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Inhibition of Neurotoxicity/Anticancer Activity of Bortezomib by Caffeic Acid and Chlorogenic Acid

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Abstract. *Background/Aim: Bortezomib, used for the treatment of multiple myeloma, has been reported to induce potent neurotoxicity. The present study investigated whether eight popular polyphenols inhibit bortezomib-induced neurotoxicity without affecting its anticancer activity. Materials and Methods: Viable cell number was determined with the MTT method. Tumor-specificity was determined by the relative cytotoxicity in human oral squamous cell carcinoma vs. normal oral cells. Neurotoxicity was determined by the relative cytotoxicity in differentiated rat neuronal PC12 cells vs. normal cells. Apoptotic cells were quantified by cell cycle analysis. Results: Bortezomib induced cell shrinkage, disruption of neurites, and accumulation of PC-12 cells in subG1. Only chlorogenic acid and caffeic acid protected PC-12 cells from bortezomib-induced neurotoxicity. Ferulic acid that has one of the two hydroxyl groups replaced by a methoxy group showed a significantly reduced neuroprotective effect. Caffeic acid and the chlorogenic acid also neutralized the anticancer potential of bortezomib. Conclusion: Caffeic acid and the chlorogenic acid may reduce the biological activity of bortezomib by forming a conjugate.*

Drugs and radiation show bimodal biological activity known as hormesis: beneficial actions at lower concentrations, and adverse actions at higher concentrations (1, 2). Previous studies reported adverse effects of anticancer drugs such as cardiotoxicity (3, 4), neurotoxicity (5, 6), and keratinocyte toxicity (7, 8). Bortezomib, a proteasome inhibitor used in the treatment of multiple myeloma, has been reported to induce potent neurotoxicity (9, 10). Since polyphenols such as curcumin (11), resveratrol (12), chlorogenic acid (13, 14), and *p*-coumaric acid (15) (Figure 1) daily taken from curry, wine, coffee, and various food materials have been reported to show neuroprotective activity, the present study was undertaken to investigate whether these compounds can protect differentiating P12 neuronal cells from the bortezomib-induced insult (9) (Figure 2) without affecting the anticancer activity. Since chlorogenic acid, which consists of caffeic acid and quinic acid, showed the most potent neuroprotective activity among these compounds (Figure 3), caffeic acid (CA), and its related compounds ferulic acid (FA), and isoferulic acid (IFA), and vanillic acid (4-hydroxy-3-methoxybenzoic acid) (VA) (16) (Figure 1), known for their neuroprotective activity (17-19), were also investigated whether they also abrogate the bortezomib-induced neurotoxicity.

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Key Words: Polyphenol, PC12, SH-SY5Y, NGF, overlay method, neurotoxicity, tumor-specificity, bortezomib, cell cycle, chlorogenic acid, caffeic acid.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO BRL (Grand Island, NY, USA); fetal bovine serum (FBS) and actinomycin D from Sigma Aldrich Inc. (St. Louis, MO, USA); chlorogenic acid (CGA), resveratrol (RSV), curcumin, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and human recombinant nerve growth factor (NGF) from Wako Pure Chem. Ind. (Osaka, Japan); *p*-coumaric acid, caffeic acid, ferulic acid, isoferulic acid and vanillic acid from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); bortezomib (Bmib) from Janssen Pharmaceutical K.K. (Tokyo, Japan); culture plastic dishes and plates (96-well) from Becton Dickinson (Franklin Lakes, NJ, USA).
Cell culture. PC12, a cell line derived from a pheochromocytoma of

