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Protection of Bortezomib-induced Neurotoxicity by Antioxidants

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Abstract. *Background/Aim: Although chemotherapy agents, such as oxaliplatin, cisplatin, paclitaxel and bortezomib frequently cause severe peripheral neuropathy, very few studies have reported the effective strategy to prevent this side effect. In this study, we first investigated whether these drugs show higher neuropathy compared to a set of 15 other anticancer drugs, and then whether antioxidants, such as sodium ascorbate, N-acetyl-L-cysteine, and vitamin B12 have any protective effect against them. Materials and Methods: Rat PC12 cells were induced to differentiate into neuronal cells by repeated overlay of serum-free medium supplemented with nerve growth factor. The cytotoxic levels of anticancer drugs against four human oral squamous cell carcinoma cell lines, three normal oral cells, and undifferentiated and differentiated PC12 cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Cells were sorted for apoptotic cells (distributed into subG_1 phase) and cells at different stages of cell cycle (G_1, S and G_2/M). Results: All 19 anticancer drugs showed higher cytotoxicity against PC12 compared to oral normal cells. Among them, bortezomib showed the highest cytotoxicity against both undifferentiated and differentiated PC12 cell and, committed them to undergo apoptosis. Sodium ascorbate and N-acetyl-L-cysteine, but not vitamin B12, completely reversed the cytotoxicity of bortezomib. Conclusion: Bortezomib-induced neuropathy might be ameliorated by antioxidants.*

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In recent years, molecular-targeted anticancer drugs with much lower side effects than classical anticancer drugs have taken over cancer therapy (1-3). Chemotherapy-induced peripheral neuropathy (CIPN) is known as one of the adverse effects of chemotherapy (4). So far, no effective preventive agent for CIPN has been reported (5). Serious peripheral neuropathy sometimes causes the discontinuation of cancer therapy. Although there are fluctuations in the intensity of peripheral nerve damage induced by anticancer drugs, axonal damage is common in previous studies with animal models (6). We have developed a method of isolation and differentiation of neuronal cells from rat pheochromocytoma PC12 cells by repeated overlay of nerve growth factor (NGF)-enriched serum-free medium, without medium change nor collagen-coating (7). This method can be applicable to evaluate the peripheral neuropathy *in vitro*.

We recently reported that among 19 anticancer drugs, bortezomib (Bmib, proteasome inhibitor) showed much higher cytotoxicity against human oral squamous cell carcinoma cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) as compared to human normal oral cells [gingival fibroblast (HGF), periodontal ligament fibroblast (HPLF), pulp cell (HPC)] [tumor-specificity index (TS)=504]. On the other hand, platinum agents (cisplatin, carboplatin, oxaliplatin) showed very low tumor-specificity (TS=3.8-10.4) (8). Oral mucositis is also one of the adverse effects of chemotherapy, thus evaluation of cytotoxicity against normal oral cells, such as HGF, is very important. At present, a limited number of research groups have searched for the protective substances against the bortezomib-induced neuropathy using the PC 12 cells, frequently used for the study of neuronal differentiation, with or without treatment with NGF (5, 6, 9-11). Furthermore, none

of them has investigated the intensity of both neuropathy and mucositis induced by anticancer drugs at the same time, or any possible fluctuations in the susceptibility of PC12 cells to anticancer drugs during the differentiation process.

In the present study, we have investigated the neurotoxicity of 15 classical anticancer drugs (including three platinum compounds) and 4 molecular-targeted drugs (including Bmib) against PC12 cells at three differentiation stages: i) day 0, ii) day 3 and iii) day 6) and inoculated at a high (H), middle (M), or low (L) cell density. Since oxidative-stress is involved in cisplatin-induced neuropathy (12), we also investigated whether two popular antioxidants, such as sodium ascorbate (vitamin C) and *N*-acetyl-L-cysteine (NAC), as well as mecobalamin (vitamin B12), prevent the Bmib-induced neurotoxicity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) from GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and actinomycin D were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Propidium iodide (PI) and human recombinant NGF were purchased from Wako Pure Chem. Ind. (Osaka, Japan); abraxane (NabPTX) from Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan); bortezomib (Bmib) from Janssen Pharmaceutical K, K. (Tokyo, Japan); ramucirumab (Rmab) from Eli Lilly Japan K. K. (Kobe, Japan); oxaliplatin (L-OHP) from Yakult Honsha Co., Ltd. (Tokyo, Japan); gemcitabine (GEM) and cisplatin (CDDP) from Nichi-Iko Pharmaceutical Co. Ltd. (Toyama, Japan); paclitaxel (PTX) from Nipro Corporation (Osaka, Japan); cetuximab (Cmab) Merck Serono K. K. (Tokyo, Japan); carboplatin (CBDCA) and docetaxel (DTX) from Sawai Pharmaceutical Co. Ltd. (Osaka, Japan); etoposide (ETP) Sandoz K. K. (Yamagata, Japan); 5-fluorouracil (5-FU) and irinotecan (IRT) from Towa Pharmaceutical Co. Ltd. (Osaka, Japan); nivolumab (Nmab) from Ono Pharmaceutical Co. Ltd. (Osaka, Japan); vinorelbine (VNR) from Kyowa Kirin Co. Ltd. (Tokyo, Japan); vinblastine (VBL) and vincristine (VCR) from Nippon Kayaku Co. Ltd. (Tokyo, Japan); eribulin mesilate (ERI) and mecobalamin (Meco) from Eisai Co. Ltd. (Tokyo, Japan). Sodium L-ascorbate (VC) and *N*-acetyl-L-cysteine (NAC) from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Culture plastic dishes and plates (96-well) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Cell culture. PC12, a cell line derived from a pheochromocytoma of rat adrenal medulla was purchased from Riken Cell Bank (Tsukuba, Japan). Human normal oral mesenchymal cells (HGF, HPLF and HPC) were established from the first premolar tooth as described previously (8). Human oral squamous cell carcinoma (OSCC) cell lines [Ca9-22 (derived from gingival tissue), and HSC-2, HSC-3, and HSC-4 (derived from tongue)] were purchased from Riken Cell Bank (Tsukuba, Japan). These cells were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS and antibiotics, under a humidified 5% CO_2 atmosphere (8). Cell morphology was checked periodically under a light microscope (EVOS FL; Thermo Fisher Scientific, Waltham, MA, USA).

Induction of differentiation toward neurons. PC12 cells were inoculated at 1×10^3 (low cell density: L), 3×10^3 (medium cell density: M) or 6×10^3 (high cell density: H) cells per each well of a 96-microwell plate and incubated for 24 h to allow complete cell attachment in DMEM+10%FBS. The medium was completely removed by suction and replaced with 0.08 ml of differentiation medium (serum-free DMEM containing 50 ng/ml NGF). Cells were then incubated for 3 days to induce neuronal differentiation characterized by neurite formation (referred to as Day 3 cells). To produce more mature cells, aliquots of day 3 cells were further incubated with 0.04 ml of newly overlaid differentiation medium (100 ng/ml NGF) for 3 days (referred to as Day 6 cells).

