TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2015) 45: 38-46

doi:10.3906/sag-1401-127

An in vitro and in vivo investigation of the cytotoxic effects of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester and bortezomib in multiple myeloma cells

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Received: 26.01.2014 • **Accepted:** 10.05.2014 • **Published Online:** 12.01.2015 • **Printed:** 09.02.2015

Background/aim: In this study, the in vitro and in vivo effectiveness of caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) in combination with bortezomib, a proteasome inhibitor, was explored in multiple myeloma (MM) cells.

Materials and methods: The cytotoxic effects of CAPE and bortezomib were determined by XTT cell proliferation assay. Apoptosis levels were analyzed with annexin V-fluorescein isothiocyanate, nuclear factor kappa beta (NF-κB) was analyzed with electrophoretic mobility-shift assay, and interleukin (IL)-6 levels were analyzed with enzyme-linked immunosorbent assay to evaluate CAPE's mechanism of action. To investigate the in vivo effectiveness of CAPE and bortezomib, an experimental plasmacytoma model was induced in BALB/c mice.

Results: Increasing concentrations of CAPE and bortezomib decreased the proliferation of ARH-77 cells in a dose-dependent manner. With doses of CAPE IC50, a significant increase in apoptosis and a significant decrease in IL-6 levels were detected. The NF-κB DNA-binding activity decreased compared to the basal ARH-77 level. The administration of CAPE alone or in combination with bortezomib increased the rate of survival compared to the control group.

Conclusion: We think that our study, which is the first to demonstrate the in vitro and in vivo effectiveness of the combined use of CAPE and bortezomib, will be a pioneer for future human applications of CAPE in MM.

Key words: Caffeic acid phenethyl ester, bortezomib, multiple myeloma, in vitro cytotoxicity, in vivo study

1. Introduction

Multiple myeloma (MM) is a hematologic cancer characterized by uncontrolled monoclonal plasma cell proliferation (1). MM is currently an incurable B-cell malignancy that accounts for nearly 10% of human hematopoietic cancers and 1% of all human cancers (2). Presently, the 2 most effective treatment choices for patients with MM are tandem high-dose chemotherapy followed by autologous stem cell infusion, or allogeneic hematopoietic stem cell transplantation after myeloablative therapy or reduced-intensity conditioning (1). The proteasome inhibitor bortezomib is a novel drug with promising efficacy, even for patients with relapsed refractory MM (3,4). Nevertheless, these choices are not appropriate in all patients, and drug resistance often increases over time. Consequently, there is a need for new treatment options that can augment the effectiveness of current treatments (5,6).

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Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) is an active component of honey bee propolis extract. Propolis is a resinous and waxy substance made by bees in the natural environment that forms the honeycomb, and, depending on its source, varies from yellow to dark brown in color. Although the chemical structure of propolis varies depending on the flora in the region where the bees reside, it is not fully understood. At present, more than 300 compounds have been found in the structure of propolis. Propolis is used as a traditional medicine in the Far East (7). Recently, CAPE has been shown to act as an antiviral (8), antiinflammatory (9), antitumoral (10), neuroprotective (11), antioxidant (12,13), antiallergic (14), and antiatherosclerotic (15) agent in diverse systems (16). CAPE has cytotoxic, apoptotic, and antiproliferative effects on various tumor cells, both in vivo and in vitro (17,18). CAPE is a well-known and well-documented inhibitor of interleukin-6 (IL-6) and the transcription factor nuclear factor kappa beta (NF-κB) (19,20). IL-6 is a key growth and survival factor for murine plasmacytoma cells, human myeloma cells, and myeloma cell lines. Additionally, IL-6 is also a major morbidity factor for patients with MM (2,21). Although the precise role of NF-κB activation in the pathogenesis of MM has not been fully characterized, MM cell adhesion to bone marrow stromal cells induces NF-κB-dependent upregulation of the transcription of IL-6, which is a growth and antiapoptotic factor in MM (22,23).

Transcription NF-κB is the generic name for a family of dimeric factors that bind to many different promoters to initiate transcription (24). The most common form of NF-κB is the p65/p50 heterodimer, while the other forms occur less frequently (25). Loss of the normal regulation of NF-κB has been found to be a major contributor to unregulated growth, resistance to apoptosis, and the predisposition to metastasize, which are observed in many cancers. Constitutive NF-κB activity has been identified in many different tumors, such as MM, acute lymphoblastoid leukemia, prostate cancer, breast cancer, and colon cancer (26–28).