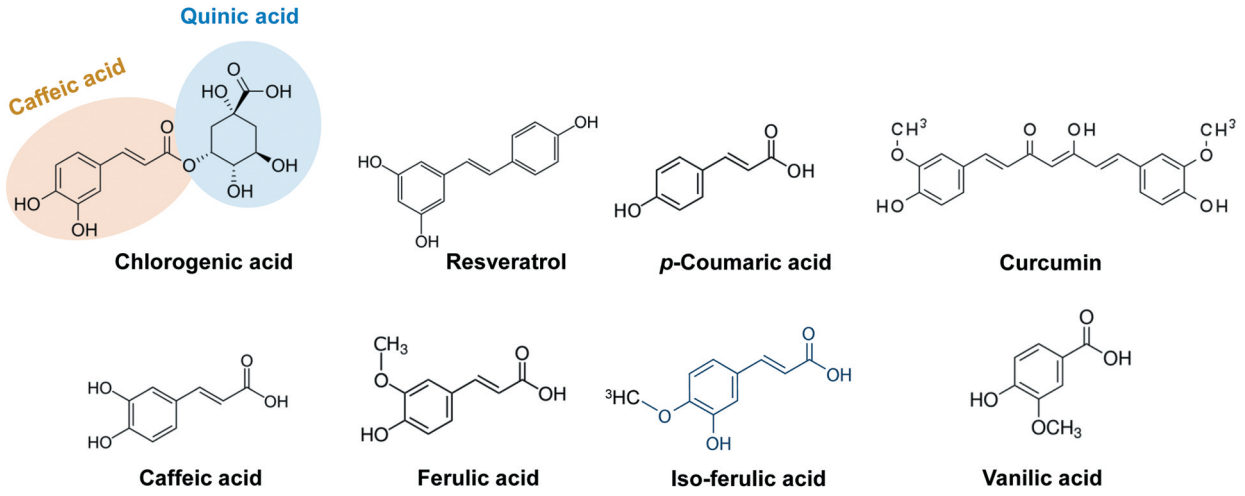


Figure 1. *Structures of polyphenols used in this study.*

rat adrenal medulla (20), SH-SY5Y, cloned from a human bone marrow biopsy derived line called SK-N-SH (21, 22), and human oral squamous cell carcinoma (OSCC) cell lines [Ca9-22 (derived from gingival tissue), HSC-2, HSC-3 and HSC-4 (derived from tongue)] were purchased from Riken Cell Bank (Tsukuba, Japan). Human gingival fibroblast (HGF), periodontal ligament fibroblast (HPLF), and pulp cell (HPC) were established from the first premolar extracted tooth and periodontal tissues of a twelve-year-old girl, according to the guideline of Institutional Board of Meikai University Ethic Committee (No. A0808) (23) and used at 10–18 population doubling level (PDL), considering their limited life span. These cells were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS and antibiotics, under a humidified 5% CO_2 atmosphere. Differentiated PC12 cells (referred to as dPC-12 cells) expressing extended neurites (Figure 4) were prepared by incubating PC-12 cells for 6 or 7 days in serum-free DMEM medium supplemented with NGF (50 ng/ml), with fresh NGF-containing medium added on day 3 (24). Cell morphology was checked periodically under a light microscope (EVOS FL; Thermo Fisher Scientific, Waltham, MA, USA).

Determination of viable cell number. Cells were treated for 48 h with the indicated concentrations of samples, and the viable cell number was determined by the MTT method, as described previously (25). In brief, cells were incubated for 2 h with MTT reagent at 0.1 mg/ml (for dPC-12 cells) or 0.2 mg/ml (for other cells). The formazan formed was dissolved with 0.1 ml DMSO and the absorbance at 560 nm (that reflects the relative viable cell number) was measured using a microplate reader (Infinite F50R; TECAN, Männedorf, Switzerland).

Calculation of tumor-specificity index (TS). TS was calculated by the following equation: TS=Mean 50% cytotoxic concentration (CC_{50}) (HGF + HPLF + HPC)/mean CC_{50} (Ca9-22 + HSC-2 + HSC-3 + HSC-4) (B/A in Table I).

Calculation of neurotoxicity (NT). Neurotoxicity was calculated by the following equation: NT=mean CC_{50} (HGF + HPLF + HPC)/ CC_{50} (dPC-12) (C/A in Table I).

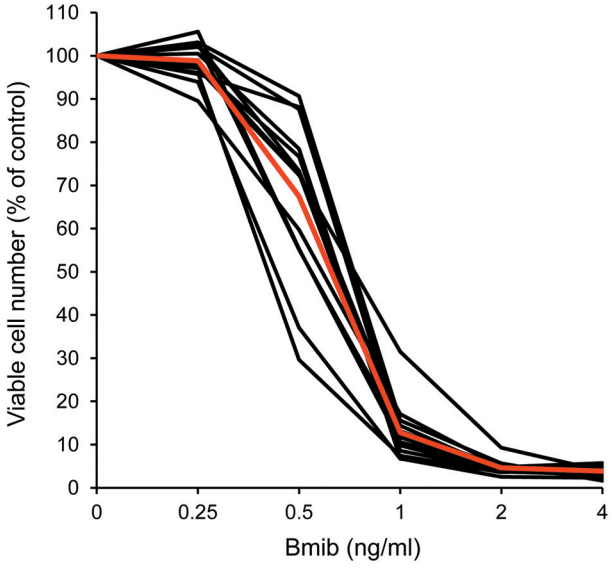


Figure 2. *Dose-response curve of growth inhibition by bortezomib. Differentiated PC12 cells (Day 6) were treated for 48 h with the indicated concentrations of bortezomib, and then the viable cell number was determined by the MTT method. Each value represents the mean of triplicate determinations. Data of thirteen experiments are shown. Red line represents the mean of 13 experiments.*

Cell-cycle analysis. Treated and untreated cells grown in 10-cm cultured dishes (approximately 10^6 cells) were harvested, fixed for 1 h with paraformaldehyde (1%), treated for 30 min with RNase A (0.2 mg/ml), stained with propidium iodide (0.01%) in the presence of 0.01% NP-40 in PBS (–) to prevent the cell aggregation, filtered through the cell strainers, subjected to cell sorting (SH800 Series; SONY Imaging Products and Solutions Inc., Kanagawa, Japan), and then analyzed with Cell Sorter

