Induction of Necrosis in Human Myeloma Cells by Kigamicin

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Abstract. *Background: Kigamicin (KGM) is a novel compound derived from Actinomycetes that was originally reported to induce necrosis in pancreatic cancer cells only under nutrient-starved conditions via inhibition of PI3-kinase. The effects of KGM on myeloma cells were investigated. Materials and Methods: Cytotoxic activity was quantified using WST8 assay. Necrosis was determined by Annexin V/PI staining. Regulatory protein levels were assessed by Western blot. LY294002 was utilized as a PI3-kinase inhibitor. Results: KGM induced necrosis in myeloma cells in nutrient rich conditions with a CC_{50} of approximately 100 nM. KGM did not induce necrosis in normal lymphocytes. Cyclin D1, p21, p-AKT and p-ERK were inhibited by KGM while LY294002 did not inhibit cell death by KGM. A melphalan-resistant myeloma cell line was more susceptible to KGM than the melphalan-sensitive parental cell line. Conclusion: KGM-induced necrosis in myeloma cells even at very low concentration. The present data warrant further investigation into the use of KGM as a potential therapeutic agent for multiple myeloma.*

Multiple myeloma is a disease of malignant plasma cells that cannot be cured by chemotherapy, although various new therapeutic agents, such as proteasome inhibitors and thalidomide derivatives, have been developed. Therefore, new therapeutic agents with unique mechanisms of action are required.

Recently, Esumi and co-workers discovered a new chemical compound derived from *Actinomycetes*, kigamicin (KGM), that induced necrosis in the pancreatic cancer cell line PANC-1, by screening for a reagent that was cytotoxic only under hypoxic conditions (1-3). KGM is thought to be an ideal therapeutic reagent for pancreatic cancer, since hypovascularity is a key feature of this type of cancer. They also found that KGM induced necrosis *via* inhibition of PI3-kinase under nutrient-starved conditions, but not under oxygen-rich conditions, indicating that it disrupted the mechanism regulating the survival pathway of PANC-1 cells under hypoxic conditions. The KGM was effective at nanomolar concentrations, suggesting low toxicity toward normal tissues when administered in clinical settings. Indeed, no toxic effects of KGM were found, even though it showed antitumor effects in an *in vivo* model (3).

It has been suggested that myeloma cells should be highly susceptible to hypoxia, since these cells exclusively require angiogenesis (4). Therefore, a therapeutic reagent that can function under serum-starved conditions would be expected to synergize anti-angiogenesis therapy towards myeloma. In an attempt to develop a new therapeutic strategy, the cytotoxic effects of KGM toward myeloma cells were analyzed.

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Key Words: Multiple myeloma, PI3-kinase, necrosis.

Materials and Methods

Cells and cell culture. The human myeloma cell line KMS-12-PE (12PE) (5) was kindly provided by Dr. Ohtsuki (Kawasaki Medical School, Kurashiki, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS at 37˚C in humidified air containing 5% CO_2. For some experiments, bone marrow samples were obtained from myeloma patients admitted to Kumamoto University Hospital after informed consent was obtained. Following centrifugation of the bone marrow aspirates through a Ficoll density gradient (Invitrogen, Carlsbad, CA, USA), the myeloma cells were purified using CD138-coated immune-magnetic beads (Miltenyi Biotec, Auburn, CA, USA) as described elsewhere (6).

Reagents. Kigamicin was obtained from Dr. Esumi (National Cancer Center Research Institute East, Japan). LY294002 was purchased from Sigma (St. Louis, MO, USA). The caspase inhibitor, ZVAD-FMK, was purchased from Invitrogen.

Analysis of cytotoxicity. Cytotoxicity was evaluated by trypan blue dye exclusion analysis. Each experiment was quadruplicated. In some experiments, the WST8 assay (Dojindo Laboratories, Kumamoto, Japan) was performed according to the manufacturer's protocol. Necrosis was evaluated by Annexin V / promidium iodide (PI) staining and analyzed using an Epics V flow-cytometer (Coulter, Miami, FL, USA).

