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Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia

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We made a disease-specific comparison of unrelated cord blood (CB) recipients and human leukocyte antigen allele–matched unrelated bone marrow (BM) recipients among 484 patients with acute myeloid leukemia (AML; 173 CB and 311 BM) and 336 patients with acute lymphoblastic leukemia (ALL; 114 CB and 222 BM) who received myeloablative transplantations. In multivariate analyses, among AML cases, lower overall survival (hazard ratio [HR] = 1.5; 95% confidence interval [CI], 1.0-2.0, $P = .028$) and leukemia-free survival (HR = 1.5; 95% CI, 1.1-2.0, $P = .012$) were observed in CB recipients. The relapse rate did not differ between the 2 groups of AML (HR = 1.2; 95% CI, 0.8-1.9, $P = .38$); however, the treatment-related mortality rate showed higher trend in CB recipients (HR = 1.5; 95% CI, 1.0-2.3, $P = .085$). In ALL, there was no significant difference between the groups for relapse (HR = 1.4, 95% CI, 0.8-2.4, $P = .19$) and treatment-related mortality (HR = 1.0; 95% CI, 0.6-1.7, $P = .98$), which contributed to similar overall survival (HR = 1.1; 95% CI, 0.7-1.6, $P = .78$) and leukemia-free survival (HR = 1.2; 95% CI, 0.9-1.8, $P = .28$). Matched or mismatched single-unit CB is a favorable alternative stem cell source for patients without a human leukocyte antigen–matched related or unrelated donor. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients. (Blood. 2009;113:1631-1638)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) with bone marrow (BM) or peripheral blood, the curative treatment of choice for acute leukemia, is limited by the inadequate supply of human leukocyte antigen (HLA)–identical related donors. Bone marrow from HLA-matched unrelated donors has been a major alternative graft source.[1-3] Umbilical cord blood (CB), an alternative stem cell source to BM or peripheral blood stem cells, has been used primarily in children,[4-10] but its use in adults is increasing.[11,12]

Clinical comparison studies of cord blood transplantation (CBT) and bone marrow transplantation (BMT) for leukemia from unrelated donors in adult recipients showed comparable outcomes.[11-13] Recipients of CBT showed delayed neutrophil recovery and lower incidence of acute graft-versus-host disease (GVHD).[11-13] Overall treatment-related mortality (TRM) was reported to be similar[12] or higher[11] compared with HLA-matched BM. Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are different disease entities that require different chemotherapy regimens for treatment. However, previous comparison studies have included both diseases because of limitation in the number of CBTs given to adults.

In addition, the study periods of previous studies encompass the pioneering period of CBT, when the general practice was to use these grafts in patients in whom there were no other curative options and when the relevance of cell dose and HLA matching had not yet been recognized.[6,7,14]

Accumulation of a larger number of CBT results enabled us to make a controlled comparison with unrelated BMTs. To avoid the inclusion of the pioneering period of CBT, the subjects were limited to those who received transplantations in and after 2000.

Methods

Collection of data and data source

The recipients' clinical data were provided by the Japan Cord Blood Bank Network (JCBBN) and the Japan Marrow Donor Program (JMDP).[15]

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Peripheral blood stem cell donation from unrelated donors is not permitted in Japan. All 11 CB banks in Japan are affiliated to JCBBN. Both JCBBN and JMDP collect recipients' clinical information at 100 days after transplantation. Patients' information on survival, disease status, and long-term complications, including chronic GVHD and second malignancies, are renewed annually by follow-up forms. This study was approved by the data management committees of JMDP and JCBBN.

Patients

Between January 2000 and December 2005, a total of 1690 adult patients at least 16 years of age with acute leukemia (999 AML, 261 CB and 738 BM; and 691 ALL, 178 CB and 513 BM) received first HSCT with myeloablative conditioning either CB or BM from unrelated donors. Of these, patients who received a single CB unit with 0 to 2 HLA mismatches, or HLA-A, -B, -C, and DRB1 allele-matched BM from unrelated donors were analyzed. HLA matching of CB was performed using low-resolution molecular typing methods for HLA-A and -B, and high-resolution molecular typing for HLA-DRB1. Of 1023 BM recipients with complete HLA high-resolution data, the following recipients with HLA HLA-A, -B, -C, and DRB1 allele mismatches were excluded: 306 recipients with 1 of 8 mismatches (39 for HLA-A, 6 for HLA-B, 137 for HLA-C, and 124 for HLA-DRB1), 150 recipients with 2 of 8 mismatches (36 for 2 class I antigens, and 114 for class I and class II antigens), 33 recipients with 3 of 8 mismatches, and 1 recipient with 4 of 8 mismatches. Of 390 recipients of CB with complete HLA data, 95 recipients with 3 mismatches and 8 patients with 4 mismatches were excluded. A total of 484 patients with AML (173 CBTs and 311 BMTs) and 336 patients with ALL (114 CBTs and 222 BMTs) were the subjects for the analyses. Eighty-five centers performed 287 CBTs analyzed in this study, and 114 centers performed 533 BMTs.

Definitions

Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells/mm^3 for 3 consecutive points; platelet recovery was defined by a count of at least 50 000 platelets/mm^3 without transfusion support. Diagnosis and clinical grading of acute GVHD were performed according to the established criteria.[16] Relapse was defined as a recurrence of underlying hematologic malignant diseases. Treatment-related death was defined as death during a continuous remission. Leukemia-free survival (LFS) was defined as survival in a state of continuous remission.

Statistical analysis

Separate analyses were performed for AML and ALL. Descriptive statistical analysis was performed to assess patient baseline characteristics, diagnosis, disease classification, disease status at conditioning, donor-patient ABO mismatches, preparative regimen, and GVHD prophylaxis. The 2-sided χ^2 test was used for categorical variables, and the 2-sided Wilcoxon rank sum test was used for continuous variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil and platelet recovery, acute and chronic GVHD, relapse, and TRM.[17] For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD and relapse were the competing events; for relapse, death without relapse was the competing event; and, for TRM, relapse was the competing event. Gray test was used for group comparison of cumulative incidence.[18] Overall survival (OS) and LFS were calculated using the Kaplan-Meier method. The log-rank test was used for group comparisons. Adjusted comparison of the stem cell source on OS and LFS was performed with the use of the Cox proportional-hazards regression model. For other outcomes, the Fine and Gray proportional-hazards model for subdistribution of a competing risk was used.[19] Adjusted probabilities of OS and DFS were estimated using the Cox proportional-hazards regression model, with consideration of other significant clinical variables in the final multivariate models. The variables considered were the patient's age at transplantation, patient's sex, donor-patient sex mismatch, donor-patient ABO mismatch, disease status at conditioning, and t(9;22) chromosome abnormality or others for ALL, cytogenetic information and French-American-British (FAB) classification of M5/M6/M7 or others for AML, the conditioning regimen, and the type of prophylaxis against GVHD. Factors differing in distribution between CB and BM recipients ($P < .10$) and factors known to influence outcomes (such as patient age at transplantation and chromosome abnormalities and FAB classification of leukemia) were included in the final models. Variables with more than 2 categories were dichotomized for the final multivariate model. The cutoff points of the variables were chosen to make optimal use of the information, with the proviso that smaller groups contain at least 20% of the patients. Variables were dichotomized as follows: patient age greater or younger than 45 years at transplantation, female donor to male recipient donor-recipient sex mismatch versus others for donor-recipient sex matching, donor-recipient ABO major mismatch versus others for ABO matching, M5/M6/M7 FAB classification versus others for classification of AML, chromosome abnormality other than favorable abnormalities for cytogenetics of AML, cyclophosphamide and total body irradiation (TBI) or busulfan and cyclophosphamide or others for conditioning regimen of AML, cyclophospohamide and TBI, or others for conditioning regimen of ALL, and cyclosporine-based versus tacrolimus-based prophylaxis against GVHD. Disease status at transplantation was categorized as first complete remission (1CR), second or later complete remission (2CR), or more advanced disease; which was included in the final model using dichotomized dummy variables. All P values were 2-sided.

The statistical power to detect hazard ratios (HRs) of 2.0 and 1.5 (a regression coefficient equal to 0.6931 and 0.4055, respectively) on Cox regression of the log hazard ratio at a .05 significance level adjusted for event rate were 99% and 78%, respectively, for 484 patients with AML and 97% and 60%, respectively, for 336 patients with ALL. The levels of statistical power for subgroup analyses were as follows: 54% and 22% for 1CR, 51% and 21% for 2CR, 96% and 58% for more advanced in AML patients, 62% and 26% for 1CR, 47% and 20% for 2CR, and 67% and 29% for more advanced in ALL patients.[20]

Results

Patient characteristics

The characteristics of the patients are shown in Table 1. There was no significant difference in recipients' age at transplantation in AML (median age, CB vs BM = 38 vs 38 years, $P = .61$) and in ALL (median age, CB vs BM = 34 vs 32 years, $P = .29$). The female/male ratio was higher (CB vs BM = 54% vs 38% in AML patients, and CB vs BM = 54% vs 38% in ALL patients, $P < .001$ and $P = .005$, respectively) in CB recipients, resulting in the lower donor-patient sex match rate (CB vs BM = 48% vs 69% in AML patients, and CB vs BM = 46% vs 65% in ALL patients, $P < .001$ and $P = .002$, respectively) in CB recipients. The proportion of ALL patients with Philadelphia chromosome abnormality was higher (CB vs BM = 38% vs 23%) in CB recipients. CB recipients were likely to have more advanced disease status at transplantation (relapse or induction failure, CB vs BM = 47% vs 31% in AML patients, and CB vs BM = 26% vs 19% in ALL patients), and the difference was significant in AML ($P = .003$). HLA-A, -B (low-resolution typing), and -DRB1 (high-resolution typing) was mismatched in 93% of both AML and ALL among CB recipients, whereas HLA -A, -B, -C, and -DRB1 were all genotypically matched for BM recipients. The ABO-matched donor-patient pair proportion was consistently lower for CB (CB vs BM = 34% vs 59% in AML patients and CB vs BM = 32% vs 58% in ALL patients).

