

# Methylation of PLK-1 Potentially Drives Bendamustine Resistance in Leukemia Cells

Toshikazu Itabashi<sup>1</sup>, Takahiro Ueda<sup>1</sup>, Ryohei Fukunaga<sup>1</sup>,  
Takeshi Asano<sup>2</sup> and Yasuhiko Itoh<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Nippon Medical School, Tokyo, Japan

<sup>2</sup>Department of Pediatrics, Nippon Medical School Chiba Hokusoh Hospital, Chiba, Japan

**Background:** Drug resistance remains a significant impediment in leukemia treatment. While Bendamustine hydrochloride (BH) stands out as a promising therapeutic agent for non-Hodgkin's lymphoma and mantle cell lymphoma, the mechanisms of resistance to BH are not yet fully understood. Our study focuses on elucidating the mechanisms behind bendamustine resistance in leukemia cells, with a specific emphasis on epigenetics.

**Methods:** Bendamustine-resistant cells were cultivated from human B cell lymphoblastic leukemia cell lines through systematic and sustained exposure to bendamustine, using the limiting dilution method. Gene expression was assessed via real-time polymerase chain reaction, while the expression of the multidrug resistance protein 1 (MDR1) was evaluated using flow cytometry.

**Results:** Bendamustine-resistant leukemia cells exhibited a decreased RNA expression level for Polo-like kinase-1 (PLK-1). Notably, after treatment with the demethylating agent 5-aza-2'-deoxycytidine, PLK-1 gene expression surged significantly, enhancing bendamustine's cytotoxicity in the resistant leukemia cells. However, MDR1 expression, as determined by flow cytometry, remained consistent between parental and bendamustine-resistant leukemia cells.

**Conclusions:** Our findings indicate that the methylation of the PLK-1 gene plays a pivotal role in modulating PLK-1 expression and is central to the development of bendamustine resistance in leukemia cells. (J Nippon Med Sch 2024; 91: 162–171)

**Key words:** bendamustine hydrochloride, leukemia, drug resistance, PLK-1, MDR1

## Introduction

Drug resistance remains a significant challenge in leukemia treatment. Compared to children with drug-sensitive leukemia cells, those with cells exhibiting *in vitro* resistance to antileukemic agents consistently display a markedly poorer prognosis<sup>1,2</sup>. Bendamustine hydrochloride (BH) has emerged as a pivotal therapeutic agent in the management of non-Hodgkin's lymphoma (NHL) and mantle cell lymphoma (MCL). BH's anti-lymphoma properties stem from intricate, intersecting mechanisms. These include inducing apoptosis via a DNA damage stress response, inhibiting mitotic checkpoints, and triggering mitotic catastrophe<sup>3,4</sup>. Because of these overlapping mecha-

nisms, BH presents only partial *in vitro* cross-resistance with other alkylators<sup>5</sup> and retains robust antitumor efficacy in anthracycline-resistant and platinum-drug resistant cell lines<sup>6</sup>.

Clinically, the combination of BH and rituximab enhances progression-free survival (PFS) and demonstrates fewer toxic effects than the conventional R-CHOP regimen (Rituximab (RIT), cyclophosphamide (CPA), doxorubicin (DOX), and vincristine (VCR)) in patients with untreated MCL<sup>3</sup>. BH also shows therapeutic efficacy in patients with recurrent NHL, even those resistant to other alkylating agents and purine analogs<sup>3,4</sup>. Yet, some patients still experience relapse, and the mechanisms driving re-

Correspondence to Takeshi Asano, Department of Pediatrics, Nippon Medical School Chiba Hokusoh Hospital, 1715 Kamagari, Inzai, Chiba 270-1694, Japan

E-mail: july1364@nms.ac.jp

[https://doi.org/10.1272/jnms.JNMS.2024\\_91-206](https://doi.org/10.1272/jnms.JNMS.2024_91-206)

Journal Website (<https://www.nms.ac.jp/sh/jnms/>)

sistance to BH remain largely undefined.

Aberrant methylation has been identified as a pivotal factor in tumorigenesis. Both genome-wide hypomethylation and regional hypermethylation of tumor suppressor gene promoters are recognized as distinct cancer markers<sup>7</sup>. In eukaryotic organisms, DNA methylation predominantly occurs at CpG sites, commonly found in gene promoters. Growing evidence suggests that epigenetic alterations play a central role in acquiring drug resistance. Notably, post-chemotherapy changes in gene expression often manifest without any associated gene mutations<sup>8-10</sup>.

Given this background, our current study sought to elucidate the mechanisms responsible for bendamustine resistance in leukemia cells, emphasizing the role of heightened methylation. Additionally, we explored the potential therapeutic impact of the novel demethylating agent, 5-aza-2'-deoxycytidine, on a newly characterized acute lymphoblastic bendamustine-resistant leukemia cell line.

## Materials and Methods

### Drugs and Chemicals

5-aza-2'-deoxycytidine and dimethyl sulfoxide were sourced from Wako Pure Chemical Industries (Osaka, Japan). Phosphate-buffered saline (without metal salt solution) was procured from Nissui (Tokyo, Japan). Roswell Park Memorial Institute medium 1640, Hanks' Balanced Salt Solution (without Ca<sup>2+</sup> Mg<sup>2+</sup>), fetal calf serum, and gentamicin were purchased from Life Technologies (Gaithersburg, MD). Vorinostat was obtained from Tokyo Chemical Industry Company (Tokyo, Japan). Doxorubicin hydrochloride was sourced from Fuji Film (Osaka, Japan). Vincristine sulfate and BH were purchased from Sigma (St. Louis, MO). Verapamil hydrochloride was also procured from Wako Pure Chemical Industries (Osaka, Japan).

### Cell Lines

The human B cell lymphoblastic leukemia parental cell line, BALL/P, was sourced from RIKEN (Tsukuba, Japan). Resistant cell lines to bendamustine (BALL/BENDA), doxorubicin (BALL/ADR), and vincristine (BALL/VCR) were established through stepwise and sustained exposure to BH, doxorubicin, and vincristine, respectively. This was accomplished using the limiting dilution method, as previously detailed<sup>8,11,12</sup>.

Prior to experimental use, the resistant cells to bendamustine, doxorubicin, and vincristine were cultured without their respective drugs for a period of 2 weeks. All cell lines were verified to be free of mycoplasma con-

tamination, as validated by the MycoAlert mycoplasma detection kit (Lonza Japan, Tokyo, Japan).

