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Research paper

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

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ABSTRACT

Gemtuzumab ozogamicin (GO), the first antibody-drug conjugate (ADC), has attracted the interest of hematologists because more than 90% of acute myeloid leukemia (AML) blasts express its target, CD33. Although GO and subsequently developed ADCs depend on lysosomes for activation, lysosome number and activity in tumor cells has not been well elucidated. In this study, we investigated whether an mTORC1/2 kinase inhibitor, PP242, which was reported to activate lysosomal function, potentiates the cytotoxicity of GO in AML cells. Eight AML cell lines (U937, THP-1, SKM-1, SKK-1, SKNO-1, HL-60, MARIMO and KO52) were treated with GO and PP242. The cytotoxic effect of GO was enhanced by concurrent treatment with a non-cytotoxic concentration (500 nM) of PP242 in most cell lines, except MARIMO and KO52 cells. We then used LysoTracker to label acidic lysosomes in U937, THP-1, SKM-1, MARIMO and KO52 cells. LysoTracker fluorescence was dramatically increased by treatment with PP242 in U937, THP-1 and SKM-1 cells, and the intensified fluorescence was retained with PP242 + GO. In contrast, PP242 did not induce a significant increase in fluorescence in MARIMO cells, consistent with the lack of combinatory cytotoxicity. LysoTracker fluorescence was also increased by PP242 in KO52 cells, which have been reported to strongly express multidrug resistance (MDR). Further, PP242 suppressed GO-induced Chk1 activation and G2/M cell cycle arrest, which in turn triggered cell cycle promotion and cell death. These results indicate that inhibition of mTORC1/2 kinase by PP242 enhanced the cytotoxicity of GO by increasing lysosomal compartments and promoting the cell cycle via suppression of GO-induced Chk1 activation. This combination may represent an attractive new therapeutic strategy for the treatment of leukemia.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder which has very poor overall survival, particularly relapsed and refractory AML [1]. The development of tumor-associated antigen-directed cytotoxic agents has opened a new door to improving treatment outcomes in these patients [2]. CD33 antigen is detected on blasts of over 90% of AML patients, making it a highly promising candidate for targeted therapy for AML [3–6].

Gemtuzumab ozogamicin (GO; Mylotarg) is a recombinant humanized anti-CD33 monoclonal antibody which is conjugated to a DNA-damaging calicheamicin derivative with an acid-labile linker [7]. Consistent with the general concept of the trafficking and processing of ADCs in the endocytic pathway [8], 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 [8–11]. The linker is then hydrolyzed in the lysosome, allowing free calicheamicin to intercalate into DNA, leading to double-stranded DNA breaks [12]. From the above predicted mechanism of action, GO, and also the other ADCs, require that the cellular function of target cells be intact to exert optimal cytotoxicity. However, some previous clinical trials of GO aimed to maximize its efficacy by concurrent treatment with conventional chemotherapy, despite the possibility that this might exacerbate bone marrow suppression and increase the rate and severity of complications [13].

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In contrast with this approach, we have focused on strengthening the activity of GO by taking advantage of its intrinsic mechanism of action, rather than by simple combination with strongly cytotoxic drugs. Building on this concept, we previously demonstrated that a low, non-toxic dose of the epigenetic modulating agent decitabine retains cellular functions and enhances the cytotoxicity of GO [14].

Lysosomal activity is one of the essential cellular functions that GO and some other ADCs requires to exert its mechanism of action. However, the number and activity of lysosomes in tumor cells is not well understood, particularly in AML blasts. Further, GO was previously shown to cause pronounced G2/M cell-cycle arrest, which correlates with phosphorylation of checkpoint kinase Chk1 and Chk2 but not with p53 and p21 induction, and modest induction of apoptosis in a subset of cases [15]. Conveniently, an mTORC1/2 kinase inhibitor, PP242, was reported to activate lysosomal function [16] and modulate cell cycle checkpoints [17].

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

2. Materials and methods

2.1. Cells and cell culture

We used eight leukemia cell lines, namely HL-60, SKM-1, SKNO-1, MARIMO and KO52 (JCRB Cell Bank; Setagaya, Tokyo, Japan), U937 (ATCC; Manassas, VA, USA), THP-1 (ECACC; Porton Down, UK) and SKK-1 (established by Dr. Hiroshi Matsuoka [18]). HL-60, MARIMO and THP-1 cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Other cell lines were cultured as described in our previous work [14].