The human MM cell line ARH-77 has been used in many in vitro cytotoxicity studies (29–31). We have previously shown the cytotoxic effect of CAPE on the ARH-77 cell line (18). However, the cytotoxic effect of the combination of CAPE and bortezomib on ARH-77 cells has not yet been investigated.

The present study aimed to investigate the in vitro and in vivo cytotoxic effects of CAPE and bortezomib in MM cells. We had already proven, for the first time, the antitumor effectiveness of CAPE, found in propolis, on MM cells in an in vitro study (18). The concurrent use of bortezomib, which is used in the treatment of MM, with CAPE has never been attempted.

2. Materials and methods

2.1. Reagents and drug

CAPE, dimethyl sulfoxide (DMSO), pristane (2,6,10,14-tetramethylpentadecane, 95% pure), and the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide (XTT) cell proliferation assay were manufactured by Sigma-Aldrich (USA). Bortezomib was manufactured by Janssen-Cilag (UK). All materials were purchased commercially.

2.2. In vitro studies

2.2.1. Cell lines and culture conditions

The ARH-77 MM cell line was used in frozen form in a liquid nitrogen system at the Gülhane Military Medical Academy Research and Development Center, Medical and Cancer Research Laboratory. The ARH-77 MM cell line was previously purchased from the American Type Culture Collection (USA). ARH-77 is a floating human plasma cell leukemia cell line that secretes IgGκ. The ARH-77 cell line was maintained at 37 °C in a 5% CO_2 medium in RPMI 1640 (GIBCO, USA), which was supported with a solution of 10% fetal bovine serum (Hyclone Laboratory, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO).

2.2.2. Cell viability assay and isobologram analysis

For the in vitro study, IC_{50} values (drug concentration that restrains cell proliferation by 50%) were obtained by the XTT method at hours 24 and 48 following administration of varying doses of CAPE and bortezomib to the MM cell line (ARH-77). In summary, 96-well plates were seeded with 2×10^4 cells/well including 100 μL of growth medium in the absence (0.1% DMSO) or presence of increasing concentrations of the drugs (CAPE concentrations of 5, 10, 20, 40, 80, and 160 μg/mL and bortezomib concentrations of 1, 10, 20, 30, 50, and 100 nM). At the end of incubation, the XTT test was used to assess cell viability. Shortly thereafter, 96-well plates were seeded with 2×10^4 cells/well including 200 mL of growth medium in the presence or absence of increasing concentrations of the drugs. The cells were incubated at 37 °C in 5% CO_2. After 72 h, cell suspensions were treated with 50 mL of XTT reagent for 4 h. Specimens were detected spectrophotometrically. Each study was repeated 3 times.

After increased doses of CAPE and bortezomib were given individually and in combination, the decrease in viability resulting from using doses of CAPE IC_{50} and bortezomib IC_{50} were evaluated with CalcuSyn isobologram analysis. The combination index (CI), a measurement of the combined action of both drugs, is used as a method of determining the median effect. A CI value of <1 shows a synergistic effect (0.1–0.5 potent synergism; <0.1 very potent synergism); a CI value of 1 shows an additive effect; and a CI value of >1 shows an antagonistic effect (3.3–10 potent antagonism, >10 very potent antagonism).

2.2.3. Electrophoretic mobility-shift assay for NF-κB

Electrophoretic mobility-shift assay was performed on ARH-77 cells stimulated with 0.1% DMSO, CAPE IC_{50}, or bortezomib IC_{50} with 0.1% DMSO for 24 h. For the isolation of nuclear and cytoplasmic proteins, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 1 mL of Buffer A containing 20 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, and 1X protease inhibitors; it was then incubated on ice for 30 min. The cells were vortexed at a high speed for 15 s, and then, following the addition of 0.25% NP40 (AppliChem, Germany), the cells were incubated on ice for another 5 min and vortexed for 5 s. Supernatants were obtained after centrifugation at 12,000 × *g* for 1 min at 4 °C. The remaining nuclear pellet was washed with 200 µL of Buffer A to remove all cytoplasmic proteins and centrifuged. The pellet was resuspended in 200 µL of Buffer B containing 20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 0.5 M NaCl, and 25% glycerol and incubated on ice for 30 min. The lysate was centrifuged for 10 min at 12,000 × *g* at 4 °C. The supernatant was accepted as the nuclear fraction.