### Cytotoxicity Assay

Cytotoxicity was evaluated using the trypan blue dye exclusion assay<sup>8,10,11</sup>. In brief, cells were prepared at a concentration of 1 × 10<sup>5</sup> cells/mL and incubated with various concentrations of anticancer drugs, including BH, vincristine, doxorubicin, 2 μmol/L of 5-aza-2'-deoxycytidine, 0.1 μmol/L of vorinostat, and 60 μmol/L of verapamil for 72 h<sup>9,11,13</sup>. Subsequently, viable cells were quantified following trypan blue staining.

### Flow Cytometric Analysis

We conducted the analysis using a BD FACS Verse flow cytometer, paired with FLOWJo software (Becton Dickinson Labware, Franklin Lakes, NJ, USA). A minimum of 10,000 events were acquired for each sample, following the manufacturer's guidelines. The anti-human multidrug resistance protein 1 (MDR1)-FITC antibody was sourced from Becton Dickinson (San Jose, CA, USA).

### RNA Extraction

Total RNA was extracted from each sample utilizing the Qiagen RNA Mini Kit (Qiagen), following the manufacturer's instructions. The integrity of the isolated RNA was then verified through 1% agarose gel electrophoresis.

### Quantitative Real-Time Polymerase Chain Reaction

To assess the mRNA expression levels of genes associated with BH metabolism<sup>5</sup>, we conducted quantitative real-time PCR (qPCR) using the ABI Prism 7500 Sequence Detection System from Applied Biosystems.

Primers were sourced from Hokkaido System Science Co., Ltd. (Sapporo, Japan). The primer sequences, along with their corresponding annealing temperatures, are detailed below:

- 18S:
  - Forward: 5'-CGCCGCTAGAGGTGAAATTC-3'
  - Reverse: 5'-TTGGCAAATGCTTTTCGCT-3'
  - Annealing temperature: 55°C
- p21:
  - Forward: 5'-CCTCATCCCGTGTTCCTTT-3'
  - Reverse: 5'-GTACCACCAGCGGACAAGT-3'
  - Annealing temperature: 57°C
- NOXA:
  - Forward: 5'-ATTTCTTCGGTCACTACACAA-3'
  - Reverse: 5'-AACGCCAACAGGAACAC-3'
  - Annealing temperature: 55°C
- PLK-1:
  - Forward: 5'-CTCAACACGCCTCCTCCT-3'
  - Reverse: 5'-GTGCTCGCTCATGTAATTGC-3'
  - Annealing temperature: 57°C

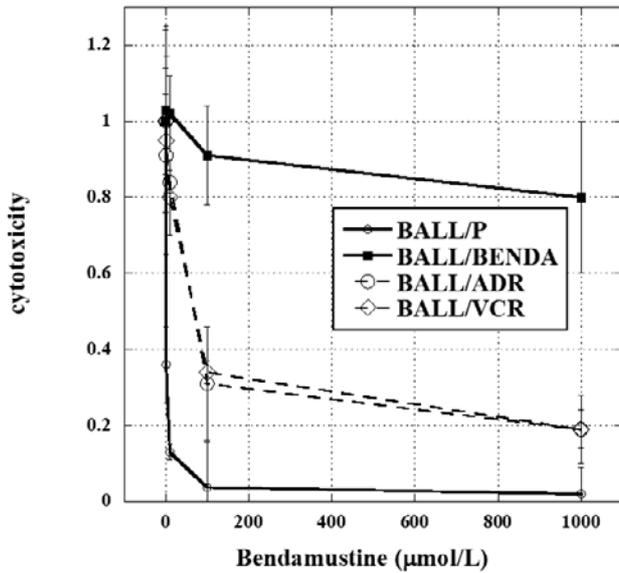


Fig. 1 Cytotoxic effects of bendamustine on BALL parental cells (BALL/P) and cells resistant to bendamustine (BALL/BENDA), doxorubicin (BALL/ADR), and vincristine (BALL/VCR).

Leukemia cells were incubated with various concentrations of bendamustine for 72 h, and cytotoxicity was evaluated by trypan blue dye exclusion assay. The cytotoxicity values were normalized to the viable cell count in BALL/P, set as a baseline of 1. The presented data represent the mean ± standard deviation derived from five independent experiments.

BALL/P vs. BALL/BENDA:  $p < 0.01$  for all bendamustine concentrations.

BALL/P vs. BALL/ADR:  $p < 0.01$  for all concentrations, except at 1 µmol/L of bendamustine.

BALL/P vs. BALL/VCR:  $p < 0.01$  for all bendamustine concentrations.

BALL/BENDA vs. BALL/ADR:  $p < 0.01$  for all concentrations, excluding 1 µmol/L of bendamustine.

BALL/BENDA vs. BALL/VCR:  $p < 0.05$  for all, except at 1 µmol/L of bendamustine.

• Aurora A:

o Forward: 5'-TCCTTGTCAGAATCCATTACCTGT-3'

o Reverse: 5'-GAATGCGCTGGGAAGAATTTG-3'

• Annealing temperature: 55°C

• Aurora B:

o Forward: 5'-AGAGTGCATCACACAACGAGA-3'

o Reverse: 5'-CTGAGCAGTTTGGAGATGAGGTC-3'

o Annealing temperature: 56°C

• Cyclin B1:

o Forward: 5'-AGTGTGACCCAGACTGCCTC-3'

o Reverse: 5'-CAAGCCAGGTCCACCTCCTC-3'

o Annealing temperature: 57°C

• Exo1:

o Forward: 5'-TTGGTCTGGAGGTCTTGGAGA-3'

o Reverse: 5'-GAATCGCTCTTCTTCGGAAGT-3'

o Annealing temperature: 57°C.

P21 and NOXA were inquired for apoptosis in response to DNA damage. Polo-like kinase 1 (PLK-1), Aurora Kinase A and B, and cyclin B1 were important genes as mitotic checkpoint regulation. Exo1 was represented as DNA repair gene<sup>5</sup>.

**Ethics Statement**

This study received approval from the Institutional Review Board at Nippon Medical School Chiba Hokusoh Hospital (approval number: 389).

**Statistical Analysis**

We employed the Kruskal-Wallis H test for our statistical analysis. To ascertain the significance of variations between groups, the Mann-Whitney U test was utilized.

