Nelarabine Resistance of Childhood T-Cell Lymphoblastic Leukemia/Lymphoma Cells

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**Background:** Nelarabine (NEL) is a new purine nucleoside analogue that has recently become available for both adults and children with relapsed or refractory T-cell acute lymphoblastic leukemia (T-ALL) and T-lymphoblastic lymphoma (T-LBL). We studied the drug resistance profile of eight children with T-ALL/LBL using an *in vitro* cytotoxic assay including NEL. Furthermore, we tried to establish a NEL resistant T-ALL cell line model to investigate the mechanism of NEL resistance.

**Results:** Four of 8 patients showed a higher 50% lethal concentration (*LC*₅₀) than the maximum clinical concentration of NEL. There were no correlations in *LC*₅₀ values between NEL and other drugs. To study the mechanism of NEL resistance, we originally established a nucleoside analogue resistant T-ALL Jurkat cell line model (Jurkat+C) induced by incubation in medium containing cytarabine (AraC) at *LC*₅₀ of the control. Jurkat+C showed resistance to NEL and Fludarabine as well as AraC but not Daunorubicin. Next, we studied the role of Equivalent Nucleoside Transporter-1 (*ENT*-1), a major cellular nucleoside transporter, in the acquired drug resistance in Jurkat+C. Nitrobenzylmercaptopenurine riboside, an *ENT*-1-specific inhibitor, showed an inhibitory effect of nucleoside analogues on *in vitro* toxicity in both Jurkat and Jurkat+C, while no differences in *ENT*-1 mRNA expression levels between Jurkat and Jurkat+C were found.

**Conclusions:** *In vitro* NEL resistance was seen in half of the tested childhood T-ALL cells. We could establish the NEL resistant T-ALL cell line model by AraC exposure. *ENT*-1 may partly act as a transporter of NEL, but not play a key role in AraC induced NEL resistance. This cell line model may be useful to provide a mechanism to explain NEL resistance. (Kitakanto Med J 2011: 61: 119~126)

**Key words:** Nelarabine, Cytarabine, T-ALL, T-LBL, *ENT*-1

**Abbreviations:**
T-cell acute lymphoblastic leukemia = T-ALL
Event-free survival = EFS
9-β-arabinofuranosylguanine = ara-G
Equivalent Nucleoside Transporter-1 = *ENT*-1
Adenosine deaminase = ADA
ara-G triphosphate = ara-GTP
Lethal concentration = *LC*₅₀
T-lymphoblastic lymphoma = T-LBL
Complete remission rate = CR
Nelarabine = NEL

Maximum plasma concentration = *C*max
Cytarabine = AraC

**Introduction**

T-cell acute lymphoblastic leukemia (T-ALL) accounts for 10% of ALL in children. Five-year event-free survival (EFS) has reached 50~60% with recent combination chemotherapy.¹⁻⁴ However, EFS decreases below 20% in relapse cases and a standard treatment strategy has not been established.⁴ Compar-
ed to B-cell precursor ALL, T-ALL has clinical features related to treatment failure, such as occurrence at higher ages, frequent central nervous involvement and cellular drug resistance.\(^6\) The usefulness of an in vitro cytotoxic assay to predict drug resistance in high risk leukemia such as T-ALL has previously been reported.\(^6\)–\(^8\)

Nelarabine (NEL) is a new purine nucleoside analogue that is the prodrug of 9-β-arabinofuranosylguanine (ara-G). NEL is converted to ara-G in plasma by adenosine deaminase (ADA) and then transported into cells. Ara-G is subsequently phosphorylated to ara-G triphosphate (ara-GTP) and inhibits DNA synthesis.\(^9\) NEL has recently become available for both adults and children with relapsed or refractory T-ALL and T-lymphoblastic leukemia (T-LBL).\(^10\)

The efficacy of NEL monotherapy was verified in 3 clinical trials.\(^11\)–\(^13\) In a pediatric trial reported by Berg et al., the rate of complete remission (CR) and CR without complete hematologic recovery (CR\(^w\)) was 48% in first relapse patients and 23% in second or higher number relapers with 650mg/m\(^2\) doses through day 1 to 5.\(^12\) Recently, clinical trials of NEL including combination chemotherapy for T-ALL have been conducted.\(^14\) However, no reports have yet appeared on studies employing an in vitro cytotoxic assay of NEL using patients’ samples.

