A Novel Tetramethylnaphthalene Derivative Selectively Inhibits Adult T-Cell Leukemia (ATL) Cells *In Vitro*

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Abstract. *Adult T-cell leukemia (ATL) is caused by infection with human T-cell leukemia virus type-1 (HTLV-1). The tetrahydrotetramethylnaphthalene derivative TMNAA has recently been identified as a selective inhibitor of HTLV-1-infected T-cell lines and adult T-cell leukemia (ATL) cells but not of uninfected T-cell lines and peripheral blood mononuclear cells (PBMCs). In the present study, more than 100 derivatives of TMNAA were synthesized and examined for their inhibitory effects on the proliferation of various T-cell lines and PBMCs. Among the compounds, MN417 is a more potent inhibitor of ATL cells than TMNAA. This compound is a novel phenanthridinone derivative with the tetrahydrotetramethylnaphthalene structure. Interestingly, PN-H and MN314-B, which are also phenanthridinone derivatives but do not have the tetrahydrotetramethylnaphthalene structure, could not distinguish between HTLV-1-infected and uninfected T-cell lines in terms of their anti-proliferative activity. These results suggest that the tetrahydrotetramethylnaphthalene structure is required for the selective inhibition of HTLV-1-infected cells.*

Adult T-cell leukemia (ATL) is a malignancy of peripheral T-lymphocytes caused by human T-cell leukemia virus type-1 (HTLV-1) (1). HTLV-1 infection is endemic mainly in Japan, Caribbean islands, South America, and South Africa.

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Key Words: HTLV-1, ATL, tetramethylnaphthalene derivative, inhibitor.

The number of HTLV-1 carriers is estimated to be 15 to 20 million worldwide (2). ATL is classified into four sub-types, such as acute, lymphoma, smoldering, and chronic. The median survival time of patients with chronic and smoldering types of ATL is more than 2 years, whereas aggressive (acute and lymphorma) types of ATL have a poor prognosis with a median survival time of 13 months (3). Although conventional anticancer chemotherapy against malignant lymphomas is usually applied to patients with the aggressive types of ATL, their mean survival time has not been sufficiently extended (4, 5). It is, therefore, mandatory to find novel and effective therapeutic agents against ATL.

There have been several attempts to develop novel inhibitors of ATL targeting various cellular factors involved in ATL cell growth. These include nuclear factor-κB (NF-κB), proteasome, histone deacetylase (HDAC), and heat shock protein-90 (HSP90). The NF-κB pathway is recognized as a positive regulator of ATL cell proliferation and survival through transcriptional activation of many pro-survival and anti-apoptotic genes, such as *Bcl-xl*, and survivin (6, 7). The NF-κB inhibitors DHMEQ and Bay 11-7082 block the activation of NF-κB and induce apoptosis of ATL cell lines and primary ATL cells (8, 9). ABT-737 and YM155 suppress the Bcl-2 family and survivin, respectively, resulting in the inhibition of cell proliferation followed by the induction of apoptosis in ATL cells (10, 11). The proteasome inhibitor bortezomib inhibits the degradation of IκB in ATL cells, resulting in suppression of NF-κB and induction of ATL cell death (12). HDAC inhibitors are a new class of compounds, which act at the transcriptional level by interfering with epigenetic chromatin modification and histone de-acetylation. Furthermore, they increase acetylation of other proteins, including nuclear transcription factors, and this function mostly mediates their anticancer activity (13). Several HDAC inhibitors, such as LBH589 and FR901228, also suppress the proliferation of ATL cells and primary ATL cells (14, 15). HSP90 proteins are expressed at a high level

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in ATL cells (16). The HSP90 inhibitor 17-DMAG decreases the level of HSP90 and suppresses the AKT/GSK-3β/β-catenin pathway in ATL cells (17).

We have recently found that 2-acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetrametylnaphthalene (TMNAA) is a potent and selective inhibitor of the ATL cell line S1T but not the non-ATL cell line MOLT-4 (18). In addition, TMNAA could also suppress the proliferation of various T-cell lines infected with HTLV-1 (19). Based on these findings, we attempted identification of more potent inhibitors of ATL cells than TMNAA. To this end, more than 100 derivatives of TMNAA were synthesized and examined for their inhibitory effect on the proliferation of ATL and non-ATL cells, including primary peripheral blood mononuclear cells (PBMCs), *in vitro*. In the present study, we show that a novel phenanthridinone derivative containing the tetrahydrotetramethylnaphthalene structure is a more potent inhibitor of ATL cells than TMNAA. The original phenanthridinone derivative was designed, synthesized and examined for its antiviral activity against hepatitis C virus (HCV) (20).