**Results**

**Establishment of Cell Lines Resistant to Bendamustine, Doxorubicin, and Vincristine**

BALL/BENDA, BALL/ADR, and BALL/VCR cell lines were established by continually exposing cells to escalating concentrations of BH, doxorubicin, and vincristine. These resistant phenotypes remained stable even after being cultivated for over 6 months in a drug-free environment.

As depicted in **Figure 1**, the bendamustine-resistant cell line (BALL/BENDA) exhibited significantly higher resistance to BH compared to the parental cell line (BALL/P). Similarly, the doxorubicin (BALL/ADR) and vincristine (BALL/VCR) resistant BALL cells also displayed enhanced resistance to BH when compared to the parental cell line (BALL/P) (**Fig. 1**).

**Degree of Resistance to BH Is Significantly Higher in BALL/BENDA Compared to BALL/ADR and BALL/VCR**

As depicted in **Figures 2, 3**, BALL/ADR and BALL/VCR cells demonstrate high resistance to doxorubicin and vincristine. While BALL/BENDA cells exhibit moderate resistance to both doxorubicin and vincristine, the resistance is statistically significant. Conversely, BALL/P cells are sensitive to both doxorubicin and vincristine.

**Flow Cytometric Analysis Revealed No Change in MDR1 Expression in BALL/P and BALL/BENDA Cells, but Increased in BALL/ADR, BALL/VCR Leukemic Cells**

As shown **Figure 4**, MDR1 expression analysis via flow cytometry in BALL/P cells (a), BALL/ADR cells (b),

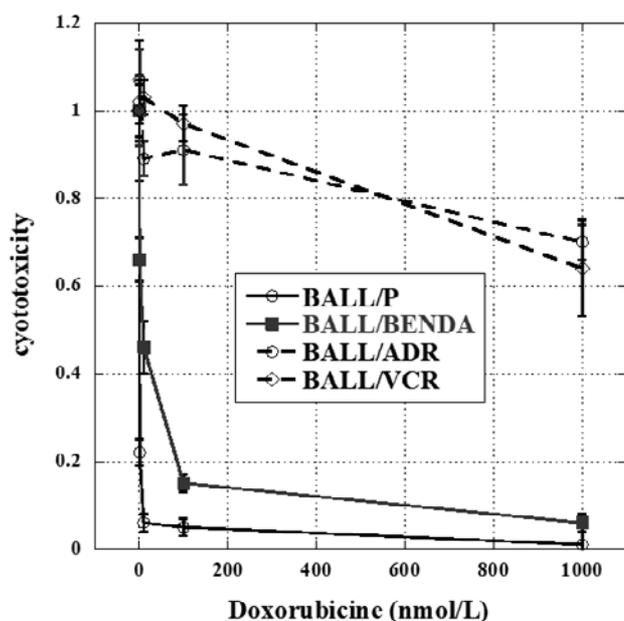


Fig. 2 Cytotoxic effects of doxorubicin on BALL parental cells (BALL/P) and cells resistant to bendamustine (BALL/BENDA), doxorubicin (BALL/ADR), and vincristine (BALL/VCR).

Leukemia cells were incubated with various concentrations of doxorubicin for 72 h, and cytotoxicity was evaluated by trypan blue dye exclusion assay. The cytotoxicity values were normalized to the viable cell count in BALL/P, set as a baseline of 1. The presented data represent the mean  $\pm$  standard deviation from three independent experiments.

BALL/P vs. BALL/BENDA:  $p < 0.05$  for all concentrations except at 1,000 nmol/L of doxorubicin.

BALL/P vs. BALL/ADR:  $p < 0.01$  for all doxorubicin concentrations.

BALL/P vs. BALL/VCR:  $p < 0.01$  for all doxorubicin concentrations.

BALL/BENDA vs. BALL/ADR:  $p < 0.01$  for all doxorubicin concentrations.

BALL/BENDA vs. BALL/VCR:  $p < 0.01$  for all doxorubicin concentrations.

BALL/VCR cells (c), and BALL/BENDA cells (d). MDR1 overexpression was evident in BALL/ADR and BALL/VCR cells but was absent in BALL/P and BALL/BENDA cells.

#### Gene Expression Linked to Metabolism in Bendamustine-Resistant Cells

We quantified seven genes potentially associated with BH resistance using qPCR<sup>5</sup>. Expression levels of these genes were standardized to 18S levels, and relative values were calculated by comparing the BH, doxorubicin, and vincristine-resistant leukemia cells (BALL/BENDA, BALL/ADR, BALL/VCR) to their parental counterpart

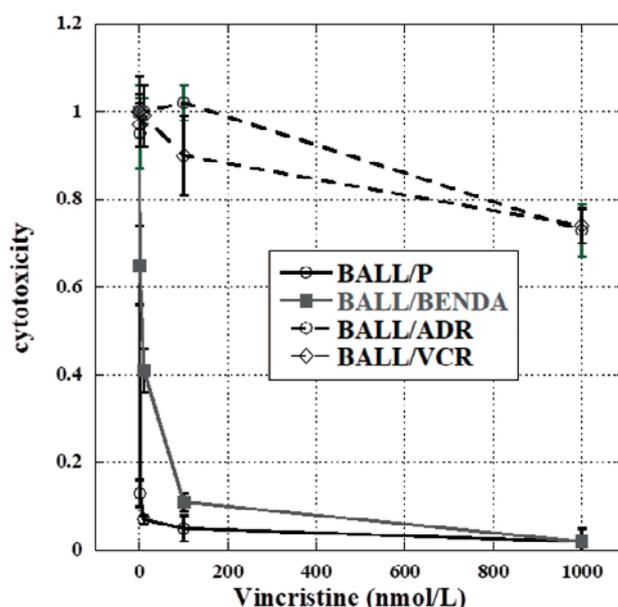


Fig. 3 Cytotoxic effects of vincristine on BALL parental cells (BALL/P) and cells resistant to bendamustine (BALL/BENDA), doxorubicin (BALL/ADR), and vincristine (BALL/VCR).

Leukemia cells were incubated with various concentrations of vincristine for 72 h, and cytotoxicity was evaluated by trypan blue dye exclusion assay. The cytotoxicity values were normalized to the viable cell count in BALL/P, set as a baseline of 1. The presented data represent the mean  $\pm$  standard deviation from three independent experiments.

BALL/P vs. BALL/BENDA:  $p < 0.05$  for all concentrations except at 1,000 nmol/L of vincristine.

BALL/P vs. BALL/ADR:  $p < 0.01$  for all of vincristine concentrations.