A preparative regimen with TBI and cyclophosphamide was used in almost all patients, and cytosine arabinoside was supplemented for CB recipients with AML (36%) in addition to TBI and cyclophosphamide. For GVHD prophylaxis, tacrolimus (CB vs BM = 29% vs 56% in AML patients, and CB vs BM = 37% vs 53% in ALL patients) and

Table 1. Characteristics of recipients of cord blood or bone marrow from unrelated donors in 484 patients with acute myeloid leukemia and 336 patients with acute lymphoblastic leukemia

	Acute myeloid leukemia			Acute lymphoblastic leukemia		
Characteristic	**U-CBT**	**U-BMT**	***P***	**U-CBT**	**U-BMT**	***P***
No. of transplantations	173	311		114	222	
Median patient age at transplantation, y (range)	38 (16-69)	38 (16-60)	.61	34 (16-58)	32 (16-59)	.29
Patient sex, n (%)						
Male	80 (46)	194 (62)	< .001	52 (46)	137 (62)	.005
Female	93 (54)	117 (38)		62 (54)	85 (38)	
Sex matching, n (%)			< .001			.002
Matched	83 (48)	215 (69)		52 (46)	145 (65)	
Male to female	44 (25)	57 (18)		35 (31)	42 (19)	
Female to male	46 (27)	37 (12)		27 (24)	35 (16)	
Unknown	0 (0)	1 (0)		0 (0)	0 (0)	
Disease classification						
AML (French-American-British)			.045			
M0	17 (10)	26 (8)				
M1	30 (17)	38 (12)				
M2	52 (30)	88 (28)				
M3	4 (2)	25 (8)				
M4	27 (16)	55 (18)				
M5	23 (13)	41 (13)				
M6	3 (2)	18 (6)				
M7	2 (1)	5 (2)				
Others/unknown	15 (9)	15 (5)				
Cytogenetics			.042			
Favorable*	19 (11)	66 (21)				
Normal	74 (43)	116 (37)				
Other	57 (33)	95 (31)				
Unknown	23 (13)	34 (11)				
ALL cytogenetics						.022
t(9;22)				43 (38)	52 (23)	
t(4;11)				2 (2)	3 (1)	
Others				22 (19)	51 (23)	
Normal				27 (24)	85 (38)	
Unknown				20 (18)	31 (14)	
Disease status			.003			.33
First CR	50 (29)	130 (42)		63 (55)	130 (59)	
Second or after CR	39 (23)	82 (26)		21 (18)	48 (22)	
Relapse/induction failure	81 (47)	95 (31)		30 (26)	42 (19)	
Unknown	3 (2)	4 (1)		0 (0)	2 (1)	
HLA matching†						
0 mismatched loci	12 (7)			8 (7)		
1 mismatched locus	35 (20)			25 (22)		
2 mismatched loci	126 (73)			81 (71)		
ABO matching			< .001			< .001
Matched	59 (34)	185 (59)		37 (32)	128 (58)	
Minor mismatch	48 (28)	57 (18)		30 (26)	48 (22)	
Major mismatch	37 (21)	59 (19)		24 (21)	41 (18)	
Bidirectional	28 (16)	8 (3)		23 (20)	3 (1)	
Unknown	1 (1)	2 (1)		0 (0)	2 (1)	
Nucleated cells infused per 10^7/kg, median (range)	2.44 (1.65-5.49)	26.3 (2.10-58.8)	< .001	2.48 (1.51-4.06)	28.2 (2.30-79.0)	< .001
Preparative regimen			< .001			.38
CY + TBI	43 (25)	142 (46)		42 (37)	92 (41)	
CY + CA + TBI	62 (36)	41 (13)		31 (27)	53 (24)	
CY + BU + TBI	7 (4)	36 (12)		3 (3)	5 (2)	
Other TBI regimen	42 (24)	33 (11)		34 (30)	54 (24)	
BU + CY	18 (10)	55 (18)		4 (4)	12 (5)	
Other non-TBI regimen	1 (1)	4 (1)		0 (0)	6 (3)	
GVHD prophylaxisis			< .001			< .001
Cyclosporine A + sMTX	103 (60)	131 (42)		65 (57)	100 (45)	
Cyclosporine A ± other	20 (12)	4 (1)		6 (5)	3 (1)	
Tacrolimus + sMTX	34 (20)	168 (54)		26 (23)	106 (48)	
Tacrolimus ± other	15 (9)	5 (2)		16 (14)	11 (5)	
Others	1 (1)	3 (1)		1 (1)	2 (1)	

U-CBT, indicates unrelated cord blood transplantation; U-BMT, unrelated bone marrow transplantation; CR, complete remission; HLA, human leukocyte antigen; CY, cyclophosphamide; CA, cytarabine; BU, oral busulfan; TBI, total body irradiation; and sMTX, short-term methotrexate.

*Favorable abnormal karyotypes are defined as t(8;21), inv16,or t(15;17).

†Number of mismatches was counted among HLA-A, -B (low-resolution typing), and DRB1 (high-resolution typing).

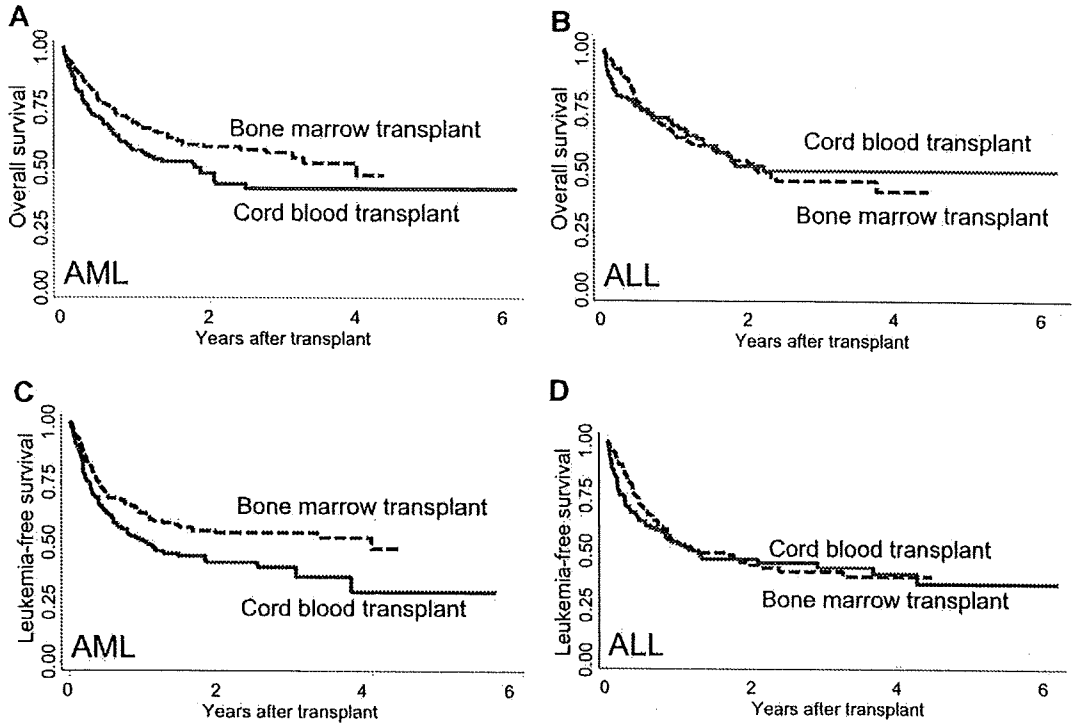

Figure 1. Adjusted OS and LFS of recipients with AML or ALL of CB or BM from unrelated donors. For patients with AML, adjusted probabilities of (A) OS (CB vs BM = 48% vs 59% at 2 years, $P = .010$) and (C) LFS (CB vs BM = 42% vs 54% at 2 years, $P = .004$) were both lower in CB recipients. For patients with ALL, the adjusted probabilities of (B) OS (CB vs BM = 52% vs 53% at 2 years, $P = .99$) and (D) LFS (CB vs BM = 46% vs 44% at 2 years, $P = .41$) were similar between CB recipients and BM recipients.

short-term methotrexate (CB vs BM = 80% vs 96% in AML patients, and CB vs BM = 80% vs 93% in ALL patients) were used preferentially in BM recipients. The median follow-up period for survivors was 1.9 years (range, 0.1-6.2 years) for CB recipients and 1.4 years (range, 0.3-4.5 years) for BM recipients.

Outcome

OS. For patients with AML, the unadjusted probabilities of OS were lower for CB recipients at 1 year (51% vs 69%) and 2 years (43% vs 60%) compared with BM recipients ($P < .001$). For patients with ALL, there were no significant differences between the 2 groups (CB vs BM = 66% vs 66% at 1 year, 49% vs 57% at 2 years, $P = .40$).

Among patients with AML, the use of CB remained a significant risk factor for overall mortality after adjustment for other factors (HR = 1.5; 95% confidence interval [CI], 1.0-2.0; $P = .028$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for overall mortality on multivariate analysis (HR = 1.1; 95% CI, 0.7-1.6; $P = .78$). The adjusted probability of OS was significantly lower for CB recipients (57% vs 69% at 1 year, and 48% vs 59% at 2 years, $P = .010$; Figure 1A) compared with BM recipients for patients with AML, whereas the adjusted probability of OS was similar (69% vs 64% at 1 year, and 52% vs 53% at 2 years, $P = .99$; Figure 1B) between the groups for patients with ALL.

Results of the subgroup analyses showed that the difference in survival among AML patients was prominent in patients demonstrating 1CR at transplantation (RR = 2.9, 95% CI = 1.4-6.2, $P = .005$; Table 3).

LFS. For patients with AML, the unadjusted probabilities of LFS were significantly lower for CB recipients at 1 year (43% vs 62%) and 2 years (36% vs 54%) compared with BM recipients ($P < .001$). For patients with ALL, the unadjusted probabilities of LFS were lower with marginal significance for CB recipients at 1 year (52% vs 58%) and 2 years (45% vs 51%) compared with BM recipients ($P = .06$).

Among patients with AML, the use of CB remained as a significant risk factor for treatment failure (ie, relapse or death) after adjustment for other factors (HR = 1.5; 95% CI, 1.1-2.0; $P = .012$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for treatment failure by multivariate analysis (HR = 1.2; 95% CI, 0.9-1.8; $P = .28$). The adjusted probability of LFS was significantly lower for CB recipients (51% vs 62% at 1 year, and 42% vs 54% at 2 years, $P = .004$; Figure 1C) compared with BM recipients for patients with AML, whereas the adjusted probability of LFS was similar (53% vs 53% at 1 year, and 46% vs 44% at 2 years, $P = .41$; Figure 1D) between the groups for patients with ALL.

Relapse

On univariate analyses, the cumulative incidence of relapse was higher for CB recipients with marginal significance in both AML (27% vs 20% at 1 year, and 31% vs 24% at 2 years) and ALL (27% vs 19% at 1 year, and 31% vs 24% at 2 years) ($P = .067$, and .085, respectively; Figure 2A,B).

On multivariate analyses adjusted by other factors, there was no significantly higher risk of relapse for CB recipients with either AML (RR = 1.2, 95% CI = 0.8-1.9, $P = .38$) or ALL (RR = 1.4, 95% CI = 0.8-2.4, $P = .19$; Table 2).