Materials and Methods

Compounds. All compound were synthesized by Dr. Hashimoto and his colleagues, according to a method previously described (18, 20). The compounds were dissolved in dimethyl sulfoxide (DMSO) (Nakalai Tesque, Kyoto, Japan) at a concentration of 20 mM to exclude the cytotoxicity of DMSO and stored at −20˚C until use.

Cell lines. Six HTLV-1-infected cell lines (S1T, Su9T01, K3T, F6T, and MT-2) and two uninfected T-cell lines (MOLT-4 and CEM) were used in the present study. They were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. S1T, Su9T01, K3T, and F6T are leukemia cell lines isolated and established from ATL patients (ATL cell lines), while MT-2 is an HTLV-1-transformed T-cell line (21, 22). PBMCs were obtained from healthy volunteers under informed consent. The cells were isolated by Ficoll-Hypaque density gradient centrifugation and stimulated with 5 μg/ml phytohemagglutinin (PHA) in RPMI-1640 medium supplemented with 20% FBS, 100 U/ml recombinant human interleukin-2 (kindly provided by Takeda Pharmaceutical Company, Osaka, Japan), and antibiotics for 3 days. The above medium without PHA was used for assay experiments with PBMCs.

Cell proliferation assay. The inhibitory effect of test compounds on cell proliferation was determined by a tetrazolium dye method (TetracolorONE®; Seikagaku Biobusiness, Tokyo, Japan). The cells (1×10^4 cells/well for cell lines and 1×10^5 cells/well for PBMCs) were cultured in the presence of various concentrations of the test compounds in a 96-well plate. After incubation at 37˚C for 96 h, the tetrazolium dye (10 μl) was added to each well. The cells were further incubated at 37˚C for 2 h. The absorbance of each well was measured at 450 and 600 nm with a microplate reader (Bio-Rad, Richmond, CA, USA). The inhibitory effect of test compounds on cell proliferation was also determined by the trypan blue exclusion at 24, 48, 72, and 96 h after incubation in the presence of compounds.

Apoptosis assay. The cells (5×10^4 cells/ml) were cultured in the absence or presence of 10 μM TMNAA, 10 μM MN417, or 10 μM cepharanthine (CEP) as a positive control. After incubation for 48 and 72 h, the cells were collected and washed twice with an annexin binding buffer. Cells (1×10^6 cells) were further stained with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) using an Annexin V-FITC Apoptosis kit (eBioscience, San Diego, CA, USA). After staining, the cells were analyzed by flow cytometry (FACSCalibur™; Becton Dickinson, San Jose, CA, USA).

Statistical analysis. The statistical significance of the assay results was determined by the Student's *t*-test.

Results

Inhibitory effect of TMNAA derivatives on cell proliferation. After discovery of TMNAA (Figure 1) as a selective inhibitor of ATL cells, 105 compounds were newly-synthesized and examined for their inhibitory effect on the proliferation of S1T and MOLT-4 cells. Among the compounds, 5-butyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-j]phenanthridin- 6(5H)-one (MN417) (Figure 1) was found to be a more potent inhibitor of S1T cell proliferation than TMNAA. TMNAA achieved more than 50% inhibition at a concentration of 4 μM, while MN417 could do so at 0.8 μM (Figure 2). Furthermore, MN417 also more strongly inhibited the proliferation Su9T01, K3T, and F6T cells in a dose-dependent fashion, compared to TMNAA (Figure 2). Both compounds were more potent inhibitors of the growth of MT-2 cells than ATL cell lines (Figure 2). In contrast, the proliferation of the uninfected T-cell lines MOLT-4 and CEM and PBMCs was not affected by TMNAA and MN417 at concentrations up to 20 and 4 μM, respectively. These results suggest that, like TMNAA, MN417 is also a potent and selective inhibitor of ATL cells' proliferation. To confirm the selective inhibition of ATL cells by MN417, the viable cell number of S1T, MT-2, MOLT-4, and CEM were also examined by trypan blue exclusion every 24 h (up to 96 h) after drug-exposure. MN417 strongly suppressed the growth of S1T and MT-2 cells in a time-dependent fashion at a concentration of 4 μM (Figure 3). Again, these compounds did not affect the growth of MOLT-4 and CEM cells at these concentrations.

