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Boromycin Abrogates Bleomycin-induced G2 Checkpoint

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The DNA-damaging agent bleomycin arrests the cell cycle at the G2 phase of Jurkat cells defective in the G1 checkpoint, and microtubule-acting colchicine arrests it at the M phase. Boromycin itself, an actinomycete metabolite, showed no effect on the cell cycle status of Jurkat cells at least up to 340 nM. However, the compound (3.4～340 nM) was found to abrogate bleomycin-induced G2 arrest even at 3.4 nM, resulting in a drastic decrease in cells at the G2 phase and increase in cells at the subG1 phase. On the other hand, boromycin did not show any effect on the colchicine-induced M phase arrest in Jurkat cells, nor on the cell cycle status of the bleomycin-treated or -untreated HUVEC, normal cells conserving both G1 and G2 checkpoints. Furthermore, boromycin potentiated anti-tumor activity of bleomycin in scid mice inoculated with Jurkat cells. These data suggest that boromycin disrupts the cell cycle at the G2 checkpoint of cancer cells selectively, leading to sensitization of cancer cells to anti-cancer reagents.

DNA damage induces signal transduction pathways known as checkpoints, which delay cell cycle progression to repair DNA[1]. Checkpoints arrest cells in the G1 phase to prevent replication of damaged DNA and in the G2 phase to prevent the segregation of damaged chromosomes during mitosis. Normal human cells can repair DNA in the G1 and G2 phases, whereas most cancer cells have mutations in genes involved in the G1 checkpoint, such as *p53*, *p16*^{TNK4}, or *Rb*[2], and can repair DNA only in the G2 phase. Accordingly, disruption of the G2 checkpoint in cancer cells is expected to increase sensitivity to DNA-damaging reagents like bleomycin, cisplatin and camptothecin, resulting in selective cell death of cancer cells by accumulation of mutation in genes. Therefore, the G2 checkpoint is a potential target for development of a novel therapy against intractable cancers.

In the G2 checkpoint, DNA damage activates the ATM (ataxia telangiectasia-mutated) and ATR (A-T and rad3-related) members of the phosphoinositide kinase family[3,4]. A signal is then transmitted through the downstream protein kinases Chk1 and Chk2[3～8], which are able to phosphorylate Cdc25 on Ser216. This phosphorylation is thought to directly prevent Cdc25 from activating the Cdc2 kinase[9]. Chk1 and Chk2 can also phosphorylate and activate Wee1, a kinase that catalyzes Cdc2 inhibitory phosphorylation[10,11]. Several G2 checkpoint inhibitors have been reported to date. For example, UCN-01, a derivative of staurosporine, abrogates DNA damage-induced S and G2 arrest, due to inhibition of Chk1 and perhaps Chk2[12]. Although Phase I clinical trials have been completed, UCN-01 was found to bind readily to human plasma proteins and to inhibit kinases other than Chk1 and Chk2, which may cause unwanted side effects in patients[13～15]. SB-218078[16] and Go6979[17] reported recently are specific to Chk1 inhibition and their binding activity to human plasma proteins was stronger than that of UCN-01. Debromohymenialdsine isolated from marine sponge was also reported to inhibit Chk1 and Chk2[18]. On the other hand, caffeine and PD0166285 inhibited the checkpoint kinases ATM and ATR[19,20] and Wee1 kinase[21],

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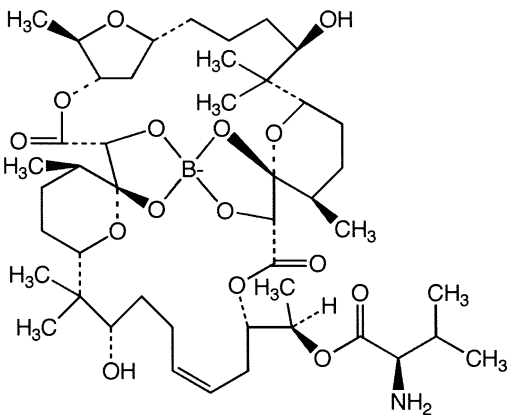
Fig. 1. The structure of boromycin.



