

## Reduced effect of gemtuzumab ozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers

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**Gemtuzumab ozogamicin (CMA-676), a calicheamicin-conjugated humanized anti-CD33 mouse monoclonal antibody, has recently been introduced clinically as a promising drug for the treatment of patients with acute myeloid leukemia (AML), more than 90% of which express CD33 antigen. However, our recent study suggested that CMA-676 was excreted by a multidrug-resistance (MDR) mechanism in P-glycoprotein (P-gp)-expressing leukemia cell lines. We analyzed the *in vitro* effects of CMA-676 on leukemia cells from 27 AML patients in relation to the amount of P-gp, MDR-associated protein 1 (MRP1), CD33 and CD34, using a multi-laser-equipped flow cytometer. The cytotoxic effect of CMA-676, estimated by the amount of hypodiploid portion on cell cycle, was inversely related to the amount of P-gp estimated by MRK16 monoclonal antibody ( $P = 0.004$ ), and to the P-gp function assessed by intracellular rhodamine-123 accumulation in the presence of PSC833 or MS209 as a MDR modifier ( $P = 0.0004$  and  $P = 0.002$ , respectively). In addition, these MDR modifiers reversed CMA-676 resistance in P-gp-expressing CD33<sup>+</sup> leukemia cells ( $P = 0.001$  with PSC833 and  $P = 0.0007$  with MS209). In CD33<sup>+</sup> AML cells from 13 patients, CMA-676 was less effective on CD33<sup>+</sup>CD34<sup>+</sup> than CD33<sup>+</sup>CD34<sup>-</sup> cells ( $P = 0.002$ ). PSC833 partially restored the effect of CMA-676 in CD33<sup>+</sup>CD34<sup>+</sup> cells. These results suggest that the combined use of CMA-676 and a MDR modifier will be more effective on CD33<sup>+</sup> AML with P-gp-related MDR.**

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### Introduction

There has been great progress in the treatment of acute myeloid leukemia (AML), and 70 to 80% of newly diagnosed patients achieve complete remission (CR). However, relapse, often associated with drug resistance, occurs in more than 50% of them. The development of multidrug resistance (MDR) involves a series of events by which leukemia cells become resistant to several structurally unrelated anti-leukemia agents. The most extensively studied type of MDR concerns P-glycoprotein (P-gp), detected on leukemia cells from patients with refractory and relapsed AML.<sup>1–6</sup>

To overcome resistance, MDR modifiers have been developed. PSC833 and MS209 restore the effect of chemotherapeutic agents in P-gp-related resistant cell lines by inhibiting the efflux of anti-leukemia agents.<sup>7,8</sup> Another promising approach is monoclonal antibody (MoAb)-based therapy, which aims at increasing the selectivity of cytotoxic agents by using conjugates of MoAbs that target to some antigens on leukemia cells.<sup>9</sup> CD33, a 67-kDa glycoprotein, is

found on the surface of myeloid cells, and belongs to the sialoadhesin family.<sup>10</sup> The exact function of CD33 is not known, but it is involved in adhesion or cell-to-cell communication at least.<sup>10</sup> CD33 is expressed on leukemia cells of more than 90% of patients with AML but not on normal hematopoietic stem cells and non-myeloid tissues.<sup>11</sup> Therefore, CD33 is an advantageous target for MoAb-based therapy of AML.<sup>12–15</sup>

Gemtuzumab ozogamicin (CMA-676) is a conjugate of calicheamicin derivative and a recombinant humanized antibody (IgG<sub>4</sub>) directed against the CD33 antigen. Calicheamicin is a very potent anti-tumor antibiotic. It binds to the minor groove of DNA in a sequence-specific manner,<sup>16–18</sup> and breaks double-stranded DNA by the abstraction of specific hydrogen atoms,<sup>19</sup> which is probably the initial step in cell death. The Food and Drug Administration of USA recently approved CMA-676 for relapsed patients with CD33<sup>+</sup> AML of 60 years or older.

Our previous studies suggested that the effect of CMA-676 was influenced by P-gp.<sup>20,21</sup> Thus, P-gp seems to play a role in the clinical resistance to CMA-676. If calicheamicin is also pumped out by P-gp in clinical samples, CMA-676 would be less effective on P-gp-expressing AML. In this study, we attempt to clarify the effect of CMA-676 on AML cells from patients in relation to MDR. Additionally, since CD34<sup>+</sup> cells have been reported to express more P-gp than CD34<sup>-</sup> cells in relapsed AML,<sup>22</sup> we also analyzed the effect of CMA-676 in relation to CD33 and CD34.

### Materials and methods

#### Cell preparation

After informed consent, leukemia cells were obtained from 27 patients with AML, one with chronic myeloid leukemia in lymphoblastic crisis (CML-BC) and one with acute lymphoblastic leukemia (ALL, L2), who were consecutively admitted to Hamamatsu University School of Medicine Hospital from July 1999 to August 2001. The diagnosis was made according to the French–American–British classification.<sup>23,24</sup> Patient characteristics are shown in Table 1. Among the AML patients, nine were newly diagnosed and 18 were relapsed cases.