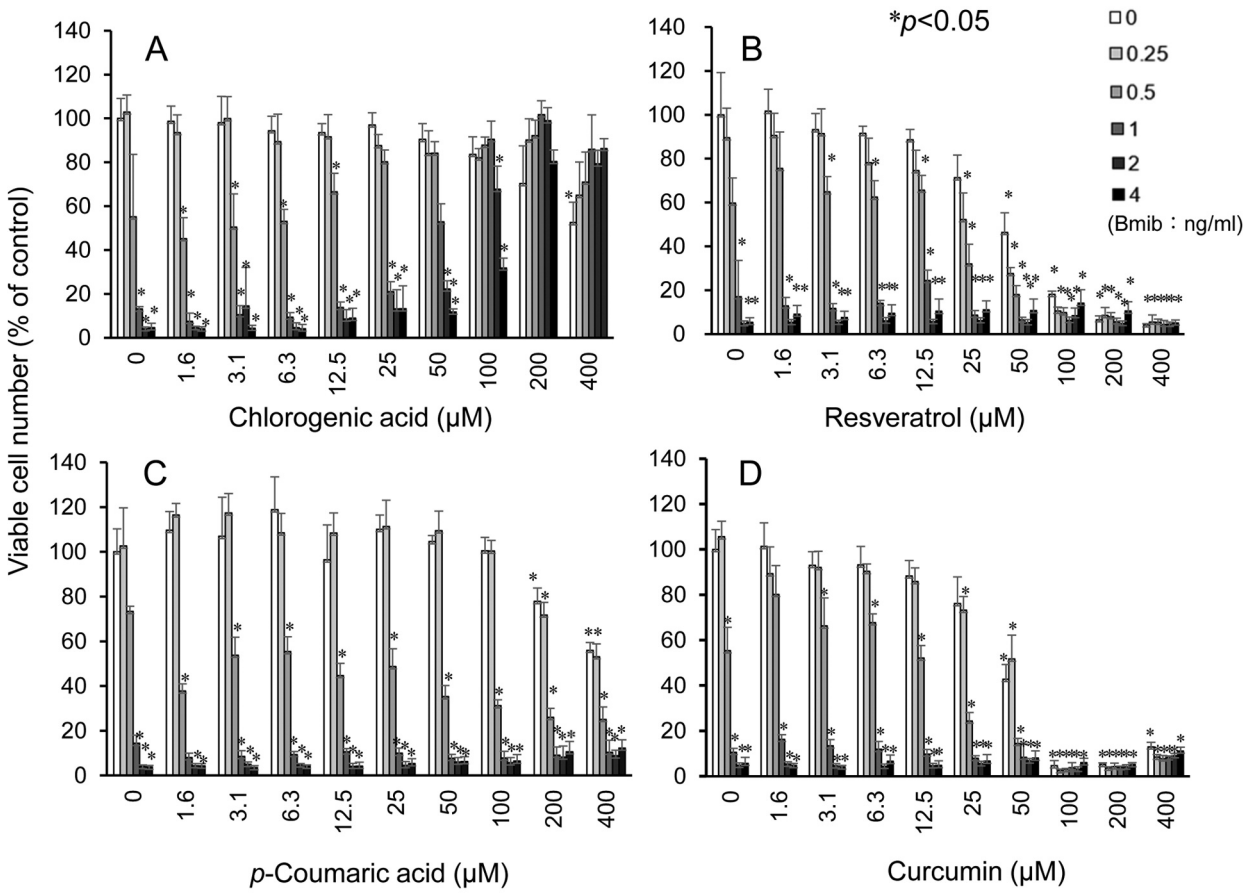


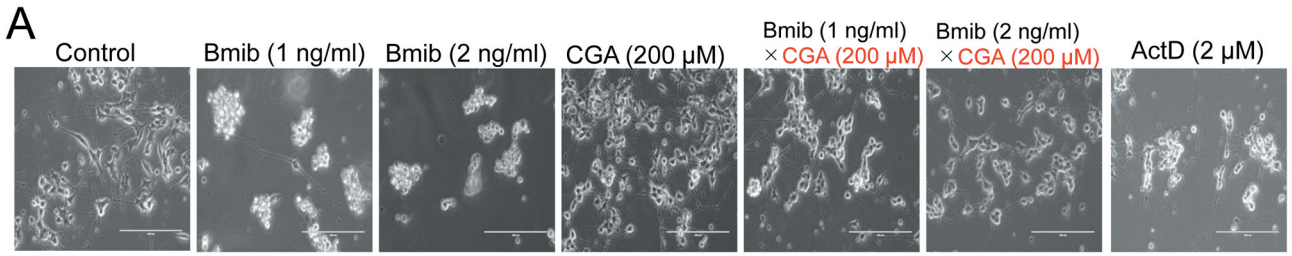
Figure 3. *Rescue of bortezomib-induced neurotoxicity by chlorogenic acid. Differentiated PC-12 (dPC-12 cells) were incubated for 48 h without (control) or with 0.25, 0.5, 1, 2, or 4 ng/ml bortezomib (Bmib) in the presence of increasing concentrations of chlorogenic acid (A), resveratrol (B), p-coumaric acid (C) or curcumin (D). The viable cell number was determined with the MTT method, and expressed as % of control. Each value represents mean±S.D. of triplicated determinations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 was considered to indicate statistically significant differences.*

Software version 2.1.2. (SONY Imaging Products and Solutions Inc.), as described previously (25).

Statistical analysis. Statistical analyses were performed using the Origin pro 2018 software (Origin Lab Corporation, MA, USA). Experimental data are presented as the mean±standard deviation (SD) of triplicate determinations. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test for multiple comparisons. A value of $p<0.05$ was considered to indicate statistically significant differences.

Results

Tumor-specificity and neurotoxicity of popular polyphenols. Among four popular polyphenols tested at the initial stage of experiments, resveratrol showed the highest tumor specificity against human oral squamous cell carcinoma cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) *vs*. human normal oral cells [gingival fibroblast (HGF), periodontal ligament fibroblast (HPLF), pulp cells (HPC)] (B/A in Table I) (TS=5.5), followed by curcumin (TS=2.0), chlorogenic acid and p-coumaric acid (Exp. 1, Table I), caffeic acid, and structurally-related compounds (vanillic acid, ferulic acid, isoferulic acid) (TS=1.0) (Exp. II in Table I). It should, however, be noted that their tumor-specificity was much lower than that of anticancer drugs, bortezomib (TS=570) (25), doxorubicin (TS>34) and melphalan (TS=13.3) (Table I). Similarly, neurotoxicity (NT) of these polyphenols against differentiated rat PC-12 cells (dPC-12 cells) (B/C in Table I) (NT=1~9.3) was much lower than that of anticancer drugs (NT=428, 30, >111, respectively) (Table I). These results demonstrated that eight candidate compounds showed background levels of anticancer activity and neurotoxicity themselves.