The NF-κB DNA-binding consensus sequences were prepared in the database at http://www.ncbi.nlm.nih.gov/nucleotide and purchased commercially (İontek, Turkey). Binding reactions were prepared with NF-κB-binding consensus sequences containing marked probes. Afterwards, these reactions were carried out in a polyacrylamide gel system and transferred to nylon membranes. To identify the mark on the probes, the nylon membranes were incubated in the reaction medium. Probes bound with NF-κB protein were visualized with chemiluminescence.

2.2.4. Measurement of IL-6 by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay was performed on ARH-77 cells stimulated with 0.1% DMSO, CAPE IC_{50}, or bortezomib IC_{50} with 0.1% DMSO for 24 h. The supernatant's IL-6 levels were measured with the enzyme immunoassay method. The absorbance readings were made with the Alisei Quality System device (SEAC Radim Group, Italy). IL-6 concentrations were calculated by drawing standard curves.

2.2.5. Measurement of apoptosis by annexin V and flow cytometry

The fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit protocol (BD Pharmingen, USA) was used for this study. CAPE IC_{50}, bortezomib IC_{50}, and, for control purposes, 0.1% DMSO were applied to ARH-77 cells for 24 h. Cells were centrifuged at 800 rpm for 5 min and the supernatant was removed. The cell pellet, at a final concentration of 1×10^6 cells/mL, was dissolved with 1X annexin V binding buffer. A 200-µL sample was taken from this suspension and vortexed after adding 5 µL of annexin V-FITC and 5 µL of propidium iodide. It was then left to incubate at room temperature for 15 min. At the end of incubation, 400 µL of annexin V binding buffer was added and vortexed. Thirty minutes after readings were made using flow cytometry (BD FACS CANTO), the results were analyzed.

2.3. In vivo studies

2.3.1. Animals

Female BALB/c mice (5–6 weeks old) were provided by the Gülhane Military Medical School Research Center, Ankara, Turkey. The mice were housed in a controlled environment with a 12-h light/dark cycle at a temperature of 24 ± 2 °C and humidity of 55 ± 10%. They were given pellet chow and water ad libitum. Ethical approval for the animal study was received from the Animal Research and Care Committee at Gülhane Military Medical School.

2.3.2. Induction and diagnosis of mouse plasmacytoma

The mouse plasmacytoma (MPC) model was prepared as previously defined (38). In brief, MPC was induced in mice by intraperitoneal injections of 0.5 mL of pristane on days 0, 60, and 120. Controls were given an equal amount of PBS. Full groups were maintained under surveillance for 400 days. One hundred days after the first injection of pristane, mice were inspected for the presence of ascites. They were examined by paracentesis. The diagnosis of plasmacytoma was made according to previously defined criteria (32).

2.3.3. Study protocol

A total of 124 mice were registered in this study and they were randomly assigned to 8 experimental groups: Group I (n = 20), pristane-induced alone; Group II (n = 20), CAPE started after the appearance of pristane-induced MPC; Group III (n = 20), bortezomib started after the appearance of pristane-induced MPC; Group IV (n = 20), CAPE and bortezomib started after the appearance of pristane-induced MPC; Group V (n = 11), CAPE given alone; Group VI (n = 11), bortezomib given alone; Group VII (n = 11), PBS given alone; and Group VIII (n = 11), nothing given.

2.3.4. Histopathological evaluation

After 400 days of observation, the mice were sacrificed by cervical dislocation and the intraabdominal organs (spleen, liver, and kidneys) were fixed with 10% formalin and embedded in paraffin. Sections 4 µm long were taken, stained with hematoxylin and eosin, and examined for plasmacytoma. Histopathological diagnosis of plasmacytoma was made according to previously specified criteria (33).

2.4. Statistical analysis

At the end of the CAPE and bortezomib cytotoxic studies, the IC_{50} values for the 2 agents were calculated using the

regression model. The IL-6 and apoptosis levels of the different groups were compared using one-way variation analysis. Dunnett and Bonferroni corrections were used as post hoc tests. The comparison of the survival times between the treatment groups was performed using Kaplan–Meier analysis; log-rank statistics were calculated. Isobologram curves were drawn and combination indexes were calculated. $P < 0.05$ was considered statistically significant. SPSS 15 was used for statistical analysis.