BALL/P vs. BALL/VCR:  $p < 0.01$  for all of vincristine concentrations.

BALL/BENDA vs. BALL/ADR:  $p < 0.01$  for all of vincristine concentrations.

BALL/BENDA vs. BALL/VCR:  $p < 0.01$  for all of vincristine concentrations.

(BH-sensitive; BALL/P) (Fig. 5a~g). Notably, bendamustine-resistant cells exhibited reduced PLK-1 mRNA expression relative to both the parental cells and the doxorubicin and vincristine-resistant cells (Fig. 5d).

Given these findings, our subsequent experiments will center on investigating the expression of PLK-1 in bendamustine-resistant leukemia cells.

#### 5-Aza-2'-Deoxycytidine Enhances PLK-1 Gene Expression in Bendamustine-Resistant Leukemia Cells

To explore the potential of 5-aza-2'-deoxycytidine—a novel demethylating agent—in reversing BH resistance, we assessed changes in the expression of the PLK-1 gene. This assessment was conducted on both BH-sensitive (BALL/P) and resistant leukemia cells to BH, doxorubi-

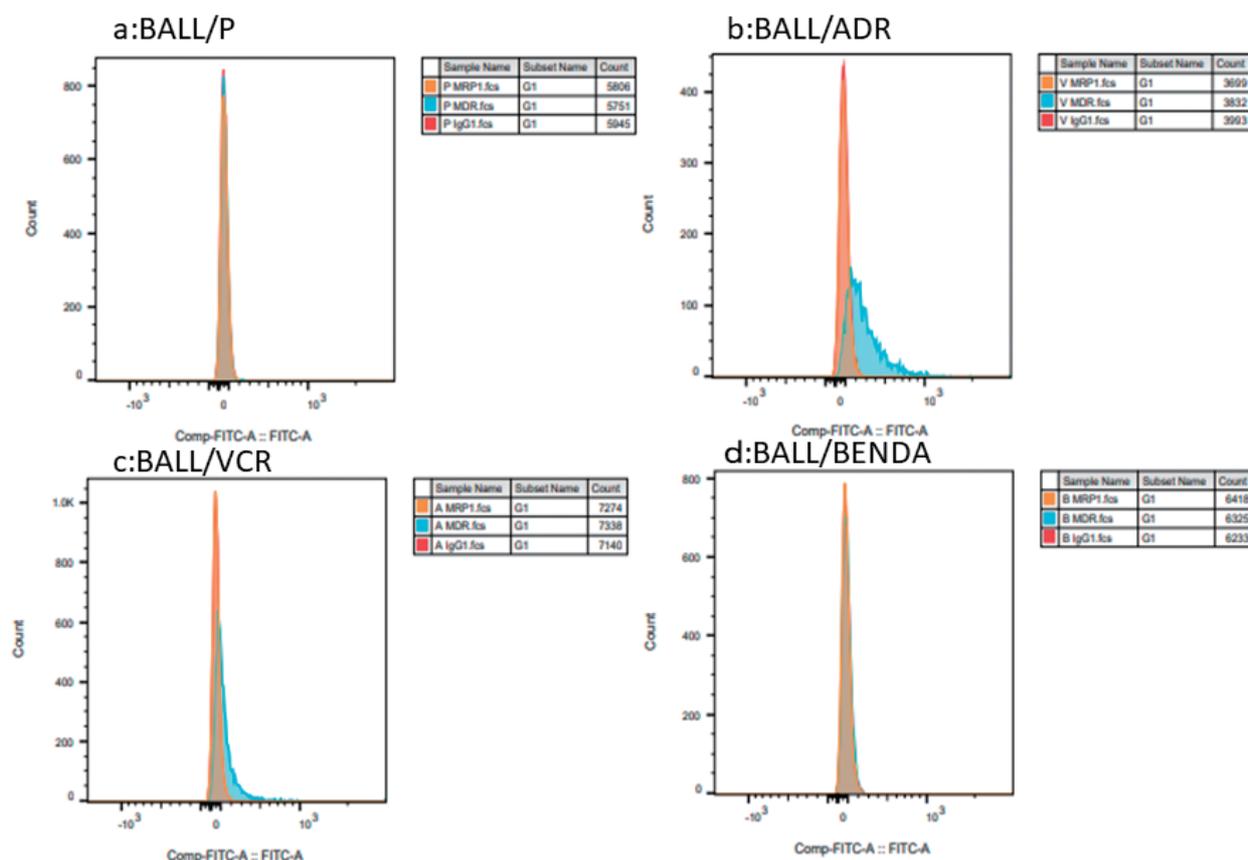


Fig. 4 MDR1 expression across various BALL cell lines.

MDR1 expression analysis via flow cytometry in BALL/P cells (a), BALL/ADR cells (b), BALL/VCR cells (c), and BALL/BENDA cells (d). Analysis was conducted using the BD FACSVerser system and interpreted with FlowJo software. The red curve denotes the negative control, while the blue curve indicates MDR1 expression.

cin, and vincristine (BALL/BENDA, BALL/ADR, BALL/VCR), with and without the treatment of 5-aza-2'-deoxycytidine.

Our results revealed a significant upregulation in the PLK-1 gene expression within bendamustine-resistant leukemia cells post 5-aza-2'-deoxycytidine treatment (Fig. 6).

#### 5-Aza-2'-Deoxycytidine Enhances Sensitivity in Bendamustine-Resistant Leukemia Cells, unlike Verapamil

We further evaluated the potential of 5-aza-2'-deoxycytidine to reinstate BH sensitivity in bendamustine-resistant cells. Treatment with 5-aza-2'-deoxycytidine markedly amplified the cytotoxic effects of BH on BALL/BENDA cells (Fig. 7b). These findings robustly suggest a synergistic action between 5-aza-2'-deoxycytidine and BH in bendamustine-resistant cells, potentially due to PLK-1 methylation.

Contrastingly, Vorinostat, a novel histone deacetylase (HDAC) inhibitor, did not modify the cytotoxic response to BH in BALL cells (Fig. 7a~d). In comparison, treat-

ment with verapamil, a renowned inhibitor of MDR1 expression, considerably augmented BH's cytotoxicity in both BALL/VCR and BALL/ADR cells. This synergism between verapamil and BH, especially in p-glycoprotein-associated BH resistance observed in BALL/ADR and BALL/VCR cells, could likely be attributed to MDR1 overexpression (Fig. 7c, d).