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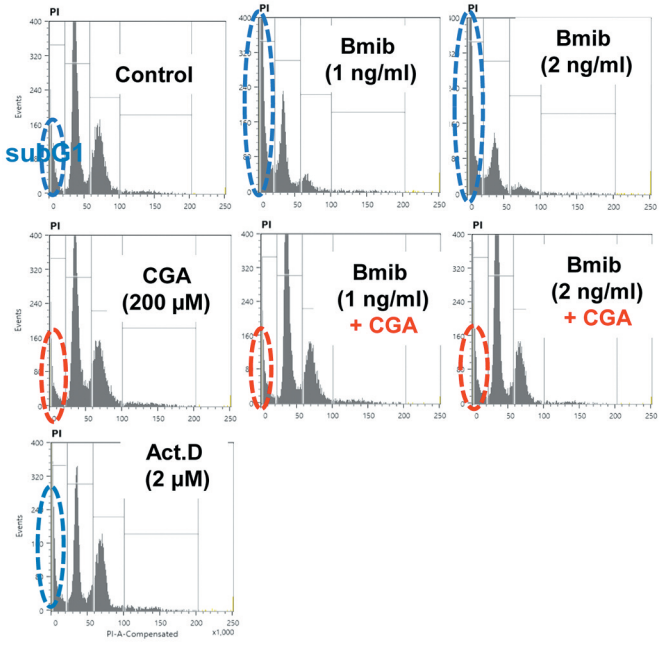


	Distribution (%)			
	subG1	G1	S	G2/M
Control	12.98	51.96	30.87	3.69
Bmib (1 ng/ml)	**60.42***	27.45	6.98	2.61
Bmib (2 ng/ml)	**67.88***	21.33	5.45	2.29
CGA (200 μM)	9.45	53.33	31.51	4.77
Bmib (1 ng/ml) + CGA (200 μM)	12.58	54.13	27.68	4.35
Bmib (2 ng/ml) + CGA (200 μM)	15.68	57.08	22.98	2.43
Act.D (2 μM)	23.10*	37.23	28.70	4.95

*$p<0.05$

Figure 4. *Inhibition of Bmib-induced apoptosis by chlorogenic acid in differentiated PC12 cells (Day 6). Differentiated PC12 cells (at Day 6) were treated for 24 h without (control) or with 1 or 2 ng/ml bortezomib (Bmib) in the presence or absence of 200 μM chlorogenic acid (CGA). (A) CGA inhibited the Bmib-induced cell shrinkage and disruption of neurites. (B) CGA inhibited the Bmib-induced accumulation of subG1 population. Actinomycin D (Act. D) (1 μM) was used as reference control of apoptosis. Each value represents mean of triplicate assays. The differences between groups were evaluated by ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

Chlorogenic acid, but not resveratrol, p-coumaric acid, and curcumin, were neuroprotective. Bortezomib dose-dependently reduced the viability of dPC-12 cells. At 1 and 2 ng/m of bortezomib, the viable cell number was reduced to 13 and 5% of control level, respectively (Figure 2). Chlorogenic acid dose-dependently reduced the neurotoxicity of bortezomib. The protective effect of chlorogenic acid was detected above 50 μM, increasing up to a plateau level at 200 μM, where complete inhibition of neurotoxicity was achieved (Figure 3A). On the other hand, resveratrol, *p*-coumaric acid, and curcumin failed to induce such neuroprotective effects throughout the dose ranges from 1.6 to 400 μM (Figure 3B, C and D). Bortezomib (1 and 2 ng/ml) induced the disruption of neurites, and cell shrinkage (A) and accumulation of subG1 population, (B), more potently than actinomycin D (reference compound of apoptosis inducer) (Figure 4). This was completely reversed by the addition of chlorogenic acid (200 μM) (Figure 4).

Caffeic acid, but not other structurally related compound, was neuroprotective. Caffeic acid inhibited the bortezomib-induced neurotoxicity above 25 μM, with a maximum inhibition achieved at 200 μM (Figure 5A). On the other

Table I. *Much lower tumor selectivity and neurotoxicity of chlorogenic acid, as compared with B-mib, melphalan and doxorubicin.*

	CC_{50} (μM)											
	Human oral squamous cell carcinoma cell lines					Human normal oral cells						
	Ca9-22	HSC-2	HSC-3	HSC-4	mean (A)	HGF	HPLF	HPC	mean (B)	Diff. PC-12 (C)	TS (B/A)	NT (B/C)
<Exp.1>												
CGA	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400	1*	1*
RSV	6.3	33.4	42.2	31.3	43.3	253	228	238	240	25.9	6	9
*p*CA	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400	1*	1*
CUR	58.4	68.3	64.5	71.8	65.8	153	124	122	133	84.7	2	2
<Exp. 2>												
CA	>400	>400	>400	>400	>400	>400	>400	>400	>400	277	1*	>1
VA	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400	1*	1*
IFA	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400	1*	1*
FA	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400	1*	1*
B-mib					0.003				1.71	0.004	570	428
Melph.	41.5	22.8	25.5	10.6	25.1	349	319	334	334	11.2	13	30
DXR	16.4	5.9	14.3	4.3	10.2	>400	321	321	>347	3.1	>34	>111

CC_{50}: 50% Cytotoxic concentration; TS: tumor specificity; NT: neurotoxicity; B-mib: bortezomib; HGF; human gingival fibroblast; HPLF: human periodontal ligament fibroblast; HPC: human pulp cell. *Since several polyphenols show very low cytotoxicity, their CC_{50} values against oral squamous cell carcinoma cell lines, normal oral cells and differentiated PC12 cells exceeded 400 μM. This made it very difficult to determine the accurate TS and NT values. Therefore, we presented their TS and NT values as 1.

hand, structurally-related ferulic acid, isoferulic acid and vanillic acid were inactive (Figure 5B, C, D). Induction of cell shrinkage, neurite disruption (Figure 6A) and accumulation of sub G1 population (Figure 6B) by bortezomib were repeatedly observed. Addition of caffeic acid at 200 μM inhibited the cell shrinkage and disruption of neurites (Figure 6A) and accumulation of subG1 population, achieving the near complete recovery of viable cells (Figure 6B).