respectively. SUGANUMA *et al.* previously reported that the short peptide corresponding to amino acids 211～221 of human Cdc25C, which is an important phosphatase in the G2 checkpoint, fused with a part of HIV1-TAT can efficiently disrupt the cell cycle G2 checkpoint that is activated by DNA damage and thereby sensitize cancer cells but not normal cells to anticancer reagents[22]. On the basis of these finding, more potent analogs of this peptide have been developed as G2 checkpoint inhibitors.

Jurkat, a human T-cell leukemia-derived cell line, depends on the G2 checkpoint to repair DNA damage because it does not have functional p53[22]. We established a cell-based assay using Jurkat cells to screen culture broths of microorganisms for G2 checkpoint inhibitors. From the screening program, boromycin, previously reported to show diverse activities such as anti-Gram positive bacterial, anti-coccidial and anti-HIV activities[23,24], was isolated from the culture of an actinomycete strain, *Streptomyces* sp. OH-1034 (Fig. 1). In this paper, we show that boromycin abrogates bleomycin-induced G2 arrest in Jurkat cells, and induces cell death at nanomolar levels without any effect on colchicine-induced M phase arrest. Furthermore, boromycin increased sensitivity of Jurkat cells to bleomycin in scid mice. The compound is expected to be developed as a novel anticancer agent.

Materials and Methods

Materials

Boromycin was isolated from the culture broth of *Streptomyces* sp. OH-1034. A human T-cell leukemia-derived cell line, Jurkat, was kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University. Human umbilical vein endothelial cells (HUVEC) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Fetal calf serum (FCS) was purchased from JRH Biosciences (Tokyo, Japan). Phenylmethansulfonylfluoride (PMSF), aprotininin, leupeptin, pepstatin A, Na_3VO_4, NaF, colchicine, Trypsin-EDTA solution, propidium iodide, ribonuclease A and NP-40 were obtained from Sigma (St. Louis, MO). Bleomycin was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Protein A-Sepharose was obtained from Amersham Bioscience Corp. (Princeton, NJ). Mouse anti-human Cdc2 antibody, mouse anti-phosphotyrosine antibody and goat anti-mouse horseradish peroxidase conjugated IgG antibody were purchased from Oncogene Research Products (San Diego, CA), Upstate Biotechnology Inc. (Charlottesville, VA), and Sigma, respectively. RPMI 1640 medium and EBM-2 medium were obtained from Iwaki (Chiba, Japan) and Sanko Junyaku (Tokyo, Japan), respectively.

Cell Culture and Cell Cycle Analysis

Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. HUVEC were cultured in EBM-2 medium supplemented with 10% FCS at 37°C in 5% CO_2. The cell cycle status was analysed according to the method described previously[22]. Briefly, Jurkat cells (5.0×10^5 cells/200 μl) were treated with boromycin (0～340 nM) in the presence or absence of bleomycin (42 μM) or colchicine (0.5 μM) for 24 hours at 37°C. The cells were then resuspended in 200 μl of 0.1% sodium citrate solution containing 50 μg/ml propidium iodide, 20 μg/ml ribonuclease A and 0.5% NP-40 (Krishan's solution). HUVEC (5.0×10^4 cells/500 μl) in a 48-well plate were treated with boromycin (70 nM) in the presence or absence of bleomycin (7.1 μM) for 24 hours at 37°C. Then the supernatant in each well was transferred to a test tube. Cells were washed with phosphate-buffer saline (PBS) and harvested by treatment with trypsin-EDTA. The cell suspension mixed with the supernatant was centrifuged at 700 g for 5 minutes. The pellet was resuspended in 200 μl of Krishan's solution. The cell cycle status was determined by analysis of the DNA content using FACSCalibur (Becton Dickinson, NJ). Cells (1.0×10^4 cells) were measured and the population in each phase (subG1, G1, S and G2/M) was calculated by the program ModiFit LT ver. 2.0 according to the manufacturer's protocol.