Table I summarizes the 50% inhibitory concentrations (IC_{50}s) of TMNAA and MN417 for various T-cell lines and PBMCs. Although there was some difference in their IC_{50} values for the five ATL cell lines, the values were significantly lower than those for the uninfected T-cell lines and PBMCs. Furthermore, MN417 appeared to be 2- to 7-fold more potent than TMNAA. To gain insight into the structural requirement for the selective inhibition, two compounds structurally-related to MN417, 5-butylphenanthridin-6(5*H*)-one (PN-H) and 5-butylbenzo[*j*]phenanthridin-6(5*H*)-one (MN314-B) (Figure 1), were examined for their anti-proliferative activity. Interestingly, PN-H and

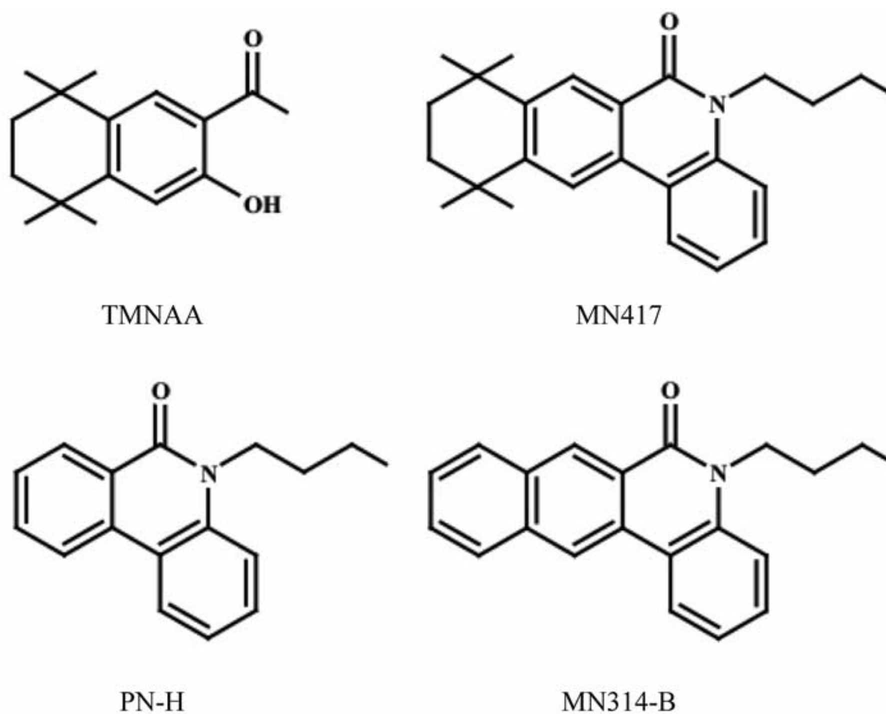

Figure 1. *Chemical structures of 2-Acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetr-ametylnaphthalene (TMNAA) 5-butyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-j]phenanthridin-6(5H)-one (MN417), 5-butylphenanthridin-6(5H)-one (PN-H), and 5-butylbenzo[j]phenanthridin-6(5H)-one (MN314-B).*

MN314-B could not distinguish between HTLV-1-infected and uninfected T-cell lines in terms of their activity (Table I), suggesting that the tetrahydrotetramethylnaphthalene structure is required for the selective inhibition of HTLV-1-infected cells.

TMNAA and MN417 did not induce apoptosis in S1T cells. Inhibitory effect of MN417 on cell proliferation was further investigated by flow cytometry. S1T cells were treated with either 10 μM TMNAA, 10 μM MN417, or 10 μM CEP and incubated for 48 and 72 h. When annexin V-positive and PI-negative cells, which indicate early apoptotic cells, were analyzed, treatment of S1T cells with TMNAA and MN417 hardly increased the proportion of apoptotic cells at 48 and 72 h. Interestingly, the proportion of PI-positive cells, which indicate dead cells, were significantly increased after incubation with these compounds for 72 h (Figure 4), indicating that both compounds induced non-apoptotoc cell death in S1T cells.