Both chlorogenic acid and caffeic acid also inhibited the cytotoxicity of bortezomib against human neuroblastoma SH-SY5Ycells (Figure 7), as well as rat dPC-12 cells (Figure 3, Figure 4, Figure 5 and Figure 6).

Chlorogenic acid reduced the anticancer activity of bortezomib. Ideally, chlorogenic acid should only protect from bortezomib-induced neurotoxicity, without affecting its antitumor potential. Bortezomib (0.25~4 ng/ml) dose-dependently reduced the viability of two human oral squamous cell carcinoma cell lines Ca9-22 (derived from gingiva) (Figure 8A and C) and HSC-2 (derived from tongue) (Figure 8B and D), confirming previous findings (25). However, contrary to our expectation, the anticancer activity of bortezomib was also abolished by addition of increasing concentrations of chlorogenic acid (Figure 8A and B) and caffeic acid (Figure 8C and D).

Discussion

In the American Society of Hematology 2020 annual meeting, the triplet regimen bortezomib, lenalidomide, and dexamethasone was recommended as the standard first-line treatment of multiple myeloma (26). However, as the population of cancer survivors increases, the long-term toxicities of chemotherapeutic agents has been gradually strengthened. More than 30% of patients receiving bortezomib alone or in combination with other drugs have been reported to develop peripheral neuropathy (bortezomib-induced peripheral neuropathy, BIPN) (27), characterized by neuropathic pain. The pathological mechanism of BIPN may include: (i) demyelination, formation of aggregates, endoplasmic reticulum stress, inhibition of dedifferentiation of Schwann cells, (ii) neuronal dysfunction [hyperactivity of wide dynamic range (WDR) neurons in the spinal dorsal horn, damage of afferent sensory neuron cell bodies located in the dorsal root ganglia (DRG) of the spinal cord, abnormality of ion channel, imbalance of tubulin dynamics, mitochondrial dysfunction, inflammation in DRG neurons], (iii) astrocyte activation, and (iv) macrophage infiltration (10). When human induced pluripotent stem cell-derived sensory neurons were exposed to a clinically relevant dose of bortezomib, alterations in microtubule-associated proteins (especially significant reduction of MAP2c) occurred, suggesting a multifaceted