To establish an effective treatment strategy for childhood T-ALL/LBL, we conducted an in vitro cytotoxic assay of clinical samples using NEL. We found that in 50% of patients who had no history of NEL treatment, the LC\(_{50}\) values of NEL were higher than the mean maximum plasma concentration (C\(_{max}\)) of NEL reported by Cohen et al.\(^15\) To investigate the mechanisms of acquiring resistance to NEL, we tried to establish an original NEL-resistant T-ALL cell line model through long-term nucleoside analogue (AraC) exposure. In this study we focused on the alteration of an Equilibrative nucleoside transporter (ENT\(^−1\)) with this cell line model. The ENT family has been well documented regarding nucleoside analogue transporters and drug resistance. ENT\(^−1\) is particularly well-known as a major nucleoside transporter of cytarabine (AraC).\(^16\)–\(^19\)

### Materials and Methods

**Patient characteristics**

Eight patients diagnosed as having T-ALL/LBL, 5 males and 3 females aged 4 to 17 years old, were studied. The disease stage and treatment courses are listed in Table 1. Five patients were relapse cases (patient 1 to 5), and other patients were primary cases (patient 6 to 8). Seven T-ALL patients were treated first with the high-risk ALL protocol of Tokyo Children’s Cancer Study Group (TCCSG-HEX), consisting of prednisolone (PSL), dexamethasone (DEX), vincristine (VCR), vindesine, daunorubicin (DNR), cyclophosphamide (CY), L-asparaginase (L-ASP), methotrexate (MTX), cytarabine (AraC), ifosfamide, 6-mercaptopurine (6-MP), and etoposide (VP-16). Patient 6 with T-LBL failed to achieve first remission following the initial chemotherapy with the advanced LBL protocol of the Japan Pediatric Leukemia/Lymphoma Study Group (JPLSG-ALB05), consisting of PSL, DEX, DNR, pirarubicin, L-ASP, CY, MTX, and 6-MP. In both treatment protocols, initial PSL monotherapy was performed to detect steroid resistance, which was reported to indicate a poor prognosis. Patient 7 had a poor response to initial PSL treatment. Patient 8 had a first induction failure. NEL was administered in patients 1, 2, 4 and 5 as reinduction chemotherapy and in patient 7 as first induction therapy. Patients 1 and 4 did not respond to NEL and failed to achieve second complete remission. Three patients died (patients 1 to 3) and 5 patients (patients 4 to 8) survived.

**Samples from patients**

Individual samples from patients 1 to 5 were obtained at first relapse. The sample from patient 6 with T-LBL was obtained from pleural effusion at disease onset. The sample from patient 7 and 8 was obtained at disease onset and after first induction failure, respectively. The samples from all patients except for patient 6 were obtained from the bone marrow or peripheral blood. The leukemic blast content of each sample was over 80%, verified morphologically and/or by flow cytometry. All samples except for that from patient 6 were treated within 24 hours of collection for an in vitro cytotoxic assay, but only the sample from patient 6 was preserved under LN\(_2\) with dimethyl sulfoxide (DMSO) including stock solution. The cell viability of patient 6 was 70% at the thawing and after 96-hours culturing measured with trypan blue staining.

Each sample was manipulated in accordance with the previous report by Hongo et al.,\(^14\) then used for an in vitro cytotoxic assay and total RNA isolation. RNA samples of patient 6 and 8 could not be obtained because of the small amount of the samples.

