# An mTORC1/2 kinase inhibitor enhances the cytotoxicity of gemtuzumab ozogamicin by activation of lysosomal function

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学位論文の内容要旨

# An mTORC1/2 kinase inhibitor enhances the cytotoxicity of gemtuzumab ozogamicin by activation of lysosomal function

## mTORC1/2 キナーゼ阻害剤はリソソーム活性化を介して gemtuzumab ozogamicin の殺細胞効果を増強する

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MAIMAITILI YIMAMU

**Background**

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder which has very poor overall survival, particularly relapsed and refractory AML. Gemtuzumab ozogamicin (GO), the first antibody-drug conjugate (ADC), has attracted the interest of hematologists because over 90% of AML express its target, CD33. GO requires several cellular steps to exert its effect: after binding with CD33 antigen, the CD33-GO complex is rapidly internalized into endosomes. These early endosomes can mature into more acidic late endosomes that ultimately fuse with lysosomes. The linker is then hydrolyzed in the lysosome, allowing free calicheamicin to intercalate into DNA, leading to double-stranded DNA breaks. Although GO and subsequent current ADCs depend on lysosomes for activation, lysosome number and activity in tumor cells has not been well elucidated, especially in AML blasts. An mTORC1/2 kinase inhibitor, PP242, was reported to activate lysosomal function, and was therefore speculated to increase free calicheamicin by improving the efficiency of linker hydrolysis.

We therefore speculated that PP242 would enhance the cytotoxicity of GO by improving the efficiency of linker hydrolysis. Here, to investigate this possibility, we examined whether PP242-induced inhibition of mTORC1/2 kinase potentiates the cytotoxicity of GO in AML cells.

**Results**

**PP242 increased the cytotoxic effect of GO in different leukemia cells**

After determined the non-cytotoxic concentration of PP242 on U937 cells by apoptosis assay and western blotting, different leukemia cell lines were then exposed to GO with or without PP242. The cytotoxic effect of GO was synergistically enhanced by concurrent treatment with 500 nM of PP242 in U937 (CI = 0.5), THP-1 (CI = 0.7), SKM-1 (CI = 0.6), SKNO-1 (CI = 0.7) and HL-60 (CI = 0.8) cells. In contrast, no combinatory effect was detected in SKK-1 (CI = 1), KO52 (CI = 1.3) or MARIMO (CI = 1.7) cells. A second supportive result is that cleaved PARP, which serves as a marker of cells undergoing apoptosis, was observed only in the cells in which a combinational effect was detected, such as U937 and THP-1, and not in MARIMO or KO52 cells.

**PP242 enhanced lysosomal activity in a cell-dependent manner**

To confirm our hypothesis, we labeled and quantified acidic compartments (lysosomes) in U937, THP-1, SKM-1, MARIMO and KO52 cells using LysoTracker Red DND-99. Fluorescence was dramatically increased by treatment with PP242 in U937, THP-1 and SKM-1, and this intensified fluorescence was retained with GO+PP242, as expected. In contrast, we observed only a slight and non-significant increase in LysoTracker fluorescence by PP242 in MARIMO, consistent with the lack of combinatory cytotoxicity. Bafilomycin A (Baf-A1), a specific inhibitor of vacuolar type H+-ATPase (V-ATPase), inhibits the acidification of lysosomes and lysosomal activity. Importantly, LysoTracker fluorescence was totally abolished by the addition of Baf-A1, indicating that LysoTracker fluorescence exactly labeled the acidic compartment, lysosomes. To further confirm our findings, we investigated the expression level of a representative lysosomal membrane marker, LAMP-1, after treatment of U937 and MARIMO cells with GO, PP242 and GO+PP242 at two time points. In agreement with the LysoTracker staining results, we observed that PP242 elevated the amount of LAMP1 protein in U937 cells but not in MARIMO cells, indicating that lysosome formations increased in U937 cells but not in MARIMO cells.