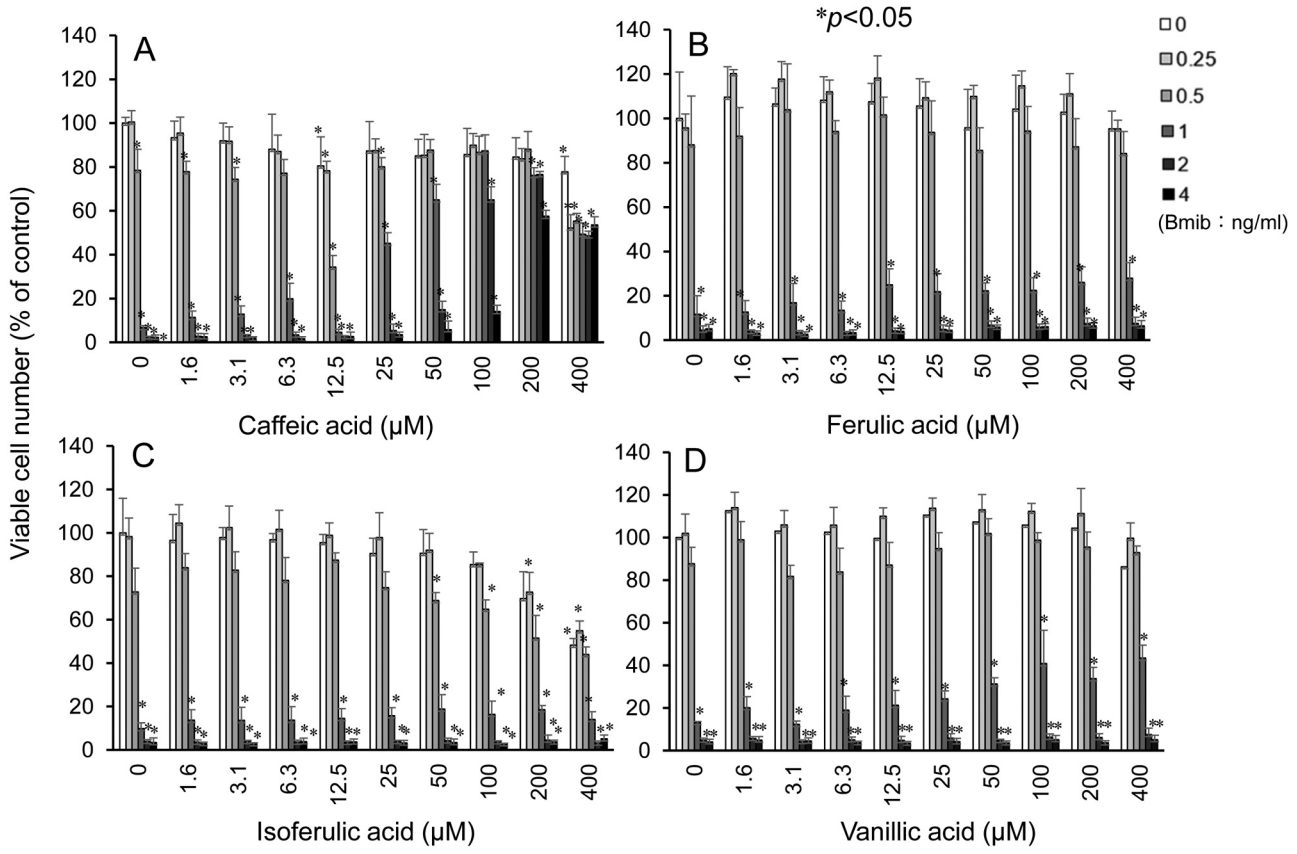


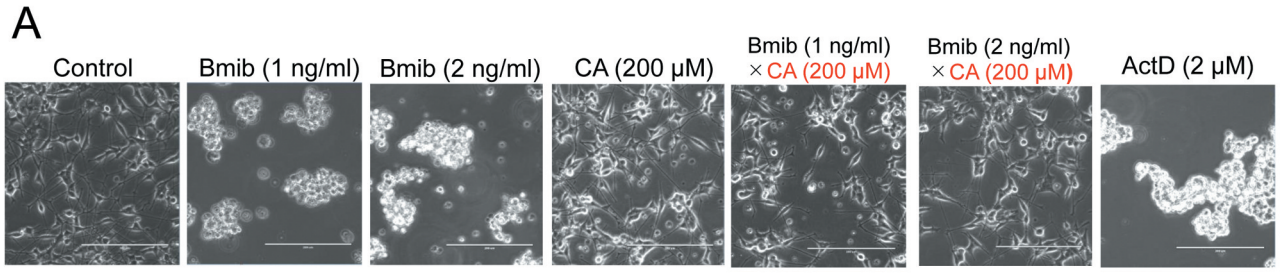
Figure 5. *Rescue of bortezomib-induced neurotoxicity by caffeic acid. Differentiated PC-12 (dPC-12 cells) were incubated for 48 h without (control) or with 0.25, 0.5, 1, 2, or 4 ng/ml bortezomib (Bmib) in the presence of increasing concentrations of caffeic acid (A), ferulic acid (B), isoferulic acid (C), or vanillic acid (D). The viable cell number was determined with the MTT method, and expressed as % of control. Each value represents mean±S.D. of triplicated determinations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

relationship between bortezomib-induced proteotoxicity and microtubule cytoskeletal architecture (28).

The present study demonstrated that bortezomib induced potent neurotoxicity against differentiated PC-12 neuronal cells (NT=428) in addition to its potent tumor-specificity (TS=570), confirming our previous findings (9, 25). Potent neurotoxicity of bortezomib was accompanied by apoptosis phenotypes such as cell shrinkage, disruption of neurites, and accumulation of subG1 population of dPC-12 cells (29-31). However, eight polyphenols tested in this study showed very little neurotoxicity (NT lower than 9) and tumor-specificity (TS lower than 6).

Among the eight test compounds, only chlorogenic acid (an ester formed between caffeic acid and the 3-hydroxyl of L-quinic acid) and caffeic acid, but not other 6 polyphenols tested, protected rat dPC-12 (Figure 3, Figure 4, Figure 5 and Figure 6) and human SH-SY5Y neuroblastoma cells (Figure 7) from potent cytotoxicity of bortezomib. This cannot be explained simply by water solubility, since chlorogenic acid is water soluble (log P=–0.36), while other polyphenols (including caffeic acid) and anticancer drugs (log P=1.33~3.14) are hydrophobic (Table II).

Instead, we noticed that the only difference between caffeic acid and ferulic acid is the number of hydroxyl groups. Caffeic acid has two hydroxyl groups in the catechol moiety, while ferulic acid and isoferulic acid have one hydroxyl group with one methoxy group. Under alkaline conditions (such as in the culture medium, pH 7.4), the boronic acid group of bortezomib and the catechol group of caffeic acid are complexed due to the protonation of the hydroxyl (OH) groups of caffeic acid, creating a high affinity bond between these two molecules (32) (Figure 9). Since dissociation of the conjugate can occur under acidic conditions (pH <5), it is difficult to prevent the conjugation during incubation in a 5% CO_2 atmosphere, which maintains the pH at neutral levels. Therefore, chlorogenic acid probably inhibits both neurotoxicity and anticancer activity of bortezomib by conjugation between these two molecules.



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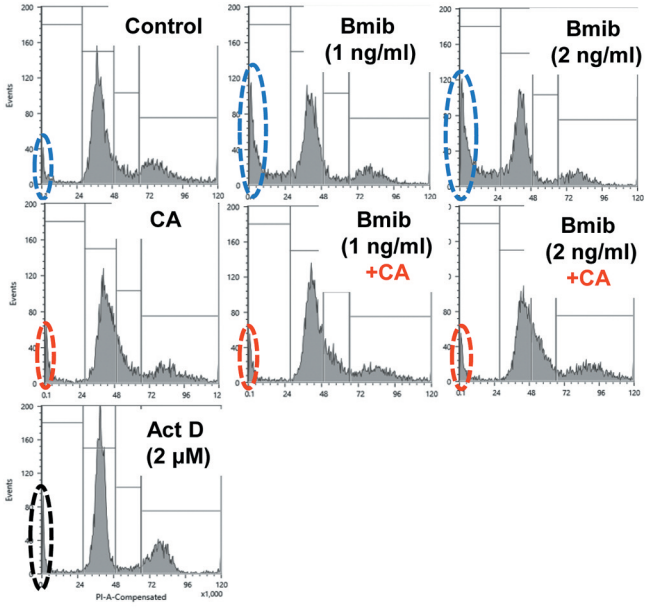

	Distribution (%)			
	subG1	G1	S	G2M
Control	5.68	57.35	12.27	25.61
Bmib (1 ng/ml)	**26.87***	50.66	6.83	16.43
Bmib (2 ng/ml)	**34.91***	46.07	6.32	13.81
CA (200 μM)	8.63	52.86	18.47	21.43
Bmib (1 ng/ml) +CA (200 μM)	8.23*	54.74	17.89	20.40
Bmib (2 ng/ml) +CA (200 μM)	8.67*	51.56	18.75	22.27
Act.D (2 μM)	8.48*	61.39	4.99	25.56