Immunoprecipitation and Immunoblotting

Jurkat cells (8.0×10^6 cells) were treated with boromycin (100 nM) in the presence or absence of bleomycin (42 μM) for 20 hours at 37°C. The cell suspension was centrifuged at 700 *g* for 5 minutes. Then the cells were washed with PBS twice, were suspended with 500 μl of lysis buffer (50 mM Tris-HCl buffer containing 150 mM NaCl, 6 mM sodium deoxycholate, 3.5 mM sodium dodecylsulfate (SDS), 1 mM EGTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 2.1 μM leupeptin, 1.5 μM pepstatin A, 1% (w/v) NP-40 and 1 μg/ml aprotininin, pH 7.4) and were allowed to stand at 4°C for 30 minutes. The cell lysate was centrifuged at 10,000 *g* for 20 minutes to collect the supernatant, which was used for immunoprecipitation sampling. The supernatant (50 μg protein/150 μl) was incubated with 0.25 μl of anti-human Cdc2 antibody and 5 μl of protein A-Sepharose at 4°C for 12 hours. The immunoprecipitate was washed twice with lysis buffer at one-third the strength above. The immunoprecipitate was then suspended with 50 μl of sample buffer (62.5 mM Tris-HCl buffer containing 70 mM SDS, 2.9 mM sucrose, 5% (w/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue, pH 6.8) and boiled for 5 minutes. A 20 μl portion of each sample was used for Western blot analysis of Cdc2 and phosphorylated Cdc2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions in 12.5% acrylamide gel according to the method of LAEMMLI[25], and the proteins were electrotransferred from the gel to the polyvinylidiene difluoride (PVDF) membrane by the established method[26]. Each membrane was incubated with anti-Cdc2 antibody (4 μg/4 ml) or anti-phosphotyrosine antibody (4 μg/4 ml), and incubated further with anti-horseradish peroxidase conjugated IgG antibody (0.8 μg/4 ml). Each membrane was washed and detected by an enhanced chemiluminescence detection system (Amersham Biosciences Corp.) according to the manufacturer's instructions. Densitometric analysis and calculation were performed using Versadoc 5000 (Bio-Rad Laboratories, Hercules, CA).

Animal Studies

Scid mice (male, 4 weeks of age) were obtained from Charles River Laboratories Japan (Kanagawa, Japan), and acclimated to laboratory conditions 1 week before tumor implantation. Scid mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Jurkat cell xenografts were established by injecting *s.c.* 5×10^8 cells. Treatment was initiated when the tumors were 10×10 mm^3 after injection of Jurkat cells. Bleomycin and boromycin were dissolved in sterilized water and DMSO, respectively, and adjusted to 10 mg/ml. Bleomycin (25 mg/kg) and boromycin (0.2 mg/kg) were administered by *i.p.* injections every 6 days and every 3 days, respectively, for 2 weeks. The weight of the tumor was measured 18 days after the initial treatment, and the body weights of the mice were monitored for indications of toxicity. Each value represented the mean tumor weight±S.E. obtained from four mice. The significance of the difference of the means was determined by using the Student's *t* test. A *P* value of <0.05 was taken as significant. Regression analyses were performed with STATVIEW (SAS Institute, Tokyo).