Cytotoxicity of anticancer drugs. Day 0 cells (undifferentiated cells), day 3 and day 6 cells prepared as described above were overlaid with 40 μl of drip-type anticancer drug diluted with differentiation medium (serum-free DMEM containing 100 ng/ml NGF). Following incubation for 48 h, the viable cell number was determined using the MTT method. In brief, 40 μl of MTT solution (final concentration=0.1 mg/ml) were added to the cells and incubated for a further 48 h period. The MTT-containing medium was subsequently suctioned out and DMSO was added to lyse the cells. Then, the absorbance at 560 nm (reflecting the relative viable cell number) was measured using a microplate reader (Infinite F50R; TECAN, Männedorf, Switzerland). The compound concentration that reduced the viable cell number by 50% (CC_{50}) was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from triplicate assays (Table I). Table II lists the maximum serum concentrations (Cmax) of anticancer drugs administered through intravenous (*i.v.*) injection to cancer patients at the indicated doses (cited from the interview form of the supplier), CC_{50} for OSCC (B) and normal oral cells (D) [cited from our recently published paper (8), and CC_{50} for PC12 (E) (determined in this study, Table I). The safety margin (chemotherapy index) of VC and NAC was calculated by dividing the CC_{50} by 50% effective concentration.

Cell-cycle analysis. Treated and untreated cells (approximately 10^6 cells) were harvested, fixed, treated with RNase A, stained with propidium iodide, filtered through cell strainers, subjected to cell sorting (SH800 Series; SONY Imaging Products and Solutions Inc., Kanagawa, Japan), and then analyzed with Cell Sorter Software version 2.1.2. (SONY Imaging Products and Solutions Inc.), as described previously (8).

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

Bortezomib showed the highest cytotoxicity against PC12 cells. PC12 cells, inoculated at low (L), middle (M) or high cell density (H), were induced to differentiate into neuronal cells to various extents by incubating with 50~100 ng/ml NGF for 0, 3 or 6 days and then exposed to various concentrations

Table I. *Cytotoxic activity of 19 anticancer drugs against PC12 cell (inoculated at different cell density and at different stages of differentiation). Each value represents the mean of triplicate determinations.*

	CC_{50} (μg/ml)														
	D0				D3				D6				Mean		
Agent	L	M	H	Mean	L	M	H	Mean	L	M	H	Mean	OSCC[a]	Normal[a]	PC12[b]
Bortezomib (Bmib)	0.0019	0.0025	0.0048	0.0031	0.00057	0.00057	0.00030	0.00048	0.0041	0.00079	0.0011	0.0020	<0.0013	0.66	0.0019
Eribulin Mesilate (ERI)	0.01	0.03	0.01	0.014	<0.02	0.02	1.1	<0.38	0.07	>0.2	>0.2	>0.12	<0.002	0.11	0.2
Vinorelbine (VNR)	<0.04	<0.04	<0.04	<0.04	<0.01	<0.01	<0.01	<0.01	0.06	0.27	0.45	0.26	<0.039	1.7	<0.10
Vinblastine (VBL)	<0.01	0.017	0.030	<0.019	<0.05	0.07	1.1	<0.41	0.2	>0.3	>0.3	>0.26	<0.0039	0.17	0.23
Vincristine (VCR)	0.046	0.034	0.031	0.037	0.4	1.6	>0.13	>0.73	<1.3	<1.3	<1.3	<1.3	<0.0039	>0.23	0.7
Docetaxel (DTX)	<0.3	<0.3	8.0	<2.9	1.4	0.6	7.3	3.1	1.2	1.6	0.8	1.2	<0.00049	42.2	<2.4
Paclitaxel (PTX)	<0.1	<0.1	5.0	<1.7	0.3	1.3	3.6	1.7	0.6	2.4	2.6	1.8	<0.0015	10.9	<1.8
Abraxane (NabPTX)	<9.7	<9.7	742.1	<253.8	100.2	310.4	<625	<345.2	13.3	<156.2	<156.2	<108.6	<0.2	154	<236
Fluorouracil (5-FU)	<4.8	1.3	5.3	<3.8	<1.2	13.7	<156.2	<57.0	1.2	24.6	12.0	12.6	10.4	1772	<24.5
Gemcitabine (GEM)	<15.6	<15.6	<15.6	<15.6	<3.9	<3.9	208	<71.9	2.4	23.8	<125	<50.4	<0.0039	1474	<45.9
Etoposide (ETP)	4.5	6.8	9.1	6.8	2.3	3.0	12.5	5.9	1.8	5.9	2.8	3.5	3.7	193	5.4
Irinotecan (IRT)	14.6	13.3	14.1	14.0	11.7	13.9	31.2	18.9	5.9	7.9	6.4	6.7	4.8	134.5	13.2
Doxorubicin (DOX)	<0.08	<0.08	0.1	<0.38	0.25	0.26	0.55	0.35	0.17	0.31	0.37	0.29	0.13	3.2	<0.22
Cisplatin (CDDP)	1.6	2.8	3.1	2.5	3.3	3.0	11.5	5.9	3.0	7.0	4.1	4.7	0.73	7.6	4.4
Oxaliplatin (L-OHP)	<1.9	<1.9	0.4	<1.4	<0.4	<0.4	9.2	<3.3	<0.4	9.7	4.5	<4.9	<4.3	29.4	<3.2
Carboplatin (CBDCA)	1.5	2.7	2.9	2.4	2.9	2.3	6.3	3.8	3.9	4.8	1.3	3.3	27.9	106	3.2
Ramucirumab (Rmab)	>6250	>6250	>6250	>6250	>6250	>6250	>6250	>6250	>6250	>6250	>6250	>6250	1370	>4902	>6250
Nivolumab (Nmab)	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	3436	4281	>2500
Cetuximab (Cmab)	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	1714	>2500	>1250

CC_{50}: 50% Cytotoxic concentration; D0, D3, D6: differentiation induction time at Day 0, 3, 6, respectively; L: low cell density; M: middle cell density; H: high cell density; OSCC: oral squamous cell carcinoma. [a]Cited from (8). [b]Mean of CC_{50} from mean 1 (D0), mean 2 (D3) and mean 3 (D6).

of anticancer drugs to determine the viable cell number. Figure 1 shows the dose-response curve. Figure 2 and Table I show the CC_{50} values calculated from Figure 1. Among the 19 anticancer drugs, Bmib showed the highest cytotoxicity against PC12 cells (CC_{50}=0.0019 μg/ml), followed by VNR (<0.10), ERI (0.2), DOX (<0.22), VBL (0.23), VCR (0.7), PTX (<1.8), DTX (<2.4), CBDCA (3.2), L-OHP (<3.2), CDDP (4.4), ETP (5.4), IRT (13.2), 5-FU (<24.5), GEM (<45.9), NabPTX (>236), Cmab (>1250), Nmab (>2500), and Rmab (>6250) (Table I). The CC_{50} values used here were the averages of the means of CC_{50} (L), CC_{50} (M) and CC_{50} (H) at D0, D3 and D6 ($PC12^{b}$ in Table I).

