A Supramolecular Substance, [2] Rotaxane, Induces Apoptosis in Human Molt-3 Acute Lymphoblastic Leukemia Cells

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Abstract
The antitumor effects of a supramolecular substance, the [2] rotaxane (TRO-A0001), and its molecular mechanisms were investigated. TRO-A0001 suppressed the proliferation of cultured human Molt-3 acute lymphoblastic leukemia cells for 12–72 h in a dose-dependent manner. Based on flow cytometry, TRO-A0001 clearly induced apoptosis after 24 h. The mitochondrial membrane potential disappeared after treatment with 1.0 µM of TRO-A0001. Expression of the cleaved forms of caspase-9 and caspase-3 was significantly increased in cells exposed to TRO-A0001, whereas the expression of XIAP, a type of inhibitor of apoptosis family, was decreased. These results suggest that [2] rotaxane TRO-A0001 may be a highly promising new antitumor medicine.

Introduction
Although progress has been made, the available chemotherapy for acute lymphoblastic leukemia (ALL) provides a limited survival rate, particularly for adults and causes severe side effects. Few molecular targeting drugs or multiple combinations of anticancer drugs are used. Therefore, the development of effective new agents is urgently required. We previously reported that TRO-A0001 induced the activation of caspases in solid cancer cells in vitro [1]. The aim of this study was to investigate the potential of TRO-A0001 as a new chemotherapeutic agent for ALL.

Materials & Methods
Materials
The [2] rotaxane, [2]bis(2-(3,5-dimethylphenylcarbonyloxy)-ethyl) ammonium trifluoromethanesulfonate-[dibenzo-24-crown-8] rotaxane (TRO-A0001) [2], was used in this study. It is covered by patent numbers JP 3887008 and US 8,207,357B.

The human acute lymphoblastic leukemia cell line Molt-3 (DS Pharma Biomedical Co., Ltd., Osaka, Japan) was cultured in RPMI 1640 medium (Life Technologies Co., CA, USA).

Cell survival assay
Cell viability was assayed by MTT. Cells (1 × 10^5 cells/well) were added to 96-well plates in RPMI 1640 (100 µL) containing 10% FBS. Cells were treated with TRO-A0001 (0.1, 0.5, 1.0, 2.5 and 5.0 µM in 0.1% DMSO) and incubated for 24 h. At the end of treatment, 10 µL of MTT was added, and analyzed the results with a microplate reader (Beckman Coulter, CA, USA).

DNA fragmentation assay
Cells treated with or without TRO-A0001 were collected by centrifugation at 200 × g for 5 min and washed once with phosphate-buffered saline (PBS). The cell pellet was suspended in 100 µL of cell lysis buffer (10 mM Tris-HCl buffer, pH 7.4 containing 10 mM EDTA and 0.5% TritonX-100), kept at 4 °C for 10 min, and the cell lysate was centrifuged at 16 000 rpm for 20 min. The supernatants were incubated with RNase A (0.5 mg/mL; Sigma-Aldrich) at 37 °C for 60 min, then incubated with proteinase K (0.5 mg/mL; Sigma-Aldrich) at 50 °C for 60 min. The supernatants were mixed with 20 µL of 5 M NaCl and 120 µL of isopropyl alcohol overnight at −20 °C. The supernatants were then collected by centrifugation at 16000 rpm for 15 min. DNA samples were dissolved in TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0), separated by 2% agarose gel electrophoresis at 100 V for 45 min and stained with 0.1 mg/L ethidium bromide solution.
Flow cytometry
Apoptosis and necrosis were measured using the Annexin V-propidium iodide Kit (Medical & Biological Lab., Nagoya, Japan) [3]. The change in the mitochondrial transmembrane potential was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, MI, USA) according to manufacturer instructions. Each population in quadrants was analyzed using a BD Accuri C6 Flow Cytometer (BD Biosciences, CA, USA).