**Cell line**

The Jurkat cell line (human T-cell lymphoblast-like cell line, kindly provided by Dr. Hando of Gunma University) was maintained in RPMI-1640 medium supplemented with 25mM HEPES buffer, 2μM-glutamine (Invitrogen, CA, USA) and 10% heat-inactivated FCS (standard RPMI). Logarithmically
### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>PNO</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Disease stage</th>
<th>Initial chemotherapy</th>
<th>Reinduction chemotherapy</th>
<th>Response to reinduction</th>
<th>SCT</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14/Male</td>
<td>T-ALL</td>
<td>1 rel</td>
<td>TCCSG HEX</td>
<td>NEL, VP16, CY</td>
<td>not achieved 2CR</td>
<td>Non CR-UBMT</td>
<td>Dead (2.5 Mo after relapse)</td>
</tr>
<tr>
<td>2</td>
<td>7/Female</td>
<td>T-ALL</td>
<td>1 rel</td>
<td>TCCSG HEX</td>
<td>NEL, VP16, CY</td>
<td>2CR</td>
<td>2CR SCT → rel</td>
<td>Dead (9 Mo after relapse)</td>
</tr>
<tr>
<td>3</td>
<td>4/Male</td>
<td>T-ALL</td>
<td>1 rel</td>
<td>TCCSG HEX</td>
<td>VCR, AraC, 6MP, MTX</td>
<td>partial response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14/Male</td>
<td>T-ALL</td>
<td>1 rel</td>
<td>TCCSG HEX</td>
<td>NEL, VP16, CY</td>
<td>2 CR → 2rel → non CR</td>
<td>Non CR-UBMT</td>
<td>Alive 7 Mo+ (in 3rd Relapse)</td>
</tr>
<tr>
<td>5</td>
<td>17/Male</td>
<td>T-ALL</td>
<td>1 rel</td>
<td>TCCSG HEX</td>
<td>TCCSG-HEX, NEL, VP16, FLU</td>
<td>2CR</td>
<td>UBM</td>
<td>Alive 7 Mo+ (on chemotherapy)</td>
</tr>
<tr>
<td>6</td>
<td>12/Male</td>
<td>T-LBL</td>
<td>PIF</td>
<td>JPLSG ALB05 (continue initial protocol)</td>
<td>non CR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>14/Female</td>
<td>T-ALL</td>
<td>Omset</td>
<td>TCCSG HEX + NEL</td>
<td>(continue initial protocol)</td>
<td>1CR</td>
<td>ICR BMRT</td>
<td>Alive 6 Mo+</td>
</tr>
<tr>
<td>8</td>
<td>11/Female</td>
<td>T-ALL</td>
<td>PIF</td>
<td>TCCSG HEX</td>
<td>FLAG-IDA</td>
<td>1CR</td>
<td>ICR BMRT</td>
<td>Alive 7 Mo+ (on chemotherapy)</td>
</tr>
</tbody>
</table>

**Abbreviations:** PNO, number of patient; T-ALL, T-cell acute lymphoblastic leukemia; T-LBL, T-cell lymphoblastic lymphoma; rel, relapse; PIF, primary induction failure; TCCSG HEX, Treatment protocol for high-risk ALL by Tokyo Children’s Cancer Study Group; JPLSG ALB05, Treatment protocol for advanced LBL by Japanese Pediatric Leukemia/Lymphoma Study Group; NEL, nelarabine; VP16, etoposide; VCR, vincristine; AraC, cytarabine; 6MP, 6-mercaptopurine; MTX, methotrexate; CY, cyclophosphamide; FLU, fludarabine; FLAG-IDA, FLU, AraC, idarubicin (IDA), and G-CSF; UBM, unrelated donor bone marrow transplantation; SCT, stem cell transplantation; CR, complete remission.