#### Pretreatment of cells with CMA-676

PSC833 and MS209 were used as MDR modifiers, and probenecid as a modifier for MDR-associated protein (MRP). Mononucleated cells ( $1 \times 10^6$ ) were isolated using Ficoll–Paque (Pharmacia, Uppsala, Sweden), washed three times and suspended in RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with L-glutamine (2 mmol/l), anti-

**Table 1** Patient characteristics and their P-gp and MRP1 expression (%), % increase of intracellular Rh123 accumulation, % inhibition of calcein-AM efflux and the effect of CMA-676 in 27 AML patients

Case No.	Sex	Age	FAB classification	Stage of disease	Blast (%) in BM	Surface marker (%)		P-gp (%)	% increase of intracellular Rh123 accumulation		MRP1 (%)	% inhibition of intracellular calcein-AM efflux by probenecid	% increase of hypodiploid portion by CMA-676 <sup>a</sup>
						CD33	CD34		by PSC833	by MS209			
1	M	22	M1	At diagnosis	14.1	78.1	79.1	3.1	5.1	7.3	0.2	0.3	14.8
2	M	67	M2	At diagnosis	84.5	88.9	80.9	11.1	7.1	5.4	5.4	12.3	21.1
3	M	32	M2	At diagnosis	87.6	67.0	84.0	8.3	9.4	8.2	4.1	9.8	2.9
4	F	57	M2	At diagnosis	42.5	26.5	80.5	17.8	11.6	11.5	2.9	3.2	0.2
5	F	52	M2	At diagnosis	31.2	69.3	17.3	0.6	9.3	0.2	1.0	0.2	11.4
6	M	50	M3	At diagnosis	90.0	53.1	4.3	9.6	17.1	14.6	3.7	5.2	3.4
7	F	34	M5	At diagnosis	86.4	82.7	7.8	5.8	2.1	3.3	0.9	ND	15.9
8	M	90	M5	At diagnosis	95.0	83.4	37.5	5.7	3.7	1.7	8.3	11.1	10.1
9	M	67	M6	At diagnosis	57.1	91.3	82.1	15.1	17.1	13.1	1.3	0.2	6.1
10	M	39	M2	Relapse	64.8	90.4	62.1	0.9	1.1	2.5	0.9	2.1	28.2
11	M	65	M2	Relapse	70.1	99.3	71.9	5.6	6.5	6.5	1.4	0.9	8.5
12	M	74	M2	Relapse	21.5	79.8	12.8	1.7	4.8	4.2	4.8	5.9	3.1
13	M	58	M2	Relapse	45.1	18.9	68.0	13.7	11.2	8.2	8.4	14.8	2.5
14	F	66	M2	Relapse	67.8	21.1	77.3	13.0	12.2	23.8	3.7	7.2	0.0
15	M	78	M2	Relapse	44.0	6.8	96.4	6.8	13.4	9.0	4.3	2.4	1.3
16	M	31	M2	Relapse	30.3	87.9	3.1	10.2	14.1	18.1	0.9	0.2	3.2
17	F	43	M3	Relapse	40.0	90.9	1.0	ND	ND	ND	ND	ND	4.4
18	F	36	M3	Relapse	20.2	98.7	2.0	1.8	3.9	7.8	1.0	0.4	10.7
19	F	38	M3	Relapse	38.7	85.4	5.2	4.5	ND	ND	9.5	ND	9.5
20	M	51	M3	Relapse	30.8	97.9	3.6	4.4	16.8	5.3	ND	ND	9.6
21	F	38	M3	Relapse	77.2	94.6	30.8	1.4	8.9	9.4	2.8	0.8	16.9
22	F	21	M3	Relapse	94.0	97.8	48.1	0.0	3.5	4.3	3.4	0.0	32.4
23	M	50	M3	Relapse	78.0	83.6	75.5	11.6	6.6	5.8	5.4	10.3	10.8
24	M	57	M3	Relapse	83.2	89.8	4.3	9.1	8.3	11.1	6.7	9.3	6.6
25	M	53	M4	Relapse	74.7	89.7	89.6	1.8	5.4	5.1	5.6	ND	17.6
26	M	67	M4	Relapse	11.0	99.4	79.1	0.0	0.0	0.0	4.0	7.2	29.5
27	M	67	M5	Relapse	22.9	93.9	12.8	0.2	2.7	1.8	1.6	0.0	6.1
Mean ± s.d.						76.5 ± 27.0	45.1 ± 35.4	6.3 ± 5.2	8.1 ± 5.1	7.5 ± 5.6	3.7 ± 2.6	4.7 ± 4.8	10.6 ± 9.0
Correlation coefficient (r) <sup>b</sup>						0.556	0.140	-0.585	-0.637	-0.570	-0.134	-0.133	
P value						0.002	0.49	0.004	0.0004	0.002	0.53	0.56	