TRM

For patients with AML, the unadjusted cumulative incidence of TRM was significantly higher for CB recipients at 1 year (30% vs 19%) and 2 years (33% vs 22%) compared with those for BM recipients ($P = .004$; Figure 2C). For patients with ALL, the

Table 2. Results of multivariate analysis of outcomes in 173 recipients of cord blood and 311 recipients of bone marrow with acute myeloid leukemia, and 114 recipients of cord blood and 222 recipients of bone marrow with acute lymphoblastic leukemia

	Acute myeloid leukemia		Acute lymphoblastic leukemia	
Outcome	RR (95% CI)	*P*	RR (95% CI)	*P*
Overall survival*				
BM	1.00		1.00	
CB	1.45 (1.04-2.01)	.028	1.06 (0.71-1.57)	.78
Leukemia-free survival†				
BM	1.00		1.00	
CB	1.48 (1.09-2.01)	.012	1.22 (0.85-1.76)	.28
Relapse‡				
BM	1.00		1.00	
CB	1.21 (0.79-1.87)	.38	1.42 (0.84-2.41)	.19
TRM§				
BM	1.00		1.00	
CB	1.47 (0.95-2.28)	.085	1.01 (0.59-1.73)	.98
Neutrophil recovery‖				
BM	1.00		1.00	
CB	0.41 (0.33-0.51)	< .001	0.37 (0.29-0.48)	< .001
Platelet recovery¶				
BM	1.00		1.00	
CB	0.34 (0.27-0.44)	< .001	0.43 (0.33-0.56)	< .001
Acute GVHD#				
BM	1.00		1.00	
CB	0.80 (0.56-1.15)	.23	0.61 (0.39-0.95)	.028
Chronic GVHD**				
BM	1.00		1.00	
CB	0.94 (0.63-1.42)	.79	1.08 (0.66-1.77)	.77
Chronic GVHD, extensive type††				
BM	1.00		1.00	
CB	0.36 (0.18-0.72)	.004	0.58 (0.28-1.20)	.14

RR indicates relative risk; CI, confidence interval; BM, bone marrow; CB, cord blood; and GVHD, graft-versus-host disease.

*For overall survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

†For leukemia-free survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

‡For relapse, other significant variables for AML were more advanced disease status at conditioning, donor-recipient ABO major mismatch, chromosome abnormality other than favorable abnormalities, and cyclophosphamide and total body irradiation or busulfan and cyclophosphamide conditioning regimen; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and cyclophosphamide and total body irradiation conditioning.

§For TRM, other significant variables for AML were patient age more than 45 years at transplantation, second or after complete remission disease status, more advanced disease status, and chromosome abnormality other than favorable abnormalities; other significant variables for ALL were patient age more than 45 years at transplantation, more advanced disease status at conditioning, and conditioning other than cyclophosphamide and total body irradiation.

‖For neutrophil recovery, other significant variables for AML were second or after complete remission disease status and more advanced disease status; other significant variables for ALL were more advanced disease status at conditioning and cyclosporine-based GVHD prophylaxis.

¶For platelet recovery; other significant variables for AML were second or after complete remission disease status, more advanced disease status, female donor to male recipient donor-recipient sex mismatch, and tacrolimus-based GVHD prophylaxis; other significant variables for ALL were more advanced disease status at conditioning and conditioning other than cyclophosphamide and total body irradiation.

#For acute GVHD, no other significant variables were identified for both AML and ALL.

**For chronic GVHD, other significant variables for AML were more advanced disease status and conditioning other than cyclophosphamide and total body irradiation or busulfan and cyclophosphamide; there were no other significant variables identified for ALL.

††For extensive chronic GVHD, there were no other significant variables identified for AML; another significant variable for ALL was patient male sex.

cumulative incidence of TRM was similar between the 2 groups (CB vs BM = 21% vs 23% at 1 year, 24% vs 25% at 2 years, $P = .83$; Figure 2D).

On multivariate analyses adjusted by other factors, the risk for TRM was higher for CB recipients compared with that for BM recipients among patients with AML (RR = 1.5, 95% CI = 1.0-2.3, $P = .085$; Table 2) with marginal significance. For patients with ALL, the risk for TRM was similar between CB and BM recipients (RR = 1.0, 95% CI = 0.6-1.7, $P = .98$).

Cause of death

Recurrence of the primary disease was the leading cause of death in each group (CB vs BM = 37% vs 33% in patients with AML and 36% vs 41% in patients with ALL). The following causes were infection and organ failure in all groups (Table 4).

Other outcomes of transplantation

Neutrophil and platelet recovery. The unadjusted cumulative incidence of neutrophil recovery or platelet recovery at day 100 was significantly lower in CB recipients for both AML (77% vs 94%) and ALL (80% vs 97%) compared with that among BM recipients ($P < .001$ for both). On multivariate analyses, neutrophil recovery was significantly lower among CB recipients for both AML (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$; Table 2).

Table 3. Results of multivariate analysis of overall survival according to disease status at transplantation

	First complete remission			Second or after complete remission			More advanced		
Overall survival	n	RR (95% CI)	P	n	RR (95% CI)	P	n	RR (95% CI)	P
AML									
UBMT	130	1.00		82	1.00		95	1.00	
UCBT	50	2.92 (1.38-6.18)	.005	39	1.24 (0.51-3.04)	.63	81	1.29 (0.84-1.98)	.25
ALL									
UBMT	130	1.00		48	1.00		42	1.00	
UCBT	63	1.60 (0.84-3.05)	.16	21	0.62 (0.22-1.74)	.36	30	0.80 (0.38-1.69)	.57

RR indicates relative risk; CI, confidence interval; UBMT, unrelated bone marrow transplantation; and UCBT, unrelated cord blood transplantation.

The unadjusted cumulative incidence of platelet recovery greater than 50 000/μL at 4 months was significantly lower among CB recipients for both AML (59% vs 85%) and ALL (61% vs 83%) compared with that of BM recipients ($P < .001$ for both). The difference was also significant on multivariate analyses for both AML (RR = 0.3, 95% CI = 0.3-0.4, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.6, $P < .001$; Table 2).

Acute GVHD. The unadjusted cumulative incidence of grade 2 to 4 acute GVHD was lower among CB recipients compared with that among BM recipients (32% vs 35% in AML, 28% vs 42% in ALL); the difference was significant in patients with ALL ($P = .39$ in AML, $P = .008$ in ALL). The difference was also significant on multivariate analyses in ALL (RR = 0.6, 95% CI = 0.4-1.0, $P = .028$). There was no significant difference in patients with AML (RR = 0.8, 95% CI = 0.6-1.2, $P = .23$; Table 2).

Chronic GVHD. The unadjusted cumulative incidence of chronic GVHD at 1 year after transplantation did not significantly differ between CB recipients and BM recipients in both AML (28% vs 32%, $P = .46$) and ALL (27% vs 30%, $P = .50$). The cumulative incidence of extensive-type chronic GVHD was significantly lower among CB recipients compared with that among BM recipients in both AML (8% vs 20%, $P < .001$) and ALL (10% vs 17%, $P = .034$). On multivariate analyses, the risk of developing chronic GVHD was similar in CB recipients and BM recipients in both AML (RR = 0.9, 95% CI = 0.6-1.4, $P = .79$) and ALL (RR = 1.1, 95% CI = 0.7-1.8, $P = .77$). The risk of developing extensive chronic GVHD was lower in CB recipients compared with BM recipients (RR = 0.4, 95% CI = 0.2-0.7, $P = .004$ in AML, and RR = 0.6, 95% CI, 0.3-1.2, $P = .14$ in ALL) and was significantly different in patients with AML (Table 2).

Discussion

The objective of our study was to investigate the outcomes of HLA-A, -B, low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit unrelated CBT in adult patients with acute leukemia compared with those of HLA-A, -B, -C, and -DRB1 (8 of 8) allele-matched unrelated BMT. Although AML and ALL are different diseases, previous comparisons of unrelated BMT and

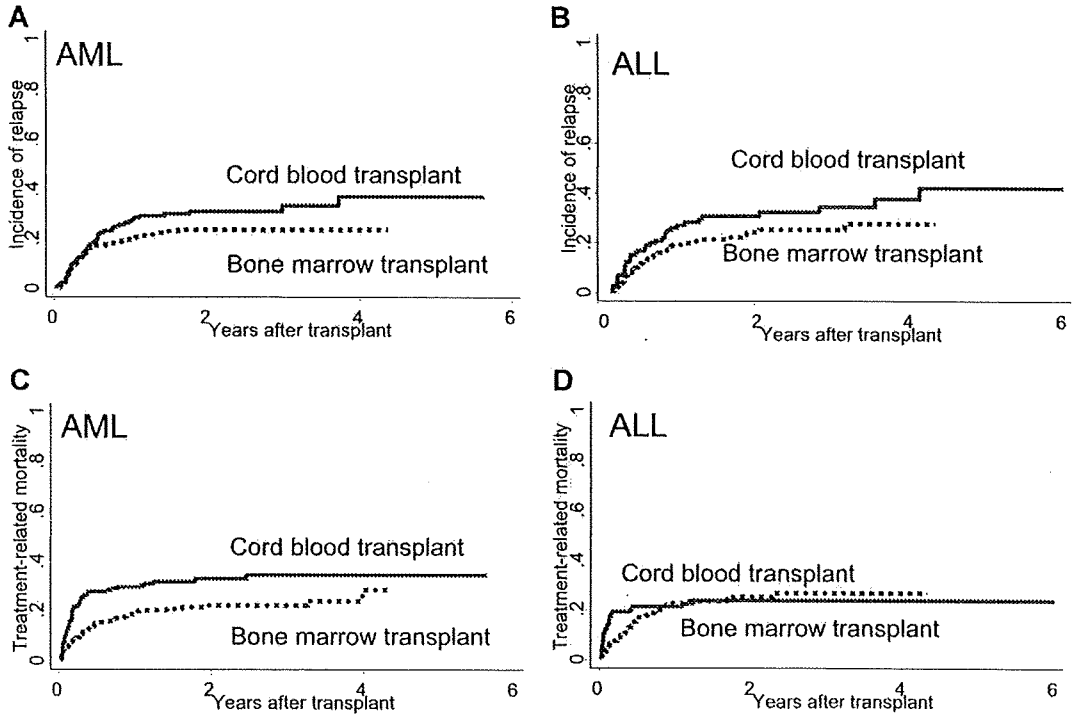

Figure 2. Cumulative incidence of relapse or TRM of recipients of CB or BM among patients with AML or ALL. For patients with AML, the cumulative incidence of (A) relapse (CB vs BM = 31% vs 24% at 2 years, $P = .068$) and (C) TRM (CB vs BM = 33% vs 22% at 2 years, $P = .004$) was higher in CB recipients. For patients with ALL, the cumulative incidence of relapse (B) was higher in CB recipients with marginal significance (CB vs BM = 31% vs 24% at 2 years, $P = .085$), but the incidence of TRM (D) was similar in CB and BM recipients (CB vs BM = 24% vs 25% at 2 years, $P = .83$).

Table 4. Causes of death after transplantation of unrelated cord blood or unrelated bone marrow among patients with acute myeloid leukemia or acute lymphoblastic leukemia

	Acute myeloid leukemia		Acute lymphoblastic leukemia	
Cause of death	UCBT	UBMT	UCBT	UBMT
Recurrence of disease	35 (37)	34 (33)	18 (36)	34 (41)
Graft failure/rejection	3 (3)	4 (4)	0 (0)	3 (4)
Graft-versus-host disease	6 (6)	7 (7)	3 (6)	5 (6)
Infection	22 (23)	19 (18)	13 (26)	11 (13)
Idiopathic pneumonia	4 (4)	4 (4)	2 (4)	6 (7)
Organ failure	17 (18)	17 (16)	8 (16)	10 (12)
Secondary cancer	0 (0)	1 (1)	0 (0)	0 (0)
Other causes	5 (5)	5 (5)	2 (4)	4 (5)
Unknown/data missing	2 (2)	13 (13)	4 (8)	10 (12)
Total	94 (100)	104 (100)	50 (100)	83 (100)

Data are presented as n (%).