Table I. *Inhibitory effect of TMNAA and related compounds on cell proliferation.*

Cell	HTLV-1	IC_{50} (μM)			
		TMNAA	PN-H	MN417	MN314-B
S1T	+	1.65±0.03[a]	35.3±5.1	0.59±0.07	14.4±5.1
Su9T01	+	14.2±5.8	34.9±2.8	6.10±1.0	34.4±4.9
K3T	+	30.7±2.8	38.3±5.3	6.60±0.5	36.4±8.6
F6T	+	34.2±5.1	44.5±9.7	6.20±1.3	13.5±1.9
MT-2	+	1.17±0.25[a]	31.5±10.4	0.16±0.08	10.9±1.4
MOLT-4	−	>100	53.3±3.2	23.1±1.2	37.7±7.8
CEM	−	>100	58.1±3.1	26.2±7.7	46.3±8.9
PBMCs	−	>100	ND	52.2	ND

IC_{50}: 50% Inhibitory concentration; ND: not determined. Except for PBMCs, all data represent mean±SD for three independent experiments. [a]Data are taken from reference 23.

Discussion

In the present study, we have demonstrated that MN417, a TMNAA derivative, is a more potent inhibitor of several ATL cell lines than TMNAA. In contrast, the two derivatives containing the same phenanthridinone structure PN-H and MN314-B (Figure 1) did not show such selective inhibition of ATL cell growth. The results of the present study indicate that the tetrahydrotetramethylnaphthalene structure is required for the selective inhibition of ATL cells. MN417 is considered to be a hybrid of TMNAA and PN-H. TMNAA is a lead compound of selective ATL inhibitors, while PN-H