3. Results

3.1. Cytotoxic effects of CAPE and bortezomib on ARH-77 cells

In this study, it was shown that increasing concentrations of CAPE (5–100 μg/mL) decreased the proliferation of ARH-77 cells in a dose-dependent manner compared to untreated controls (Figure 1). As a result, the IC_{50} value of CAPE was determined to be 28 and 21 μg/mL at 24 and 48 h, respectively. Increasing intracellular concentrations of bortezomib (1–100 nM) also inhibited cell proliferation in a dose-dependent manner compared to untreated controls (Figure 2). The IC_{50} value of bortezomib was calculated as 13 and 11 nM for ARH-77 cells at 24 and 48 h, respectively.

3.2. Combination effects of CAPE and bortezomib on ARH-77 cell proliferation

Combination studies of CAPE with bortezomib were also carried out to determine whether there was an increase in the antiproliferative effects of CAPE on ARH-77 cells. Cell proliferation data showed that both the increased dosage of bortezomib with the IC_{50} constant dose of CAPE and the increased dosage of CAPE with the IC_{50} constant dose of bortezomib increased the cytotoxic effectiveness compared to 0.1% DMSO in ARH-77 cells. The increased dosage of bortezomib and the IC_{50} constant dose of CAPE had very strong synergism at both 24 h (CI (ED_{50}): 0.00067) and 48 h (CI (ED_{50}): 0.00295) in the ARH-77 cell line (Figure 3A). Similarly, the increased dosage of CAPE and the IC_{50} constant dose of bortezomib had strong synergism at both 24 h (CI (ED_{50}): 0.58040) and 48 h (CI (ED_{50}): 0.51044) in the ARH-77 cell line (Figure 3B).

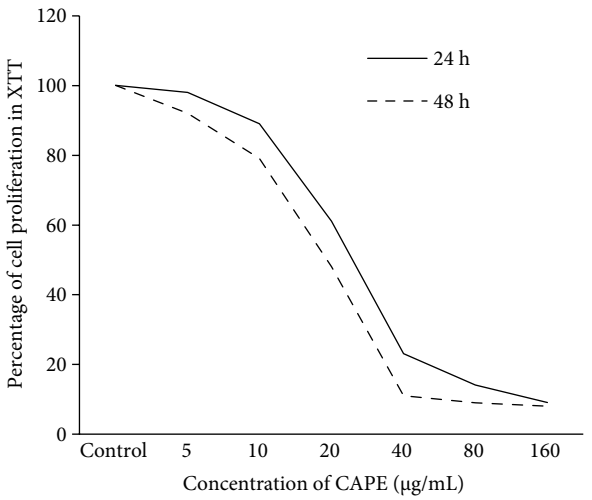

Figure 1. Percentage of alive cells at different CAPE concentrations.

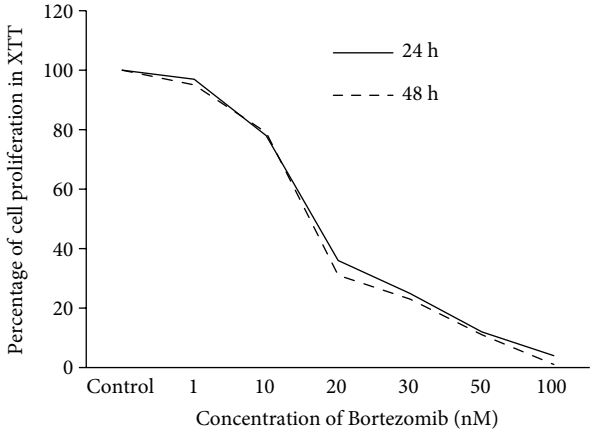

Figure 2. Percentage of alive cells at different bortezomib concentrations.

3.3. Effect of CAPE on apoptosis

To investigate whether there was any decrease in cell viability caused by apoptosis, we analyzed apoptosis using annexin V with flow cytometry. A significant increase in apoptosis was observed in the ARH-77 MM cells given CAPE IC_{50} doses ($P < 0.001$). These results show that CAPE does induce apoptosis in ARH-77 cells ($P < 0.001$) (Table 1).