#### Discussion

Currently, BH serves as a pivotal therapeutic component in both initial and salvage treatments for various low-grade B-cell NHLs and for MCL, a typically aggressive and challenging disease subtype<sup>5,14,15</sup>. Although the mechanism of BH action has been studied, its resistance mechanism has been less explored. Nonetheless, the emergence of clinical resistance to BH is a defining factor that potentially sets the life trajectory for patients with B-cell NHL and MCL. Hence, it is pressing to decipher the resistance mechanisms to BH.

To address this, our study systematically established a BH-resistant cell line via continuous in vitro exposure to

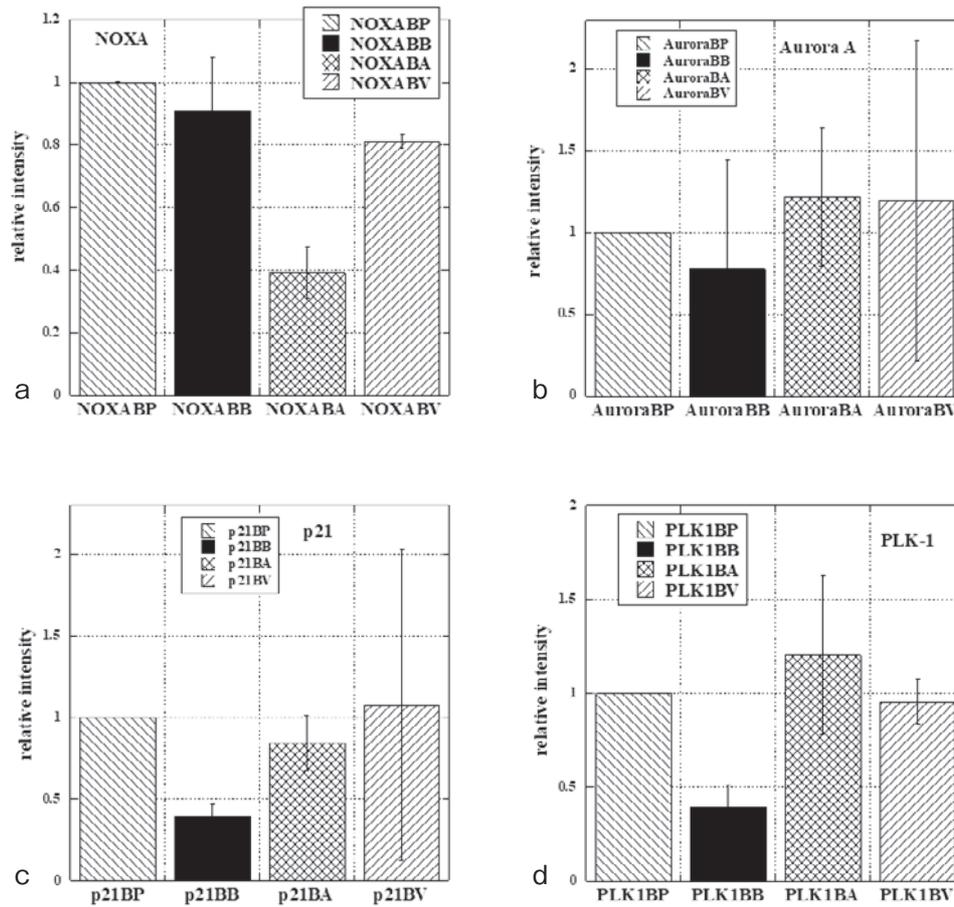


Fig. 5 RNA across various BALL cell lines.

(a) Noxa gene RNA expression was measured using qPCR. After normalization to 18S expression, relative values were calculated based on the expression level of NOXA gene in BALL/P cells, set as a baseline of 1. The presented data represent the mean  $\pm$  standard deviation from five independent experiments.

Noxa expression in BALL/P vs BALL/ADR, BALL/VCR:  $p < 0.05$

Noxa expression in BALL/ADR vs BALL/VCR:  $p < 0.05$

NOXABP: Noxa expression in BALL/P cells

NOXABB: Noxa expression in BALL/BENDA cells

NOXABA: Noxa expression in BALL/ADR cells

NOXABV: Noxa expression in BALL/VCR cells

(b) Aurora A gene RNA expression was measured using qPCR.

No significant differences were observed among the cell lines.

AuroraBP: Aurora A expression in BALL/P cells

AuroraBB: Aurora A expression in BALL/BENDA cells

AuroraBA: Aurora A expression in BALL/ADR cells

AuroraBV: Aurora A expression in BALL/VCR cells

(c) p21 gene RNA expression was measured using qPCR.

p21 expression in BALL/P vs BALL/BENDA:  $p < 0.05$

p21 expression in BALL/BENDA vs BALL/ADR:  $p < 0.05$

p21BP: p21 expression in BALL/P cells

p21BB: p21 expression in BALL/BENDA cells

p21BA: p21 expression in BALL/ADR cells

p21BV: p21 expression in BALL/VCR cells

(d) PLK-1 gene RNA expression was measured using qPCR.