All anticancer drugs showed higher cytotoxicity against PC12 compared to oral normal cells. Vinca alkaloids (VNR, VBL, VCR) and halichondrine (ERI) were highly cytotoxic to PC12 cells (CC_{50}=0.7-0.10 μg/ml) as well as normal oral cells (CC_{50}=1.7-0.11 μg/ml). Bmib showed 347.3-times higher cytotoxicity against PC12 (CC_{50}=0.0019 μg/ml)

Table II. *Correlation between clinical data of maximum serum concentration (C_{max}) and cytotoxicity of the 19 anticancer drugs determined in this study. C_{max} was obtained from the interview form of supplier. CC_{50} for OSCC (B) and normal oral cells (D) cultured under the same conditions are cited from our recently published paper (8), although this is not ideal to compare experiments that have not been performed together. CC_{50} for PC12 (E) are derived from Table I. Bold number indicates the potent neurotoxicity.*

Agent	Dose mg/m^2	Serum conc. Cmax μg/ml	CC_{50} (OSCC) B μg/ml	CC_{50} (normal cell) D μg/ml	CC_{50} (PC12) E μg/ml	Ratio (OSCC) Cmax/B	Ratio (normal cell) Cmax/D	Ratio (PC12) Cmax/E
Bortezomib (Bmib)	1.3	0.22	<0.0013	0.66	0.0019	**>169.2**	0.33	**115.8**
Eribulin Mesilate (ERI)	1.4	0.52	<0.002	0.11	><0.2	**>260**	**4.73**	**2.60**
Vinorelbine (VNR)	25	1.1	<0.039	><1.7	<0.10	**>28.2**	0.65	**11.0**
Vinblastine (VBL)	3	0.48	<0.0039	><0.17	><0.2	**>123.1**	**2.82**	**2.40**
Vincristine (VCR)	1.4	0.085	<0.0039	>0.23	><0.67	**>358.9**	<0.37	0.13
Docetaxel (DTX)	60	2.0	<0.00049	42.2	<2.4	**>4082**	0.05	>0.83
Paclitaxel (PTX)	100	0.43	<0.0015	10.9	<1.8	**>287**	0.04	>0.23
Abraxane (NabPTX)	260	20.0	<0.2	154	<236	**>100**	0.13	>0.08
Fluorouracil (5-FU)	1000	15.3	10.4	1772	<24.5	1.5	0.01	>0.62
Gemcitabine (GEM)	1000	21.8	<0.0039	1474	<46	**>5590**	0.01	>0.47
Etoposide (ETP)	100	4.4	3.7	193	5.42	1.2	0.02	0.81
Irinotecan (IRT)	180	9.1	4.8	135	13.22	1.9	0.07	0.69
Doxorubicin (DOX)	60	3.3	0.13	3.2	<0.22	**25.4**	1.0	**>15**
Cisplatin (CDDP)	100	17.1	0.73	7.6	4.4	**23.4**	**2.3**	**3.89**
Oxaliplatin (L-OHP)	85	0.63	<4.3	29.4	<3.2	>0.1	0.02	>0.19
Carboplatin (CBDCA)	400	5.9	27.9	106	3.2	0.2	0.06	**1.84**
Ramucirumab (Rmab)	8	16	1370	>4902	>6250	0.012	<0.003	<0.003
Nivolumab (Nmab)		69	3436	4281	>2500	0.020	0.016	<0.03
Cetuximab (Cmab)	400	287	1714	>2500	>1250	0.17	<0.11	<0.23

compared to normal oral cells (CC_{50}=0.66 μg/ml). Bmib showed comparable cytotoxicity against OSCC and PC12 cells, whereas platinum agents (L-OHP and CBDCA) showed higher cytotoxicity against PC12 cells (CC_{50}≤3.2, 3.2 μg/ml) compared to OSCC (CC_{50}=4.3, 27.9 μg/ml) (Table I).

Bmib showed the highest neurotoxicity throughout differentiation process. The sensitivity of PC12 cells to anticancer drugs ERI, VNR, VBL, VCR, 5-FU, GEM, DOX, CDDP and L-OHP changed depending on the degree of cell differentiation.

Bmib showed the highest cytotoxicity against undifferentiated PC12 cells (at Day 0) (CC_{50}=0.0031μg/ml), followed by ERI (0.014), VBL (<0.019), VCR (0.037), VNR (<0.04), DOX (<0.38), L-OHP (<1.4), PTX (<1.7), CBDCA (2.4), CDDP (2.5), DTX (<2.9), 5-FU (<3.8), ETP (6.8), IRT (14.0), GEM (<15.6), NabPTX (<253.8), Cmab (>1250), Nmab (>2500), and Rmab (>6250).

Bmib showed the highest cytotoxicity against differentiating PC12 cells (at Day 3) (CC_{50}=0.00048 μg/ml), followed by VNR (<0.01), DOX (0.35), ERI (<0.38), VBL (<0.41), VCR (>0.73), PTX (1.7), DTX (3.1), L-OHP (<3.3), CBDCA (3.8), CDDP (5.9), ETP (5.9), IRT (18.9), GEM (<71.9), 5-FU (<57.0), NabPTX (<345.2), Cmab (>1250), Nmab (>2500), and Rmab (>6250).

Bmib again showed the highest cytotoxicity against more mature PC12 cells (at Day 6) (CC_{50}=0.0020 μg/ml), followed by ERI (>0.12), VNR (0.26), VBL (>0.26), DOX (0.29), DTX (1.2), VCR (<1.3), PTX (1.8), CBDCA (3.3), ETP (3.5), CDDP (4.7), L-OHP (<4.9), IRT (6.7), 5-FU (12.6), GEM (<50.40), NabPTX (<108.60), Cmab (>1250), Nmab (>2500), and Rmab (>6250).

These data clearly showed that Bmib showed the highest cytotoxicity regardless of differentiation stage.

Bmib exerts high neurotoxicity regardless of target cell density. We assessed the possibility that increasing cell density could reduce the neurotoxicity of anticancer drugs. At a low cell density, Bmib showed the highest cytotoxicity (CC_{50}=0.0022 μg/ml), followed by ERI (<0.033), VNR (<0.036), VBL (<0.079), DOX (0.14), PTX (<0.33), VCR (<0.57), L-OHP (<0.9), DTX (<0.95), 5-FU (<2.40), CDDP (2.60), CBDCA (2.77), ETP (2.86), GEM (<7.31), IRT (10.73), NabPTX (<41.07), Cmab (>1250), Nmab (>2500), and Rmab (>6250).