<sup>a</sup>Defined as the difference of the proportion of hypodiploid portion between samples incubated with or without CMA-676.<sup>b</sup>Correlation coefficient and P value between the % increase of hypodiploid portion by CMA-676.

biotics and 10% fetal calf serum (FCS). The cells were incubated with or without 100 ng/ml of CMA-676 in the presence or absence of 2  $\mu$ mol/l of PSC833 (Novartis Pharma, Basle, Switzerland), 5  $\mu$ mol/l of MS209 (Mitsui Pharmaceuticals, Chiba, Japan) or 2 mmol/l of probenecid (Sigma, St Louis, MO, USA) in a humidified CO<sub>2</sub> incubator at 37°C for 12 h. After incubation, the cells were washed three times to remove the cell surface-unbound CMA-676, PSC833, MS209 and probenecid, and then re-incubated for 24 h to internalize the cell surface-bound CMA-676 as previously described.<sup>20</sup> The viability of cells before the incubation was >99% by propidium iodide density analyzed by flow cytometry.

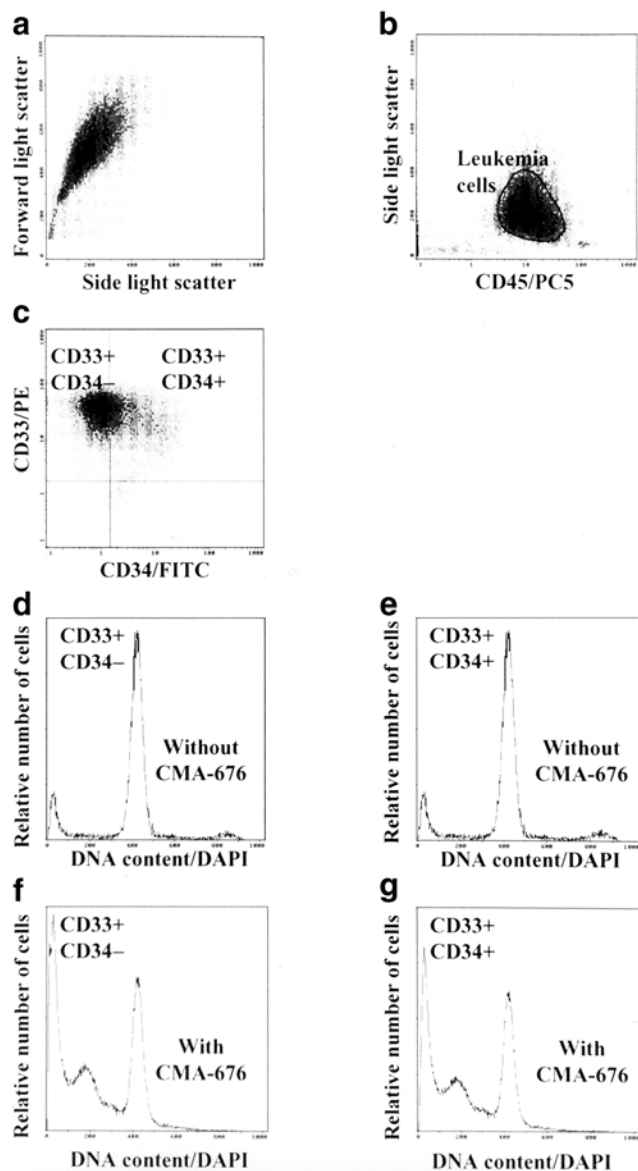
#### Staining of CD33, CD34 and CD45 antigens

The cells were stained with 20  $\mu$ l (0.8  $\mu$ g/ml) of phycoerythrin (PE)-conjugated anti-CD33 MoAb (Becton Dickinson, Mountain View, CA, USA), 20  $\mu$ l (3.3  $\mu$ g/ml) of fluorescein isothiocyanate (FITC)-conjugated anti-CD34 MoAb (Becton Dickinson) and 10  $\mu$ l (0.1  $\mu$ g/ml) of phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-CD45 MoAb (Beckman Coulter, Fullerton, CA, USA) at 4°C for 1 h, and then washed three times. In the flow cytometric analysis, leukemia cells were first gated by side scattering (SSC) and CD45 expression.<sup>25</sup> The purity

of the gated leukemia cells was more than 99.7% and the contamination of erythrocytes was minimal.