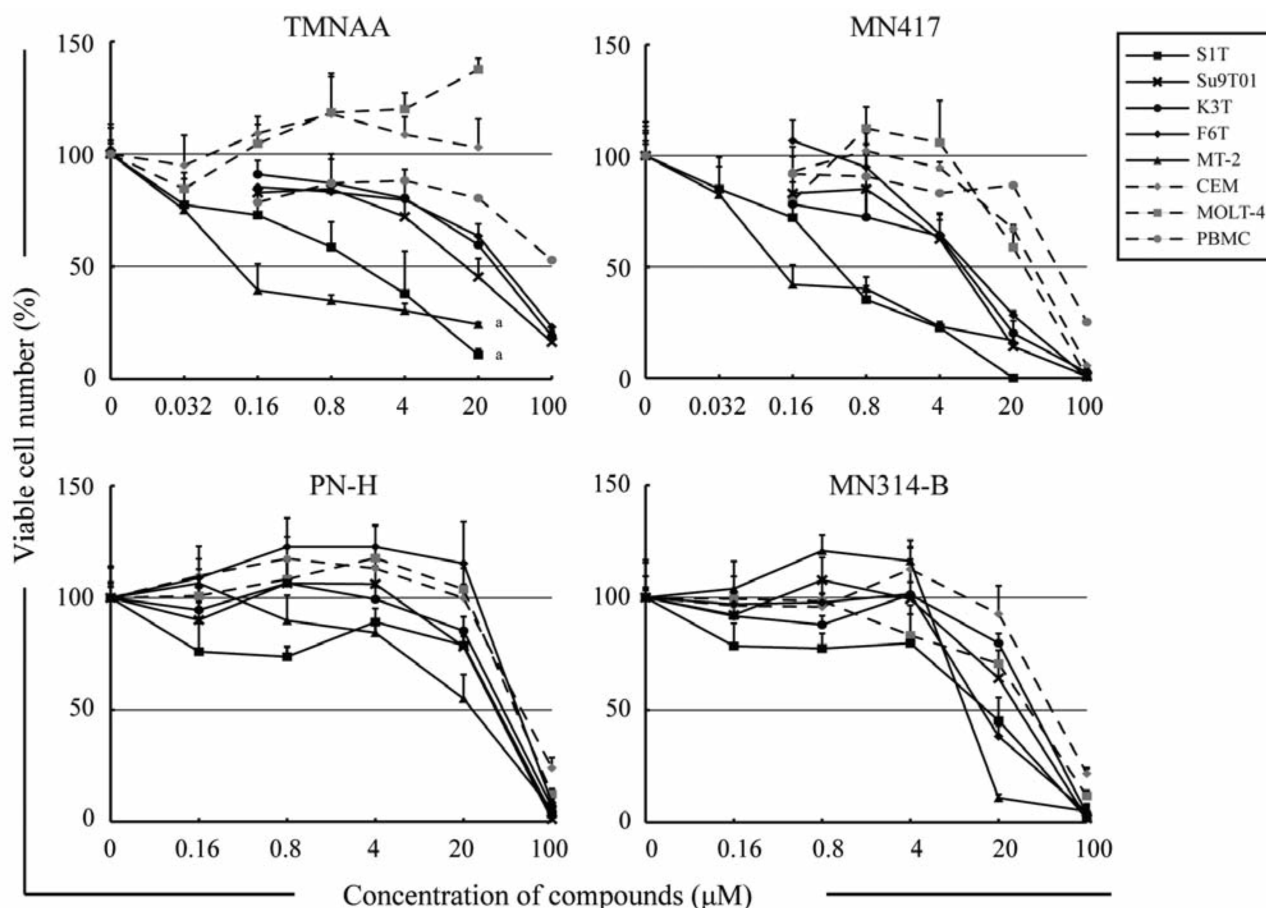

Figure 2. *Inhibitory effect of compounds on cell proliferation. The cells (1×10^4 cells/well) were incubated in the presence of various concentrations of compounds for 96 h. The number of various cells was determined by the tetrazolium dye method. Except for PBMCs, all data represent mean±SD for three independent experiments.* [a]*Data are taken from reference 23.*

has been shown to inhibit the replication of HCV (unpublished data). In fact, MN417 is a weak inhibitor of HCV replication (24). MN314-B, which is structurally related to MN417, proved to be a selective inhibitor of HCV replication in sub-genomic and full-genomic HCV replicon cells (20, 24). Interestingly, both compounds did not display significant inhibition of the hepatocellular carcinoma cell line Huh-7, which also suggests that MN417 is optimized as a selective inhibitor of ATL cells.

The viral transcriptional activator protein Tax plays an important role in T-cell immortalization (25). Tax activates not only HTLV-1 replication but also modulates the activity of various cellular factors, such as NF-κB and AP-1. However, S1T cells do not express HTLV-1 Env or Tax, indicating that Tax is not a target molecule of TMNAA and MN417 for inhibition of S1T cell growth. On the other hand, the minus strand HTLV-1 pro-virus encodes HBZ, also considered to be a key factor in leukemogenesis of ATL. Like Tax, HBZ modulates the activity of NF-κB and AP-1. In addition, HBZ RNA promotes T-cell proliferation by E2F1, which plays an important role in the G_1-to-S phase transition of trans-activating target genes (26, 27). Since the expression of HBZ is conserved in any kind of ATL cells (26), the effect of TMNAA on HBZ expression was examined in S1T cells and MT-2 cells. HBZ levels in these cells were not altered by the presence of TMNAA (data not shown). Furthermore, TMNAA and MN417 did not suppress the production of HTLV-1 antigens in MT-2 cells (23 and unpublished data). These results suggest that the molecular target of TMNAA is a host cellular factor rather than viral gene products.

Several dysregulations of cellular processes have been shown in HTLV-1-related T-cell disorders (28). At present, the target molecule of TMNAA or MN417 for inhibition of ATL cells remains unknown. As described above, while S1T and MT-2 cells are derived from different sources (ATL *vs.*