UCBT indicates unrelated cord blood transplantation; and UBMT, unrelated bone marrow transplantation.

unrelated CBT did not separate these 2 diseases. Our report is the first to show the result of disease-specific analyses with a sufficient number of patients.

For AML patients, the recipients of CB were more likely to have advanced leukemia at the time of transplantation, as reported previously, suggesting that CB was used as an alternative stem cell source in the later phase of unrelated donor searches, especially in adults.[11,12,14] A larger proportion of CB recipients with ALL had the Philadelphia chromosome abnormality, which correlates with highly aggressive ALL and usually requires urgent transplantation, in which CB has an advantage over BM.[21]

Different outcomes of mortality were found between AML and ALL in a controlled comparison using multivariate analyses. Whereas significantly lower OS and LFS rates were observed in CB recipients with AML, rates of overall mortality and treatment failure were similar between CB and BM recipients with ALL. The relapse rate was not different between CBT and BMT in patients with both AML and ALL, which was consistent with previous reports.[11-13] In adult patients with ALL, a previous report showed no difference in the outcome of related compared with unrelated BM or peripheral blood transplantation in 1CR.[22] Favorable disease status at transplantation could be a more important factor affecting outcome rather than the type of stem cell source or donor type in patients with ALL. It is notable that TRM in HLA allele-matched unrelated BM recipients with AML was quite low in our study. This is probably associated with the low incidence of acute and chronic GVHD in the Japanese population, which is thought to be the result of genetic homogeneity.[23-26] Among patients with AML, although the difference was not statistically significant, a higher trend of TRM observed in CB recipients might be associated with higher overall and TRM rates in CB recipients. Reasons for higher TRM could include the graft source and delayed neutrophil recovery. Better supportive care is required after CBT for patients going through a prolonged neutropenic period. Development of better graft engineering or better conditioning regimens would help to decrease the TRM rate in CB recipients. Because relapse was the major cause of death in all groups, any attempt to decrease TRM should preserve the antileukemia effect to improve OS and LFS. Another reason for the higher TRM could be a higher risk patient population, higher risk for both disease status and comorbid conditions, requiring rapid transplantation. Searching for unrelated donors earlier and providing transplantation earlier in the disease course could help to decrease TRM in CB recipients.

Neutrophil and platelet recovery was slower in CB recipients with either AML or ALL, consistent with the results of previous reports.[11,12,27] Multiple studies have reported lower incidence of acute GVHD in CB recipients.[8-10,12,13] In our study, particularly in patients with ALL, the risk of developing grade 2 to 4 acute GVHD in CB recipients was lower compared with BM recipients, which was reported to be lower compared with the incidence reported from Western countries.[23-25] The risk of developing chronic GVHD was similar between CB and BM recipient with either disease, but the risk of developing extensive-type chronic GVHD was lower in CB recipients; the difference was significant in patients with AML. It is notable that there was no increase in the incidence of acute or chronic GVHD in CB recipients among patients with either AML or ALL, despite HLA disparity.

For differences in outcomes between AML and ALL, one possibility is a difference of treatment before conditioning therapy. Most AML patients received a more intense treatment for induction and consolidation therapy compared with that for ALL. There was no adjustment made for previous treatment, and this could be the reason for higher mortality in CBT, which requires a longer time for neutrophil recovery. Another possible cause of the difference in outcomes is the difference in conditioning regimens. Preparative regimens were similar between CB and BM recipients among ALL patients. However, in patients with AML, the proportion of standard regimens, such as cyclophosphamide and TBI or busulfan and cyclophosphamide, was smaller among CB recipients. These differences in the distribution of preparative regimens were also seen in a previous report.[11] Although the final model was adjusted for conditioning regimens, we cannot rule out the possibility of an effect that larger CB recipients received additional or different chemotherapeutic agents compared with BM recipients among patients with AML. Although the difference was small, the median age of CB recipients with AML was 4 years older than CB recipients with ALL (median age, 38 vs 34 years, $P = .021$), which might have affected the higher mortality rate among CB recipients with AML. It is also possible that some unknown biologic aspects have contributed to these differences, and this would require further evaluation in future studies.

Further subgroup analyses indicated that the superiority of HLA allele-matched BM versus CB for OS was mostly found in patients with AML showing 1CR at conditioning. However, because of the limited numbers of patients in these subgroup analyses and the possibility of an unidentified bias in stem cell source selection, our findings should be verified by further analysis in a larger population.

In conclusion, we found different outcomes between patients with AML and ALL, indicating the importance of disease-specific analyses in alternative donor studies. HLA-A, -B low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit CB is a favorable alternative stem cell source for patients without a suitable related or 8 of 8 matched unrelated BM donor. In the absence of a suitable donor, unrelated CBT should be planned promptly to transplant the patient while in a better disease status and better clinical condition. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients.

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Authorship

Contribution: Y.A. and R.S. designed the study and wrote the paper; Y.A. analyzed results and made the figures; S. Kato and Y.M. designed the research; T.-N.I., H.A., and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai, H.S., Y. Kouzai, M.K., and T.F. submitted and cleaned the data; and S.O., M. Tsuchida, K.K., Y.M., and Y. Kodera reviewed and cleaned the Japan Marrow Donor Program data and reviewed the results.

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A complete list of members from the Japan Marrow Donor Program and the Japan Cord Blood Bank Network can be found in the Supplemental Appendix (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

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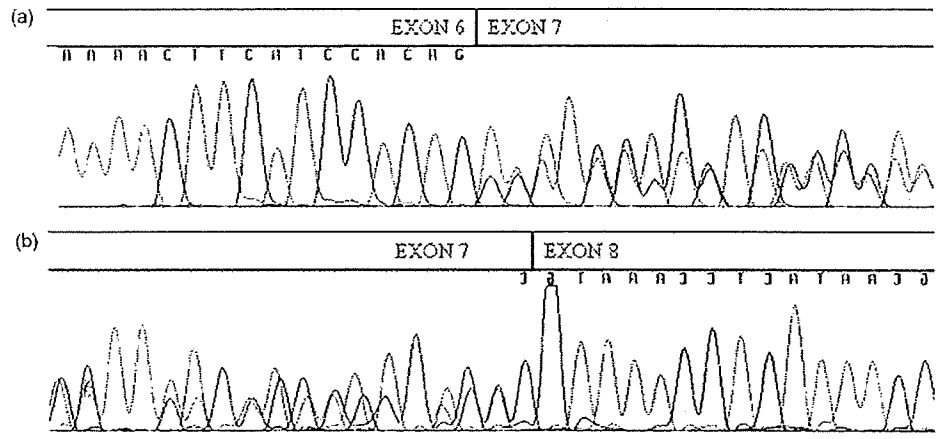

Fig. 1. Direct sequencing of c-ABL shows the pattern of deletion of exon 7: (a) forward sequencing around the initiation site of exon 7 and (b) reverse sequencing around the termination site of exon 7.

Conflict of interest statement

All authors have no conflict of interest.

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Compliance with taking imatinib mesylate in patients with chronic myeloid leukemia in the chronic phase

The development of imatinib mesylate (IM) has redefined the management of chronic myeloid leukemia (CML) [1]. Most newly diagnosed patients with chronic-phase disease, treated with IM, achieve durable complete cytogenetic response, and it is possible to assess the minimal residual disease at the molecular level. After 6 years of an introduction of IM for CML treatment, some recommendations for IM therapy in CML patients have appeared, and criteria of CML patients who failed or responded suboptimally to IM are currently being evaluated [2]. Although there is clinical evidence for IM therapy, compliance regarding taking IM has not been fully determined [3,4]. This study aims to obtain more insight into the IM-compliance in CML patients in the chronic phase.

We evaluated 52 patients with CML in the chronic phase who were taking IM between 1 April 2006 and 31 March 2007. The IM-taking-compliance was calculated by the following formulation:

$$\text{The estimated IM-taking-compliance (\%)} = \left(\frac{\text{total dose of IM obtained at pharmacy}}{\text{total dose of IM prescribed at the hospital during the above period}}\right) \times 100.$$

We also denoted age, sex, the presence of prior therapeutic history, and period of IM therapy (separated into >1 year of IM therapy and <1 year). Data were statistically analyzed using Student's *t*-test and a *P*-value of less than 0.05 was considered to indicate a statistically significant difference.

Of the 52 CML patients treated with IM, there was no significant difference of IM-taking compliance by gender ($P=0.4820$), however, we noticed two male patients who had only 56.3 and 60.6%, respectively (Fig. 1A). When CML patients were separated into three age groups, no significant difference in IM-taking compliance was notable: $93.4 \pm 13.2\%$ in the group aged ≤ 40 years, $98.4 \pm 2.1\%$ in the group aged 41–60 years, and $95.9 \pm 8.2\%$ in the group aged ≥ 61 years (Fig. 1B). There were no significant differences of IM-taking compliance between those with or without prior therapeutic history ($P=0.4931$) (Fig. 1C), or between those with IM therapy period for less than 1 year or longer than 1 year ($P=0.2430$) (Fig. 1D). The estimated

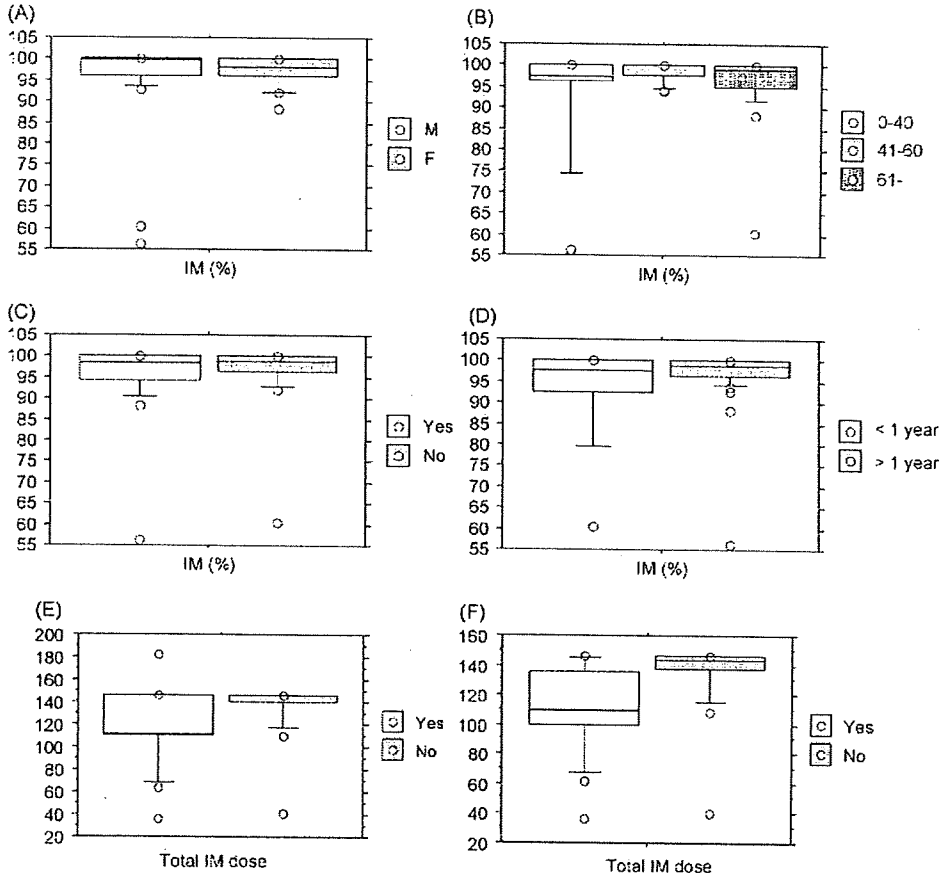

Fig. 1. Compliance of taking imatinib mesylate (IM) in chronic myeloid leukemia patients. Two male patients showing poor IM-compliance are notable. IM-taking compliance is separated by gender (A), age group (B), the presence of a prior therapy other than IM (C), or duration of IM treatment (D). IM (%): IM-taking compliance. Prescribed (E) and estimated (F) IM dose in chronic myeloid leukemia patients who had been treated for more than 1 year. A significant low dose in prescribed and estimated IM dose in patients with a prior therapeutic history other than IM (Yes), compared to those without prior therapy (No). Each *boxplot* and bar show the mean and 50^{th}, and 90^{th} percentiles of the distribution.

