Cancer is currently a leading cause of death in throughout the world. Therefore there is an urgent need to develop anticancer agents. Most anticancer drugs work only after being absorbed into a cancer cell, the drugs having relatively low molecular weights and stable structure that can be easily incorporated via a diffusion system or transportation in the cell membrane. However, anticancer drugs that are easily absorbed are also often easily excreted from the cell, and the effect may be insufficiently controlled. Also their use may be accompanied by severe adverse drug reactions and multiple drug resistances (1). Therefore, a new seed compounds for future development of anticancer drugs are required. We have synthesized a novel supramolecular substance, [2] rotaxane (TRO-A0001) (Fig. 1A), which is a unique polymer compound devoid of a covalently bonded moiety (2 – 4).

In the therapy to control cancer cells through induction of apoptosis, it is considered important to focus on the mitochondria-mediated pathway, converging on caspase-3 and other proteases in the therapeutical means (5 – 7). The principal purpose of this study was to determine whether this [2] rotaxane has bioactivity to inhibit the growth of cancer cells and whether its mechanism is related to action against caspase-3 or another protease.

Abstract. We studied the influence of novel supramolecular substance, [2] rotaxane (TRO-A0001), on caspase signaling and cell viability in cancer cell lines. TRO-A0001 suppressed concentration-dependently cell proliferation. Expression of the cleaved-form caspase-3 and PARP was significantly increased in cells exposed to TRO-A0001. The expression of Bax was increased by TRO-A0001. Furthermore, the down-regulation of Bax by siRNA resulted in growth activation significantly. The morphological analysis demonstrated that TRO-A0001 increased the levels of apoptotic cells in human cancer cell lines. These results suggest that TRO-A0001 induces apoptosis in cancer cells and holds potential as a new anti-tumor medicine.

Keywords: [2] rotaxane, supramolecular compound, apoptosis
DLD-1 (European Collection of Cell Cultures, UK), mouse melanoma cell line B16/BL6, and mouse rectum carcinoma cell line Colon-26 (Institute of Development, Aging, and Cancer, Tohoku University, Japan) were used in this study. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin (50 units/ml)-streptomycin (50 μg/ml) (Gibco) at 37°C in a humidified atmosphere of 5% CO2.

For quantitative study of viable cells and proliferation, we used the MTT assay and analyzed the results with a microplate reader (Beckman Coulter, CA, USA). Cells (5.0 × 10^3 cells/well) were added to 96-well plates in RPMI (50 μL) containing 10% FBS. After incubating for 12 h, cells were treated with various concentrations of TRO-A0001 (2.5, 5.0, and 10.0 μM) in 0.2% DMSO and incubated for another 12 – 48 h. The control cultures were treated with 0.2% DMSO alone in RPMI. The number of viable cells after treatment with different concentrations of TRO-A0001 was determined from the standard curve of the number of cells and absorbance value.

Western blotting methods were used for analyzing caspase-9, caspase-3, caspase-7, poly (ADP-ribose) polymerase (PARP), and Bax proteins (antibody purchased from Cell Signaling Technology, MA, USA). Cell lysates treated with TRO-A0001 for 24 h were centrifuged at 4°C for 15 min (14,000 × g), and supernatants were used for protein samples. Equal amounts of protein samples (2.5 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA).

Bax and non-targeting control small interfering RNA (siRNA) were purchased from Cell Signaling Technology. Cells were seeded in 6-well culture plates and allowed to grow for 24 h (60% – 80% confluence) in

![Diagram of TRO-A0001 and Dibenzo-24-crown 8-ether](image-url)

**Fig. 1.** Effect of TRO-A0001 on the growth in tumor cells. A) Chemical structure of [2] Rotaxane (TRO-A0001) and the structural constituents of the wheel and axis portion of the molecule. B) Effect of TRO-A0001 on the growth of melanoma and colon cancer cell lines. Cells were exposed to TRO-A0001 of various concentrations (2.5, 5.0, 10.0 μM), and control (0 μM) cultures were treated with 0.2% DMSO in RPMI for 12 – 48 h. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *P < 0.01, compared with control group incubated for each time.
complete medium. After incubating, cells were transfected for 24 – 48 h using TransIT-siQUEST Transfection Reagent (Mirus, Madison, WI, USA) with 100 nM control or Bax siRNA. After transfection, cells were harvested and seeded in 96-well culture plates to measure cell viability as described below.

Terminal Deoxynucleotidyl Transferase-dUTP Nick End Labeling (TUNEL) assay showed apoptotic cells. To examine cell death caused by TRO-A0001, we used the Click-iT TUNEL Alexa Fluor 594 Imaging Assay (Life Technologies, Invitrogen, Carlsbad, CA, USA). TUNEL-positive cells stained red and nuclei stained blue with Hoechst 33342. To provide a positive TUNEL group, we added DNase to another control group. Photographs were obtained using a fluorescent microscope (Keyence, Osaka).

Results were analyzed by one-way analysis of variance, followed by the Tukey–Kramer post hoc test to determine differences among groups. All values are expressed as the mean ± S.E.M. P < 0.01 was considered statistically significant.