### Table 2: In vitro cytotoxic assay in T-ALL/LBL patients, median LC50 in μg/ml (95% Confidence Interval)

<table>
<thead>
<tr>
<th>NEL</th>
<th>AraC</th>
<th>FLU</th>
<th>PSL</th>
<th>DEX</th>
<th>VCR</th>
<th>L-ASP</th>
<th>DNR</th>
<th>VP16</th>
<th>4HC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Patient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.38</td>
<td>0.02</td>
<td>0.02 - 20</td>
<td>0.02 - 20</td>
<td>0.05 - 50</td>
<td>0.01 - 10</td>
<td>0.002 - 2</td>
<td>0.05 - 50</td>
<td>0.1 - 100</td>
</tr>
<tr>
<td>2</td>
<td>0.03</td>
<td>0.02</td>
<td>0.35</td>
<td>&lt; 0.2</td>
<td>&lt; 0.02</td>
<td>&lt; 0.05</td>
<td>0.15</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>3.17</td>
<td>0.01</td>
<td>0.18</td>
<td>NR</td>
<td>NR</td>
<td>2.40</td>
<td>(3.14 - 184.1)</td>
<td>0.03</td>
<td>(0.01 - 193)</td>
</tr>
<tr>
<td>4</td>
<td>1.47</td>
<td>0.04</td>
<td>0.88</td>
<td>8.90</td>
<td>0.99</td>
<td>0.38</td>
<td>0.84</td>
<td>0.12</td>
<td>0.32</td>
</tr>
<tr>
<td>5</td>
<td>4.15</td>
<td>0.41</td>
<td>0.19</td>
<td>NR</td>
<td>NR</td>
<td>3.29</td>
<td>(11.9 - 3.97)</td>
<td>2.05</td>
<td>(0.08 - 20)</td>
</tr>
<tr>
<td>6</td>
<td>1.38</td>
<td>0.03</td>
<td>0.07</td>
<td>0.73</td>
<td>0.17</td>
<td>0.03</td>
<td>0.16</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8.33</td>
<td>0.24</td>
<td>0.08</td>
<td>NR</td>
<td>NR</td>
<td>0.34</td>
<td>0.14</td>
<td>0.01</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>8</td>
<td>NR</td>
<td>0.06</td>
<td>0.04</td>
<td>NR</td>
<td>NR</td>
<td>0.01</td>
<td>0.02</td>
<td>0.002</td>
<td>0.51</td>
</tr>
</tbody>
</table>

**Abbreviations:** PSL, prednisolone; DEX, dexamethasone; AraC, cytarabine; NEL, nelarabine; VCR, vincristine; L-ASP, l-asparaginase; DNR, daunorubicin; VP-16, etoposide; 4HC, 4-hydroxycyclophosphamide; NR, not reduced to 50% in tested drug concentration; NA, not available.
growing cells were used for an in vitro cytotoxic assay, RNA preparation and induced drug low-sensitivity culture, as mentioned later.

**In vitro cytotoxic assay**

Patients’ samples and Jurkat cells were tested for drug sensitivity using 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) assay using a Cell-Counting–Kit–8 (Dojindo, Kumamoto, Japan), as previously described. The cells were incubated over 3 days in RPMI medium supplemented with 10% FCS with various drugs. NEL (Arranon, GlaxoSmithKlein), AraC (Cytoside, Nippon–Shionogi), FLU (Fludara, Genzyme), PSL (Predomin, Shionogi), DEX (Decadron, Banyu), VCR (Oncovin, Nippon–Kayaku), L–ASP (Leunase, Kyowa–Kirin), DNR (Daunomycin, Meiji–Seika), VP–16 (Lastet, Nippon–Kayaku) and 4–hydroperoxycycl phosphamide (4HC, an active metabolite of CY, kindly provided by Shionogi) were tested. The tested range of each drug was defined according to the previous reports, and are listed in Table 2.

Drug sensitivity was expressed as the LC$_{50}$ (50% lethal concentration in vitro) of each drug. The LC$_{50}$ was calculated by nonlinear regression with a 3–parameter logistic equation using GraphPad PRISM software (GraphPad Software, Inc., USA).

**Establishment of a NEL resistant T–ALL cell line**

To explore the mechanism of NEL resistance, we tried to establish a NEL resistant T–ALL cell line model with long-term nucleoside analogue exposure. We cultured Jurkat cells in standard RPMI containing approximately the LC$_{50}$ concentration of NEL AraC, or FLU, (2.0μg/mL, 0.025μg/mL and 1.0μg/mL, respectively) for the control Jurkat cells. After 24–hour incubation in drug–containing medium, cells were washed out and cultured in standard RPMI medium without the drug for another 24 hours. We repeated these steps in total 3 times and then cultured the cells in a drug–containing medium for 3 weeks. We established a logarithmically constant growing sub–cell line of Jurkat in the drug–containing medium. We named the sub–cell line Jurkat + N, Jurkat + C and Jurkat + F incubated with NEL, AraC and FLU, respectively. Jurkat + C showed the highest LC$_{50}$ of NEL in these sub–cell lines, and then was used in further experiments (data not shown).