**PP242 affected cell cycle by suppression of GO-induced Chk1 activation**

We found that, when GO was administered alone, almost all cells were blocked in G2/M phase without a significant increase in sub-G1, as reported previously. The addition of PP242, however, reduced the G2/M population and increased that of sub-G1. Inhibition of mTOR signaling is reported to prevent activation of checkpoint kinase Chk1 following DNA damage, which is one of the key regulators of DNA damage-induced cell cycle arrest. Thus, we considered that the mechanism of the enhancement of cytotoxicity seen with the addition of PP242 likely includes change of cell cycle. To prove this, we first investigated the impact of PP242 on the cell cycle using another time course experiment. Two groups of cells were treated with GO for 24 h to facilitate the induction of G2/M arrest, and were then additionally cultured for 1, 2, 3, 6, 9 and 12 hours with or without PP242. Whole cell lysates were then assayed by western blot. Results

clearly showed that the addition of PP242 attenuated GO-induced phosphorylation of Chk1 in all tested cell lines whereas phosphorylation of Chk2 was not affected.

**PP242 proceeded cell cycle to mitosis, resulting in DNA double-strand breaks**

After confirming the impact of PP242 on Chk1 phosphorylation, we assessed the phosphorylation of Histone H3, a mitosis marker, to further elucidate the effect of PP242 on Chk1-mediated cell cycle arrest. For this purpose, we used the same lysates as those prepared in the time course experiment described above. These lysates were assayed by western blot for phosphorylated and total histone H3 protein. Phosphorylated histone H3 was increased in a time-dependent manner in all tested cell lines treated with GO + PP242, whereas this phosphorylated histone H3 was not increased significantly during treatment with GO alone. Further, phosphorylation of H2AX (γ-H2AX), which is a marker of double-strand breaks, was significantly induced by the addition of PP242 in the same manner as with H3 phosphorylation.

**Conclusion**

We demonstrated that PP242 induces lysosomal activation and progression of cell cycle, thereby potentiating the cytotoxicity of GO. This finding in turn suggests that an mTOR kinase inhibitor may be an attractive 'partner' for GO and other subsequent ADCs in the exertion of their anti-cancer activity.

神戸大学大学院医学(系)研究科（博士課程）

| 論文審査の結果の要旨 | | | |
|---|---|---|---|
| 受付番号 | 甲 第2825号 | 氏　名 | MAIMAITILI YIMAMU |
| 論文題目<br>Title of Dissertation | An mTORC1/2 kinase inhibitor enhances the cytotoxicity of gemtuzumab ozogamicin by activation of lysosomal function<br>mTORC1/2 キナーゼ阻害剤はリソソーム活性化を介して gemtuzumab ozogamicin の殺細胞効果を増強する | | |
| 審査委員<br>Examiner | 主　査<br>Chief Examiner　河野 誠司<br>副　査<br>Vice-examiner　古屋敷 智之<br>副　査<br>Vice-examiner　矢野 寛章 | | |