#### Cell cycle distribution analysis

Cell cycle distribution was identified and quantified with flow cytometry by staining leukemia cells with 4'-6'-diamidino-2-phenyl-indole (DAPI) (Sigma). After staining with MoAbs, the cells were permeabilized with 1 ml of 70% ethanol at 4°C for 30 min, treated with RNase A (1.0 mg/ml) (Sigma), and stained with DAPI (1.5  $\mu$ g/ml final concentration). DAPI was excited by a ultra-violet laser (325 nm), and its green fluorescence was collected through a 525/20 nm band pass filter set. The signal was processed using linear amplification. Forward scattering (FSC) and SSC as well as CD33, CD34 and CD45 surface phenotypes were simultaneously determined by the 488 nm excitation line of an argon laser using EPICS Elite (Beckman Coulter), a multi-laser-equipped flow cytometer, which enabled us to analyze the cell cycle distribution together with the amounts of CD33 and CD34 on the CD45-gated leukemia cells (Figure 1). At least 30 000 CD45-gated leukemia cells were counted.



**Figure 1** Multi-color flow cytometric assay of the cell cycle distribution and cell surface markers including CD33, CD34 and CD45 in the same sample by a multi-laser-equipped flow cytometer. (a) Two-dimensional histogram of forward and side scattering (FSC and SSC). (b) Two-dimensional histogram of SSC and the expression of CD45. Leukemia cells have a low SSC and low CD45 intensity, and are gated by a round window. (c) The expression of CD33 and CD34 on leukemia cells gated as shown in (b). Leukemia cells are grouped in two or three subpopulations, based on the expression of CD33 and CD34. (d, e) Cell cycle distributions of CD33<sup>+</sup>CD34<sup>-</sup> and CD33<sup>+</sup>CD34<sup>+</sup> cells incubated without CMA-676, respectively. (f, g) Cell cycle distributions of CD33<sup>+</sup>CD34<sup>-</sup> and CD33<sup>+</sup>CD34<sup>+</sup> cells incubated with CMA-676, respectively.

#### Flow cytometric analysis for P-glycoprotein (P-gp) and MRP1

For P-gp analysis, cells were reacted with 10  $\mu$ l (1.0  $\mu$ g/ml) of biotinylated MRK16 mouse IgG<sub>2a</sub> MoAb (Kyowa Medics, Tokyo, Japan) or a subclass-matched control MoAb (Fab') (Kyowa Medics), and then stained with streptavidine-RED670 (Gibco BRL) as previously described.<sup>26</sup> The cells were further stained with 20  $\mu$ l (10  $\mu$ g/ml) of FITC-conjugated anti-CD45

MoAb (Beckman Coulter) as previously described.<sup>25</sup> FSC, SSC, CD45 surface phenotype and the number of MRK16 reacted cells were simultaneously determined by the 488 nm excitation line of an argon laser using an Elite flow cytometer, which enabled us to evaluate the amount of MRK16 positivity on the CD45-gated leukemia cells. At least 10000 of CD45-gated leukemia cells were counted.

For MRP1 analysis, cells were first fixed with a mixture of 2% formaldehyde solution and acetone, and then incubated with 10  $\mu$ l (2.0  $\mu$ g/ml) of MRPm6 mouse IgG<sub>1</sub> MoAb (Nitirei, Tokyo, Japan), which specifically reacted with MRP1, or with a subclass-matched control IgG<sub>1</sub> MoAb (Fab') (Nitirei) for 1 h. After washing three times, the cells were stained with FITC-conjugated rabbit anti-mouse F(ab')<sub>2</sub> antibody (Dako Japan, Tokyo, Japan) as previously reported.<sup>27</sup> The cells were further stained with 10  $\mu$ l (0.1  $\mu$ g/ml) of PC5-conjugated anti-CD45 MoAb. P-gp-negative NB4 and P-gp-positive NB4/MDR were used as controls for the P-gp assay. MRP1-negative K562 and MRP1-positive K562 were used as controls for the MRP1 assay.

The degree of dissociation between the fluorescence intensity (FI) of the cells which reacted with MRK16 or MRPm6 MoAb and the FI of the cells reacting with the respective control MoAb was analyzed by the channel-by-channel subtraction method.<sup>26,28,29</sup> The cell count in each channel of a control histogram was subtracted from the cell count in the corresponding channel of the test histogram, and the percent accumulation was calculated from the sum of differences for all channels, using the Immuno-4 routine of the Epics cytometer software (Beckman Coulter), and the degree of dissociation was defined as the amount (%) of P-gp or MRP1.<sup>26</sup>

#### Functional analysis of P-gp and MRP

P-gp function was determined by intracellular rhodamine-123 (Rh123) accumulation and its enhancement by MDR modifiers. Cells (1  $\times$  10<sup>6</sup>/ml) were pre-incubated in phosphate-buffered saline containing 5% FCS (5% FCS-PBS) in the presence or absence of 2  $\mu$ mol/l PSC833 or 5  $\mu$ mol/l MS209 for 1 h. After further incubation in the presence of 200 ng/ml Rh123 (Sigma), the cells were stained with 10  $\mu$ l (0.1  $\mu$ g/ml) of PC5-conjugated anti-CD45 MoAb at 4°C for 1 h. The degree of dissociation between the FI of the leukemia cells which accumulated Rh123 in the presence or absence of PSC833 or MS209 was analyzed by the channel-by-channel subtraction method, and defined as the% increase of intracellular Rh123 accumulation by PSC833 or MS209, as previously described.<sup>26,30</sup>