**Real–time quantitative PCR analysis**

Expressions of ENT–1 mRNA in patient samples and the cell line were determined employing reverse transcription (RT), followed by real–time quantitative PCR (RQ–PCR). Total RNA was extracted from cells using a TOTALLY RNA kit (Ambion, TX, USA). RT was performed from 300 ng of total RNA using the High Capacity RNA to eDNA Master Mix (Applied Biosystems, CA, USA). The specific primer sets for RQ–PCR were as follows: ENT–1 (SLC29A1) (forward: 5’–TGAAGGCACTTCTAT- CAAAGCCCATC–3’, reverse: 5’–CTCAACAGT- CAGGCTGGA–3’) ; GAPDH (forward, 5’–GCAC- GTCAAGGCTGAGAC–3’; reverse, 5’– TGTTGAAGACGCCCAGTGGG–3’). RQ–PCR was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR Green as a dsDNA–specific binding dye and SYBR Premix EX Taq II (Takara–Bio, JAPAN). For ENT–1 and GAPDH quantification, the initial denaturation step at 95°C for 30 seconds was followed by 40 cycles with a denaturation step at 95°C for 5 seconds, and an annealing and extension step at 60°C for 34 seconds. The specificity of amplification was verified by dissociation curve analysis following the amplification steps. The threshold of cycles (Ct) was recorded and calculated for each sample. The relative ENT–1 mRNA expression level was estimated by the dCt method to compare with GAPDH.

**NBMPR inhibition study**

To investigate the role of the ENT–1 transporter function with the nucleoside analogue in Jurkat and Jurkat+C cells, we used nitrobenzylmercapturine riboside (NBMPR) (Sigma–Aldrich, MO, USA), which is an ENT–1 specific inhibitor. Jurkat cells were preincubated in standard RPMI including 10 to 100μM of NBMPR for 30 minutes, and then an in vitro cytotoxic assay was carried out. The maximum concentration of NBMPR was set at 100μM because the cytotoxicity of NBMPR itself was found at 200μM NBMPR (data not shown). A previous dose–response study by Griffith M. et al. showed the complete inhibition of ENT–1 in 100μM.

**Statistical analysis**

Correlations between the sensitivity of patient samples to NEL and other drugs were tested employing Spearman’s correlations. The 95% confidential interval (CI) of LC$_{50}$ was calculated by nonlinear regression with a 3–parameter logistic equation using GraphPad PRISM software. The LC$_{50}$ differences between Jurkat and Jurkat+C were analyzed using Mann–Whitney’s U–test. P–values less than 0.05 were considered to be significant.

**Results**

**In vitro cytotoxic assay in patient samples**

The LC$_{50}$ values of each drug in the patients and
Jurkat cell are listed in Table 2. The LC50 values of NEL ranged from 1.38 to over 100 μg/mL, below 0.01 to 0.41 μg/mL for AraC and 0.03 to 0.88 μg/mL for FLU. No one except for patients 2 and 4 responded to PSL and DEX. In patients 1, 2, 7, and 8, the LC50 values of NEL exceeded 5 μg/mL, which was the mean maximum plasma concentration (Cmax) of NEL on 1500 mg/m2 administration to adults reported by Cohen et al.15 We considered that these four patients were resistant to NEL. Spearman’s correlations showed no significant relationships between the LC50 of NEL and other drugs.

*In vitro nucleoside resistance induction in Jurkat + C*

To investigate the mechanism of NEL resistance, we established a NEL resistant T-ALL cell line model by long-term AraC exposure (Jurkat + C) from a Jurkat cell line and tested the response to nucleoside analogues. The LC50 values of NEL, AraC and FLU in Jurkat + C are presented in Figure 1. Jurkat + C showed a high resistance profile to each drug. The LC50 value of NEL was 37.1 μg/mL (27.5-fold of control p < 0.01, Mann–Whitney U-test), 26.2 μg/mL for FLU (11.1-fold of control, p < 0.001), and 0.60 μg/mL for AraC (25.8-fold of control, p < 0.01) in Jurkat + C. There was no difference in the LC50 of DNR between Jurkat and Jurkat + C.

**ENT-1 mRNA expression**

We measured ENT-1 mRNA expression, whose alteration was reported to be involved in AraC resistance.16-19 The mRNA level of ENT-1 in Jurkat + C was almost the same as that in Jurkat (Figure 2A). The ENT-1 mRNA expression levels in patient samples were 0.11 ± 0.11 to 0.68 ± 0.52-fold of Jurkat (Figure 2B).