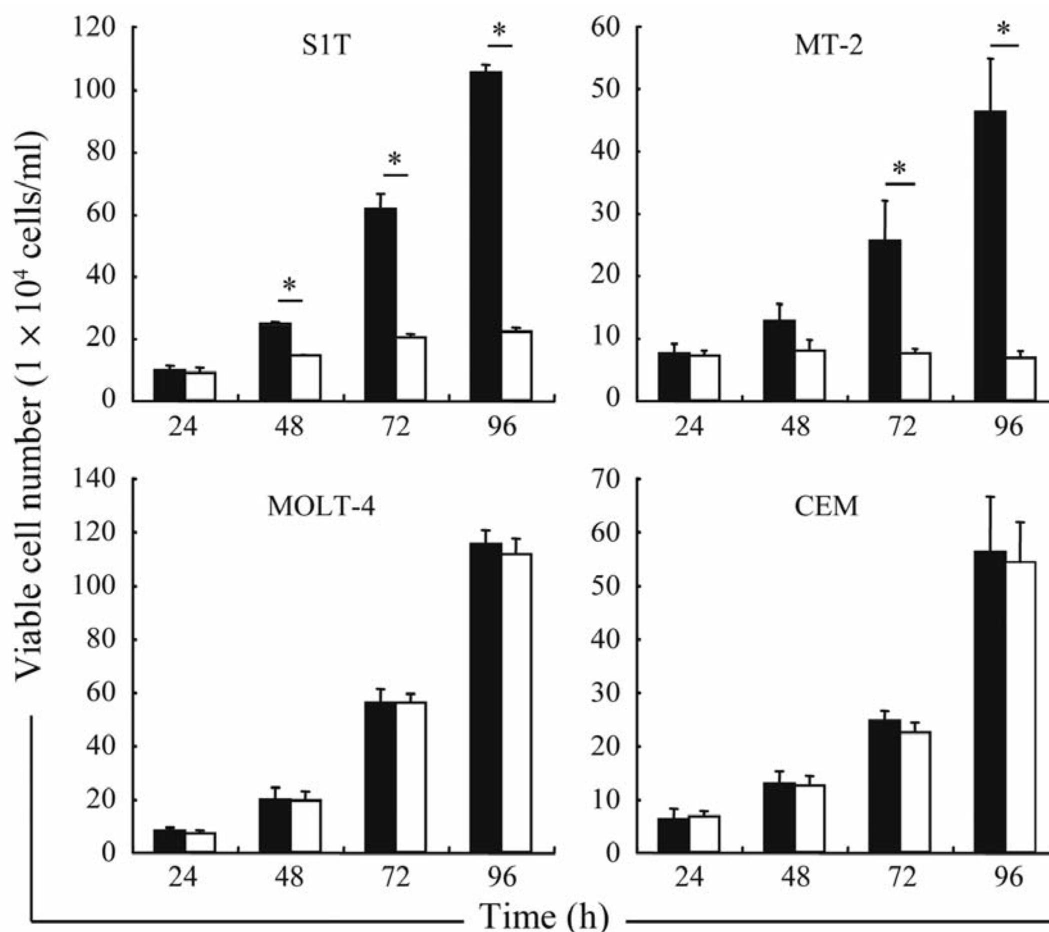

Figure 3. *Time-dependent suppression of cell proliferation by MN417. The cells (1×10^4 cells/well) were incubated in absence (closed column) or presence (open column) of MN417 (4 μM). After incubation for 24, 48, 72, and 96 h, the number of viable cells was determined by trypan blue exclusion. Statistical analysis was determined by the Student's t-test (*p<0.01).*

HTLV-1-transformed T cells), TMNAA and MN417 are more potent inhibitors of these cell lines than other ATL cell lines (Table I), (21, 22). Therefore, to gain insight into the molecular mechanism of TMNAA in S1T cells, we performed comprehensive gene expression analysis. It was previously reported that gene expression of several molecules, such as TSLC1, TCF8, and FSCN1, were significantly modulated in ATL cells compared with $CD4^+$ T cells (29-31). Interestingly, gene expression of these molecules was not altered in TMNAA-treated S1T cells (data not shown). Furthermore, TMNAA-treated and untreated S1T and MOLT-4 cells indicated that the gene ontology (GO) categories GO:0045935 (positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process), GO:0051173 (positive regulation of nitrogen compound metabolic process), GO:0031328 (positive regulation of cellular biosynthetic process), and GO:0009891 (positive regulation of biosynthetic process) were highly up-regulated in TMNAA-treated S1T cells but not in untreated S1T cells or TMNAA-treated MOLT-4 cells (data not shown). On the other hand, the categories GO:0005925 (focal adhesion), GO:0005924 (cell-substrate adherens junction), GO:0030055 (cell-substrate junction), GO:0005912 (adherent junctions), GO:0070161 (anchoring junction), and GO:0016323 (basolateral plasma membrane) were significantly down-regulated (data not shown). These results suggest that the target molecule of TMNAA for inhibition of ATL cells may differ from those of other anti-ATL compounds.

In conclusion, the present study clearly demonstrated that the novel tetrahydrotetramethylnaphthalene derivatives are selective inhibitors of ATL cells *in vitro*. Although their molecular target still remains unknown, both TMNAA and MN417 may have a potential as promising chemotherapeutic agents against ATL.