Quantitation of Proteins

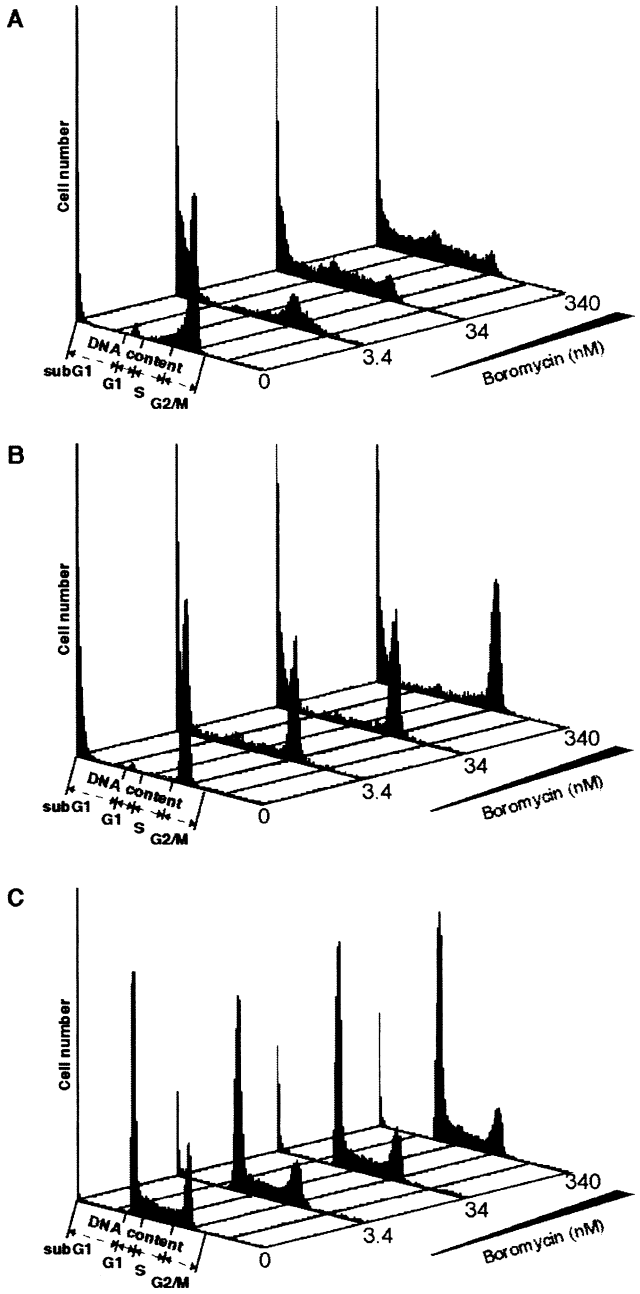
Concentrations of protein were determined by the BCA protein assay reagent (Pierce), with bovine serum albumin as the standard.

Results

Jurkat cells were cultured for 24 hours, and the cell cycle status was analysed. The distribution ratios in the subG1, G1, S and G2/M were 4.5%, 50.9%, 21.9% and 21.2%, respectively (Fig. 2C). When Jurkat cells were incubated in the presence of boromycin (3.4～340 nM), the distribution ratios in the subG1, G1, S and G2/M phases were fundamentally unchanged; 5.4～6.0%, 47.2～50.1%, 21.5～23.2% and 18.1～20.2% (Fig. 2C), respectively. These data indicated that boromycin alone shows no effect on the cell cycle of Jurkat cells. When Jurkat cells were treated with bleomycin (42 μM), the ratios of cell numbers distributed in subG1, G1, S and G2/M phases were 16.7%, 2.7%, 9.1% and 65.5%, respectively (Fig. 2A), indicating that Jurkat cells were arrested at the G2 phase by bleomycin. In combination with boromycin (3.4～340 nM), the cell cycle pattern began to change shape even at 3.4 nM. The distribution ratio in subG1 was drastically increased to 52.9～57.2%, indicating that boromycin induced cell death in DNA-injured Jurkat cells. On the other hand, when Jurkat cells were incubated in the presence of colchicine, the distribution ratios of subG1, G1, S and G2/M phases were 42.2%, 2.1%, 2.9% and 50.6%, respectively (Fig. 2B). In combination with boromycin (3.4～340 nM), the cell cycle distributions were almost unchanged; 42.7% of colchicine-treated Jurkat cells remained in G2/M phase at 340 nM boromycin. These findings strongly suggested that boromycin abrogated DNA damage-induced G2 arrest in Jurkat cells.