**Effect of NBMPR on NEL, AraC, and FLU sensitivity**

Next, we attempted to investigate the activity of ENT-1 as a nucleoside analogue transporter using an ENT-1–specific inhibitor, NBMPR. In the Jurkat cell line, NBMPR inhibited the cytotoxic activity of NEL, AraC and FLU, but not DNR (Figure 3). This
Fig. 3 Nitrobenzylmercaptopurine riboside (NBMPR) effects on drug resistance in Jurkat and Jurkat+C. The broken line with closed marks indicates the relative cell survival curve of control Jurkat. The solid line with open marks indicates the relative cell survival curve of Jurkat+C. The triangles indicate relative survival of cells cultured with 100μM of (NBMPR) and the circles indicate NBMPR-free.

effect was shown in a dose-dependent manner (data not shown). The ENT-1–blocking effect of NBMPR was the strongest in AraC. The LC50 values of AraC were 0.03 (0.01–0.05, 95% CI) μg/mL without NBMPR, 0.51 (0.38–0.67) μg/mL (17-fold of control) with 10μM NBMPR, and 4.17 (0.40–0.83) μg/mL (161.4-fold of control) with 100μM NBMPR. Nevertheless, the cytotoxic activity of AraC was not suppressed completely in Jurkat incubated with 10μM NBMPR and even 200μM NBMPR (data not shown). Concerning NEL, the LC50 value was 1.08 (0.83–1.39) μg/mL without NBMPR, 3.44 (2.34–5.06) μg/mL (3.2-fold to control) with 10μM NBMPR and 8.99 (6.62–12.2) μg/mL (8.3-fold of control) with 100μM NBMPR. The LC50 of FLU was 2.03 (1.44–2.86) μg/mL without NBMPR, 9.38 (4.73–18.6) μg/mL (4.6-fold of control) with 10μM NBMPR, and 82.0 (42.8–156.9) μg/mL (40.3-fold of control) with 100μM NBMPR.

In Jurkat+C, NBMPR also enhanced drug resistance to NEL, AraC, and FLU but not DNR. The LC50 values of AraC were 1.68 (1.04–2.70) μg/mL without NBMPR, 5.64 (4.49–7.10) μg/mL (3.36-fold of control) with 10μM NBMPR and 9.87 (5.84–11.4) μg/mL (5.9-fold of control) with 100μM NBMPR. The LC50 values of NEL were 113.5 (81.1–158.8) μg/mL without NBMPR, 118.5 (76.0–184.7) μg/mL with 10μM NBMPR and 129.9 (66.2–12.2) μg/mL (1.1-fold of control) with 100μM–NBMPR. The LC50 values of FLU could not be calculated using the logistic model in the tested range, but the live cell ratio at 200μg/mL was still higher in NBMPR–containing medium. NBMPR had no influence on DNR sensitivity in Jurkat+C

Fig. 4 Schematic model of the NEL metabolic pathway
NEL is converted to Ara-G in plasma and culture medium by adenosine deaminase (ADA) and is then transported into cells through ENT-1 and/or other nucleoside transporters such as ENT-2 or the concentrative nucleoside transporter (CNT) family. Ara-G is subsequently phosphorylated to ara-G triphosphate (ara-GTP) and inhibits DNA synthesis. Ara-GTP is transported out of the cell through drug efflux pumps such as p-glycoprotein.
either.

**Discussion**

We have investigated an in vitro cytotoxic assay with NEL including clinical samples of childhood T-ALL/LBL. It might be difficult to distinguish sensitive from resistant T-ALL/LBL using an in vitro NEL cytotoxic assay. However, we compared the LC$_{50}$ values in patient samples to the mean Cmax of NEL. The average Cmax for the usual clinical dose of NEL (1,500 mg/m$^2$, 2-hour infusion) was $5 \pm 3 $ μg/mL.$^{15,22}$

In patients 1, 2, 7 and 8, the LC$_{50}$ of NEL exceeded 5 μg/mL. From this result, we assumed that these four patients out of the eight were resistant to NEL. The rate of NEL resistance in this study is similar to that of a clinical trial reported by Berg et al., showing that using NEL monotherapy for relapsed or refractory childhood T-ALL/LBL, the rate of CR+CR$^*$ was 48%. The in vitro cytotoxic assay might provide useful information to establish an appropriate use of NEL.