（要旨は１，０００字～２，０００字程度）

Gemtuzumab ozogamicin(GO), the first antibody-drug conjugate (ADC), has attracted the interest of hematologists because over 90% of acute myeloid leukemia (AML) express its target, CD33. GO requires several cellular steps to exert its effect: after binding with CD33 antigen, the CD33-GO complex is rapidly internalized into endosomes. These early endosomes can mature into more acidic late endosomes that ultimately fuse with lysosomes. The linker is then hydrolyzed in the lysosome, allowing free calicheamicin to intercalate into DNA, leading to double-stranded DNA breaks. Although GO and subsequent current ADCs depend on lysosomes for activation, lysosome number and activity in tumor cells has not been well elucidated, especially in AML blasts. An mTORC1/2 kinase inhibitor, PP242, was reported to activate lysosomal function, and was speculated to increase free calicheamicin. In the present study, the candidate, MAIMAITILI YIMAMU, postulated that PP242 would enhance the cytotoxicity of GO by improving the efficiency of linker hydrolysis. To investigate this, he examined whether PP242-induced inhibition of mTORC1/2 kinase potentiates the cytotoxicity of GO in AML cells. After determined the non-cytotoxic concentration of PP242 on U937 cells by apoptosis assay and western blotting, different leukemia cell lines were then exposed to GO with or without PP242. The cytotoxic effect of GO was synergistically enhanced by concurrent treatment with 500 nM of PP242 in U937 (CI = 0.5), THP-1 (CI = 0.7), SKM-1 (CI = 0.6), SKNO-1 (CI = 0.7) and HL-60 (CI = 0.8) cells. In contrast, no combinatory effect was detected in SKK-1 (CI = 1), KO52 (CI = 1.3) or MARIMO (CI = 1.7) cells. A second supportive result is that cleaved PARP was observed only in the cells in which a combinational effect was detected, such as U937 and THP-1, and not in MARIMO or KO52 cells. To confirm his hypothesis, he labeled and quantified acidic compartments (lysosomes) in U937, THP-1, SKM-1, MARIMO and KO52 cells using LysoTracker Red DND-99. Fluorescence was dramatically increased by treatment with PP242 in U937, THP-1 and SKM-1, and this intensified fluorescence was retained with GO+PP242, as expected. In contrast, he observed only a slight and non-significant increase in LysoTracker fluorescence by PP242 in MARIMO, consistent with the lack of combinatory cytotoxicity. Baf-A1, a specific inhibitor of vacuolar type H+-ATPase (V-ATPase), inhibits the acidification of lysosomes and lysosomal activity. Importantly, LysoTracker fluorescence was totally abolished by the addition of Baf-A1, indicating that LysoTracker fluorescence exactly labeled the acidic compartment, lysosomes. To further confirm our findings, he investigated the expression level of a representative lysosomal membrane marker, LAMP-1, after treatment of U937 and MARIMO cells with GO, PP242 and GO+PP242 at two time points. In agreement with the LysoTracker staining results, he observed that PP242 elevated the amount of LAMP1 protein in U937 cells but not in MARIMO cells, indicating that lysosome formations increased in U937 cells but not in MARIMO cells.

He found that, when GO was administered alone,almost all cells were blocked in G2/M phase without a significant increase in sub-G1. The addition of PP242, however, reduced the G2/M population and increased that of sub-G1.Inhibition of mTOR signaling is reported to prevent activation of checkpoint kinase Chk1 following DNA damage, which is one of the key regulators of DNA damage-induced cell cycle arrest. Thus, he considered that the mechanism of the enhancement of cytotoxicity seen with the addition of PP242 likely includes change of cell cycle. To prove this, we first investigated the impact of PP242 on the cell cycle using another time course experiment. Two groups of cells were treated with GO for 24 h to facilitate the induction of G2/M arrest, and were then additionally cultured for 1, 2, 3, 6, 9 and 12 hours with or without PP242. Whole cell lysates were then assayed by western blot. Results clearly showed that the addition of PP242 attenuated GO-induced phosphorylation of Chk1 in all tested cell lines whereas phosphorylation of Chk2 was not affected. After confirming the impact of PP242 on Chk1 phosphorylation, he assessed the phosphorylation of Histone H3, a mitosis marker, to further elucidate the effect of PP242 on Chk1-mediated cell cycle arrest. For this purpose, he used the same lysates as those prepared in the time course experiment described above. These lysates were assayed by western blot for phosphorylated and total histone H3 protein. Phosphorylated histone H3 was increased in a time-dependent manner in all tested cell lines treated with GO + PP242, whereas this phosphorylated histone H3 was not increased significantly during treatment with GO alone. Further, phosphorylation of H2AX (γ-H2AX), which is a marker of double-strand breaks, was significantly induced by the addition of PP242 in the same manner as with H3 phosphorylation.

In summary, he demonstrated that PP242 induces lysosomal activation and progression of cell cycle, thereby potentiating the cytotoxicity of GO. This finding in turn suggests that an mTOR kinase inhibitor may be an attractive 'partner' for GO and other subsequent ADCs in the exertion of their anti-cancer activity. The candidate, MAIMAITILI YIMAMU, having completed studies on the effect of PP242 enhances the cytotoxicity of GO by activation of lysosomal function in AML cell lines, and having postulated a novel ADC chemotherapy strategy using mTOR inhibitors which have a high potential to be used with other ADC drugs, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).