3.4. CAPE inhibits NF-κB-binding activity and IL-6 levels

To investigate the mechanisms underlying CAPE-induced apoptosis, we examined the effect of CAPE on the expression of NF-κB and the NF-κB-dependent upregulation of IL-6. The NF-κB DNA-binding activity in ARH-77 MM cells that were given doses of CAPE IC_{50} and bortezomib IC_{50} were found to be decreased compared to basal ARH-77 levels (Figure 4). Similarly, a significant reduction was detected in IL-6 levels ($P < 0.001$). These results show that NF-κB inhibition by CAPE does induce apoptosis in ARH-77 cells.

3.5. Assay of in vivo examination

The appearances of normal mice and mice with plasmacytoma are compared in Figure 5. Abdominal distention is observed due to intraabdominal ascitic fluid in mice with experimentally induced plasmacytoma. The appearances of the intraabdominal organs (liver, kidneys, and spleen) of the normal mice and mice with plasmacytoma are compared in Figure 6.

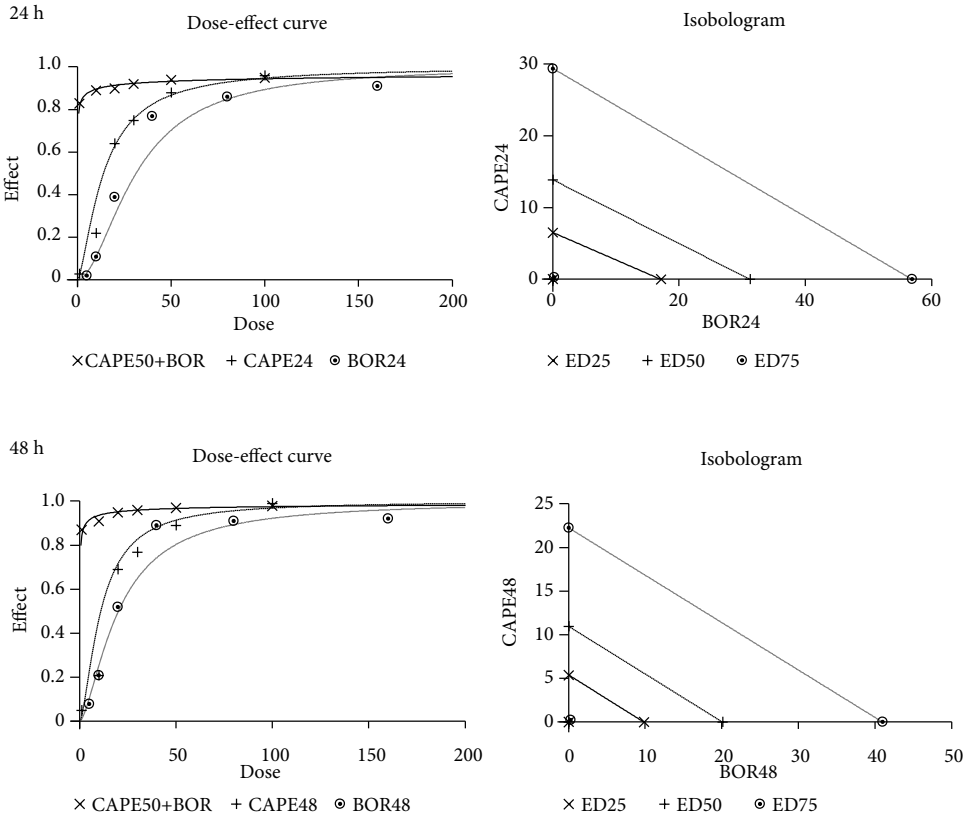

Figure 3A. Increased dosage of bortezomib and IC_{50} constant dose of CAPE on the ARH-77 cell line.