Western blot analysis. Cell lysates were obtained by treating the cells with M-per reagent (Invitrogen) according to the manufacturer's protocol. For extraction of the nuclear proteins, an N-Per kit was

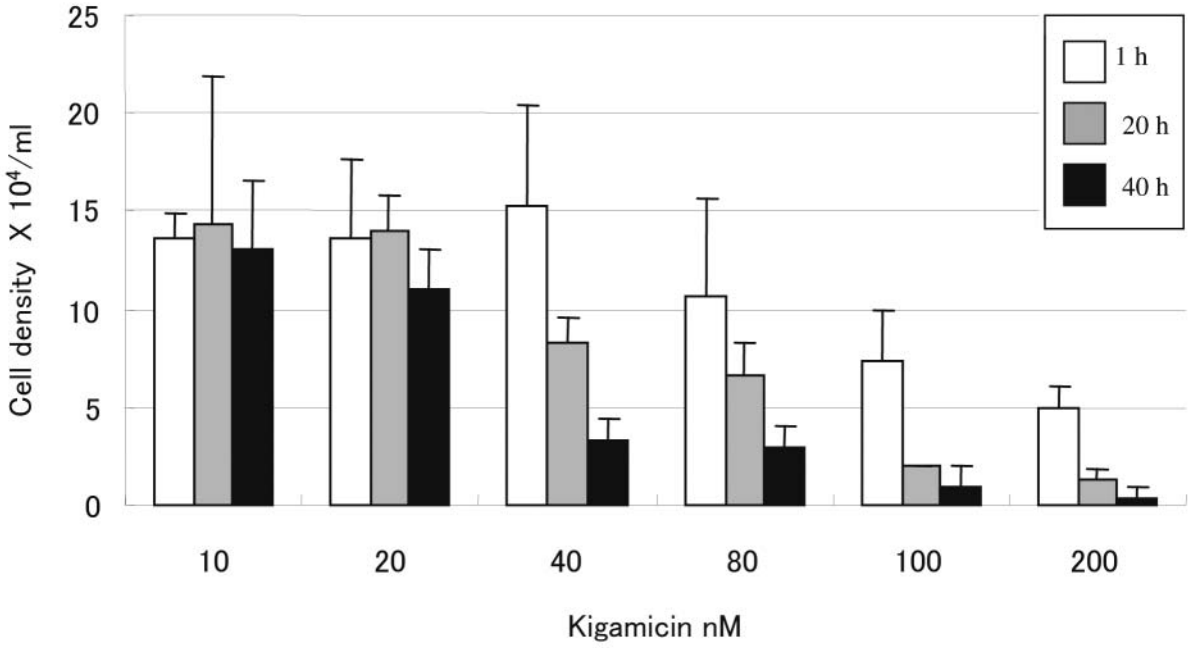


Figure 1. *Cytotoxic effects of kigamicin (KGM) on a human myeloma cell line, 12PE. Viable 12PE cells were analyzed by trypan blue dye exclusion analysis, after incubation with KGM at various concentrations for the indicated periods. Cells were initially cultured at a cell density of 15x10⁴/ml before addition of KGM.*

utilized (Pierce Biotechnology Inc., Rockford, IL, USA). The cell lysates were separated in 10% acrylamide gels, transferred to polyvinylidine difluoride (PVDF) membranes and stained with appropriate antibodies. Antibodies against phospho-AKT, total AKT, cyclin D1, caspase-3, p21, Bcl-2, p50, p65, BiP, total ERK and phosphorylated ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies and a chemiluminescence detection kit (GE Healthcare Biosciences, Chalfont St. Giles, UK) were utilized for visualization.

Results

Effect of KGM on myeloma cells. The 12PE cells were exposed to KGM at various cell densities under nutrient-rich conditions, and dose and time-dependent growth inhibition was demonstrated (Figure 1). The concentration of KGM required to induce cytotoxicity was extremely low (<100 nM). On the other hand, a pancreatic cancer cell line, PANC-1, did not show growth inhibition under nutrient-rich conditions, even at a concentration of 100 μM, as reported previously (3) (data not shown). KGM started to reduce the viable cell number at a concentration of 100 nM as early as 1 hour after the initiation of treatment (Figure 1). Morphological examination of May-Giemza stained cells revealed that KGM induced degeneration of the cytoplasm, while the nuclei remained intact, suggesting that the cytotoxic effects were independent of apoptosis (Figure 2). Indeed, Annexin V/PI analysis revealed that treatment of the myeloma cells with KGM induced marked simultaneous staining of Annexin V and PI without an Annexin V(+)/PI(–) fraction (Figure 3A), indicating that the cytotoxic effect caused by KGM was necrosis, rather than apoptosis.