MRP function was determined by calcein acetoxymethyl ester (calcein-AM) (Dojin Chem., Kumamoto, Japan) efflux and its inhibition by probenecid according to a previous report.<sup>31</sup> After being pre-incubated in 5% FCS-PBS and 0.1  $\mu$ mol/l of calcein-AM at 37°C for 30 min, cells were re-suspended in 5% FCS-PBS and allowed to efflux at 37°C for 1 h in the presence or absence of 2 mmol/l probenecid. The cells were then stained with 10  $\mu$ l (0.1  $\mu$ g/ml) of PC5-conjugated anti-CD45 MoAb at 4°C for 1 h. The degree of dissociation between the FI of the leukemia cells which retained calcein-AM in the presence or absence of probenecid was analyzed by the channel-by-channel subtraction method, and defined as the% inhibition of intracellular calcein-AM efflux by probenecid.

*Statistical analysis*

The effect of CMA-676 and MDR modifiers were quantified as changes in the cell cycle distribution. The Student's paired *t*-test was used to determine the statistical significance of the differences in the amounts of the hypodiploid portion, G0/G1 phase and S-G2/M phase between samples from different subsets or between samples incubated under different conditions. Correlations between the effect of CMA-676 and the other variables including the amount of CD33, CD34, P-gp and MRP were established using the Pearson correlation coefficients in StatView software (SAS Institute, Cary, NC, USA).

**Results**

*Flow cytometric analysis of cell cycle distribution, CD33 and CD34 on leukemia cells*

Cell cycle distribution patterns and expression patterns of CD33 and CD34 are shown in Figure 1. Leukemia cells could not be discriminated from normal counterparts by a two-dimensional histogram of FSC and SSC (Figure 1a), but easily showed up as a low SSC and low CD45-expressing population by a histogram of SSC and CD45 (Figure 1b). Then, expression patterns of CD33 and CD34 were plotted among leukemia cells gated like Figure 1b (Figure 1c). In addition, cell cycle distribution was analyzed on leukemia cells fractionated according to the expressions of CD33 and CD34. The cell cycle distributions of CD33<sup>+</sup>CD34<sup>-</sup> leukemia cells are shown in Figure 1d and f, and those of CD33<sup>+</sup>CD34<sup>+</sup> leukemia cells in Figure 1e and g.

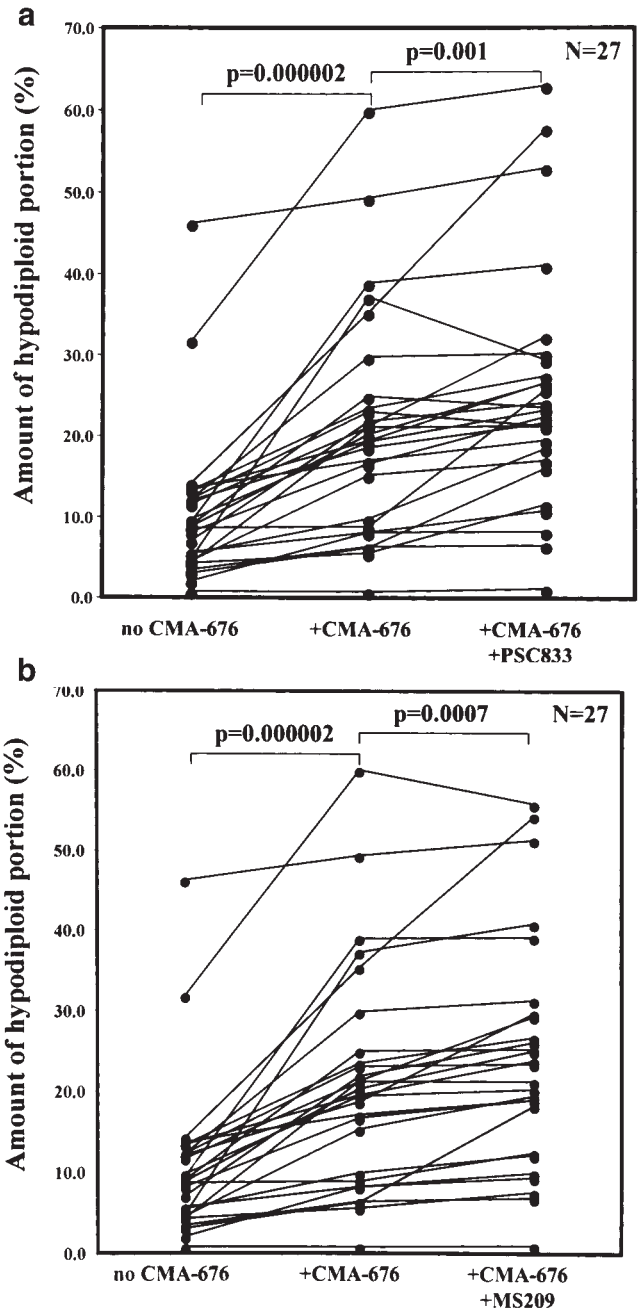
*Effect of CMA-676 and MDR modifiers on cell cycle distribution*