PLK-1 expression in BALL/BENDA vs BALL/P, BALL/ADR, BALL/VCR:  $p < 0.01$

PLK1BP: PLK-1 expression in BALL/P cells

PLK1BB: PLK-1 expression in BALL/BENDA cells

PLK1BA: PLK-1 expression in BALL/ADR cells

PLK1BV: PLK-1 expression in BALL/VCR cells

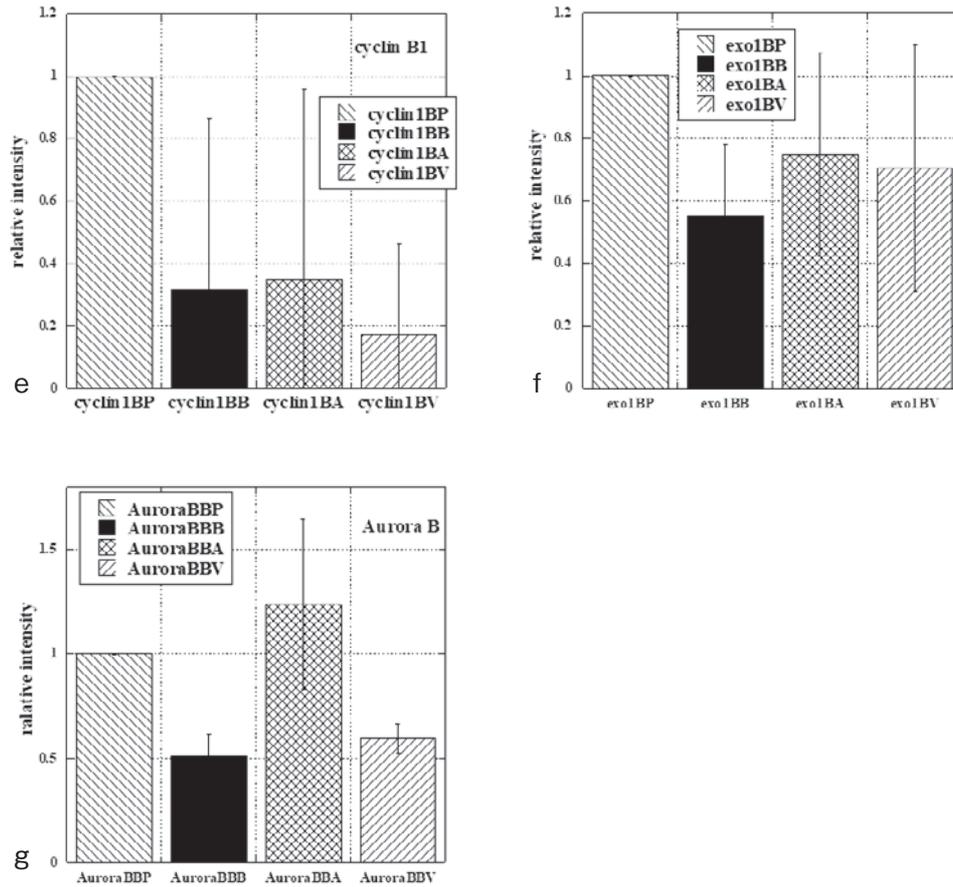


Fig. 5 RNA across various BALL cell lines. (continued)

- (e) cyclin B1 gene RNA expression was measured using qPCR.  
 Cyclin 1 expression in BALL/P vs BALL/VCR:  $p < 0.05$   
 cyclin1BP: cyclin 1 expression in BALL/P cells  
 cyclin1BB: cyclin 1 expression in BALL/BENDA cells  
 cyclin1BA: cyclin 1 expression in BALL/ADR cells  
 cyclin1BV: cyclin 1 expression in BALL/VCR cells
- (f) exo 1 gene RNA expression was measured using qPCR.  
 No significant differences were observed among the cell lines.  
 exo1BP: exo 1 expression in BALL/P cells  
 exo1BB: exo 1 expression in BALL/BENDA cells  
 exo1BA: exo 1 expression in BALL/ADR cells  
 exo1BV: exo 1 expression in BALL/VCR cells
- (g) Aurora B gene RNA expression was measured using qPCR.  
 Aurora B expression in BALL/P vs BALL/BENDA, VCR:  $p < 0.02$   
 AuroraBBP: Aurora B expression in BALL/P cells  
 AuroraBBB: Aurora B expression in BALL/BENDA cells  
 AuroraBBA: Aurora B expression in BALL/ADR cells  
 AuroraBBV: Aurora B expression in BALL/VCR cells

BH and compared with already established doxorubicin and vincristine-resistant cell lines. We evaluated mRNA levels of genes linked to BH metabolism, MRD expression, and analyzed the potential restoration of BH sensitivity using agents like 5-aza-2-deoxyxytidine, vorinostat, and verapamil.

BALL/ADR cells and BALL/VCR cells also showed resistance to bendamustine, but the degree of resistance

was lower than that of BALL/BENDA cells, suggesting a different mechanism of resistance against bendamustine. Therefore, regarding gene expression, we selected genes in BALL/BENDA that showed significantly different expression from the three cell lines, such as bendamustine-sensitive cells (BALL/P), BALL/ADR, and BALL/VCR.

The expression of EXO1 in BALL/BENDA showed no significant difference compared to BALL/P, BALL/ADR

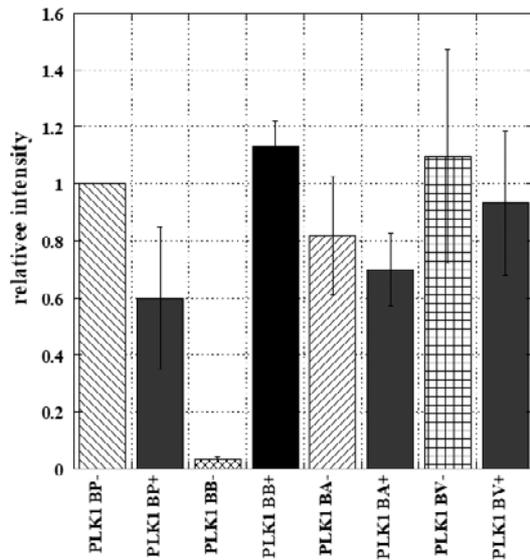


Fig. 6 PLK-1 mRNA expression in BALL parent cells, and bendamustine, doxorubicin, and vincristine-resistant cells with or without 5-aza-2'-deoxycytidine treatment.

Leukemia cells were incubated with (+) or without (-) 5-aza-2'-deoxycytidine (2 μmol/L) for 72 h, and PLK-1 RNA expression was measured using qPCR. After normalization against the 18S expression levels, relative values were calculated based on the expression level of PLK-1 gene in BALL parental cells without 5-aza-2'-deoxycytidine treatment, set as a baseline of 1. The presented data represent the mean ± standard deviation from three independent experiments.

PLK-1 expression in BALL/BENDA vs BALL/P, BALL/ADR, BALL/VCR without 5-aza-2'-deoxycytidine treatment:  $p < 0.01$

PLK-1 expression in BALL/BENDA without 5-aza-2'-deoxycytidine treatment vs with 5-aza-2'-deoxycytidine treatment:  $p < 0.001$

PLK1BP-: PLK-1 expression in BALL/P cells without 5-aza-2'-deoxycytidine treatment

PLK1BP+: PLK-1 expression in BALL/P cells with 5-aza-2'-deoxycytidine treatment

PLK1BB-: PLK-1 expression in BALL/BENDA cells without 5-aza-2'-deoxycytidine treatment

PLK1BB+: PLK-1 expression in BALL/BENDA cells with 5-aza-2'-deoxycytidine treatment

PLK1BA-: PLK-1 expression in BALL/ADR cells without 5-aza-2'-deoxycytidine treatment

PLK1BA+: PLK-1 expression in BALL/ADR cells with 5-aza-2'-deoxycytidine treatment

PLK1BV-: PLK-1 expression in BALL/VCR cells without 5-aza-2'-deoxycytidine treatment

PLK1BV+: PLK-1 expression in BALL/VCR cells with 5-aza-2'-deoxycytidine treatment

and BALL/VCR. The expressions of p21 in BALL/BENDA were significantly decreased compared to in

BALL/P and BALL/ADR ( $p < 0.05$ ), but the expressions of p21 in BALL/BENDA were not significantly different from the expressions in vincristine resistant cells (BALL/VCR). On the other hand, PLK-1 expression was significantly decreased in only BALL-BENDA compared in BALL/P, BALL/ADR, and BALL/VCR, and other three cell lines showed no significant difference in expression of PKL-1 each other. We considered that PLK-1, a key gene in BH metabolism, might be considered as key gene for bendamustine resistance.