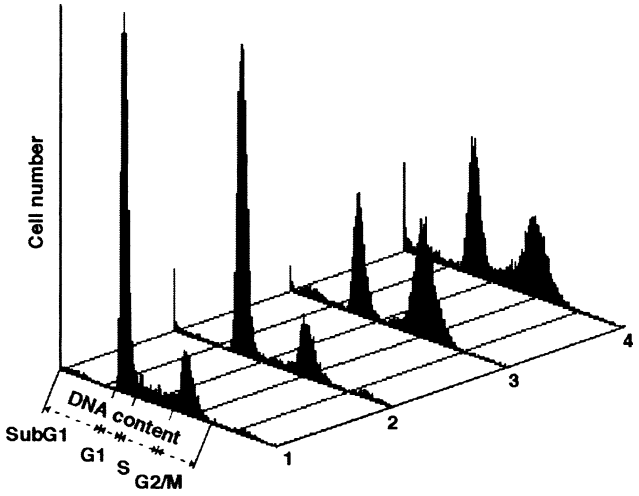
The effect of boromycin was evaluated using a normal

Fig. 2. Abrogation of bleomycin-induced G2 arrest by boromycin in Jurkat cells.



Jurkat cells treated with boromycin (0~340 nM) in the presence of 42 μM bleomycin (A) or 0.5 μM colchicine (B) or in the absence of a drug (C). Distribution in the cell cycle was analyzed by FACS 24 hours after incubation.

Fig. 3. Effect of boromycin on the cell cycle status of HUVEC treated with bleomycin.

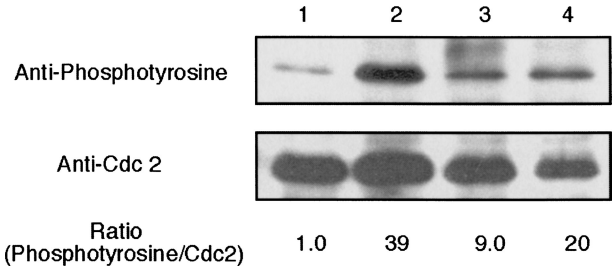


HUVEC (5.0×10^4 cells) were cultured in the absence or presence of boromycin alone, bleomycin alone, or boromycin in combination with bleomycin. Distribution in the presence or absence of drugs over 24 hours and the cell cycle status were analyzed by FACS. 1) Control, 2) boromycin (0.5 μM), 3) bleomycin (7.0 μM) and 4) boromycin (0.5 μM) and bleomycin (7.0 μM).

HUVEC line (Fig. 3). The distribution ratios of subG1, G1, S and G2/M phases in a cell cycle without a compound (control) were 4.3%, 59.2%, 12.8% and 16.9%, respectively. HUVEC treated with boromycin (71 nM) gave almost the same distribution ratios of subG1 (3.8%), G1 (64.1%), S (9.1%) and G2/M (16.8%) as the control, indicating that boromycin alone shows no effect on the cell cycle of normal cells. When HUVEC were cultured in the presence of bleomycin (7.0 μM), the distribution ratios of subG1, G1, S and G2/M changed to 7.7%, 28.2%, 4.7% and 50.4%, respectively, indicating that bleomycin induced G1 and G2 arrest in normal cells. In combination with bleomycin (7.0 μM) and boromycin (71 nM), the ratio of G2 phase slightly decreased from 50.4% to 36.9%, correspondingly the G1 ratio increased from 28.2% to 34.4%, and there was no change in subG1 ratios. These results indicated that boromycin abrogated G2 arrest but not G1 arrest in HUVEC, and showed no effect on the survival of normal cells.