IM total dose during the indicated period did not significant different in gender ($P=0.3233$), age groups, and the presence of prior therapeutic history ($P=0.7847$). Since we noticed two CML patients with poor IM-taking compliance in this study, we statistically analyzed again, excluding these two patients. There were no significantly differences in gender ($P=0.2482$), age group, the presence of prior history ($P=0.3761$), or IM therapy duration ($P=0.3392$). The estimated IM total doses during the period did not significant different whether or not the presence of prior therapeutic history (109.45 g (299.9 mg/day) versus 112.64 g (308.6 mg/day); $P=0.7847$).

We next analyzed IM-taking-compliance in 40 CML patients who had been treated with IM for ≥1 year, excluding one poor IM-compliance patient. There were no significant difference in gender ($P=0.8881$), age group, the presence of prior therapeutic history ($P=0.0963$). However, there was a significantly lower prescribed IM dose (112.62 g (308.5 mg/day) versus 136.23 g (373.2 mg/day); $P=0.0136$) (Fig. 1E) and estimated intake of IM (109.45 g (299.9 mg/day) versus 134.47 g (368.4 mg/day); $P=0.0079$) (Fig. 1F) in CML patients with a prior therapeutic history than those without a prior therapy. The recommended IM dose (≥400 mg/day) was prescribed in 15/20 CML patients without a prior therapeutic history of any drug other than IM, while only 6/20 patients with prior history achieved the recommended IM dose ($P=0.0044$), indicating that a significant number of CML patients with a prior therapeutic history did not receive a suitable IM dose, possibly due to hematologic suppression as a result of previous treatment.

One CML patient (diagnosed in 1999) who had a history of interferon-α (IFN) treatment was given 400 mg of IM beginning in December 2001. He obtained complete molecular remission in April 2005. In August 2006, he decided to reduce the IM dose and we noticed this by the increasing amount of BCR-ABL copy numbers in his blood. Guidance to take an adequate IM dose resulted in re-induction of molecular remission. Another patient with poor IM-compliance was due to complications accompanying underlying Diabetes Mellitus and renal failure.

IM represents a major advance in the treatment of patients with CML. Recently, however, some reports of poor IM

therapy compliance have appeared [3,4]. Although the number of CML patients in this survey is small, some CML patients were found to have poor compliance. Therefore, it is essential to consider IM-compliance in managing CML patients, since discontinuation or dose-reduction of IM by CML patients, unknown to the physician, resulted in underestimating IM responsiveness. Branford et al., investigating CML patients with doubling of BCR-ABL transcripts during IM treatment, reported that the occurrence of BCR-ABL mutation and relapse rate increased significantly [5]. Thus, it is essential to monitor BCR-ABL transcripts in CML patients. In this study, we found one patient with poor IM-compliance who had molecular relapse after 2 months. This case suggests that when we notice an increasing level of BCR-ABL transcripts in molecularly stable CML patients receiving IM treatment, we must consider IM-compliance first, before investigating other aspects conceiving possible relapse of the disease.

Comparing patients with or without a prior therapeutic history, e.g., interferon, we noticed that there was a significantly lower prescribed IM dose in the former group. We hypothesize that prior therapy, including hydroxyurea and/or interferon, may have induced hematologic adverse events preventing prescription of an optimal IM dose. Be that as it may, the IM-compliance of CML patients receiving IM for more than 1 year did not significantly differ between those with or without prior therapy ($97.4 \pm 3.3\%$ versus $98.7 \pm 1.5\%$; $P = 0.0963$); thus although clinicians may hesitate to increase the IM dose to a level close to the optimal recommended dose, most patients do take the prescribed IM dose.

Conflict of interest statement

We declare that we have no conflict of interest.

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Cough mixture abuse, folate deficiency and acute lymphoblastic leukemia

Cough mixture abuse is an emerging substance abuse problem in Asia. Up to 40% of abusers suffer from folate deficiency, and it can lead to peripheral neuropathy and megaloblastic anemia [1,2]. Open neural tube defects also occurred in infants of female cough mixture abusers [3]. We report the first cases of acute leukemia in this population.

Case one: A 20-year-old man presented with precursor B cell acute lymphoblastic leukemia (ALL), with positivity to Tdt, CD10, CD19, CD22, CD79a, CD34 and HLA-DR and aberrant expression of CD13 and CD33. Cytogenetic study showed complex karyotype (46, XY, add(1)(q43), add(9)(q34), add(21)(q22) [12]). The disease remained refractory to hyper-CVAD treatment and he suffered from recurrent septicemia. A hemopoietic stem cell transplantation (HSCT) from an unrelated donor was planned in partial remission. At HSCT assessment, he volunteered history of regular cough mixture abuse (500 ml daily × 3 years) with occasional ketamine and cocaine usage. Dental examination showed moderate dental carries. A remission was achieved after HSCT but disease relapsed 6 months later. A second HSCT was planned.

Case two: A 25-year-old man presented with cervical lymphadenopathy and anemia. A marrow showed pre-B ALL, positive for CD10, CD19, CD22, CD79a, CD34, Tdt, but

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PRECLINICAL STUDIES

Development and pharmacologic characterization of deoxybromophospha sugar derivatives with antileukemic activity

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Summary Here, we synthesized two phospha sugar derivatives, 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TMPP) and 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DMPP) by reacting 3-methyl-1-phenyl-2-phospholene 1-oxide with bromine, and investigated their potential as antileukemic agents in cell lines. Both agents showed inhibitory effects on leukemia cell proliferation, with mean IC_{50} values of 6.25 μmol/L for TMPP and 23.7 μmol/L for DMPP, indicating that inhibition appeared to be dependent on the number of bromine atoms in the structure. Further, TMPP at 10 μmol/L and DMPP at 20 μmol/L induced G2/M cell cycle block in leukemia cells, and TMPP at 20 μmol/L induced apoptosis in these cells. TMPP treatment effected a reduction in both cell cycle progression signals (FoxM1, KIS, Cdc25B, Cyclin D1, Cyclin A, and Aurora-B) and tumor cell survival ($p27^{Kip1}$ and $p21^{Cip1}$), as well as induced the activation of caspase-3 and -9. Further, treatment with TMPP significantly reduced the viability of AML specimens derived from AML patients, but only slightly reduced the viability of normal $ALDH^{hi}$ progenitor cells. We also observed that FoxM1 mRNA was overexpressed in AML cells, and treatment with TMPP reduced FoxM1 mRNA expression in AML cells. Here, we report on the synthesis of TMPP and DMPP and demonstrate that these agents hinder proliferation of leukemia cells by FoxM1 suppression, which leads to G2/M cell cycle block and subsequent caspase-3-dependent apoptosis in acute leukemia cells. These agents may facilitate the development of new strategies in targeted antileukemic therapy.

Keywords Leukemia · Deoxybromophospha sugar · Cell cycle · Apoptosis

Abbreviations

TMPP	2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide
DMPP	2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide
AML	acute myeloid leukemia
ALDH	Aldehyde Dehydrogenase
FoxM1	The forkhead box M1
CFU-GEMM	colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte

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CFU-GM	colony forming unit-granulocyte, macrophage
BFU-E	burst forming unit-erythroid

Introduction

Phosphorus compounds perform vital functions in the growth, sustenance, and reproductive processes of all living organisms. Organophosphorus compounds in particular have been found to possess potential applications in both life sustaining and life extinguishing processes. These characteristics suggest the possible clinical use of these compounds as drugs with lower toxicity yet higher efficacy than existing drugs.

We previously synthesized pentofuranose analogs of phospha sugars from phospholenes as potentially bioactive agents which structurally resemble AZT or ribavirin [1, 2]. Phospha sugars are analogs of normal sugars in which the central oxygen atom of the hemiacetal ring has been replaced by a phosphorus atom. Replacement of the oxygen atom in the hemiacetal ring of normal sugars by a carbon or heteroatom leads to the formation of pseudo-sugars, several of which have been heavily investigated in the fields of synthetic, biological, and medical chemistry [3]. Novel nucleoside derivatives of pseudo- or hetero-sugars reported to date include *aza*-sugar (nitrogen instead of an oxygen atom; amino sugar) [4–7], *thio*-sugar (or *thia*-sugar; sulfur instead of an oxygen atom) [8, 9], and *carba*-sugar (oxygen atom replaced by a methylene group) [10, 11]. Further, the potential bioactivity of hetero-sugar nucleosides and glycosides (e.g., glycosidase and nojirimycin) has also been reported [12].

Given this potential for bioactivity, phospha sugar chemistry is one of the most rapidly developing areas of research [15–18]. One report has suggested that acetyl derivatives of the glucopyranose pattern of phospha sugar have potential as anticancer agents [19]. *Carba*-sugar derivatives are known to be effective in hampering some enzyme activity [10, 11]. Given that *aza*-sugar compounds are known to influence carbohydrate processing in the human body, extensive, on-going research and development using the compound has been employed to combat virus infection, cancer, and tuberculosis [13, 14]. Against this background, we synthesized 2,3-dibromo-3-methyl-1-phenylphosholane 1-oxide (DMPP) and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TMPP).

Acute myeloid leukemia (AML) is characterized by the excess production of leukemic blasts arrested at various stages of granulocytic and monocytic differentiation, and it is this stage which determines the AML subtype (French-American-British [FAB] classification, AML: M1 to M5) [20]. To effectively cure a patient with AML, this proliferation of leukemic cells must be halted. Given that chemotherapy rarely eradicates the leukemic clones, efforts are now being made to find innovative new therapies which inhibit the proliferation of AML cells [21]. However, the effect of cell cycle progression and apoptosis resistance on the pathogenesis of AML remains to be defined.

In the present study, we investigated the antileukemic effect of the phospha sugar derivatives TMPP and DMPP in regulating proliferation and apoptosis in a series of leukemic cell lines, and in AML patient samples.