Effect of KGM on lymphocytes. Primary myeloma cells and normal bone marrow mononuclear cells were exposed to KGM at a concentration of 500 nM, and analyzed by Annexin V/PI staining after 18 h. Interestingly, the proportion of CD138-expressing myeloma cells decreased from 33.5% to 7.7%, while the CD138-negative normal lymphocytes were less affected (Figure 3B).

Effect of KGM on cell cycle regulatory proteins and phosphorylated ERK. Western blot analysis was performed on 12PE cells exposed to KGM at a concentration of 200 nM for 5 h and on control cells without KGM. KGM inhibited the expression of AKT, phosphorylated AKT and cyclin D1 (Figure 4A). It was notable that phosphorylated ERK decreased with KGM treated, but total ERK remained the same. Also, p21 expression in the cytoplasm and the nucleus was inhibited while nuclear expression of NF-κB (p65 and p50) and CHOP was unaffected by KGM treatment (Figure 4B).

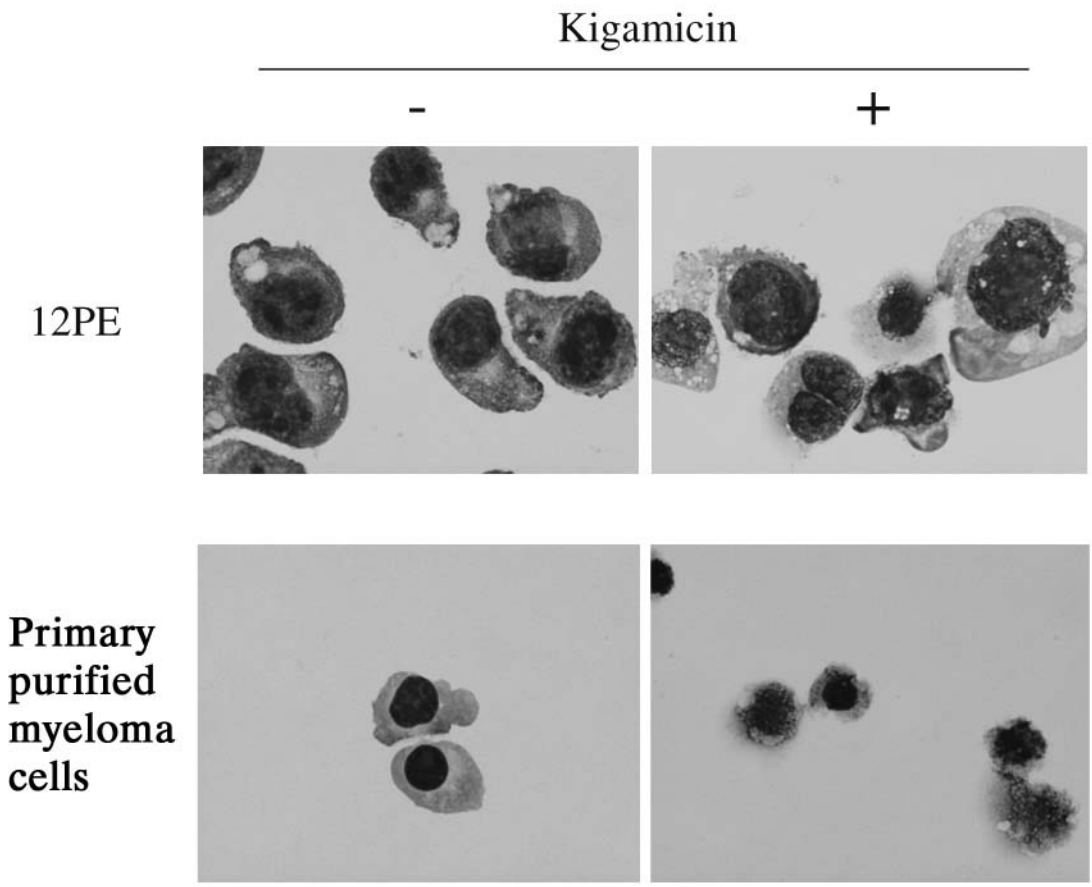


Figure 2. *Morphological changes in myeloma cells following treatment with kigamicin (KGM). After culture with 500 nM KGM for 18 hours, myeloma line, 12PE cells and primary purified myeloma cells showed vacuolization in the cytoplasm with an intact nucleus, which is a feature of necrosis as distinct from apoptosis. May-Giemza staining. Magnification: x1000.*

Involvement of caspase in the cell death induced by KGM. To further elucidate the mechanism of cell death, the molecular chaperone BiP, was analyzed by western blot which revealed that its expression was only slightly increased by KGM (Figure 4A), while caspase-3 was slightly activated and the expression of Bcl-2 was not affected.