*$p<0.05$

Figure 6. *Inhibition of Bmib-induced apoptosis by chlorogenic acid in differentiated PC12 cells (Day 6). Differentiated PC12 cells (at Day 6) were treated for 24 h without (control) or with 1 or 2 ng/ml bortezomib (Bmib) in the presence or absence of 200 μM caffeic acid (CA). (A) CA inhibited the Bmib-induced cell shrinkage and disruption of neurites. (B) CA inhibited the Bmib-induced accumulation of subG1 population. Actinomycin D (Act. D) (1 μM) was used as reference control of apoptosis. Each value represents mean of triplicate assays. The differences between groups were evaluated by ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

There remains to be investigated whether catecholamines, which have two hydroxyl groups, may also inhibit the biological activity of bortezomib.

Conclusion

Coffee is a very popular daily drink. Coffee beans are rich in chlorogenic acid, which are decomposed to give caffeic acid and quinic acid upon roasting (33). The present study demonstrated that both chlorogenic acid and caffeic acid reduced not only the neurotoxicity but also the anticancer activity of bortezomib by formation of conjugation. Thus, it is necessary to monitor the clinical effects of bortezomib especially on the patients with multiple myeloma who drink coffee.

Conflicts of Interest

The Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Authors' Contributions

RM and HS performed most experiments of the present study and wrote the manuscript. YI and SA performed the cell cycle analysis. MS and YU reviewed the manuscript.TN, YO and HT provided the interpretation of experimental results and edited the manuscript. All Authors read and approved the final version of the manuscript.

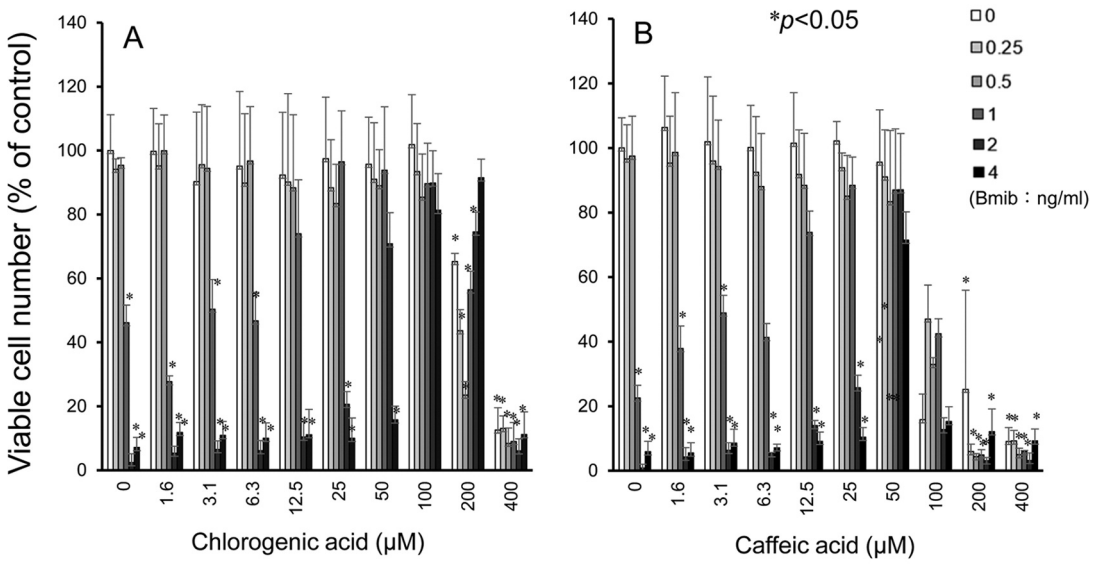

Figure 7. *Rescue of bortezomib-induced cytotoxicity against human neuroblastoma SH-SY5Y cells by chlorogenic acid and caffeic acid. SH-SY5Y cells were incubated for 48 h without (control) or with 0.25, 0.5, 1, 2, or 4 ng/ml bortezomib (Bmib) in the presence of increasing concentrations of chlorogenic acid (A) or caffeic acid (B). The viable cell number was determined with the MTT method, and expressed as % of control. Each value represents mean±S.D. of triplicated determinations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