Materials and methods

Chemical synthesis

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (DMPP) was prepared by reacting 3-methyl-1-phenyl-2-phospholene 1-oxide with bromine in the presence of manganese dioxide. 2,3,4-Tribromo-3-methyl-1-phenylphospholane 1-oxide (TMPP) was prepared similarly by reacting 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide with bromine. The reaction mixtures were extracted with chloroform, washed with saturated NaCl solution, and dried with anhydrous sodium sulfate. These reaction agents were then dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St Louis, MO) and diluted in culture medium immediately before use. The final concentration of DMSO in all experiments was less than 0.01%, and all treatment conditions were compared with vehicle controls.

Cells and cell cultures

Human leukemia cell lines HL60, NB4, U937, NOMO-1, CEM, MOLT4, SUP-B15, K562, and Meg-01 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). YRK2 and SHG3 cells were harvested in our laboratory from bone marrow samples of AML (M5a) and CML patients. Samples of these cells, except for SUP-B15 cells, were cultured in RPMI 1640 media containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL, Gaithersburg, MD). SUP-B15 cells were cultured in RPMI 1640 containing 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL).

Primary samples

This study analyzed six adult patients (age range, 34 to 78 years) with M1 (n=21), M2 (n=56), M4 (n=32) and M5 (n=18) leukemia (FAB classification). Primary leukemia

cell specimens were obtained from patients before the start of any treatment, and normal hematopoietic cells (including bone marrow and peripheral blood mononuclear cells) were extracted from healthy donors after obtaining informed consent. Mononuclear cells (MNCs) were purified by Ficoll-Hypaque density-gradient centrifugation, and any remaining erythrocytes in the neutrophil pellet were removed by hydrolysis. The MNCs were then counted, and viability was determined by trypan blue exclusion.

Cell proliferation assay

Cells were seeded in 24-well flat-bottomed microplates at a density of 3×10^4 per well and incubated at various concentrations of TMPP or DMPP for 5 days. The cells were then washed with phosphate-buffered saline (PBS), harvested, and suspended in a 0.4% trypan blue solution for trypan blue exclusion assay, in which viable cells were counted with a hemocytometer at the indicated incubation day. For the MTT assay, the cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^5 per well. The cells were incubated at various concentration of TMPP or DMPP for 24 h. After incubation, 10 μl 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) was added to each well at a final concentration of 1 mg/ml. After incubation at 37°C for 4 h, absorbance was measured at a wavelength of 560 nm using a microplate reader.

Cytotoxic assay

Cytotoxic activity of TMPP and DMPP against various leukemia cells (IC_{50} value) was determined. Cells were seeded in triplicate at 5,000 to 10,000 per well in 24-well plates. The day after plating, TMPP or DMPP were added to the wells by titrating over six or more dilutions from 10 to 1,000 μmol/L. Control cells without treatment were seeded as well. The cells were then grown for five days, and viable cells were counted. After washing with PBS, cells were placed in isotonic solution and counted immediately. IC_{50} was determined by a sigmoidal dose-response calculation (XLFit 3, IDBS, Inc., Emeryville) and represented the concentration of TMPP or DMPP that produced 50% of maximum response.

Cell cycle analysis

Propidium iodide (PI) (Sigma Chemical Company, St. Louis, MI) staining was used to analyze DNA content. TMPP- or DMPP-treated cells were cultured at 37°C in 2 ml of complete medium containing 1×10^6 cells. After incubation for 48 h, the cells were washed twice with cold PBS, fixed with 70% ethanol overnight, treated with 100 μg/ml RNase A, and then stained with 50 μg/ml PI. For apoptosis analysis, the relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France). The percentage of cells in the apoptotic sub-G1 phase, as well as G1, S, and G2/M phases, was calculated using the ModFit program (Becton, Dickinson and Company, San Jose, CA).

Western blotting

The TMPP-treated leukemia cells were harvested, washed with cold PBS, and resuspended in lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, and 1 mM dithiothreitol supplemented with one Complete Mini protease inhibitor tablet (Boehringer Mannheim GmBH, Indianapolis, IN) per 20 ml lysis buffer immediately before use. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked with 0.5% milk in PBS for 1 h at room temperature. After being washed in Tris-buffered saline Tween (TBS-T), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences Inc., Arlington Heights, IL) for 1 h and exposed to X-ray film at room temperature. The signal was detected by chemiluminescence using an ECL detection kit (Amersham Bioscience Inc.). The following commercially available antibodies and dilutions were used for western blotting: rabbit polyclonal anti-FoxM1 antibody (MPP2 K-19, 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-$p27^{Kip1}$ antibody (1:1000) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-$p21^{Cip1}$ antibody (1:1000) (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-Cdc25B2 antibody (1:500) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Cyclin D1 antibody (1:500) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Cyclin A antibody (1:500) (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-KIS antibody (1:500) (ABGENT, Inc., San Diego, CA), rabbit polyclonal anti-Aurora-B antibody (1:500) (ABGENT, Inc.), mouse monoclonal anti-bcl-2 antibody (BD Biosciences Pharmingen, San Diego, CA), mouse monoclonal anti-caspase-9 antibody (BD Biosciences Pharmingen), mouse monoclonal anti-caspase-3 (CPP32) antibody (BD Biosciences Pharmingen), and mouse monoclonal anti-PARP antibody (BD Biosciences Pharmingen). To ensure equal protein volume loading, similar experiments were performed using a mouse monoclonal anti-actin antibody (C-4; ICN Biomedicals, Inc., Aurora, OH) as an internal control.

Immunofluorescent staining

U937 cells were cytocentrifuged onto glass slides, fixed in 4% paraformaldehyde, premeabilized in 0.5% Triton X-100, stained with anti-FoxM1 rabbit polyclonal antibody (Santa Cruz, CA), and incubated with fluorescence-linked anti-rabbit immunoglobulin G (Santa Cruz, CA). The cells were viewed by phase-contrast or fluorescence microscopy (IMT-2; OLYMPUS, Tokyo, Japan).

Isolation of primitive hematopoietic cells by Aldehyde Dehydrogenase (ALDH) activity

Following the purification process, the MNCs were further fractionated according to ALDH activity by staining with Aldefluor reagent (StemCo Biomedical, Inc., Durham, NC) according to the manufacturer's specifications. Aldefluor substrate (0.625 μg/mL) was added to between 2 and 7×10^6 cells/mL suspended in proprietary Aldefluor assay buffer, then incubated for 20 to 30 min at 37°C to allow the conversion of Aldefluor substrate to a fluorescent product. The amount of intracellular fluorescence was measured by flow cytometry. $ALDH^{hi}$ cells were selected by FACS (Becton, Dickinson and Company).

Viability of AML and $ALDH^{hi}$ cells with regard to TMPP administration, and expression of FoxM1 mRNA

AML cells were obtained from PB or BM of AML patients, and normal $ALDH^{hi}$ progenitor cells were obtained from BM of healthy volunteers. The AML and normal $ALDH^{hi}$ cells were untreated or treated with TMPP at concentrations ranging from 0.25 to 16 μmol/L. To determine cell number, AML cells and $ALDH^{hi}$ progenitor cells were treated with TMPP, and any nonviable cells were identified after 3 min of staining with 0.4% trypan blue (Sigma). The number of unstained (nonviable) cells was counted using a hemocytometer.

Quantification of FoxM1 mRNA in AML and $ALDH^{hi}$ cells

AML cells were obtained from PB or BM of AML patients, and normal $ALDH^{hi}$ progenitor cells were obtained from BM of healthy volunteers. The AML and normal $ALDH^{hi}$ cells were untreated or treated with TMPP at 5 μmol/L for 48 h. Total RNA was extracted from these cells using an RNeasy system (Quiagen, Tokyo, Japan), and 2 μg RNA was reverse transcribed using a 1st strand cDNA synthesis kit (Roche, Indianapolis, IN). Oligonucleotide sequences for each primer were as follows: FoxM1, sense 5'-GGGCGCACGGCGGAAGATGAA-3', antisense 5'-CCACTCTTCCAAGGGAGGGCTC-3'; and G3PDH; sense 5'-GAACGGGAAGCTCACTGGCATGGC-3', antisense 5'-TGAGGTCCACCACCCTGTTGCTG-3'. In each experiment, RT-PCR was performed in duplicate. The real-time RT-PCR was performed using SYBER-Green dye on an ABI PRISM 7700 Sequence detector (Perkin-Elmer/Applied Biosystems, Foster City, CA). For real time using SYBER-Green, dissociation curve was obtained for melting curve analysis to confirm PCR product specificity.

Statistical analysis

Experiments were repeated at least three times and results were combined and represented graphically as the mean values ± the standard deviation (SD). The significance of the *in vitro* results was determined using Student's *t* test.

Results

In vitro antiproliferative effects of TMPP/DMPP in leukemia cells

Antiproliferative effects of TMPP and DMPP (Fig. 1) were tested in U937 and YRK2 cells (Fig. 2). Both cell lines showed a significant reduction in cell proliferation over the time course examined at 10 μmol/L TMPP or 20 μmol/L DMPP. U937 cells treated with DMPP and TMPP showed a growth inhibition of 14.8% and 5.6%, respectively, at 3 days' treatment (Fig. 2(a)). YRK2 cells treated with DMPP and TMPP showed a growth inhibition of 19.7% and 2.8%, respectively, at the same time point (Fig. 2(b)). Cytotoxic effects of TMPP and DMPP were tested in several human

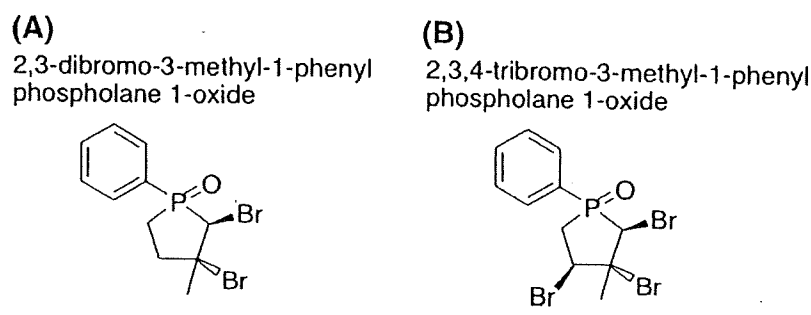


Fig. 1 Chemical structure. Chemical structure of DMPP (a) and TMPP (b)

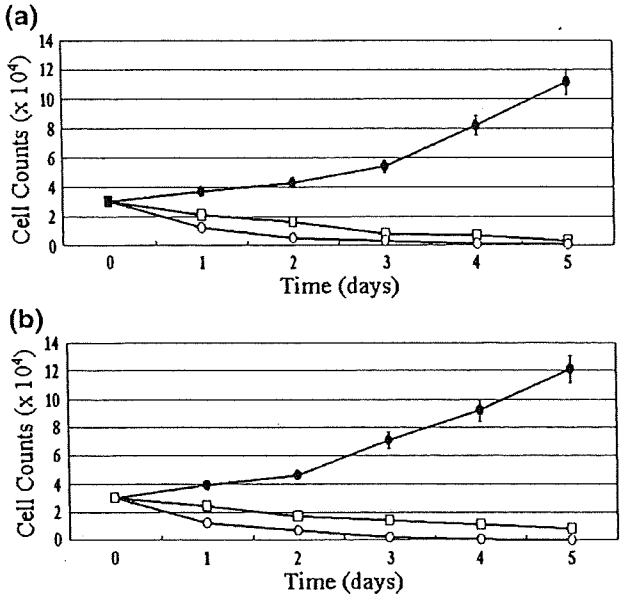


Fig. 2 Effect of TMPP/DMPP on leukemia cell proliferation. U937 (a) and YRK2 (b) leukemia cells were either untreated (•), treated with 10 μmol/L TMPP (○), or treated with 20 μmol/L DMPP (□) for 5 days. After incubation, the cells were harvested and counted. Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments

leukemia cell lines (HL60, NB4, U937, YRK2, NOMO-1, CEM, MOLT4, SUP-B15, K562, Meg-01, and SHG3) (Table 1). Results showed that the effects of TMPP were three to five times stronger than DMPP with regard to IC_{50} value. Interestingly, the strength of the effect depended on the number of bromine atoms introduced into the molecule.