To elucidate the role of caspase or PI3-kinase in the cytotoxic effects of KGM, the 12PE cells were simultaneously treated with KGM and either a pan-caspase inhibitor, ZVAD-FMK, or a pan-PI3-kinase inhibitor, LY294002. Although the cytotoxicity of KGM was slightly inhibited by ZVAD-FMK or LY294002 when analyzed by flow cytometry (Figure 5A), there was no inhibition of cell death, as evaluated by the WST8 assay (Figure 5B), indicating that both caspase and PI3-kinase were only partly involved in the cell death pathway induced by KGM.

Effect of KGM on melphalan resistance. We previously established a melphalan-resistant myeloma cell line, KHM-11^{EMS}, which is more than 100-fold more resistant to melphalan than the parental cell line KHM-11 (7). In an attempt to elucidate the clinical significance of KGM for treatment of drug-resistant myeloma, the sensitivity of KHM-11^{EMS} cells to KGM was analyzed. As shown in Figure 6, the melphalan-resistant cells showed even higher sensitivity to KGM than the parental melphalan-sensitive cells.

Discussion

A role for KGM as an inhibitor of nutrient recycling functions was suggested by its effect on nutrient-starved PANC-1 cells. Lu *et al.* (3) have shown that AKT was not phosphorylated in PANC-1 cells under nutrient-rich conditions, while it was phosphorylated under nutrient-starved conditions, suggesting a unique feature of PANC-1 cells in showing better proliferation under nutrient-starved conditions. On the other hand, myeloma cells usually proliferate within bone marrow where nutrients may be