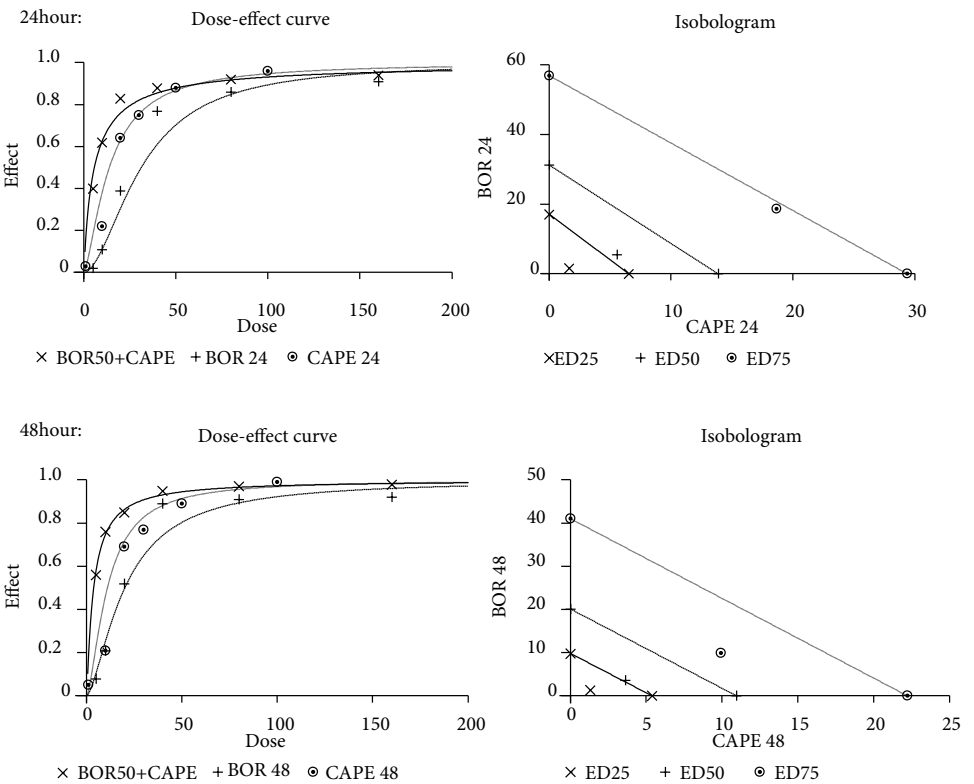

Figure 3B. Increased dosage of CAPE and IC_{50} constant dose of bortezomib on the ARH-77 cell line.

Table 1. Comparison of percentage of apoptotic cells between groups.

	n	Mean	SDP*	
Control	8	1.9000	0.34059	
Bortezomib	8	5.6000	0.48166	<0.001 [a,b,c]
CAPE	8	13.2000	0.98793	

* ANOVA test result.
Statistically significant differences between: [a]control versus bortezomib group, [b]control versus CAPE group, [c]bortezomib group versus CAPE group.

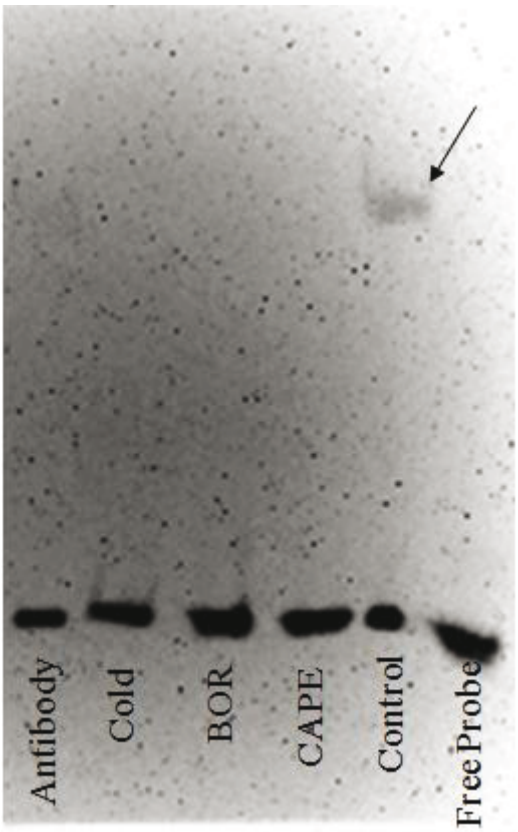

Figure 4. While NF-κB signal was obtained in the control group consisting of only the ARH-77 cell line (shown by an arrow), NF-κB binding signal could not be obtained in ARH-77 cells treated with CAPE and bortezomib.