Cell cycle analysis in leukemia cells treated with TMPP/DMPP

As shown in Fig. 3(a), treatment of U937 cells with 10 μmol/L TMPP resulted in an increase in the number of cells in the G2/M and S phases, and a slight decrease in the number in the G1 phase, in a time-dependent manner as measured by flow cytometry. Treatment of U937 cells with 20 μmol/L DMPP showed similar results. However, TMPP more strongly induced cell cycle arrest than DMPP, which is consistent with the inhibition pattern observed by MTT assay. Further, treatment of the U937 cells with 10 μmol/L TMPP for 40 h resulted in a marked accumulation of cells in the G2/M phase, and a concentration of 20 μmol/L TMPP significantly increased the number of apoptotic cells (Fig. 3(b)). The number of cells in the G2/M phase increased to 1.5 to 2 times that of the control level in all leukemia cell lines following treatment with 10 μmol/L TMPP (Fig. 3(c)), and the apoptotic fraction increased from 72±3% to 83±2% following treatment with 20 μmol/L TMPP compared to control levels (Fig. 3(d)). These findings indicate that exposure of leukemia cells to TMPP leads to G2/M block of cell cycle progression at a concentration of 10 μmol/L and to apoptosis at 20 μmol/L.

TMPP-induced cell cycle arrest and apoptosis in leukemia cells

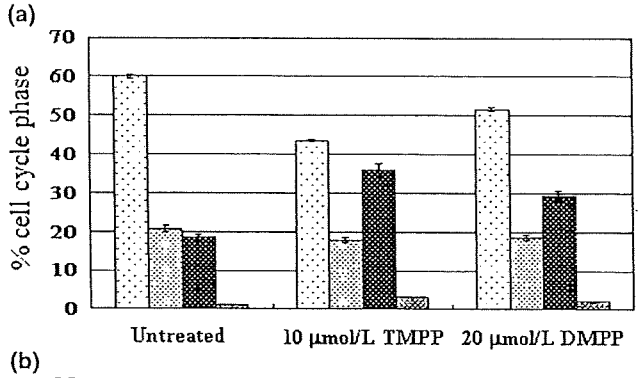
Given our previous finding (in press) that FoxM1 regulates transcription of essential cell cycle regulatory genes, we examined whether exposure of leukemia cells to TMPP affected expression of cell cycle regulators such as FoxM1, KIS, Cdc25B, $p27^{Kip1}$, $p21^{Cip1}$, Cyclin D1, Cyclin A, and Aurora-B kinase. Protein extracts were isolated from U937 and YRK2 cells treated with TMPP, and western blot analysis was performed to measure protein levels of these cell cycle regulators (Fig. 4(a)). Results showed that TMPP treatment reduced FoxM1, KIS, Cdc25B, Cyclin D1, Cyclin A and Aurora-B protein levels in a dose-dependent manner in U937 and YRK2 cells compared to untreated leukemia cells. However, exposure to TMPP actually increased levels of $p27^{Kip1}$ and $p21^{Cip1}$ protein. These results indicate that TMPP regulates the expression of cell cycle regulatory proteins.

We also examined the effect of 48-h treatment with TMPP in U937 and YRK2 cells in relation to expression of several apoptotic regulatory proteins. As shown in Fig. 4(b), the treatment of leukemia cells with TMPP resulted in reduction of BCL-2 protein expression in a dose-dependent manner. Further, with regard to caspase-9 and -3, treatment with TMPP induced significant cleavage of procaspase-9 and -3 in a dose-dependent manner. Moreover, TMPP treatment increased the levels of the cleaved form of PARP in a dose-

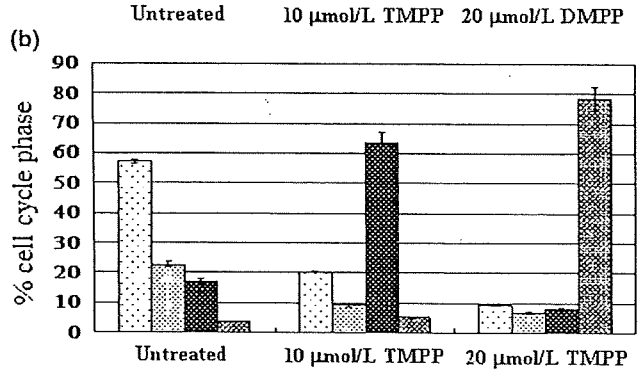
Table 1 TMPP/DMPP inhibition of AML cell proliferation

Cell lines	TMPP IC_{50}(μmol/L)	DMPP IC_{50}(μmol/L)
HL60	4.8±0.7	18±2.3
NB4	3.2±0.9	15±1.4
U937	6.2±1.1	22±1.8
YRK2	5.3±1.3	28±2.6
NOMO-1	5.5±0.8	18±2.1
CEM	6.9±0.3	29±2.4
MOLT4	6.7±1.2	26±1.8
SUP-B15	7.1±1.0	24±2.8
K562	9.1±0.8	28±1.7
Meg-01	8.6±1.4	27±1.9
SHG3	5.4±0.6	26±2.1

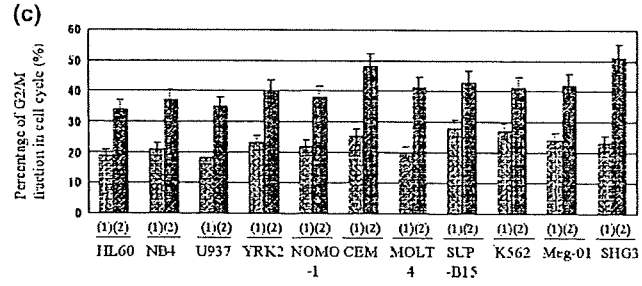
The cytotoxic activity of TMPP and DMPP. IC50 values were determined for each cell line. Data are shown as mean ± S.D. in triplicate culture and are representative of three independent experiments



(%)	Untreated	10 μM TMPP	20 μM DMPP
G1	60.1 ± 1.7	43.6 ± 1.2	51.5 ± 1.3
S	20.6 ± 2.1	17.7 ± 1.1	18.6 ± 1.2
G2/M	18.5 ± 1.1	35.9 ± 2.5	29 3 ± 1.5
Apoptosis	1.1 ± 0.4	3.2 ± 0.5	2.1 ± 0.4



(%)	Untreated	10 μM TMPP	20 μM TMPP
G1	57.2 ± 2.3	20.4 ± 2.6	9.2 ± 2.4
S	22.7 ± 1.9	9.2 ± 2 9	6.9 ± 0.8
G2/M	17.2 ± 1.8	63.7 ± 3.2	8.1 ± 2.2
Apoptosis	3.7 ± 1.3	5.3 ± 1.7	78.2 ± 6.1



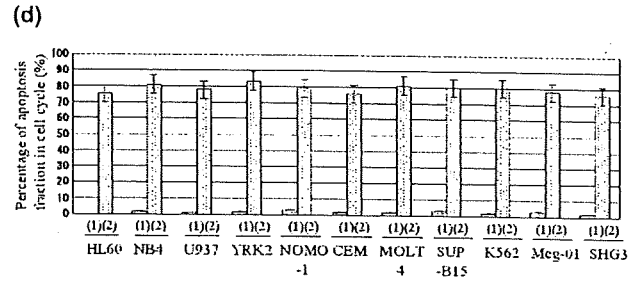


Fig. 3 Effect of TMPP/DMPP on cell cycle and apoptosis of leukemia cells. Cell cycle analysis of U937 cells. FACS analysis was performed on U937 cells treated with 10 μmol/L TMPP or 20 μmol/L DMPP for 16 h (a). FACS analysis was then performed on U937 cells treated with 10 and 20 μmol/L TMPP for 40 h (b). The G2/M (c) and apoptosis (d) fractions were analyzed in all leukemia cell lines treated with 10 and 20 μmol/L TMPP for 16 h and 40 h, respectively. Bars represent untreated cells (1) and cells treated with TMPP (2). Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments

dependent manner. In contrast, TMPP treatment did not affect procaspase-8 levels (data not shown). These results indicate that treatment of leukemia cells with TMPP induced down-regulation of anti-apoptotic proteins and was associated with the activation of caspase cascades.

Localization of FoxM1 in leukemia cells

As shown in Fig. 5(a), U937 cells were inhibited the proliferation by TMPP-dose-dependent manner. Interestingly, immunofluorescent staining in U937 cells revealed that FoxM1 was constitutively present in the nucleus. TMPP treatment significantly attenuated the cytoplasmic signals, and inhibited the transfer of FoxM1 protein from cytoplasm to nucleus (Fig. 5(b)). Moreover, FoxM1 expression in cytoplasm was reduced by TMPP-dose dependent manner (Fig. 5(c)). These findings showed that TMPP regulated the subcellular localization of FoxM1.