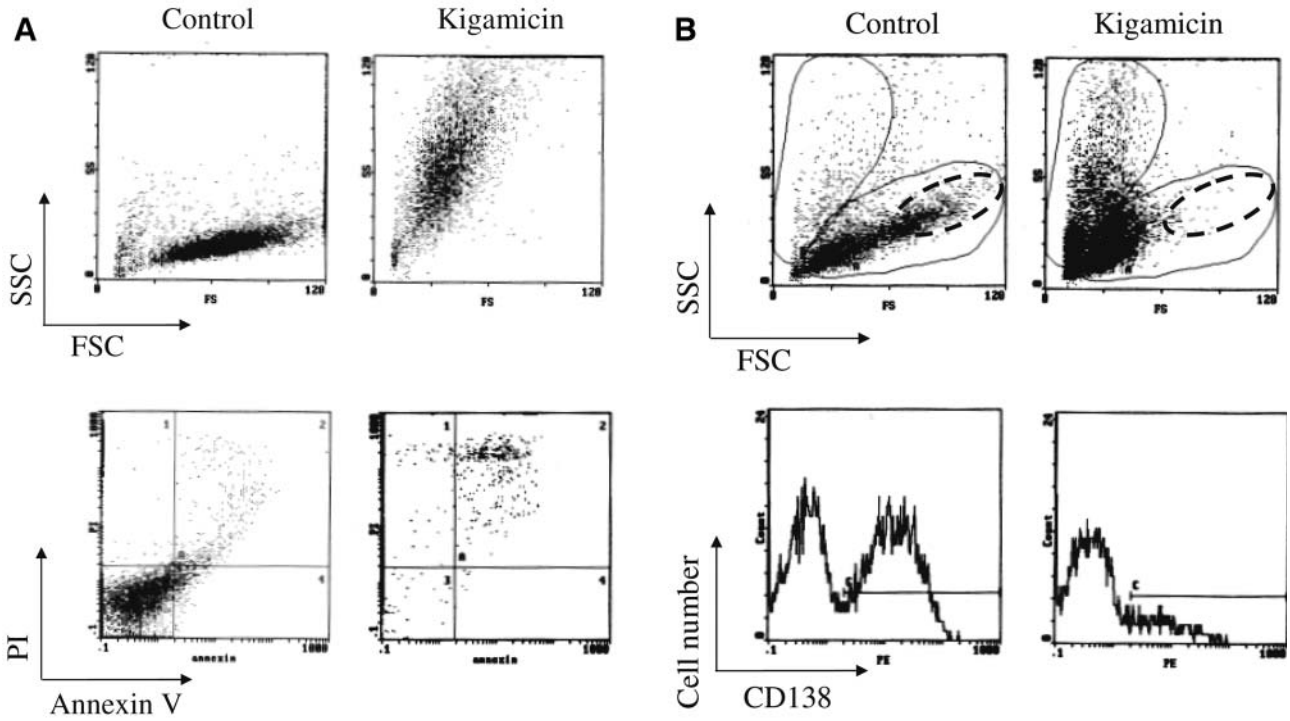
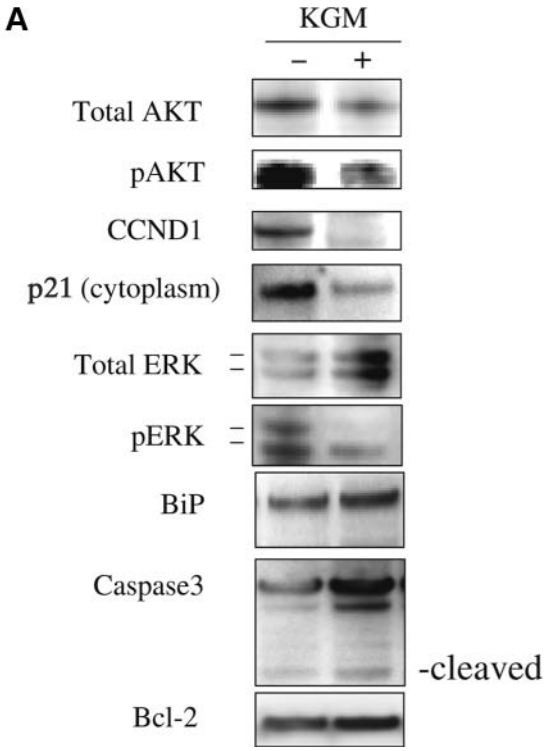


Figure 3. *Induction of necrosis in primary myeloma cells. A) Primary purified myeloma cells treated with 500 nM KGM for 18 h showed positive for both Annexin V and PI. SSC: side scatter, FSC: forward scatter. B) Whole mononuclear cells obtained from bone marrow of a myeloma patient were cultured with 500 nM KGM for 18 hours. The population of normal lymphocytes is still present after the KGM treatment, while the myeloma cells (dotted circle) have disappeared. The histogram of CD138-expressing cells shown at the bottom reveals a decrease in the proportion of CD138-expressing myeloma cells from 33.5% to 7.7%.*



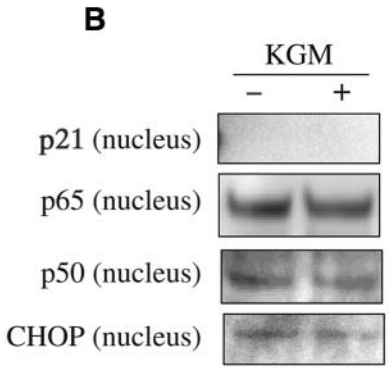


Figure 4. *Western blot analysis. A) The levels of total AKT, phospho-AKT (p-AKT), cylin D1 (CCND1), BiP, caspase-3, Bcl-2, total ERK, phosphorylated ERK and cytoplasmic p21 were analyzed after treatment with 500 nM KGM for 5 h. B) The expression levels of p21, p65, p50 and CHOP within the nucleus.*