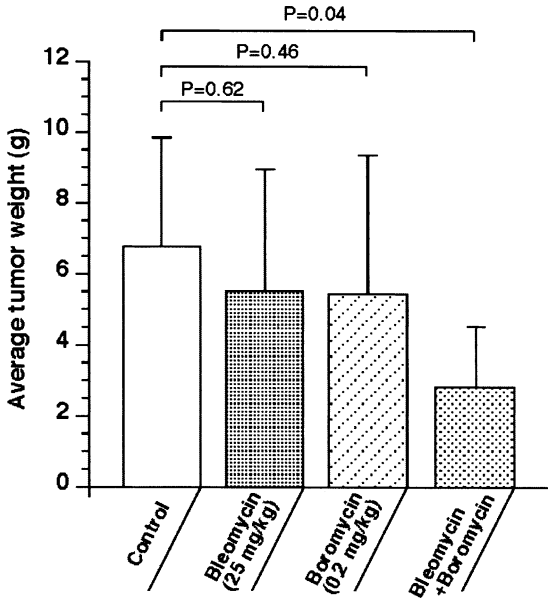
Jurkat cells were cultured for 20 hours in the absence or presence of boromycin (100 nM), and the amounts of total Cdc2 and phosphorylated Cdc2 were analyzed (Fig. 4). The phosphorylated Cdc2 was very low or almost undetectable in the absence of boromycin (control), whereas total Cdc2

Fig. 4. Effect of boromycin on the phosphorylation of Cdc2.



The cell lysates were prepared from Jurkat cells (8.0×10^6 cells) cultured in following conditions: No treatment (lane 1); 42 μM of bleomycin (lane 2); 100 nM of boromycin (lane 3); 42 μM of bleomycin in combination with 100 nM of boromycin (lane 4) for 20 hours at 37°C. Each cell lysate was immunoprecipitated with anti-Cdc2 antibody. The immunoprecipitants were resolved by SDS-PAGE, and analysed by Western blot using anti-Cdc2 antibody or anti-phosphotyrosine antibody as described in the "Materials and Methods."

Fig. 5. Potentiation of bleomycin activity by boromycin in scid mice inoculated with Jurkat cells.



Jurkat cell xenografts were established by *s.c.* injection of 5×10^8 cells in the scid mouse. Treatment was initiated when the tumors were 10×10 mm^3 after injection of Jurkat cells. Bleomycin (25 mg/kg) and boromycin (0.2 mg/kg) were administered by *i.p.* injections every 6 days and every 3 days, respectively, for 2 weeks. Weights of tumors were measured 18 days after initial treatment, and average tumor weight was calculated. The significance of difference of the means was determined as described in the "Materials and Methods".

was observed normally (Fig. 4 lane 1). The levels of phosphorylated Cdc2 slightly increased in the presence of boromycin (Fig. 4 lane 3). When the ratio (phosphorylated Cdc2 *vs.* total Cdc2) of the control was defined as 1, the ratio was calculated as 9.0 in boromycin-treated cells. On the other hand, when Jurkat cells were treated with bleomycin (42 μM), the amounts of phosphorylated Cdc2 drastically increased, and the ratio in bleomycin-treated Jurkat cells increased to 39.0 (Fig. 4 lane 2). These data indicated that bleomycin arrested the cell cycle of Jurkat cells at G2 phase by G2 checkpoint kinases. However, in combination with boromycin (100 nM) and bleomycin (42 μM), the ratio decreased from 39.0 to 20.0 (Fig. 4 lane 4), almost the same ratio (9.0) as in boromycin-treated cells. These results suggested that boromycin abrogated bleomycin-induced G2 phase arrest and moved to the M phase.