At a middle cell density, Bmib showed the highest cytotoxicity (CC_{50}=0.0013), followed by ERI (>0.064), VNR (<0.11), VBL (>0.13), DOX (<0.22), DTX (<0.84), VCR (<0.97), PTX (<1.26), CBDCA (3.26), L-OHP (<4.01), CDDP (4.28), ETP (5.23), IRT (11.68), 5-FU (13.20), GEM

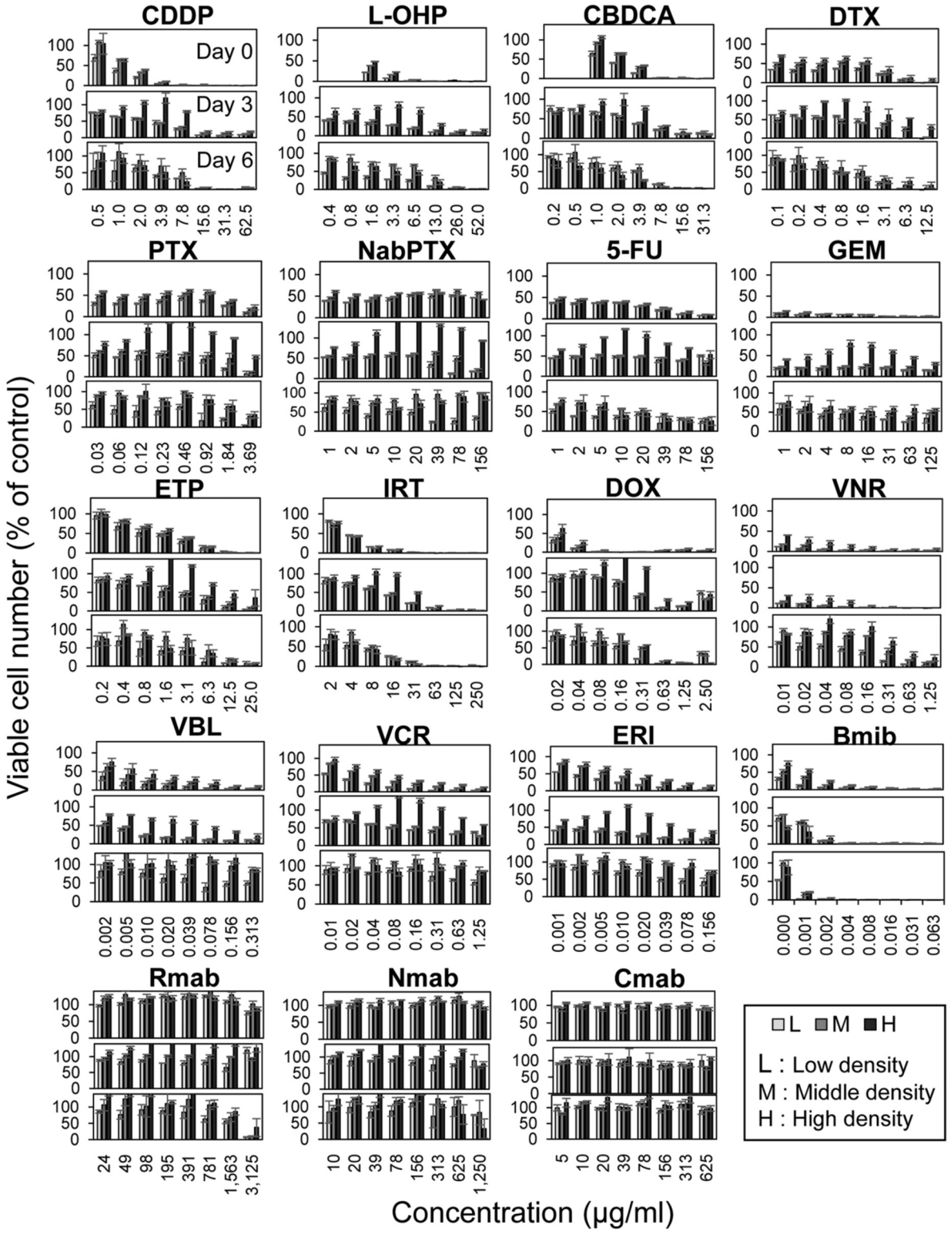


Figure 1. *Dose-response curve of growth inhibition by 15 classical anticancer drugs and 4 molecular-targeted drugs (bortezomib and 3 monoclonal antibodies). PC12 cells as Day 0 (undifferentiated cells), Day 3 (differentiating cells) and Day 6 (more mature cells) at three different cell densities (L, M and H) were overlaid with 40 μl of drip-type anticancer drug diluted with a differentiation medium (serum-free DMEM containing 100 ng/ml NGF), and then the viable cell number [% of control (without treatment)] was determined by the MTT method. Each value represents the mean±S.D. of triplicate determinations.*

(<14.43), NabPTX (<158.76), Cmab (>1250), Nmab (>2500), and Rmab (>6250).

At a high cell density, Bmib showed the highest cytotoxicity (CC_{50}=0.0021), followed by VNR (<0.17), DOX (<0.31), ERI (>0.43), VCR (0.47), VBL (>0.49), CBDCA (3.50), PTX (3.71), L-OHP (4.69), DTX (5.35), CDDP (6.19), ETP (8.16), IRT (17.24), 5-FU (<57.85), GEM (<116.19), NabPTX (<507.76), Cmab (>1250), Nmab (>2500), and Rmab (>6250) (Figure 2).

When the number of cells at the inoculation time increased, the PC12 cells at Day 6 showed strong resistance to most anticancer drugs, except for Bmib, as evidenced by the elevated CC_{50} values, compared to PC12 cells at Day 0. Resistance of PC12 cells at Day 6 to VCR was the most remarkable one (more than 34-fold), followed by VBL (16-fold), DOX (10-fold), ERI (8-fold), VNR (7-fold), L-OHP (3-fold), 5-FU (3-fold), GEM (3-fold), CDDP (2-fold), CBDCA and PTX (1-fold). Bmib, ERI, VNR, VBL, DOX and VCR were highly neurotoxic. As expected, monoclonal antibodies that act as molecular-target drugs (Rmab, Nmab and Cmab) showed very low levels of neurotoxicity, regardless of PC12 cell differentiation stage.

When the concentration of seeded cells increased from low to high density, the resistance to anticancer drugs except for three compounds (described below) was slightly increased. The increase of resistance was the highest in 5-FU (24-fold), followed by GEM (16- fold), ERI (13-fold), NabPTX (12-fold), PTX (11-fold), DTX (6-fold), VBL (6-fold), L-OHP (5-fold), VNR (5-fold), ETP (3-fold), CDDP (2-fold), IRT (2-fold), and DOX (2-fold). Sensitivity of PC12 cells at all differentiation stages showed an almost constant sensitivity to Bmib, VCR and CBDCA, regardless of cell density. Molecular targeted drugs (Rmab, Nmab and Cmab) were not neurotoxic, regardless of inoculation cell density (Figure 2).