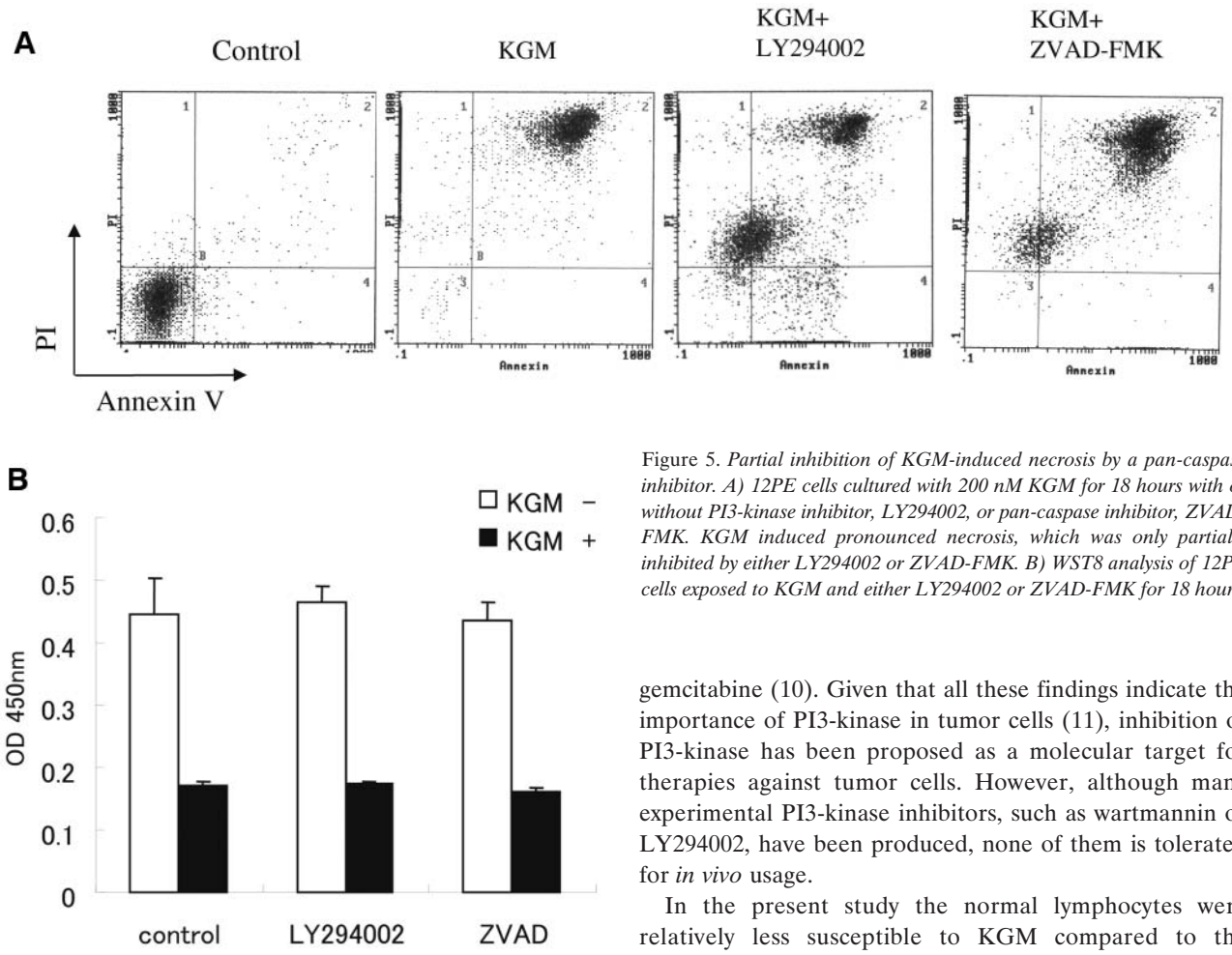


Figure 5. *Partial inhibition of KGM-induced necrosis by a pan-caspase inhibitor. A) 12PE cells cultured with 200 nM KGM for 18 hours with or without PI3-kinase inhibitor, LY294002, or pan-caspase inhibitor, ZVAD-FMK. KGM induced pronounced necrosis, which was only partially inhibited by either LY294002 or ZVAD-FMK. B) WST8 analysis of 12PE cells exposed to KGM and either LY294002 or ZVAD-FMK for 18 hours.*

sufficiently supplied. Indeed, we found rapid induction of cell death following treatment of myeloma cells with nutrient-depleted medium (data not shown). However in the present study, KGM efficiently and rapidly induced necrosis in both myeloma cell lines and primary myeloma cells even under nutrient-rich conditions.

Lu *et al.* (3) have reported that KGM inhibits phosphorylated AKT expression, suggesting that the cytotoxic effects of KGM are mediated by PI3-kinase and KGM inhibited PI-3 kinase, regardless of the nutrient conditions in the present and previous studies. In breast tumors, HER2 induces the activation of PI3-kinase, which eventually results in phosphorylation of AKT. Moreover, this process is accompanied by multidrug resistance (8). Aoudjit *et al.* have reported that activation of PI3-kinase induces drug resistance to paclitaxel and vincristine through up-regulation of integrins (9). In pancreatic cancer cells, activation of PI3-kinase is related to drug resistance to gemcitabine (10). Given that all these findings indicate the importance of PI3-kinase in tumor cells (11), inhibition