2.2. Reagents and antibodies

Gemtuzumab ozogamicin (Pfizer Inc, NY, USA) was dissolved at a stock concentration of 1 mg/ml in dH2O. PP242 (Sigma-Aldrich; St. Louis, MO, USA) and Bafilomycin A1 (Sigma-Aldrich) were dissolved at a stock concentration of 10 mM and 10 μM, respectively, in dimethyl sulfoxide (DMSO). Other reagents and antibodies were as follows: Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque Inc.; Kyoto, Japan); LysoTracker Red DND-99 (Invitrogen; Carlsbad, CA, USA); Protease Inhibitor CockTail (Sigma-Aldrich); PhosSTOP Phosphatase Inhibitor Cocktail (Roche; Indianapolis, IN, USA); Vectashield anti-fade mounting medium with DAPI (Vector Laboratories; Burlington, CA, USA); RNase A solution (WAKO; Osaka, Japan); Anti-human CD33 -FITC (eBioscience; San Diego, CA, USA); Mouse IgG1 κ-FITC (eBioscience); Anti-phospho-Akt (Ser473) (#4060; CST; Beverly, MA, USA); Anti-Akt (#9272; CST); Anti-phospho-p70 S6 Kinase (Thr389) (#9205; CST); Anti-p70 S6K (#9202; CST); Anti-phospho-Chk1 (Ser345) (#2348; CST), Anti-Chk1 (G-4) (#B1516; Santa Cruz Biotechnology; Santa Cruz, CA, USA); Anti-phospho-Chk2 (Thr68) (#2197; CST), Anti-Chk2 (#05-649; Millipore, Billerica, MA, USA); Anti-phospho-Histone H3 (Ser10) (#06-570; Millipore), Anti-Histone H3 (#9715; CST); Anti-phospho-Histone H2 AX (Ser139) (#2577; CST), Anti-Histone H2 AX (#2595; CST); Anti-PARP (#9542; CST); and Anti-LAMP-1 antibody (#SAB3500285; Sigma-Aldrich).

2.3. Apoptosis assay

Different AML cell lines were seeded at a density of 2.5×10^5 cells/mL and subjected to treatment with 2.5 μg/ml GO (0.5 μg/ml for THP-1 and SKNO-1 cells) with or without 500 nM PP242. Cells were collected and apoptosis was analyzed with a BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA) using an Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol. Specific apoptosis was calculated as follows: Specific Apoptosis = 100 × [(experimental apoptosis - spontaneous apoptosis)/(100% - spontaneous apoptosis)] [19]. Combination index (CI) can be calculated as: $CI = (E_A + E_B)/E_{AB}$, where E_A and E_B represent the effects of individual drugs and E_{AB} means the effect resulting from the combination of two drugs. CI values were interpreted as additive (CI = 1), synergistic (CI < 1) and antagonistic (CI > 1) [20].

2.4. Cell cycle analysis

Following treatment, cells were harvested and fixed with 70% ice-cold ethanol, then incubated with 25 μg/ml RNase A solution for at least 1 h at 37 °C. Cellular DNA content following cell staining with propidium iodide (PI) was measured by flow cytometry.

2.5. Confocal microscopy

For labeling of lysosomal compartments, cells were seeded at a concentration of 2.5×10^5 cells/ml and treated with 2.5 μg/ml GO (0.5 μg/ml for THP-1 cells) with or without PP242 for 6 h. The cells were stained with 300 nM LysoTracker Red DND-99 for 10 min, then fixed with 4% paraformaldehyde. Cells not treated with GO or PP242 were stained as a control. The cells were then transferred to a poly-L-lysine-coated glass bottom dish (Matsunami Glass Ind Ltd., Osaka, Japan), and coverslips were mounted using vectashield anti-fade mounting medium with DAPI. Fluorescence images were captured with a LSM700 confocal microscope (Zeiss, Germany). At least 30 cells were counted in each control and experimental group. Average LysoTracker fluorescence intensity was then measured using the ZEN 2 (blue edition) software.

2.6. Western blot analyses

Cell pellets were lysed in lysis buffer [25 mM Tris −HCl pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl, 1% Triton and 0.1% SDS] followed by brief sonication. To maintain the integrity and phosphorylation status of proteins, 1% Protease Inhibitor Cocktail and 1% PhosSTOP were freshly added. Protein content was determined by Bradford protein assay and separated on SDS-PAGE, transferred onto PVDF, then probed using the indicated primary and secondary antibodies. Each primary antibody was detected using Amersham ECL plus Western Blotting Detection Reagents (GE Healthcare; Buckinghamshire, UK).

3. Results

3.1. PP242 increased the cytotoxic effect of GO in different leukemia cells

To keep cellular function intact, we first determined the non-cytotoxic concentration of PP242 on U937 cells by apoptosis assay. At up to 500 nM, PP242 did not show cytotoxic effects after incubation for 24 h (Fig. 1A). We also confirmed the dual inhibitory effect of PP242 by western blotting using antibodies specific for p-p70S6K/p70S6K and p-Akt/Akt, which are downstream of mTORC1 and mTORC2, respectively. The results show that 500 nM was sufficient to inhibit both mTORC1 and mTORC2 activities (Fig. 1B). Different leukemia cell lines were then exposed to GO with or without PP242. As shown in Fig. 1C, 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 [21], was observed only in the cells in which a combinational effect was detected, such as U937 and THP-1 (Fig. 1D), and not in