Relationship between neurotoxicity and reported maximum serum concentration of 19 anticancer drugs. Table II shows the CC_{50} of 19 anticancer drugs against human oral squamous cell carcinoma (B), normal oral cells (D) (8) and PC12 cells (E) (derived from Table I), with the data of maximum serum concentration (Cmax) of cancer patients following *i.v.* administration at the indicated doses (mg/m^2) (cited from the interview form from the supplier pharmaceutical companies). GEM, DTX, VCR, PTX, ERI, Bmib and NabPTX are expected to be highly toxic in human oral squamous cell carcinoma (Cmax/B=>5590, >4082, >358.9, 287, 260, 169.2 and 100, respectively) when administered at C_{max}. ERI, VBL and CDDP are expected to have a highly toxic effect on human oral normal cells (Cmax/D=4.73, 2.82, and 2.3, respectively). Bmib, DOX, VNR, CDDP, ERI and VBL, CBDCA are expected to have a highly toxic effect on PC12 cells (Cmax/E=115.8, 15, 11, 3.89, 2.6, 2.4 and 1.84, respectively) (Table II).

Antioxidants neutralized the neurotoxic effect of bortezomib. We confirmed that when PC12 cells were cultured for 6 days with NGF, they were morphologically differentiated into neurocytes with characteristic neurites (Figure 3D). Addition of Bmib (1 ng/ml) resulted in cell shrinkage (characteristic to apoptotic cells), disrupted the neurites (Figure 3E) and reduced the cell viability (black bar in Figure 3A-C). Simultaneous addition of VC (0.031-0.125 mM) (Figure 3A and 3F) and NAC (0.04-1.25 mM) (Figure 3B and 3G) almost completely eliminated the cytotoxicity of Bmib, regaining the normal morphology. The safety margin as defined by the chemotherapy index (calculated by 50% cytotoxic concentration/50% effective concentration) of VC and NAC was approximately 6.0 (=0.375/0.063) and 43.5 (=10.0/0.23), respectively (Figure 3A and 3B). This indicates that the effective dose range of NAC was much broader than that of VC. On the other hand, Meco (0.004~1 mM) did not show any such protective effect (Figure 3C and 3H).

Cell cycle analysis of undifferentiated PC12 cells (Day 0) treated for 48 h with these compounds is shown in Figure 4A. Figure 4B summarized the mean values of cell cycle analyses of triplicate assays. We found that treatment with actinomycin D (Act.D) (1 μM) and Bmib (1 ng/ml) increased the subG$_1$ population (marker of apoptosis) from 2.0% (control) to 30.4% and 17.6%, respectively, indicating the induction of apoptosis (Figure 4B). Simultaneous addition of VC (0.125 or 0.25 mM) or NAC (5 or 10 mM) reduced the subG$_1$ cell accumulation (apoptosis induction) by 70, 85, 85 and 86%, respectively (Figure 4C). On the other hand, Meco failed to produce such a protective effect (Figure 4B).

Cell cycle analysis with differentiated PC12 cells (Day 6) (Figure 5A) shows similar results except that the percentage of S-phase cells was reduced by nearly a half, indicating the growth retardation. The apoptosis induction by Bmib (1 ng/ml) (62.1% of subG$_1$ cells) was nearly 4-fold compared to actinomycin (15.0%) (Figure 5B). Simultaneous addition of VC (0.125 or 0.25 mM) and NAC (5 or 10 mM) reduced the number of apoptotic cells (subG$_1$ cells) by 94, 87, 98 and 86%, respectively, while the protective effect of Meco was much lower (23%) (Figure 5C).

Discussion

The present study demonstrated that Bmib showed the highest cytotoxicity against PC12 cells among the 19 anticancer drugs we tested. The interview form issued by Janssen Pharmaceutical K, K. describes that the C_{max} of Bmib following *i.v.* administration to cancer patients is 115-fold higher than CC_{50} of Bmib against PC12 cells. If a cancer patient was to be treated with 1.3 mg/m^2 Bmib, both OSCC and neuronal cells would be seriously damaged, in contrast,