Polo-like kinase 1 (Plk1) is a serine/threonine kinase that is known to regulate multiple key steps of mitosis, including mitotic entry, centrosome maturation, formation of the bipolar spindle, mitotic exit, and a target of the DNA damage checkpoint<sup>16</sup>.

Our findings revealed that bendamustine-resistant cells, specifically BALL-BENDA, exhibit reduced PLK-1 expression, a key gene in BH metabolism. Notably, upon administering a demethylating agent, PLK-1 expression surged, implicating PLK-1 methylation in drug resistance. Recent literature suggests that other epigenetic mechanisms, such as histone deacetylation, play roles in the deactivation of genes associated with chemotherapy sensitivity and resistance<sup>9</sup>. Yet, in our bendamustine-resistant cells, vorinostat, an HDAC inhibitor, did not increase cytotoxicity.

Takimoto-Shimomura et al.<sup>12</sup> illustrated that the upregulation of ABCB1 and GST, prevalent in drug resistance pathways, aided the onset of resistance to BH. Additionally, they proposed that developing resistance to BH might concurrently escalate the risk of cross-resistance to other anti-lymphoma chemotherapeutics, such as DOX, melphalan, and VCR. ABC transporters, membrane-spanning transporters, facilitate the transit of diverse substrates across cell membranes. P-glycoprotein, or MDR1, the pioneer transporter identified, mediates resistance to various cancer therapeutics and has been linked to BH resistance<sup>17</sup>.

Utilizing BALL-ADR and BALL-VCR cells, known to express MRD1, we discerned their moderate resistance to BH. Notably, while the PLK-1 expression remained stable in these cells, the cytotoxicity of BH significantly amplified upon administering verapamil, an MDR1 inhibitor. Conversely, BALL-BENDA, expressing lower MDR1 levels, exhibited no drug sensitivity alteration with verapamil. The previously cited MDR contribution to BH resistance<sup>13</sup> was not evident in the bendamustine-resistant cell lines established in our research. Conclusively, BH resistance appears to be orchestrated by both PLK-1 and

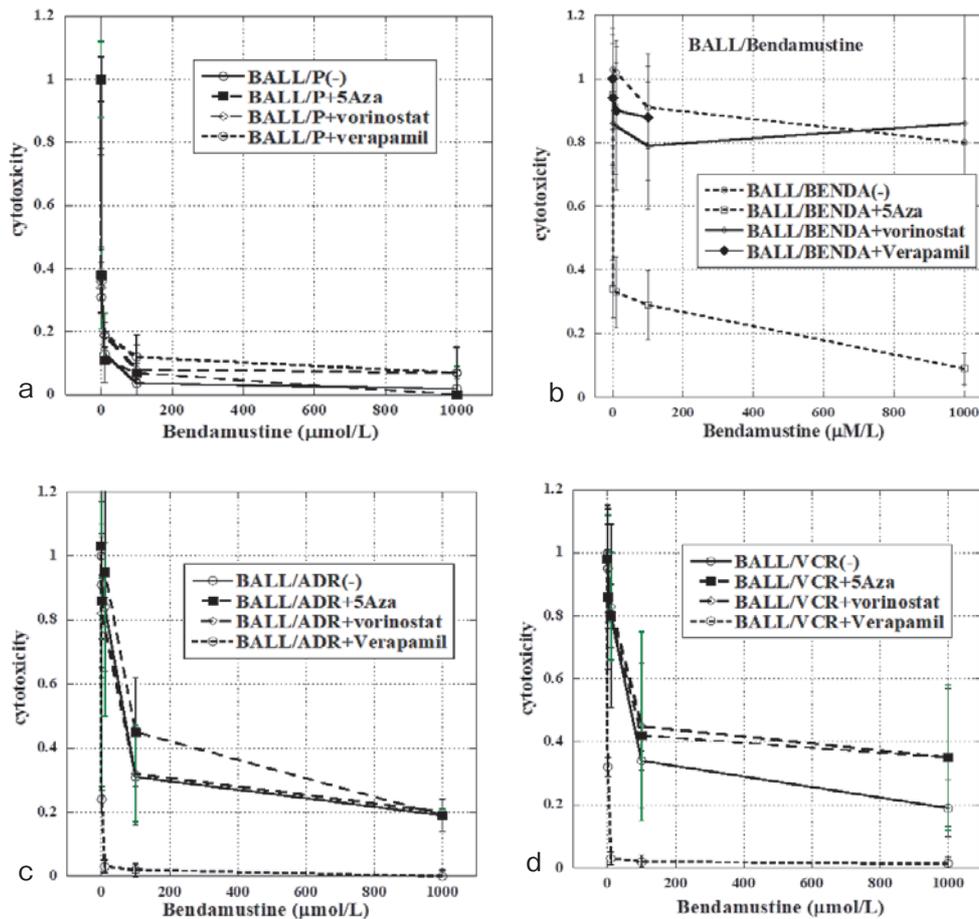


Fig. 7 Cytotoxicity in BALL, parental cells and (a) bendamustine (b), doxorubicin (c), vincristine-resistant cells (d) with or without 5-aza-2'-deoxycytidine, vorinostat, verapamil treatment.

Leukemia cells were incubated with various concentrations of bendamustine for 72 h with or without 5-aza-2'-deoxycytidine (2  $\mu\text{mol/L}$ ), vorinostat (0.1  $\mu\text{mol/L}$ ), verapamil (60  $\mu\text{mol/L}$ ) and cytotoxicity was evaluated by trypan blue dye exclusion assay. Relative cytotoxic values were calculated based on the viable cell count in BALL cells without treatment, set as a baseline of 1. The presented data represent the mean  $\pm$  standard deviation from three independent experiments.