In leukemia cells from AML patients, the hypodiploid portion on cell cycle was significantly increased by CMA-676 ( $10.4 \pm 9.4$  vs  $21.1 \pm 13.9\%$ ,  $P = 0.000002$ ) as compared with respective control samples. G0/G1 phase was decreased by CMA-676 from  $84.5 \pm 10.0$  to  $74.1 \pm 14.8\%$ , and S-G2/M phase was also relatively decreased from  $5.1 \pm 3.6$  to  $4.5 \pm 3.9\%$ . Transient S-G2/M phase expansion, recognized in AML cell lines,<sup>20</sup> was not observed in these clinical samples (data not shown). The increase of the hypodiploid portion by CMA-676 was significantly correlated with the CD33 level ( $P = 0.002$ ) but not with the CD34 level ( $P = 0.49$ ) (Table 1). The hypodiploid portion slightly increased upon CMA-676 use (6.7%) in the CML-BC cells, that had minimal CD33 expression, but not in CD33-negative ALL cells (0.5%).

Furthermore, the hypodiploid portion increased more by the combined use of CMA-676 and PSC833 ( $25.3 \pm 14.5\%$ ,  $P = 0.001$ ) or MS209 ( $24.5 \pm 14.2\%$ ,  $P = 0.001$ ) than by CMA-676 alone (Figure 2). On the other hand, the combined use of CMA-676 and probenecid did not increase the hypodiploid portion as compared with CMA-676 alone in the 22 cases analyzed ( $19.0 \pm 12.9\%$ ,  $P = 0.79$ ). PSC833, MS209 or probenecid alone had no effect on the cell cycle of AML cells (data not shown).

*Relationship between the effect of CMA-676 and P-gp or MRP1*

In leukemia cells from AML patients, the increase of the hypodiploid portion by CMA-676 was inversely correlated with the



**Figure 2** Enhancement of the cytotoxic effect of CMA-676 by PSC833 or MS209. The amount of the hypodiploid portion after treatment with or without CMA-676 in the presence or absence of PSC833 (a) or MS209 (b). Statistical analyses were performed using the Student's paired *t*-test.

amount of P-gp expression ( $P = 0.004$ ). It was also inversely correlated with the % increase of intracellular Rh123 level by PSC833 ( $P = 0.0004$ ) or MS209 ( $P = 0.002$ ) (Table 1). However, it was neither correlated with the expression nor the function of MRP (Table 1).

*Effect of CMA-676 on CD34<sup>+</sup> leukemia cells in CD33<sup>+</sup> AML*

The effect of CMA-676 was also investigated on AML cells fractionated according to the expression of CD33 and CD34

in 13 AML cases. By the treatment with CMA-676, the hypodiploid portion on cell cycle was significantly more increased in CD33<sup>+</sup>CD34<sup>-</sup> cells (from 11.5 ± 9.3% to 30.1 ± 18.2%) as compared with CD33<sup>+</sup>CD34<sup>+</sup> cells (from 6.1 ± 4.1% to 15.1 ± 8.0%) ( $P = 0.002$ ) (Figure 3). The hypodiploid portions were still smaller in CD33<sup>-</sup>CD34<sup>+</sup> cells than CD33<sup>+</sup>CD34<sup>-</sup> or CD33<sup>+</sup>CD34<sup>+</sup> cells after the treatment with CMA-676 in our preliminary experiments (data not shown). In CD33<sup>+</sup>CD34<sup>+</sup> cells, the hypodiploid portion was slightly more increased by the combination of CMA-676 and PSC833 (19.2 ± 10.9%) as compared with CMA-676 alone (15.1 ± 8.0%) ( $P = 0.028$ ).