TMPP inhibited AML cells derived from AML patients

AML cells from PB or BM donated by AML patients accounted for between 78% and 92% of MNCs, and hematopoietic progenitor cells from bone marrow donated by healthy volunteers were obtained according to ALDH activity using the Aldefluor substrate and FACS. Elevated ALDH expression is an intrinsic property in many types of stem cells. Murine xenotransplantation models have shown that cells with elevated ALDH activity are able to repopulate *in vivo* [22]. $ALDH^{hi}$ hematopoietic progenitor cells, including $CD34^{+}$, $CD133^{+}$, c-kit^{+}, and Lin^{-} cells, were selected according to side scatter (SSC) and FITC

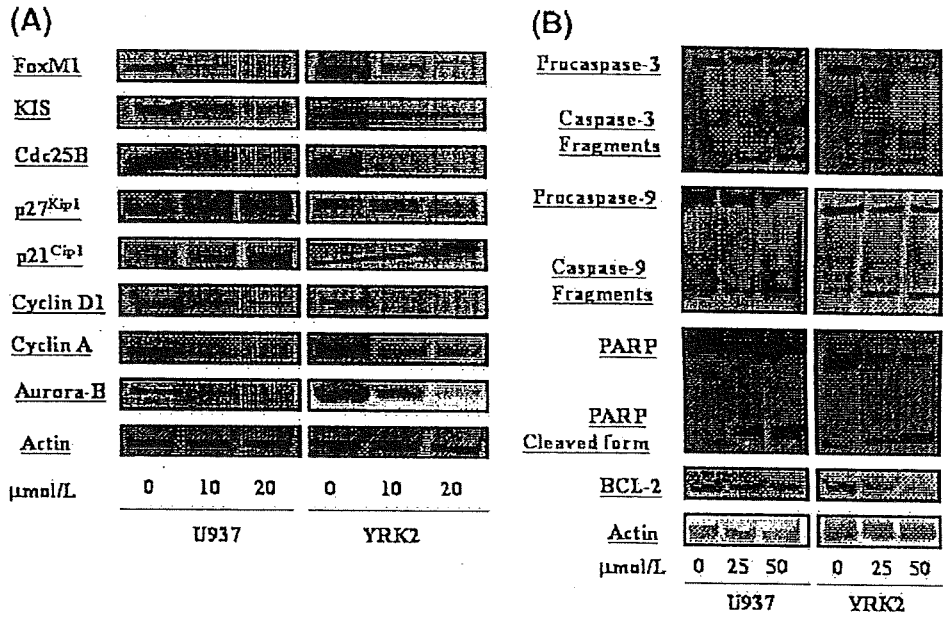


Fig. 4 Effect of TMPP treatment on cell cycle and apoptosis-related proteins. Western blot analysis was performed on TMPP-treated and untreated cells to investigate the expression of cell cycle-related proteins (a). Western blot analysis was performed on TMPP-treated and untreated cells to investigate the expression of apoptosis-related proteins (b)

properties. The $ALDH^{hi}$ selected populations in healthy volunteers represented 1.24±0.27% of MNCs. With regard to AML cells from AML patients (M2: AML #1, #2, #5; M4: AML #6, #8), cell viability was reduced by TMPP in a dose-dependent manner (Fig. 6(a)), particularly at a molarity of 4 μM. In contrast, the viability of $ALDH^{hi}$ cells obtained from healthy volunteers was only slightly reduced by TMPP (Fig. 6(b)). Moreover, high levels of FoxM1 mRNA were detected in all (127/127) primary leukemia specimens tested, and the mean ratios of FoxM1 to G3PDH in AML specimens were 1.72±0.48 (M1; 1.82, M2; 1.95, M4; 1.62, M5; 1.47). In contrast, the mean ratio of FoxM1 to G3PDH in normal $ALDH^{hi}$ cells was 1.12± 0.15. These results demonstrated that increased levels of FoxM1 mRNA were detected in nearly all of these samples from various leukemia specimens compared to normal $ALDH^{hi}$ cells (Fig. 6(c)). To assess the expression of FoxM1 in clinical specimens treated with TMPP, AML cells from AML patients (M2: AML #1, #2, #5; M4: AML #6, #8) and normal $ALDH^{hi}$ cells were treated with TMPP

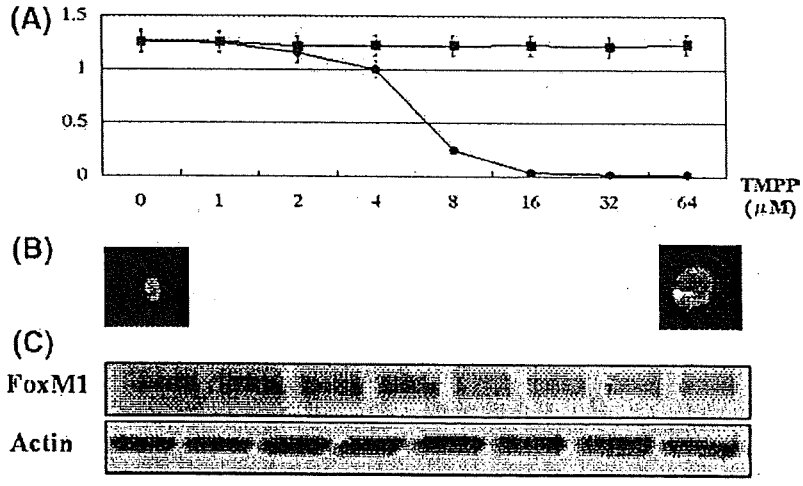


Fig. 5 TMPP inhibited leukemia cell proliferation through the regulation of cytoplasmic localization of FoxM1. (a) U937 cells were untreated (■) and treated with TMPP (●) at the indicated concentration for 72 h. The cell proliferation was measured by MTT assay. Data shown as mean ± S.D. in triplicate culture and are representative of three independent experiments. (b) U937 cells were treated with TMPP at the indicated concentration for 24 h, cytocentrifuged, and fixed with 4% paraformaldehyde for 10 min. The fixed cells were immunostained using antibody of FoxM1. Control is the left panel (fluorescence image). The treatment with 64 μmol/L TMPP is the right panel (fluorescence image). Original magnification × 400. (c) The changes of FoxM1 protein expression was evaluated by TMPP-dose dependent manner in U937 cells

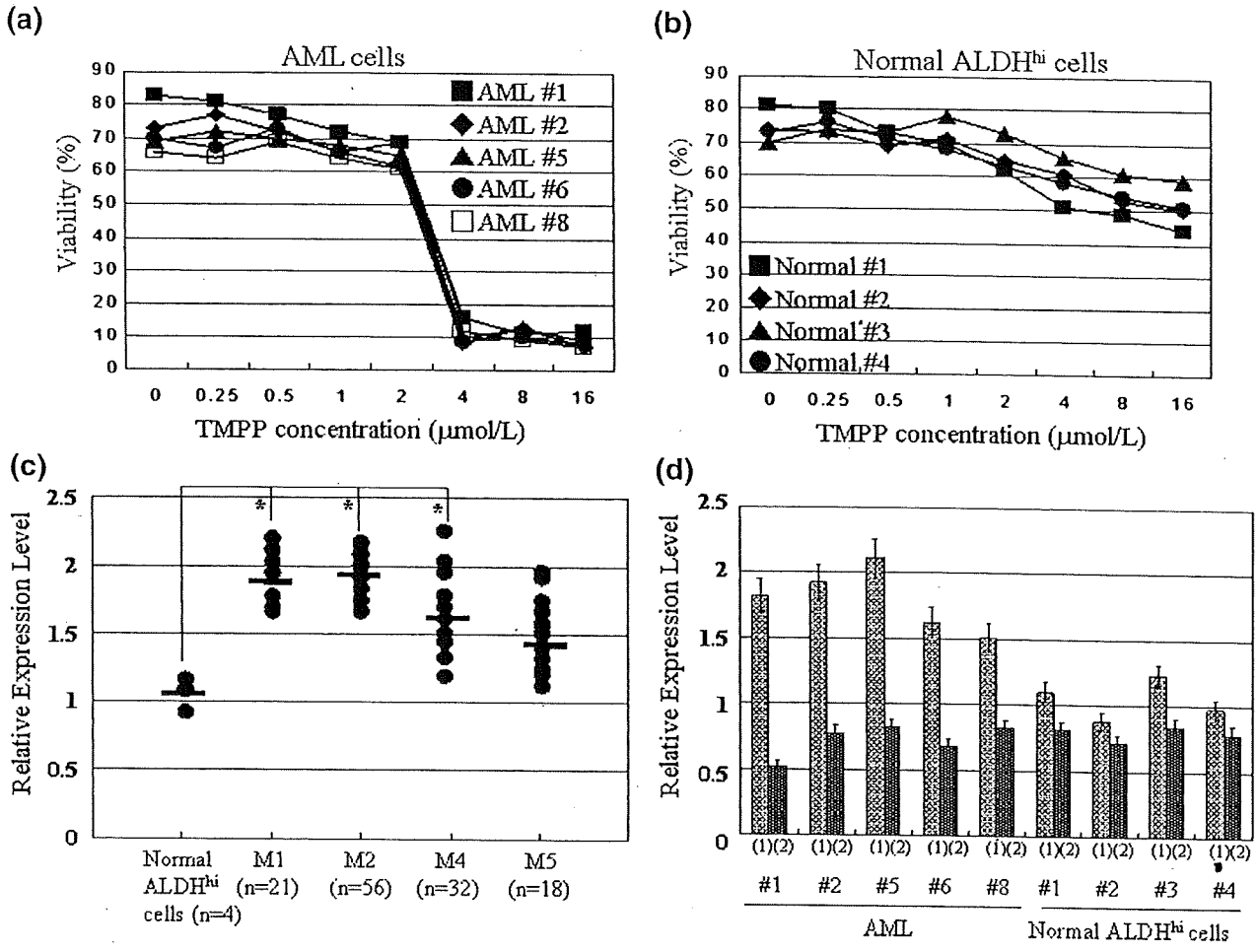


Fig. 6 Effect of TMPP treatment on AML cells and ALDH^{hi} hematopoietic progenitor cells, and effect of overexpression of FoxM1 on AML cell proliferation. AML cells derived from AML patients (M2; #1, #2, #5, M4; #6, #8) (a) and ALDH^{hi} cells derived from healthy volunteers (n=4) (b) were treated with TMPP at the indicated concentrations for 48 h. (c) FoxM1 mRNA expression levels in primary AML cells. (d) AML cells derived from AML patients (M2; #1, #2, #5, M4; #6, #8) were treated with 5 μM TMPP for 48 h. After treatment, the cells were harvested and Quantitative RT-PCR was performed for measurement of FoxM1 mRNA. (1) untreated, (2) treated with TMPP. Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments. *P<0.05 comparing FoxM1 mRNA expression

(Fig. 6(d)). FoxM1 expression in AML cells showed mean 28.6% (#1), 40.2% (#2), 38.9% (#5), 42.0% (#6), and 54.3% (#8) inhibition, relative to untreated cells. In contrast, the normal ALDH^{hi} cells showed mean 75.8± 6.75% expression inhibition at 5 μM TMPP for 48 h. These results indicate that FoxM1 mRNA were overexpressed in acute leukemia specimens, and reduction of FoxM1 mRNA by TMPP treatment inhibited cell proliferation.

Discussion

Here, we synthesized two phospha sugar derivatives, TMPP and DMPP, and evaluated their antileukemic effects in AML cell lines, AML cells derived from AML patients, and normal hematopoietic progenitor cells *in vitro*. Results showed that both TMPP and DMPP suppressed the proliferation of leukemia cells by downregulation of FoxM1, FoxM1, which is a member of the forkhead box family, is required for coupling DNA replication with mitosis, expression, at least in part, leading to G2/M cell cycle block and subsequent caspase-3-dependent apoptosis. Further, we identified FoxM1 as a target molecule of therapeutic regimens for leukemia patients. Relative to other therapeutic agents, TMPP/DMPP represent a structurally distinct type of small-molecule agent with unique mechanistic features.

TMPP and DMPP were found to have antileukemic effects in AML cell lines (mean IC_{50} 6.25 μmol/L and 23.7 μmol/L, respectively). In the MTT assay, TMPP more strongly inhibited the proliferation of leukemia cells than DMPP, an effect attributable to TMPP having more bromine atoms than DMPP. Further, concentrations of 10 μmol/L TMPP and