA CCT TGT TT-3′ (forward), 5′-CAG ATT TAT CCA AAT CCT CAC CA-3′ (reverse). A Light Cycler Universal Probe Master (Roche) specific for each sequence was applied: No. 60 (for GAPDH), No. 63 (for DR4), No. 18 (for DR5), No. 14 (for c-FLIP long form), and No. 53 (for c-FLIP short form), respectively. RT-PCR data were analyzed for at least three different experiments.

Statistical analysis

Quantitative data are presented as mean±SD. The statistical significance of differences between mean values was assessed by Student's t-test. p-Values of <0.05 were considered statistically significant.

Data analysis for combination treatment

The nature of the effect of particular dose combinations of the agents was determined by isobolographic analysis, according to the method as described previously (Berenbaum, 1977, 1978). In brief, the point representing that combination would lay on, below, or above the straight line joining the doses of the two agents that, when given alone, produce the same effect as that combination, representing additive, synergistic, or antagonistic effects, respectively. Synergism was also determined by calculating the combination

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index (CI) using the median effect analysis for fixed agent dose combinations (Chou, 2006):

$$CIx=[D]_1/[Dx]_1+[D]_2/[Dx]_2+\alpha[D]_1[D]_2/[Dx]_1[Dx]_2,$$

where CIx is the CI value for x% effect, $[Dx]_1$ and $[Dx]_2$ are the doses of agents 1 and 2 required to exert x% effect alone, whereas $[D]_1$ and $[D]_2$ are the doses of agents 1 and 2 that elicit the same x% effect in combination with the other agent, respectively. Alpha describes the mode of interaction: $\alpha=0$ for mutually exclusive (similar modes of action), $\alpha=1$ for mutually nonexclusive drugs (independent modes of action). CI=1 indicates additivity, CI<1 synergism, and CI>1 antagonism.

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