Western blotting
The expression levels of caspase-3, caspase-9, and XIAP were determined as described by Hara et al. [1]. In brief, proteins were isolated from Molt-3 cells after treatment with TRO-A0001, and protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, CA, USA).

Results and Discussion
TRO-A0001 suppressed the proliferation of Molt-3 cells for 12–72 h in a concentration-dependent manner (0.5–10.0 µM) by MTT assay. These results were similar to previous studies in human melanoma and colon cancer cells [1]. The IC₅₀ value for TRO-A0001 in Molt-3 cells, after treatment for 24 h, was 0.51 µM. We studied in leukemia cells, K562 and KG-1 cells, and got simi-
lar effect with Molt-3 cells (Fig. 1a). Molt-3 cells were treated with TRO-A0001 for 12 h followed by DNA fragmentation analyses. Significant DNA ladders were observed in Molt-3 cells treated with 5.0 µM of TRO-A0001 (Fig. 1b). Within 24 h of treatment with 5.0 µM, cells clearly exhibited significant morphological changes and chromosomal condensation, indicative of apoptotic cell death. Based on the results of flow cytometry, TRO-A0001 clearly induced apoptosis following 24 h of treatment (Fig. 1c) in a concentration-dependent manner. A significant increase in the percentage of early apoptotic cells was observed with 1.0 µM, and in late apoptotic cells was observed with 10.0 µM.

The involvement of the mitochondrial membrane potential (ΔΨm) in Molt-3 cells treated with TRO-A0001 was investigated [4]. ΔΨm disappeared after treatment with 1.0 µM TRO-A0001 (Fig. 1d). This result suggests the activation of caspase-9 cascade in the intrinsic apoptotic pathway. Looking specifically at the activation of caspase-9 and its substrate caspase-3, we found that the levels of cleaved caspase-9 and cleaved caspase-3 were significantly increased by TRO-A0001 (1.0–5.0 µM). Simultaneously, levels of full-length caspase-9 and caspase-3 were decreased (Fig. 1e, Table 1). X-linked inhibitor of apoptosis protein (XIAP) is also known as inhibitor of apoptosis protein 3 (IAP3). It has been reported that XIAP inhibits caspase-3, -7, and -9 [5]. In this study, the expression level of XIAP was reduced by TRO-A0001 in a concentration-dependent manner (1.0–5.0 µM). These results suggest that TRO-A0001 induces cell death in Molt-3 cells via apoptosis, through activation of the mitochondrial pathway and suppression of XIAP.

**Table 1** Expression levels of caspase-9, -3, and XIAP in Molt-3 cells following treatment with TRO-A0001 for 6h.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Caspase-9</th>
<th>Cleaved Caspase-9</th>
<th>Caspase-3</th>
<th>Cleaved Caspase-3</th>
<th>XIAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 µM</td>
<td>0.387 ± 0.087 **</td>
<td>2.624 ± 0.764 **</td>
<td>0.445 ± 0.218 *</td>
<td>1.381 ± 0.151 *</td>
<td>0.573 ± 0.100 **</td>
</tr>
<tr>
<td>2.5 µM</td>
<td>0.211 ± 0.067 **</td>
<td>3.489 ± 0.629 **</td>
<td>0.240 ± 0.088 **</td>
<td>1.590 ± 0.069 **</td>
<td>0.528 ± 0.096 **</td>
</tr>
<tr>
<td>5.0 µM</td>
<td>0.287 ± 0.147 **</td>
<td>5.113 ± 1.003 **</td>
<td>0.185 ± 0.030 **</td>
<td>1.981 ± 0.196 **</td>
<td>0.320 ± 0.125 **</td>
</tr>
</tbody>
</table>

All the results were normalized to the expression of the β-actin

The numerical value is the ratio compared with the control (0 µM)

Values represent the mean ± S.D. (n = 4)

* Statistical significance compared with control (t-test: * p < 0.05, ** p < 0.01)

**Conflict of Interest**

The authors declare no conflicts of interest.

**References**