After incubation with TRO-A0001 for 48 h, viable cells were significantly decreased by TRO-A0001 concentration-dependently (2.5 – 10.0 μM) in all cancer cell lines (Fig. 1B). IC50 values after incubation with TRO-A0001 for 24 h were 2.58 μM in G361, 7.81 μM in DLD-1, 6.39 μM in B16/BL6 (data not shown), and less than 2.5 μM in Colon-26 cells.

Expression levels of caspases in the signaling pathway were measured as a marker of apoptosis. In the experiment to determine expression levels of caspases, cleaved caspase-3 and cleaved PARP were up-regulated by TRO-A0001 (2.5 – 10.0 μM) in all cancer cells (Fig. 2: A – D). On the other hand, full length caspase-3 was not changed as compared with the control samples, and full length PARP was decreased in colon cancer cell lines (Fig. 2: C, D). The expression of full length caspase-3 was decreasing in melanoma cells, although full length PARP indi-
cated a different expression in G361 cells (Fig. 2: A, B). These results suggest that TRO-A0001 induced injury or death of melanoma and colon cancer cells by activating an apoptotic pathway to impair nuclear function.

Expression levels of Bax as a key for cellular induced apoptosis through the mitochondrial pathway was measured. The levels of Bax were increased by TRO-A0001, compared with the controls. Bax increases the membrane’s permeability, which leads to the increase of cytochrome c from mitochondria, activation of caspase-9, and initiation of the caspase activation pathway for apoptosis. Caspase-9 levels in human cancer cells were remarkably decreased in the 10.0-μM samples compared to the control, but the different result in the expression levels of cleaved caspase-9 was shown in G361 cells (Fig. 2: A, C). Furthermore, the expression levels of caspase-7 were different between each cell.

To confirm that the down-regulation of Bax blocked TRO-A0001-induced apoptosis, we used Bax siRNA transfection methods and examined the influence on cell viability. Our results showed that transfection of Bax siRNA significantly improved cell viability compared to control siRNA after 36-h treatment with TRO-A0001 in DLD-1, B16/BL6, and Colon-26 cells, although G361 cells did not show a significant improvement in viability (Fig. 3A). In G361, as shown Fig. 2A, expression levels of Bax were extremely low in the control samples (0 μM), so we thought the Bax siRNA transfection did
not influence the viability of G361 cells treated with TRO-A0001. Altogether, these results suggested that the down-regulation of Bax was also one of the anti-apoptotic events in the TRO-A0001-induced cell death. TUNEL imaging assay showed a significant increase in the number of apoptotic cells after TRO-A0001 treatment (Fig. 3B). The numbers of TUNEL-positive cells were higher in the TRO-A0001-treated groups than in the control group.

Caspases play an important role in regulating apoptotic signal transmission. Caspases are synthesized as relatively inactive zymogens and are activated by cleavage via upstream proteases in an intracellular cascade (9). The initiator caspases are capable of cleaving and activating downstream effector caspases, such as caspase-3, -6, and -7, and activation results in apoptosis (10). Apoptosis is a regulated suicide program. Induction of apoptosis is mediated either through death receptors or at the mitochondrial level, and both systems can activate caspase-3 (11, 12).

In this study, TRO-A0001 caused cell death characterized by activation of the caspase signal pathway (Figs. 1B, 2). Activated caspase-3 is responsible for the proteolytic degradation of PARP, which occurs at the onset of apoptosis. PARP cleavage indicates the initiation of nuclear DNA damage, resulting in DNA fragmentation and the characteristic changes observed in apoptosis. PARP (116 kDa) is a nuclear enzyme that helps cells to maintain their viability so they can repair their DNA, while cleavage of PARP (89 kDa) facilitates cellular disassembly (13–15). Our expression analysis of cleaved caspase-3 suggested that TRO-A0001 activated caspase-3 and signaled to the downstream to mediate cleavage of PARP. The expression of cleaved PARP was increased in all cancer cells, resulting in apoptosis. The results on the expressions of caspase-9 and caspase-7 may be due to different properties in each cell line. Also, many TUNEL-positive cells were detected in TRO-A0001 (10.0 μM)-treated human melanoma and colon cancer cells (Fig. 3B). Thus, cells with high levels of caspase-3 activation exhibited cell death driven by TRO-A0001, and the mechanism of cell death considered a lead to apoptosis. Furthermore, as shown in Fig. 3A, the knock-down of Bax markedly increased cell viability, in contrast with the anti-proliferative effect induced by TRO-A0001 in DLD-1, B16/B6, and Colon-26 cells, indicating that siRNA has a tendency to cause the recovery of G361 cells.

In the exposure to each constituent element of TRO-A0001, dibenzo-24-crown-8-ether and NK7-40-2 did not affect cell viability (data not shown). It is suggested that the individual rotaxane structure has influenced in the growth of cells. The supra-molecular substance has an inner-molecular movement such as a shuttling behavior on axis quantum mechanically. Then, the anti-proliferative effect may participate in the specific molecular behavior in TRO-A0001.

In conclusion, [2] rotaxane, TRO-A0001, is able to induce apoptosis through caspase signaling activation in tumor cells and may be a useful anti-tumor drug.

References