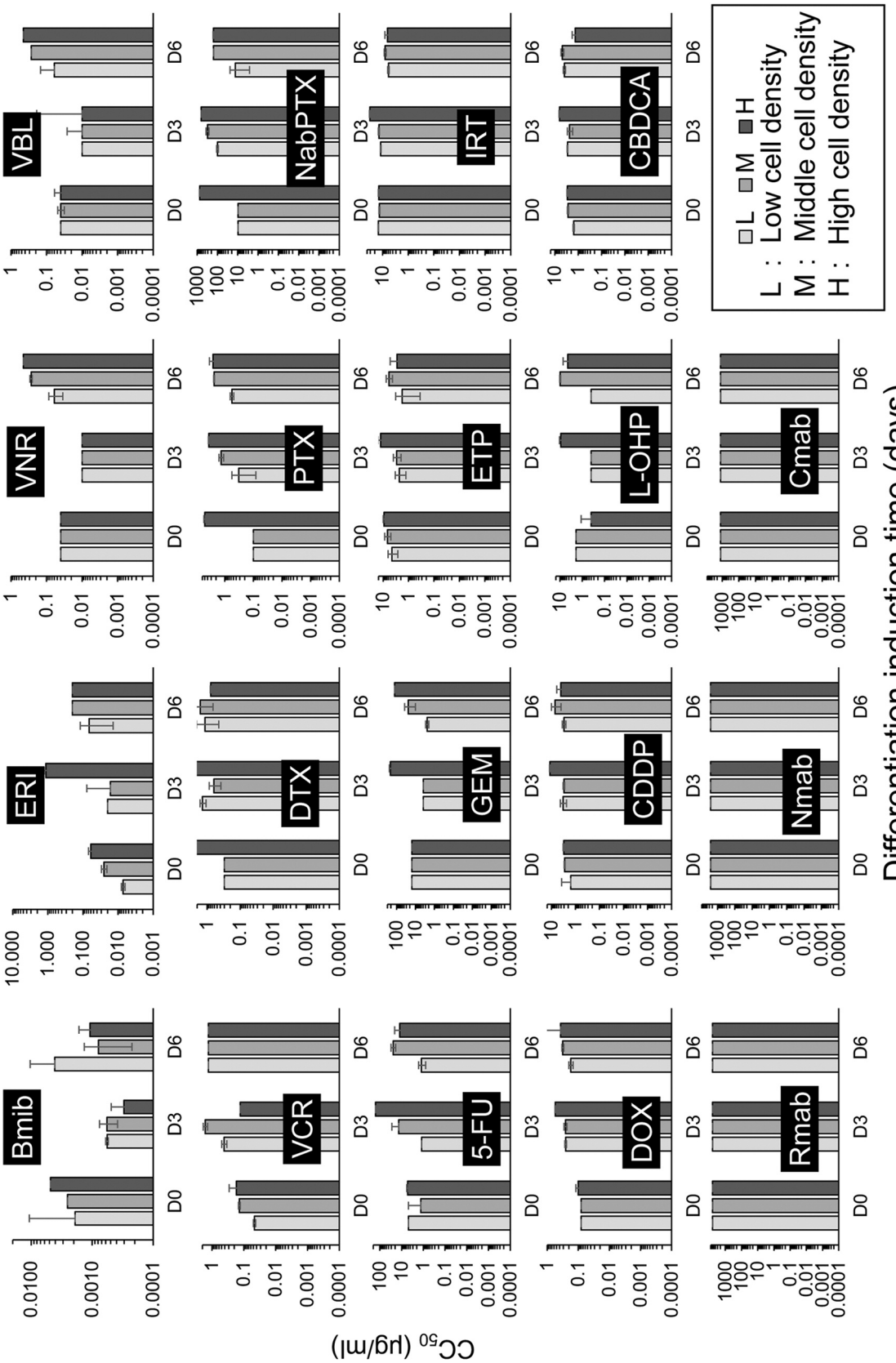


Figure 2. *Calculation of CC_{50} values for PC12 cells at three stages (Days) after inoculation at three different cell densities. Each value represents the mean±S.D. of triplicate determinations. D0: Day 0; D3: Day 3; D6: Day 6; L: low; M: medium; H: high.*

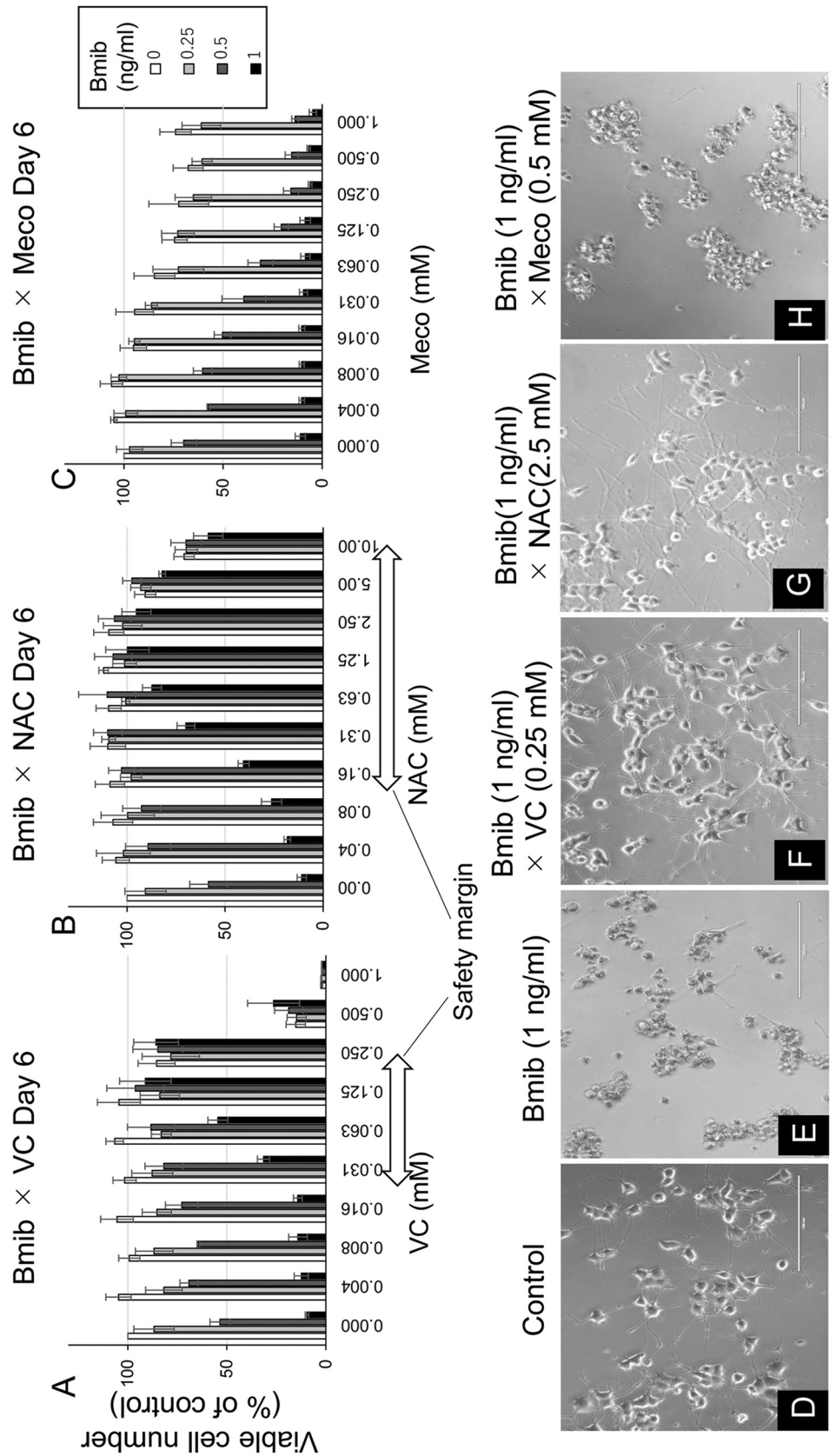
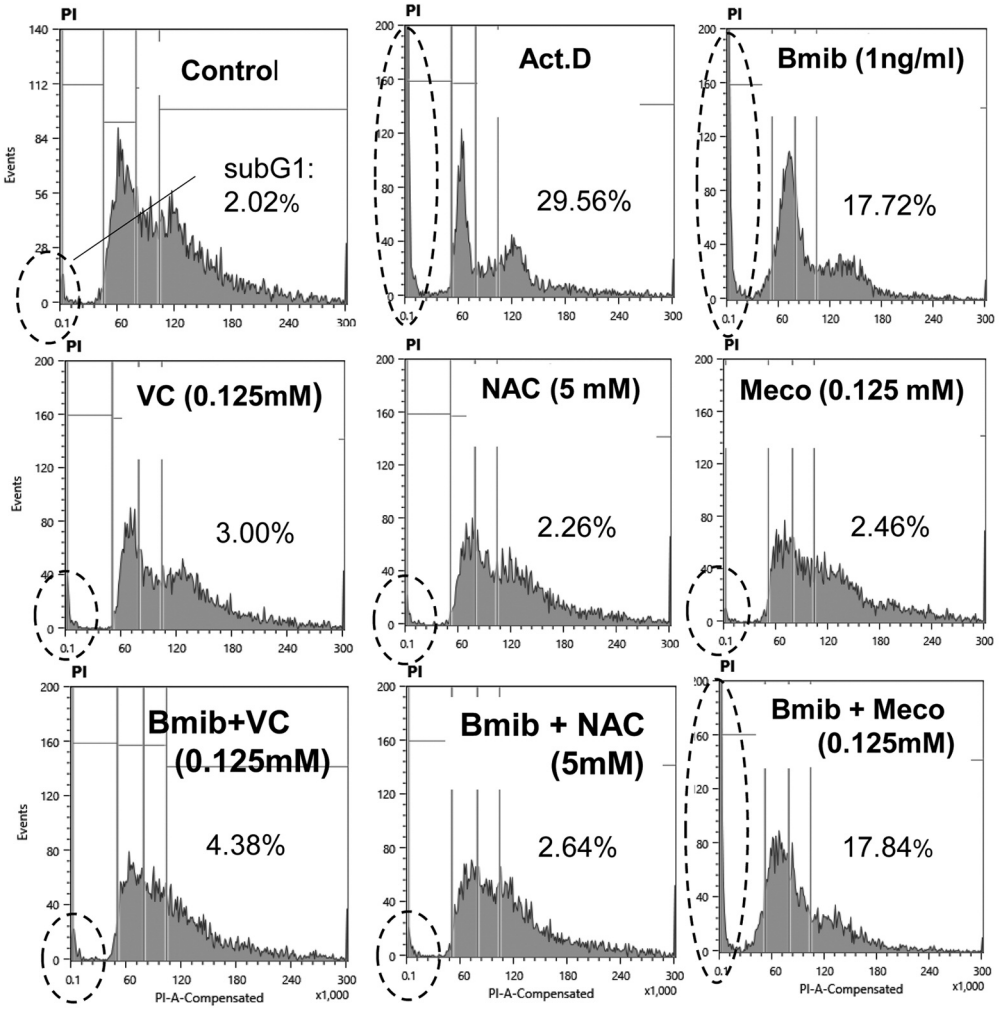