of PI3-kinase has been proposed as a molecular target for therapies against tumor cells. However, although many experimental PI3-kinase inhibitors, such as wartmannin or LY294002, have been produced, none of them is tolerated for *in vivo* usage.

In the present study the normal lymphocytes were relatively less susceptible to KGM compared to the myeloma cells. This could indicate that KGM may have more potent toxicity toward myeloma cells than normal bystander cells. Since phosphorylated AKT plays a role as a cell proliferation inducer, normal lymphocytes, which are thought to be dormant cells, should escape from the cytotoxic effects induced by PI3-kinase inhibitors. Therefore, it is expected that KGM will be a novel clinically applicable PI3-kinase inhibitor.

Significant reductions in cyclin D1 and p21 were found to be induced by KGM. Since AKT regulates the cell cycle by modifying cyclin D1 (12), our finding of cyclin D1 down-regulation by KGM was to be expected. Given the strong regulation of cyclin D1 expression by the t(11;14) translocation in 12PE cells (5), KGM overcomes even enforced expression of cyclin D1. In this regard, KGM could be effective for tumor cells retaining strongly driven expression of cyclin D1, such as mantle cell lymphoma cells where t(11;14) overdrives expression of cyclin D1. On the other hand, AKT has also been reported to phosphorylate p21 and inhibit its antiproliferative effects by retaining it within the cytoplasm (13). However, we did not detect any translocation of p21 from the cytoplasm to the nucleus in