In the plasmacytoma group, plasma cell infiltration was observed in the peritoneal surfaces of the abdominal cavity. Those plasma cells were mature (Figure 7). Additionally, fibrosis, calcification, and inflammation were also associated with the plasma cells. However, no differences were observed for fibrosis or inflammation in either the vehicle- or CAPE-treated animals. In other tissues and organs, including the liver, spleen, and kidneys, no damage was observed during histopathological examination.

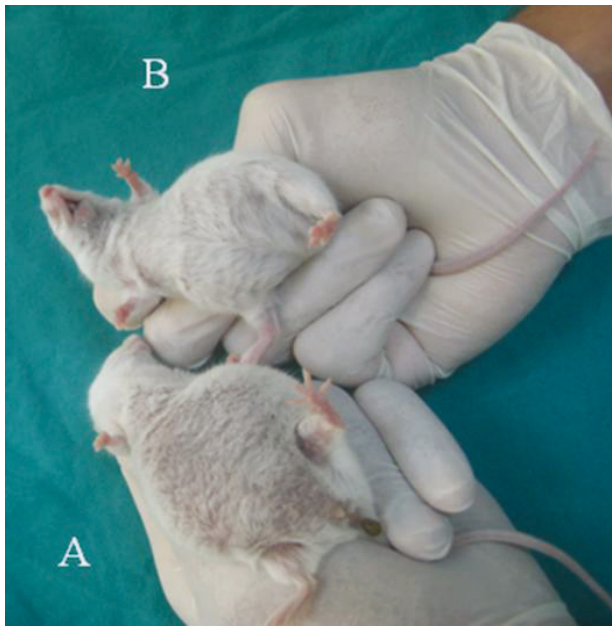

Figure 5. Abdomen was markedly distended in mouse with plasmacytoma (A) as compared to normal mouse (B).

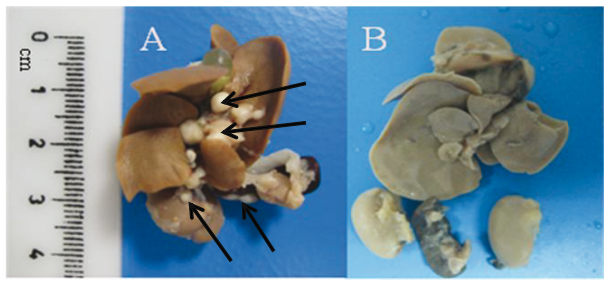

Figure 6. A) Macroscopic appearance of multiple oil granulomas (arrows) on the surface of abdominal organs in mouse with plasmacytoma. **B)** Normal appearance of abdominal organs including liver, kidneys, and spleen in the control group. Magnification of images 1/1.

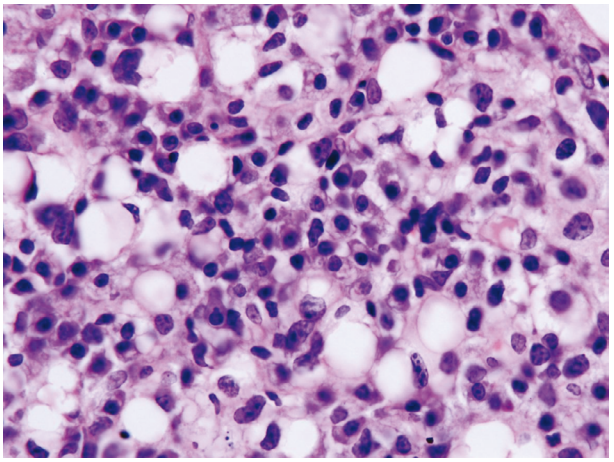

Figure 7. Atypical plasma cell infiltration in tissue samples obtained from mice with experimentally induced plasmacytoma.

The mean survival rate of mice with experimentally induced plasmacytoma is shown in Table 2. Although not statistically significant, the survival rate was longer in groups in which CAPE or bortezomib were administered, and particularly for the group in which CAPE and bortezomib were administered together.

4. Discussion

In this study, we investigated the in vitro and in vivo cytotoxic effects of CAPE, which is still an experimental drug, and bortezomib, which is currently used as a drug. Inclusion of bortezomib in our study enabled us to evaluate the synergistic effects of the agents in terms of cytotoxic effects and to use CAPE as a positive control when needed.