Jurkat cell xenografts were established on scid mice, and *in vivo* tumor growth was compared in the treatment of bleomycin (25 mg/kg) alone or in combination with boromycin (0.2 mg/kg) (Fig. 5). The average tumor weight of the control mice (no treatment) was estimated to be 6.78 ± 3.0 g 18 days after initial treatment. When bleomycin (25 mg/kg) was administrated by *i.p.* injection, the average tumor weight was calculated to be 5.50 ± 3.54 g ($P=0.62$). In the group treated with boromycin (0.2 mg/kg) alone, the average tumor weight was estimated to be 5.45 ± 3.85 g ($P=0.46$). These data indicated that bleomycin- and boromycin-treatment groups did not show significant differences in tumor weights as compared with the control group. On the other hand, bleomycin in combination with boromycin markedly decreased tumor weights to 2.83 ± 1.32 g ($P=0.04$), indicating that boromycin potentiated bleomycin activity against the growth of Jurkat cells in scid mice. The body weights in the combination therapy were almost the same as those of non-implanted scid mice during the experiments (data not shown), indicating that the combination therapy gave no remarkable side effects.

Discussion

As demonstrated in this paper, boromycin inhibited bleomycin-induced G2 phase arrest, leading to cell death in Jurkat cells, but showed no effect on colchicine-induced M phase arrest. These data suggested that boromycin abrogated the G2 checkpoint in the cell cycle specifically. To confirm this point, Cdc2 phosphorylation levels involved in the G2 checkpoint were measured. Boromycin reduced the phosphorylation levels of Cdc2 driven by bleomycin-induced DNA damage (Fig. 4), and the cells moved to the M phase compulsorily. In HUVEC, a normal cell line, bleomycin induced G1 and G2 arrests, and in combination with boromycin, induced a slight decrease in G2 phase cells and increase in G1 and S phase cells (Fig. 3). Therefore, we concluded that boromycin specifically abrogated the cell cycle G2 checkpoint. Furthermore, boromycin proved active *in vivo*, potentiating bleomycin-derived anti-tumor activity against the growth of Jurkat cells in scid mice. These findings strongly suggest that boromycin sensitizes G1 checkpoint defective cancer cells to DNA-damaging agent induced death, while having no obvious cytotoxic effects on normal cells.

Boromycin belongs to a group of polyether antibiotics (Fig. 1). Generally, polyether compounds, such as monensin, nonactin, valinomycin and so on, were known for their ionophore activity. PARK *et al.* previously reported that monencin itself caused cell cycle arrest and apoptosis in various lymphoma cells including Jurkat cells due to the exchange of Na^+ for H^+ at 0.5 μM for 72 hours[27]. LAKATOS *et al.* also reported that boromycin inhibited Ca^{2+} and Na^+ transport systems at micromolar levels in the erythrocytes, leucocytes, synaptosomes and cardiomyocytes[28]. In our assay system, boromycin abrogated G2 phase arrest in bleomycin-treated Jurkat cells at a much lower concentration of 3.4 nM, and induced cell death very rapidly within 24 hours. Importantly, boromycin itself did not affect the cell cycle status of Jurkat cells up to 340 nM for 24 hours. Furthermore, boromycin itself did not show anti-tumor effects on scid mice inoculated with Jurkat cells when boromycin (0.2 mg/kg) itself was administered by *i.p.* injections every 3 days for 2 weeks (Fig. 5). These results indicate that the mechanism of action of boromycin is not related to ionophore activity. Chk1, Chk2, ATM, ATR and Wee1 have been reported as target molecules of known G2 checkpoint inhibitors. Boromycin did not inhibit these G2 checkpoint related kinases (ATM, Chk1, Chk2 and Wee1) and PKCs at 100 nM (data not shown). It is expected that boromycin inhibited a novel target different from those of G2 checkpoint inhibitors reported until now. The combination effect of other DNA-damaging reagents such as cisplatin, camptothecin, and irradiation, and so on against solid cancer remains to be investigated.

Acknowledgments

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