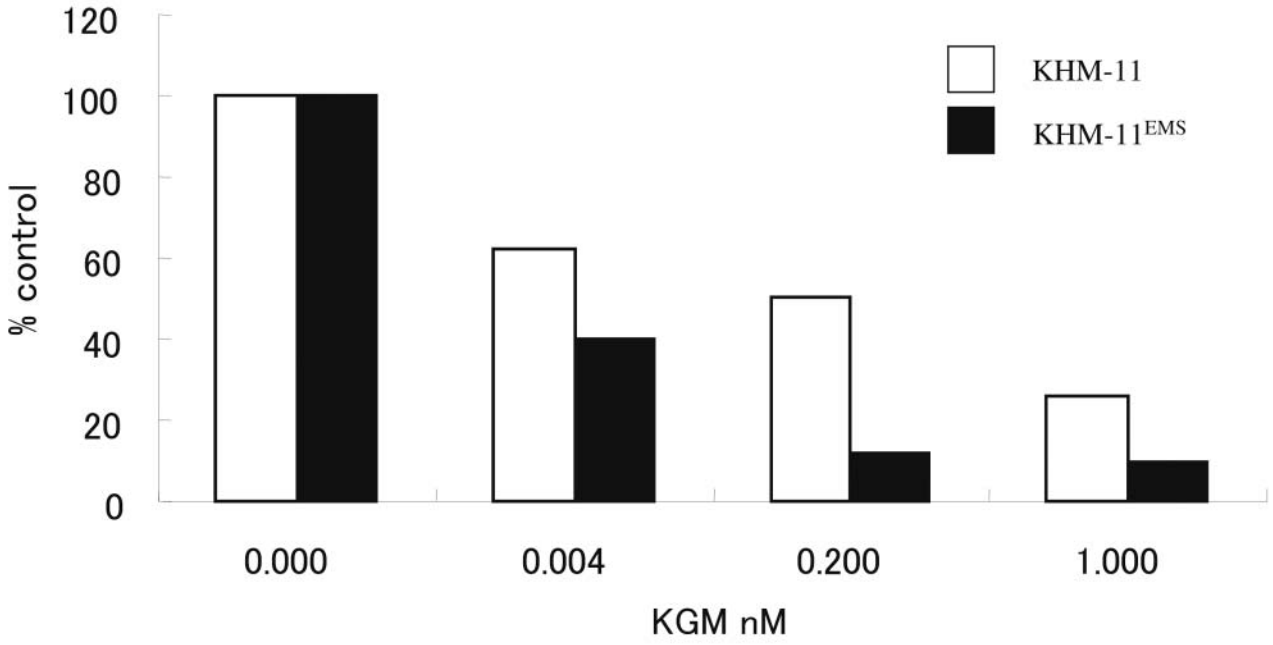


Figure 6. *Growth inhibitory effects of KGM towards melphalan-sensitive and melphalan-resistant myeloma cell lines. A melphalan-sensitive cell line, KHM-11 (white bars), and a melphalan-resistant cell line, KHM-11^{EMS} (solid bars), were cultured with KGM at the indicated concentrations for 18 h before the viability was analyzed by the WST8 method.*

response to KGM, indicating that KGM simply inhibits the expression of p21, rather than inhibiting its translocation. Taken together, these results indicate that cell cycle alterations could be one of the most important roles of KGM besides the inhibition of PI3-kinase, although the mechanism remains unclear. This explanation is compatible with the finding that a PI3-kinase inhibitor, LY294002, did not inhibit KGM cytotoxicity in our experiments.

We have previously found that endoplasmic reticulum stress mediated apoptosis in myeloma cells (14). Our finding that KGM slightly up-regulated BiP indicated that KGM induced a low level of ER stress. However, given our observation that KGM clearly induced necrosis, ER stress, which induces apoptosis, was not the major cytotoxic pathway of KGM. Since ER stress-related apoptosis is finally mediated by caspase-3, our observation of only partial inhibition of cell death by caspase inhibitor also suggested that ER stress is not involved in the cell death induced by KGM.

Accumulating evidence indicates that phosphorylated ERK plays an important role in the growth of myeloma cells (15-17), and KGM notably inhibited phosphorylation of ERK, suggesting that KGM also inhibited the MEK/ERK pathway. Since increased necrosis rather than apoptosis was found with KGM treatment, inhibition of phosphorylated ERK may be a secondary event during cell death by KGM.

Interestingly, we found even higher sensitivity in melphalan-resistant than melphalan-sensitive cells to KGM. KGM induced cell death in the melphalan-resistant myeloma cells at an extremely low concentration, 0.004 nM. This finding indicates that KGM could overcome resistance to melphalan although the mechanisms explaining preferential induction of cell death in melphalan-resistant cells are unclear.

KGM induced death in myeloma cells even at very low concentrations while sparing normal lymphocytes which encourages us to utilize KGM in clinical settings. Further analysis of KGM in preparation for future clinical trials is currently underway.

Acknowledgements

The authors appreciate the technical work by Shinya Endoh at Kumamoto University School of Medicine.

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Received June 21, 2007
Revised October 5, 2007
Accepted October 30, 2007