## Discussion

CMA-676 has recently attracted great attention because it selectively ablated CD33<sup>+</sup> leukemia cells in some patients with refractory or relapsed AML.<sup>15</sup> However, CMA-676 is less effective on MDR-expressing cells.<sup>15,20</sup> Linenberger *et al*<sup>15</sup> evaluated the relationship between 3,3'-diethyloxycarbocyanine iodide (DiOC<sub>2</sub>) efflux levels of leukemia cells and clinical response of CMA-676 in patients undergoing a phase II study, and reported that the elimination of leukemia appeared to be correlated with a low capacity of leukemia cells to extrude DiOC<sub>2</sub>. Naito *et al*<sup>20</sup> from our institution investigated the cytotoxic effect of CMA-676 on HL60, NOMO-1, NB4, NKM-1, K562, Daudi and their multidrug-resistant sublines, and reported that CMA-676 was less effective on P-gp-expressing multidrug-resistant sublines than the parental cell lines and that MDR modifiers, PSC833 and MS209, restored the cytotoxic effect of CMA-676 in these P-gp-expressing sublines.<sup>20</sup>

In the present study, we tried to clarify the relationship between MDR and the effect of CMA-676 using 27 clinical AML samples. A relatively high number of acute promyelocytic leukemia (APL) samples (33.3%) were included in this study, which was due to the fact that our institution is the center for the treatment of APL in Japan and APL patients had been referred to us especially for arsenic trioxide therapy during the study period.

In this study, we studied the effect of CMA-676 by cell cycle analysis, because our previous study using CD33<sup>+</sup> AML cell

lines had shown a transient increase of S-G2/M phase by CMA-676 prior to the increase of the hypodiploid portion and the decrease of G0/G1, S-G2/M phases. We could not show these transient increases of S-G2/M phase in clinical samples. There was some discrepancy between the present study using clinical samples and the previous study using cultured cell lines; this may result from the fact that the cell cycle of cell lines was generally more synchronous than that of clinical samples. Nevertheless, the increase of the hypodiploid portion was also observed in the present study.

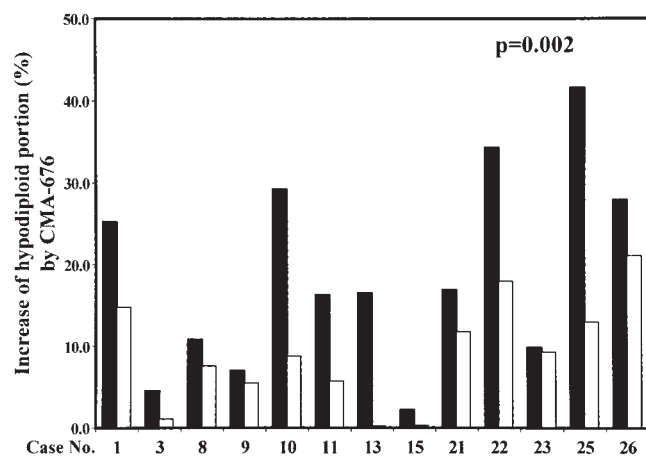
We confirmed that CMA-676 is less effective on leukemia cells from patients with a high amount of P-gp expression or increased P-gp function. MDR-1-associated P-gp is a membrane glycoprotein that actively pumps out cytotoxic agents and decreases intracellular drug accumulation.<sup>1-6</sup> Although to date it has not been well investigated whether calicheamicin itself is pumped out by P-gp, our study suggests that calicheamicin, which is released from CMA-676 after cellular internalization, is likely to be pumped out by P-gp in clinical leukemia cells also. Currently, CMA-676 is mostly used for the treatment of refractory or relapsed AML. However, such patients often express more P-gp.<sup>6</sup> Accordingly, CMA-676 treatment alone may not exert its full cytotoxic effect on refractory or relapsed AML patients.

PSC833, a non-immunosuppressive cyclosporine analog, induces a significant increase in intracellular daunorubicin accumulation by the inhibition of P-gp function.<sup>7</sup> Advani *et al*<sup>22</sup> conducted a phase II study of PSC833 in combination with mitoxantrone, etoposide and cytarabine in poor risk AML patients, and reported that 32% achieved CR. MS209, a novel quinoline derivative, directly interacts with P-gp and inhibits the transport of chemotherapeutic drugs, and reverses the resistance of vincristine- or adriamycin-resistant cell lines.<sup>8</sup> In the present study, we showed that these MDR modifiers significantly restored the cytotoxic effect of CMA-676 in P-gp-expressing leukemia cells from patients. In the case of CMA-676, MDR modifiers theoretically exert their effect only on CD33<sup>+</sup> cells. Therefore, combined use of CMA-676 and a MDR modifier will be an ideal therapeutic approach for patients with P-gp-expressing AML.

We found no positive correlation between the effect of CMA-676 and the amount of MRP1 or the function of MRP. MRP, as well as P-gp, has been reported to be over-expressed on some leukemia cells and associated with lower intracellular drug accumulation in AML.<sup>33</sup> However, the impact of MRP on clinical outcomes in AML remains to be proven. Our data suggest that MRP is not associated with CMA-676 resistance in AML, but further investigations are required to clarify this association.