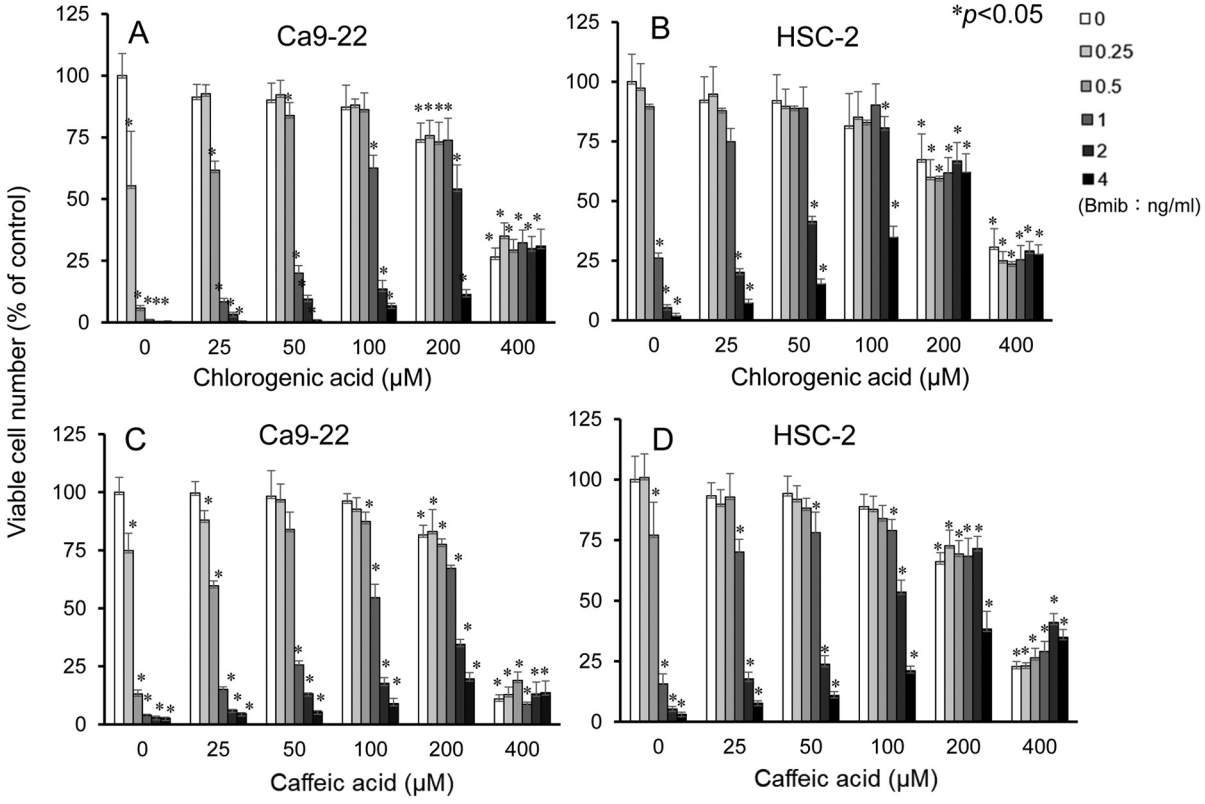

Figure 8. *Abrogation of antitumor potential of bortezomib by chlorogenic acid and caffeic acid. Human oral squamous cell carcinoma cell lines Ca9-22 (A, C) or HSC-2 cells (B, D) were incubated for 48 h without (control) or with 0.25, 0.5, 1, 2 or 4 ng/ml bortezomib (Bmib) in the presence of increasing concentrations of chlorogenic acid (A, B) or caffeic acid (C, D). The viable cell number was determined with the MTT method, and expressed as % of control. Each value represents mean±S.D. of triplicated determinations. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

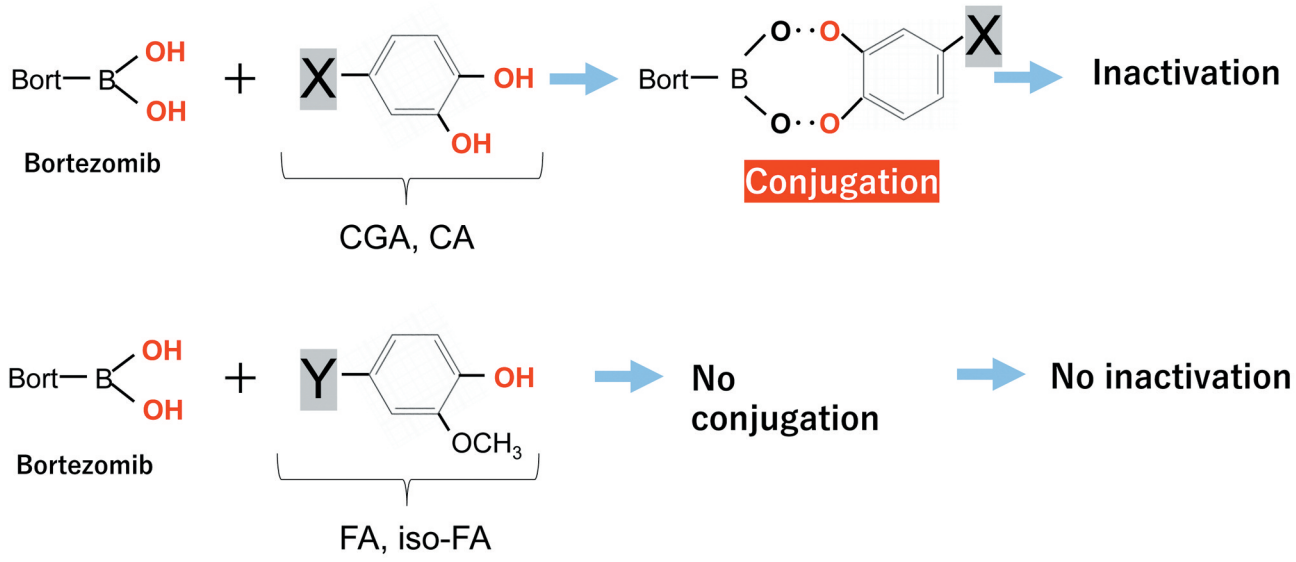

Figure 9. *Simple complexation of the boronic acid group of bortezomib and the catechol group of chlorogenic acid or caffeic acid. This figure was produced based on reference 32. Bortezomib can conjugate with chlorogenic acid or caffeic acid, resulting in the inactivation of bortezomib. On the other hand, bortezomib cannot conjugate with ferulic acid or isoferulic acid, and bortezomib is not inactivated.*

Table II. *LogP value of chlorogenic acid and anticancer drugs (Cited from chemospider).*

	LogP value
Chlorogenic acid	–0.36
Resveratrol	3.14
Curcumin	2.92
p-Coumaric acid	1.88
Quinic acid	–1.98
Caffeic acid	1.42
Ferulic acid	1.64
Isoferulic acid	1.64
Vanillic acid	1.33
Bortezomib	2.45
Melphalan	1.79
Doxorubicin	2.81

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