Figure 3. *Rescue of bortezomib-induced neurotoxicity by adding antioxidants. Viable cell number was expressed as % of control (without treatment). The safety margin of VC and NAC was calculated by dividing the CC_{50} by 50% effective concentration. Vitamin C (VC) (A) and N-acetyl-L-cysteine (NAC) (B), but not mecobalamin (vitamin B12, Meco) (C), reversed the bortezomib (Bmib)-induced cytotoxicity against differentiated PC12 cells (at Day 6). NAC showed broader safety margin than VC. It should be noted that effective concentration of NAC was one order higher than VC. Control differentiated cells expressed representative neurites (D), but treatment with Bmib resulted in the disruption of neurites (E). Simultaneous treatment with VC (F) or NAC (G), but not Meco (H), reversed the normal phenotype of differentiated cells with neurites. Scale bar: 200 μm.*

A



B

	Distribution (%)			
	subG1	G1	S	G2/M
Control	2.0	32.4	19.0	48.6
Act.D (1 μM)	30.4*	26.2	8.8	31.5
Bmib (1 ng/ml)	17.6*	33.2	20.8	30.3
VC (0.125 mM)	2.5	24.8	18.5	55.8
VC (0.25 mM)	3.7	24.9	18.5	54.8
NAC (5 mM)	2.5*	23.4	20.4	56.0
NAC (10mM)	3.6*	25.1	20.0	53.8
Meco (0.125 mM)	3.7	26.0	19.2	53.5
Bmib + VC (0.125 mM)	6.6*	29.7	22.9	43.6
Bmib + VC (0.25 mM)	4.4	28.2	19.3	50.7
Bmib + NAC (5 mM)	4.4	26.2	23.2	49.0
Bmib + NAC (10 mM)	4.2*	26.9	22.3	49.2
Bmib + Meco (0.125 mM)	18.7*	35.4	18.6	27.8

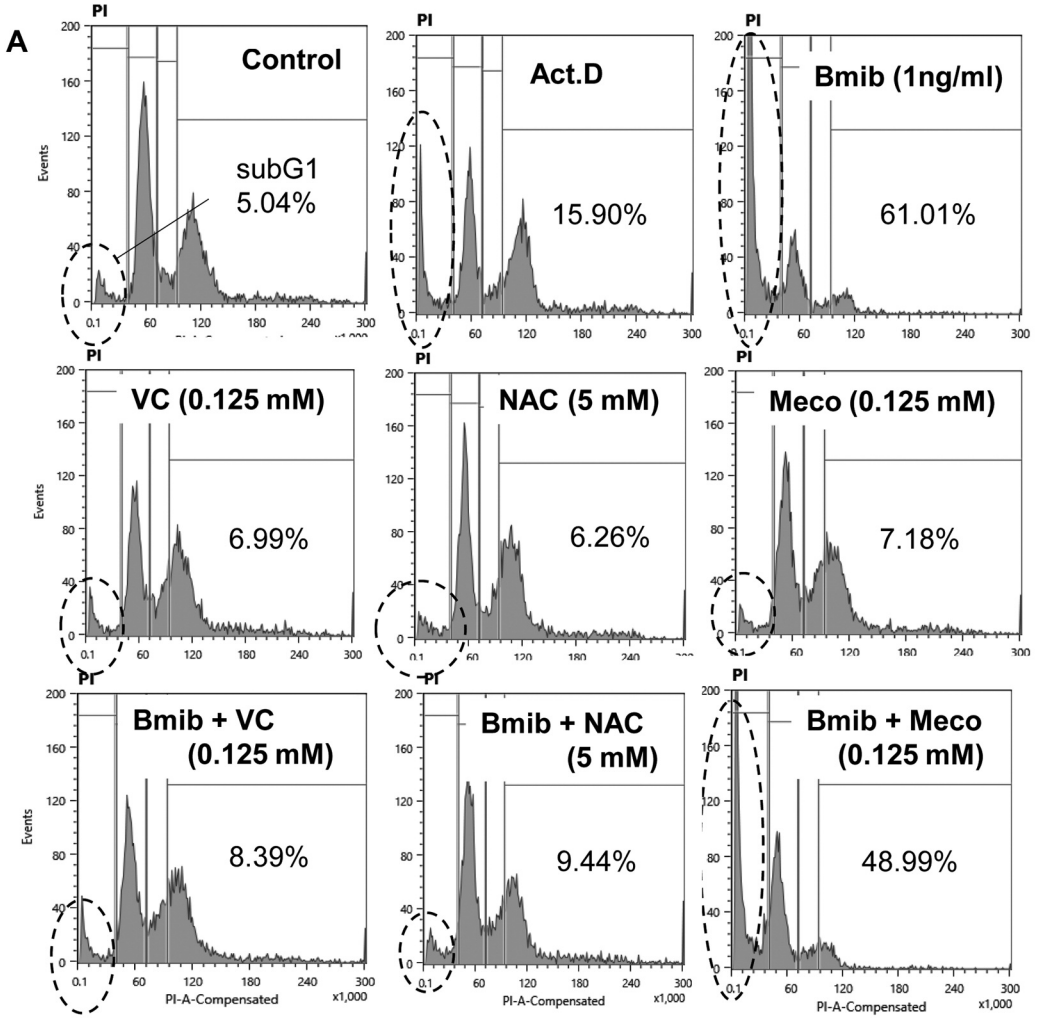
C

	subG1 (%)			
		- Control		Reduction (%)
Bmib (1ng/ml)	17.6	15.5	100	-
Bmib + VC (0.125 mM)	3.7	4.6	30	70
Bmib + VC (0.25 mM)	6.6	2.4	15	85
Bmib + NAC (5 mM)	4.4	2.4	15	85
Bmib + NAC (10 mM)	4.4	2.2	14	86
Bmib + Meco (0.125 mM)	4.2	16.6	107	-7