BALL/BENDA:  $p < 0.01$  for all of with vs without 5-Aza-2'-deoxycytidine concentrations.

BALL/ADR:  $p < 0.01$  for all of with or without verapamil concentrations.

BALL/VCR:  $p < 0.01$  for all of with or without verapamil concentrations.

MDR1, with PLK-1 playing a more dominant role in the BALL-BENDA cell line. This might be attributed to BH's dual anticancer mechanisms. Our data proposes that PLK-1 gene methylation influences its expression changes, and this methylation is pivotal for BH resistance in leukemia cells, even more than MDR1 expression. Future research should assess the clinical implications of downregulated PLK1 expression in MCL and NHL.

In conclusion, bendamustine-resistant leukemia cell lines were crafted and compared against cell lines over-expressing MDR. Bendamustine-resistant lines displayed diminished PLK-1 gene expression, which was revived by 5-aza-2'-deoxycytidine. Intriguingly, the drug sensitiv-

ity of the resistant line with restored PLK-1 expression mirrored the parental line. There was no evident change in MDR expression in bendamustine-resistant leukemia cell lines, even with verapamil administration. Our study underscores the PLK-1 gene methylation's critical role in shaping its expression and its significance in the evolution of bendamustine resistance in leukemia cells.

**Author contributions:** TA, TU, and TI conceived and designed the in vitro experiments. TI, RF, and TA executed the in vitro experiments. TI drafted the initial manuscript, while TA and TU contributed to writing the paper. TU, TA, and YI provided critical manuscript revisions and supervised the en-

tire study. All authors participated in data analysis and approved the final version of the manuscript.

**Funding:** This study received partial support from grants provided by JSPS KAKENHI (Grant-in-Aid for Scientific Research C: 16K10044), the Promotion and Mutual Aid Corporation for Private Schools of Japan, and the Science Research Promotion Fund.

**Conflict of Interest:** The authors declare no conflicts of interest.

### References

- Asano T. Drug resistance in cancer therapy and the role of epigenetics. *J Nippon Med Sch.* 2020;87(5):244–51.
- Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *N Engl J Med.* 2004;351(6):533–42.
- Friedberg JW, Cohen P, Chen L, et al. Bendamustine in patients with rituximab-refractory indolent and transformed non-Hodgkin's lymphoma: results from a phase II multicenter, single-agent study. *J Clin Oncol.* 2008;26(2):204–10.
- Roue G, Lopez-Guerra M, Milpied P, et al. Bendamustine is effective in p53-deficient B-cell neoplasms and requires oxidative stress and caspase-independent signaling. *Clin Cancer Res.* 2008;14(21):6907–15.
- Leoni LM, Bailey B, Reifert J, et al. Bendamustine (Trenda) displays a distinct pattern of cytotoxicity and unique mechanistic features compared with other alkylating agents. *Clin Cancer Res.* 2008;14(1):309–17.
- Strumberg D, Harstrick A, Doll K, Hoffmann B, Seeber S. Bendamustine hydrochloride activity against doxorubicin-resistant human breast carcinoma cell lines. *Anticancer Drugs.* 1996;7(4):415–21.
- Dunsmore KP, Winter SS, Devidas M, et al. Children's Oncology Group AALL0434: a phase III randomized clinical trial testing nelarabine in newly diagnosed T-Cell acute lymphoblastic leukemia. *J Clin Oncol.* 2020;38(28):3282–93.
- Asano T, Nakamura K, Fujii H, et al. Altered expression of topoisomerase IIalpha contributes to cross-resistant to etoposide K562/MX2 cell line by aberrant methylation. *Br J Cancer.* 2005;92(8):1486–92.
- Yamanishi M, Narazaki H, Asano T. Melatonin overcomes resistance to clofarabine in two leukemic cell lines by increased expression of deoxycytidine kinase. *Exp Hematol.* 2015;43(3):207–14.
- Asano T, Narazaki H, Fujita A. Genome-wide DNA methylation profiling of CpG islands in a morpholino anthracycline derivative-resistant leukemia cell line: p38 $\alpha$  as a novel candidate for resistance. *Pharmacol Res Perspect.* 2016;5(1):e00285.
- Yoshida K, Fujita A, Narazaki H, Asano T, Itoh Y. Drug resistance to nelarabine in leukemia cell lines might be caused by reduced expression of deoxycytidine kinase through epigenetic mechanisms. *Cancer Chemother Pharmacol.* 2022;89(1):83–91.
- Takimoto-Shimomura T, Nagoshi H, Maegawa S, et al. Establishment and characteristics of a novel mantle cell lymphoma-derived cell line and a Bendamustine-resistant subline. *Cancer Genomics Proteomics.* 2018;15(3):213–23.
- Donmez Y, Akhmetova L, Iseri OD, Kars MD, Gunduz U. Effect of MDR modulators verapamil and promethazine on gene expression levels of MDR1 and MRP1 in doxorubicin-resistant MCF-7 cells. *Cancer Chemother Pharmacol.* 2011;67(4):823–8.
- Montillo M, Ricci F, Tedeschi A, Vismara E, Morra E. Bendamustine: new perspective for an old drug in lymphoproliferative disorders. *Expert Rev Hematol.* 2010;3(2):131–48.
- Rummel MJ, Al-Batran SE, Kim SZ, et al. Bendamustine plus rituximab is effective and has a favorable toxicity profile in the treatment of mantle cell and low-grade non-Hodgkin's lymphoma. *J Clin Oncol.* 2005;23(15):3383–9.
- Wu CP, Hsiao SH, Sim HM, et al. Human ABCB1 (P-glycoprotein) and ABCG2 mediate resistance to BI 2536, a potent and selective inhibitor of Polo-like kinase 1. *Biochem Pharmacol.* 2013;86(7):904–13.
- Roninson IB, Chin JE, Choi KG, et al. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc Natl Acad Sci U S A.* 1986;83(12):4538–42.

(Received, August 20, 2023)

(Accepted, October 4, 2023)

(J-STAGE Advance Publication, December 8, 2023)

Journal of Nippon Medical School has adopted the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>) for this article. The Medical Association of Nippon Medical School remains the copyright holder of all articles. Anyone may download, reuse, copy, reprint, or distribute articles for non-profit purposes under this license, on condition that the authors of the articles are properly credited.