学 位 論 文 の 要 旨

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学　位　論　文　名	Japanese Apricot Extract(MK615) Potentiates Bendamustine-induced Apotosis via Impairment of the DNA Damage Response in Lymphoma Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Bendamustine is a bifunctional alkylating agent that combines the alkylating properties of 2-chloroethylamine and the antimetabolite properties of a benzimidazole ring. Bendamustine acts primarily as an alkylating agent that induces interstrand DNA cross-linking and subsequent strand breaks. Although bendamustine both as monotherapy and in combination with rituximab is useful against indolent lymphomas, combined chemotherapy with other therapeutic agents will be needed for the treatment of refractory malignancies such as aggressive lymphomas.

We found that bendamustine made favorable combinations with MK615, an extract of Japanese apricot. Japanese apricot contains many chemicals, including citric acid, malic acid, cyanogenic glycosides, and triterpenoids (e.g., ursolic acid). Several triterpenoids in MK615 are thought to exhibit antineoplastic effects. In the present investigation, we examined the mechanisms that underlie the synergism of MK615 and bendamustine.

MATRERIALS AND METHODS

MK615 (Misatol) was obtained from AdaBio Co, Ltd. Since Misatol is a sticky extract, an equal volume of phosphate-buffered saline (PBS) was added to Misatol®GL. The 50% diluted Misatol®GL was used as MK615 solution.

Human B-cell lymphoma (BALM3, SU-DHL-4, U698M, and SKW4), lymphoblastoid (BALM1) and myeloma cells (RPMI8226) were cultured in suspension in RPMI1640 medium supplemented with 10% fetal bovine serum and 80μg/ml gentamicin at 37℃ in a humidified atmosphere of 5% CO_2 in air. The cells were seeded at 1 x 10^5 cells/ml in a 24-well multidish.

After culture with or without the test compounds for the indicated times, cell numbers were counted in a Model Z1 Coulter Counter. Viable cells were examined by either a modified MTT assay or the trypan blue dye exclusion test using an automated cell counter.

Cells (1 x 10^4 per dish) were plated into 1.1 ml of a semisolid methylcellulose medium with 0.8% methylcellulose and 20% fetal bovine serum in triplicate for 14 days. A solution of 0.1ml of phosphate-buffered saline (PBS) containing various concentrations of MK615 and/or bendamustine was added to the semisolid medium.

Cytospin slide preparations were stained with May-Grunwald-Giemsa after exposure to bendamustine and/or MK615. Cells were collected and DNA was extracted using an Apoptotic DNA Ladder Detection kit. Equal amounts of DNA were analyzed by electrophoresis. Cells were labeled with FITC labeled-Annexin V using an Apoptotic Detection kit. After staining, cells were washed and analyzed by flowcytometry. Active caspase-3 was examined by Western blot analysis using anti-cleaved caspase-3 antibody.

Cells were packed after being washed with cold PBS and then lysed at a concentration of 1 x 10^7 cells/ml in lysis buffer. Equal amounts of protein were separated on 10 % SDS-polyacrylamide gels. The proteins were electrophoresed on gels and transferred to a membrane using anti-pChk1, Chk1, pChk2, Chk2, and β-actin antibodies.

Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized with 0.3 % Tween-20 for 15min. After fixation, cells were washed three times with PBS and then blocked with blocking buffer (1% bovine serum albumin in PBS) for 60min. Cells were incubated with primary antibodies (anti-Rad51 and anti-pH2AX) for 60min, washed with the blocking buffer and then incubated for 60min with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 594-conjugated anti-rabbit secondary antibodies. Confocal images were obtained using an inverted microscope.

RESULTS AND DISCUSSION

Bendamustine had synergistic effects with MK615 in inhibiting the viability of BALM3 cells. Similar results were obtained in the other lymphoma cell lines. RPMI8226 myeloma cells were less sensitive to bendamustine and the combination with MK615 was less effective. A colony-forming assay indicated that the combination of bendamustine and MK615 completely suppressed colony formation by BALM3 cells. The combination of bendamustine and ursolic acid inhibited the growth of BALM3 cells, but was slightly less effective.

When BALM3 cells were exposed to bendamustine and MK615 for 24h, a morphological analysis revealed shriveled cells, chromatin condensation, nuclear fragmentation and

cytoplasmic blebbing, while these morphological changes were hardly observed in cells treated with bendamustine alone. The induction of apoptosis was confirmed by gel electrophoresis of DNA from cells exposed to bendamustine and MK615, induction of cleaved caspase-3, and the expression of Annexin V. MK615 greatly enhanced the bendamustine-induced Annexin V expression. The induction of Annexin V expression by bendamustine plus MK615 was significantly inhibited by the general caspase inhibitor Z-VAD-FMK. These results indicate that combined treatment effectively induced caspase-dependent apoptosis in BALM3 cells.

Alkylating agents including bendamustine activate a DNA damage response. Bendamustine induced marked phosphorylation of the checkpoint kinases Chk1 and Chk2 in BALM3 cells at 6-24h. MK615 substantially inhibited the phosphorylation of Chk1 and Chk2 at 12-24h, although this inhibition by MK615 was not observed at 6h. Ursolic acid also markedly inhibited phosphorylation of the kinases, just like MK615.

Chk1 and Chk2 are phosphorylated by Ataxia-telangiectasia mutated protein (ATM) and ATM- and Rad3-related protein (ATR). Both VE-821 (ATR inhibitor) and KU-60019 (ATM inhibitor) significantly enhanced the bendamustine-induced growth inhibition of BALM3 cells, whereas these inhibitors hardly affected MK615-induced growth inhibition. The combination of VE-821 and KU-60019 enhanced bendamustine-induced growth inhibition more effectively than VE-821 or KU-60019 alone. Similar results were obtained in other lymphoid cells.

BALM3 cells treated with bendamustine exhibited an early rise in a marker of DNA damage, the phosphorylation of histone: 2AX (γH2AX) and Rad51 nuclear foci which are the sites of repair of DNA damage. MK615 enhanced the bendamustine-induced γH2AX. However, MK615 did not enhance bendamustine-induced Rad51 foci. These results suggest that MK615 suppresses Rad51 assembly and stimulates its degradation, independent of DNA damage.

The present results indicate that MK615 inhibited the bendamustine-induced activation of both the ATM and ATR pathways. The formation of nuclear foci of Rad51 induced by bendamustine was effectively inhibited by MK615.

Accumulating evidence suggests that targeting ATR and ATM can selectively sensitize cancer cells, but not normal cells, to DNA damage. Combination therapy with bendamustine and ATM/ATR inhibitors may be useful against malignant lymphoma.

CONCLUSION

Bendamustine is highly sensitive to B-lymphoma cells, and the combined treatment with MK615 was more evident in B-lymphoma cells. These results suggest that the combined therapy may be useful in B-lymphoma.