Day 0

Figure 4. *Inhibition of Bmib-induced apoptosis by antioxidants in undifferentiated PC12 cells. (A) Representative cell cycle distribution pattern of undifferentiated PC12 cells (at Day 0) after incubation for 48 h with the indicated compounds either alone or in combination. Mean values of distribution (%) into subG$_1$, G$_1$, S and G$_2$+M phase (B) and % decrease in subG$_1$ cell population (C) were calculated from triplicate assays. It should be noted that VC and NAC, but not Meco, inhibited the Bmib-induced apoptosis (subG$_1$ cell accumulation). The differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

A



B

	Distribution (%)			
	subG1	G1	S	G2/M
Control	5.5	43.6	10.7	42.5
Act.D (1 μM)	15.0*	33.8	9.3	43.7
Bmib (1 ng/ml)	62.1*	24.3	4.2	10.9
VC (0.125 mM)	8.2*	37.4	11.4	45.5
VC (0.25 mM)	11.1*	34.6	12.0	44.6
NAC (5 mM)	6.6*	38.0	13.8	44.4
NAC (10 mM)	7.6*	41.2	10.8	42.8
Meco (0.125 mM)	7.2*	40.8	16.7	38.7
Bmib + VC (0.125 mM)	8.6*	37.2	15.3	42.0
Bmib + VC (0.25 mM)	12.7*	40.3	10.6	38.6
Bmib + NAC (5 mM)	6.6*	38.0	13.8	44.4
Bmib + NAC (10 mM)	13.2*	44.4	12.6	33.3
Bmib + Meco (0.125 mM)	49.0*	36.1	7.2	10.4

C

	subG1 (%)			
		- Control		Reduction (%)
Bmib (1 ng/ml)	62.1	56.6	100	
Bmib + VC (0.125 mM)	8.6	3.1	6	94
Bmib + VC (0.25 mM)	12.7	7.2	13	87
Bmib + NAC (5 mM)	6.6	1.1	2	98
Bmib + NAC (10 mM)	13.2	7.7	14	86
Bmib + Meco (0.125 mM)	49.0	43.5	77	23

Day 6

Figure 5. *Inhibition of Bmib-induced apoptosis by antioxidants in differentiated PC12 cells (Day 6). Differentiated PC12 cells (at Day 6) were similarly treated with samples (A), and mean values of percent of cell cycle (B) and % decrease in subG$_1$ cell population (C) were calculated as described in Figure 4. It should be noted that (i) Bmib induced higher proportion of subG$_1$ cell population than day 0 cells (Figure 4) and (ii) VC and NAC, but not Meco, reproducibly inhibited the Bmib-induced apoptosis (subG$_1$ cell accumulation). The differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons. *p<0.05 compared to control.*

normal oral cells would suffer a much lower damage. From the ratio of Cmax/CC_{50} against PC12 cells, the intensity of neurotoxicity induced by anticancer drugs would be expected in the following order: Bmib (115.8), DOX (15.0), VNR (11.0), CDDP (3.89), ERI (2.6), VBL (2.4), CBDCA (1.84). It is thus necessary to reconsider the optimal administration doses of these drugs. On the other hand, other drugs showed much less neurotoxicity. It should be noted the DTX, PTX, NabPTX and GEM showed much higher cytotoxicity against OSCC as compared to PC12 cells.

Bmib is a therapeutic agent for multiple myeloma. It induces the intracellular accumulation of ubiquitinated proteins by inhibiting the proteasome complex and, thus, leads to apoptosis in tumor cells (13). Bmib is a first-generation drug with reported peripheral neuropathy as a side effect. Although second and third-generation drugs with reduced peripheral neuropathy are being developed, Bmib is still the first-choice drug for multiple myeloma (14).

It is therefore urgent to search for protective substances against Bmib-induced neurotoxicity. As far as we know, there is only one paper that has dealt with this issue, using Bmib-treated differentiating PC12 cells as a model. The authors have reported some protective properties of amifostine, however, with no direct evidence of apoptosis inhibition (9). We found that Bmib induced apoptosis against both undifferentiated (D0) and differentiated (D6) PC12 cells, regardless of inoculation cell density. We also found that antioxidants, VC and NAC effectively neutralized the Bmib-induced neurotoxicity. Since both VC and NAC have been reported to protect cells from the oxidative stress observed in cardiac autonomic neuropathy (15), liver injury (16), ischemia reperfusion injury (17) and critical limb ischemia (18), the oxidative stress may be involved in the present Bmib-induced neurotoxicity. Metabolomics and DNA microarray analysis may provide the evidences of oxidative stress that may be involved in the Bmib-induced neurotoxicity.

Both VC (used to treat severe sepsis and septic shock) and NAC (used to treat idiopathic pulmonary fibromatosis) are known to have fewer side effects, based on no significant overall differences in baseline characteristics and the change in forced vital capacity (FVC) compared to control groups (19, 20).

Simultaneous addition of VC or NAC efficiently eliminated the cytotoxicity of Bmib. The safety margin of VC and NAC was approximately 6.0 and 43.5, respectively, indicating that the effective dose range of NAC was much broader than that of VC, possibly due to the dual actions of VC as an antioxidant as well as a prooxidant, in the presence of oxygen (21). This point can be clarified by the experiment using hydrogen water that has only reducing activity.

Cell cycle analysis demonstrated that treatment of differentiated PC12 cells (Day 6) with Bmib (1 ng/ml) induced the accumulation of $subG_1$ cell population (a marker of apoptosis), and simultaneous addition of VC or NAC reduced the $subG_1$ accumulation (Figure 5). We also found that Meco failed to offer such a protective effect. This finding is contradictory to a previous report showing that Meco inhibited the Bmib-induced apoptosis in a rat primary culture of dorsal root ganglion cells (22). The discrepancy between the present and previous reports may be due to the difference of assay systems, either the *in vitro* or the *in vivo* ones. Since VC has also been reported to inhibit the antitumoral effect of Bmib (23), a balance between neuroprotection and anti-tumor potential should be taken into consideration in the clinical application.

In conclusion, the present study demonstrated that bortezomib showed the highest neurotoxicity among 19 anticancer drugs *via* induction of apoptosis in PC12 cells. Sodium ascorbate and *N*-acetyl-L-cysteine, completely reversed the cytotoxicity of bortezomib, suggesting the clinical application of these two antioxidants could ameliorate the bortezomib-induced neuropathy.

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

YI and HS performed the most experiments of the present study and wrote the manuscript. KB and SA performed the cell cycle analysis and reviewed the manuscript. SH, MS, TK and NH provided the interpretation of experimental results and edited the manuscript. All Authors read and approved the final version of the manuscript.

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