Drug resistance to some anti-leukemia agents has been associated with CD phenotypes in AML. We previously demonstrated that CD33<sup>+</sup>CD34<sup>+</sup> AML cells expressed P-gp more strongly than CD33<sup>+</sup>CD34<sup>-</sup> cells.<sup>6</sup> Therefore, we studied the effects of CMA-676 in association with CD phenotypes. We used a multi-laser-equipped flow cytometer, which could simultaneously detect six parameters, ie cell cycle distribution, CD33, CD34, CD45, FSC and SSC. This system enabled us to analyze hypodiploid portions on cell cycle in relation to CD phenotypes.

CMA-676 was less effective on CD34<sup>+</sup> leukemia cells, even when they expressed a sufficient amount of CD33, although not dependent on the amount of CD34. In a phase II study of CMA-676, Sievers *et al*<sup>34</sup> also reported that the expression of CD34 was associated with shorter survival after CMA-676 treatment. The reason why CMA-676 was less effective on



**Figure 3** Different effects of CMA-676 on CD33<sup>+</sup>CD34<sup>-</sup> and CD33<sup>+</sup>CD34<sup>+</sup> cells in AML cells from 13 patients. Each bar represents the % increase of the hypodiploid portion after treatment with CMA-676 in CD33<sup>+</sup>CD34<sup>-</sup> (black bars) and CD33<sup>+</sup>CD34<sup>+</sup> leukemia cells (white bars). Statistical analysis was performed using the Student's paired *t*-test.

CD34<sup>+</sup> cells is not clear at present. One explanation is that CD34<sup>+</sup> cells have been reported to express more P-gp than CD34<sup>-</sup> cells in AML patients.<sup>22</sup> In preliminary experiments we compared the amount of P-gp and the stimulation of Rh123 accumulation by PSC833 or MS209 between CD33<sup>+</sup>CD34<sup>+</sup> cells and CD33<sup>+</sup>CD34<sup>-</sup> cells. In the six patients analyzed (cases 1, 3, 9, 11, 18 and 25 in Table 1), CD33<sup>+</sup>CD34<sup>+</sup> cells expressed more P-gp than CD33<sup>+</sup>CD34<sup>-</sup> cells ( $P = 0.048$ ). Increase of Rh123 accumulation by PSC833 or MS209 in CD33<sup>+</sup>CD34<sup>+</sup> cells was more than that of CD33<sup>+</sup>CD34<sup>-</sup> ( $P = 0.019$  and  $P = 0.032$ , respectively). We and other investigators have reported similar results previously.<sup>6,35</sup> Secondly, it may be explained by the immaturity of CD34<sup>+</sup> cells. While CD34 is undoubtedly expressed on immature hematopoietic cells as well as leukemia blasts,<sup>36</sup> its amount does not necessarily represent the immaturity of cells.<sup>37</sup> There are some populations of progenitors that lack CD34 expression, but have hematopoietic reconstituting capacity.<sup>37</sup> The immaturity may be rather dominated by the co-expressing antigens such as AC133 or negative staining for dyes such as Hoechst 33342.<sup>38,39</sup> Such immature cells have been reported to be protected from various substrates including dyes and toxic agents.<sup>39,40</sup> The third possibility is that the internalization of CD33 may be decreased in CD34<sup>+</sup> cells. Another possibility is that drug resistance mechanisms unrelated to P-gp may additionally make CMA-676 less effective on CD33<sup>+</sup>CD34<sup>+</sup> cells. Higher expression of bcl-2 and Axl in AML is correlated with CD34, and associated with poor response to chemotherapy.<sup>41,42</sup> Furthermore, P-gp expressing cells may simultaneously express other drug resistance mechanisms.<sup>43,44</sup> We could not analyze all of these drug-resistant mechanisms because of the limited number of samples, but showed that MRP1 was not associated with CMA-676 resistance. In two patients (cases 7 and 10 in Table 1) who were given CMA-676 *in vivo* in a phase I study, the CMA-676 treatment eradicated CD33<sup>+</sup>CD34<sup>-</sup> leukemia cells but not CD33<sup>+</sup>CD34<sup>+</sup> cells. This may also indicate that CD34<sup>+</sup> cells are relatively resistant to CMA-676.

Although CMA-676 is presumably less clinically effective on CD33<sup>+</sup>CD34<sup>+</sup> AML, it would be a promising drug for CD33<sup>+</sup> AML without CD34. APL cells reportedly express less P-gp and CD34 than other AMLs, even at relapse.<sup>6,45,46</sup> Therefore, CMA-676 would probably be effective on relapsed or refractory APL.

The present study has demonstrated that the effect of CMA-676 depends on the amounts of P-gp and the presence of CD34 as well as CD33. We may be able to predict the clinical effect of this drug by these parameters. In CD33<sup>+</sup> AML with P-gp, combined use of CMA-676 and a MDR modifier may be expected to be more beneficial. However, in CD34 co-expressing AML, the combination effect may be limited. Accordingly, *in vitro* tests for P-gp and CD34 as well as CD33 would help estimate the *in vivo* effect of CMA-676 in patients with AML.

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