In our study, we demonstrated that CAPE and bortezomib have in vitro cytotoxic effects on the ARH-77 MM cell line and that, when they were used in combination, there was a strong synergistic effect. In addition, our in vitro studies showed that CAPE inhibited NF-κB DNA-binding activity in a dose- and time-dependent manner in ARH-77 cells. It also significantly reduced IL-6 levels and induced apoptosis.

NF-κB is misregulated in many different types of human cancers and is found to be chronically active in many cancers (34,35). NF-κB activity protects cancer cells from apoptosis and induces their proliferation. NF-κB inhibition, in connection with increased apoptosis, establishes a balance between dead and viable cells (36). Therefore, we think that the reduction of NF-κB DNA-binding activity caused by CAPE is significant.

IL-6 is the major growth and survival factor for MM cells (37). Although the role of NF-κB in MM pathogenesis is not definite, it has been shown that stimulation of the transcription of the growth and antiapoptotic factor IL-6 is dependent on NF-κB (38). For this reason, it is extremely significant that CAPE reduced IL-6 levels in the MM cell lines.

There is only one study that has investigated the in vitro cytotoxic effects of CAPE on ARH-77 MM cell lines (18). In that study, which had results that supported our own findings, Koru et al. demonstrated that CAPE, in a 100 μg/mL concentration over 72 h, inhibited cell growth by 90.4% and displayed a cytotoxic effect of 80.4%. In the same study, apoptosis stimulation was observed in 92.3% of the cells treated with a 22.5 μg/mL concentration of CAPE over 72 h and CAPE was also shown to inhibit IL-6

Table 2. Mean survival in mice of plasmacytoma groups (days). PCT = Plasmacytoma, BOR = bortezomib.

Group	N	Mean	Standard error
PCT	10	269.000	12.234
PCT + CAPE	10	284.300	10.305
PCT + BOR	10	287.500	9.762
PCT + CAPE + BOR	10	298.500	1.423
PBS	10	298.600	1.328
CAPE	10	285.600	13.661
BOR	10	281.100	17.930
No treatment	10	300.000	0.000
P*		0.139	

*: Log-rank test result.

secretion at the IC_{50} concentration. In addition, various in vitro studies have been conducted investigating the cytotoxic effects of CAPE. Chen et al. investigated the relationship between CAPE-induced alterations of the redox state and apoptosis in human leukemic HL-60 cells. In that study, CAPE was shown to inhibit cell growth in 70.3% of cells at a 6 μg/mL concentration by the end of 48 h as compared to the control group and caused DNA fragmentation at the same concentration after 6 h (17).

In the in vitro studies conducted by Onori et al., CAPE was shown to reduce the growth of cholangiocarcinoma by NF-κB inhibition and apoptosis induction (39). Other studies have also shown CAPE to inhibit NF-κB and to have cytotoxic effects on various cancer cell lines (40,41).

According to the in vivo experimental results of our study, although not statistically significant, CAPE was shown to increase the survival rate of mice with experimentally induced plasmacytoma alone or in combination with bortezomib. There are no available in vivo studies investigating the effects of CAPE on MM cells. However, results supporting our study have been reported in various studies investigating the in vivo effects of CAPE on various cancer models (42,43). Among them is the in vivo study by Onori et al. in which, after intraperitoneal CAPE at 10 mg/kg was administered to BALB/c mice, tumor growth was found to be reduced within 77 days, the latent period of the tumor was increased 2-fold, and no differences were observed in the fibrosis or inflammation in either the carrier- or CAPE-treated animals with no other tissue/organ damage.

In conclusion, CAPE was shown to prevent MM cell line proliferation when administered alone and was shown to have a very strong synergistic cytotoxic effect when administered in combination with bortezomib. In mice with experimentally induced plasmacytoma, although not statistically significant, CAPE used alone or in combination with bortezomib was shown to prolong the life span compared to the control group. We were not surprised to see the difference between in vitro studies performed in the laboratory (artificial conditions) and in vivo studies performed in a living organism. However, the significance of these findings will become more apparent once an in vivo study is conducted with a larger number of test animals or with xenograft models. Our study is the first to prove the in vivo and in vitro effectiveness of the combined use of CAPE and bortezomib. We think that our study will serve as a pioneer study for future human studies regarding CAPE.

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