Neither NEL nor AraG is substantially bound to plasma protein ($<25\%$) (Arranon$,^\text{®}$ prescribing information, http://us.gsk.com/products/assets/us-arranon.pdf). When comparing plasma NEL concentration and the in vitro LC$_{50}$ value, the difference in the protein concentration between patient’s plasma and in vitro culture medium does not seem to be influenced.

We established an original NEL-resistant T-ALL cell line model from Jurkat by long-term exposure of AraC to clarify one of the possible mechanisms of NEL resistance. The cytotoxic effect of NEL depends on the intracellular ara-GTP concentration.$^{25}$ The possible factors which affect the intracellular ara-GTP concentration are: 1) plasma ADA activity, 2) nucleoside transporter activity, 3) intracellular enzyme activity, and 4) drug efflux activity, as shown in Figure 4. In this study, we focused on nucleoside transporters in the first step to investigate the mechanisms of AraC induced resistance to NEL. The equilibrative nucleoside transporter (ENT) family has been well documented regarding nucleoside analogue transporters and drug resistance. ENT-1 is the major transporter of AraC and it has been reported that the lower expression of ENT-1 or ENT-1 mutations was involved in AraC resistance.$^{16-19}$

In this study, the ENT-1 mRNA expression level in Jurkat+C was similar to that in Jurkat. Therefore, the AraC induced NEL resistant T-ALL model we established in this study may not be involved in ENT-1 mRNA expression levels. In addition, ENT-1 mRNA expression levels in patient samples did not correlate with the LC$_{50}$ values of NEL. Next, we studied the role of transporter activity of ENT-1 in NEL cytotoxicity using NBMPR, a specific inhibitor of ENT-1. Because it has been previously reported that in vitro cytotoxicity of FLU in B-cell chronic lymphocyte leukemia was not correlated to mRNA expression of ENT family but related to biologic transporter activity.$^{24}$ In Jurkat, the ENT-1 blocking effect was the strongest for AraC cytotoxicity (161.4-fold of control), the second was for FUL (40.4-fold) and the third was for NEL (8.3-fold) with 100μM NBMPR. The differences among nucleoside analogues may possibly be dependent on ENT-1-mediated influx. These results indicate that ENT-1 may partly act as a transporter not only for AraC but also for NEL and FLU. In Jurkat+C, NBMPR still enhanced further drug resistance to AraC (5.9-fold), NEL (1.15-fold) and FLU (unable to calculate because out of the testing range). ENT-1 may not play a key role in the mechanism of acquiring NEL resistance in Jurkat+C because the ENT-1 expression was not reduced in Jurkat+C compared to the Jurkat cells, and that the NBMPR-blocking effect was still noted in Jurkat+C.

In previous studies, drug efflux mechanisms such as p-glycoprotein have been reported to be involved in multi–drug resistance including nucleoside analogues and anthracyclines.$^{6,7,25}$ In this study, NBMPR did not show any effect on DNR sensitivity in either Jurkat or Jurkat+C. This suggests that the resistance mechanism in Jurkat+C may not rely on the drug efflux system, but possibly on the nucleoside analogue metabolic pathway.

In this study, the LC$_{50}$ values of NEL in all patients were higher than in Jurkat, which is considered to be sensitive to NEL. The LC$_{50}$ values of NEL in patient samples were still higher than the mean LC$_{50}$ of NEL (0.37μg/mL) in the 15 series of T-ALL cell lines reported by Beesley et al.$^{26}$

In conclusion, childhood T-ALL/LBL cell may have in vitro NEL resistance in the patients relapsed after the T-ALL standard protocol or with first induction failure. An in vitro cytotoxic assay may be useful to establish the appropriate use of NEL. In an original NEL–resistant T-ALL cell line model which we established, ENT-1 partly acts as a NEL transporter and the NEL resistance seems to be independent of ENT-1. To elucidate the mechanisms of NEL resistance, further studies will be needed to focus on the nucleoside analogue metabolic pathway to clarify a more effective use of NEL and improve the prognosis of childhood T-ALL/LBL patients.

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Conflicts of interest statement
There is no financial or other potential conflict of interest for any author.

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