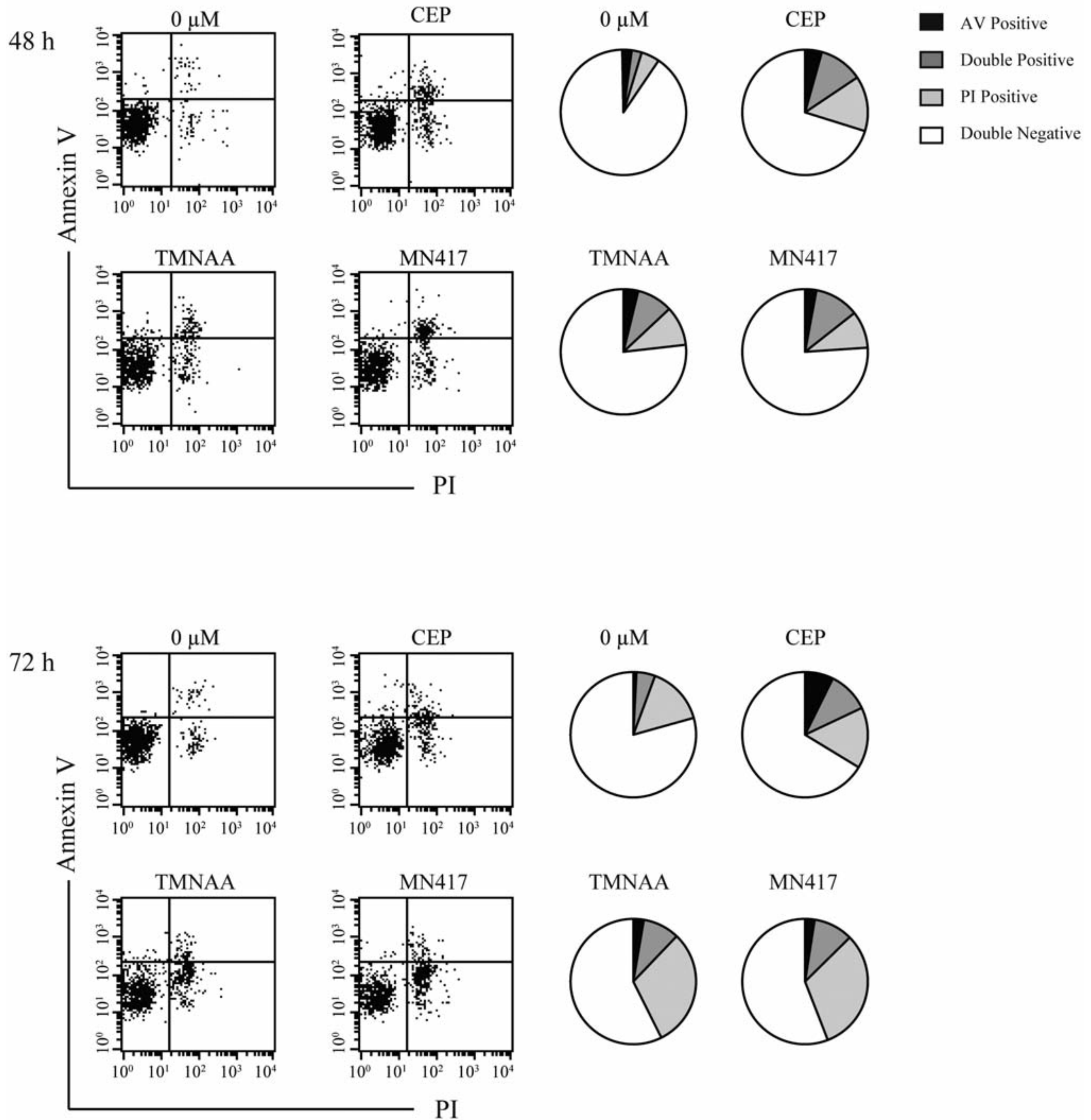

Figure 4. *Induction of S1T cell death by TMNAA and MN417. S1T cells (5×10^4 cells/ml) were incubated in the presence of 10 µM TMNAA, 10 µM MN417, or 10 µM CEP for 48 and 72 h. After incubation, the cells were stained with Annexin V (AV)-FITC and PI and analyzed by flow cytometry. The percentage of AV-positive, PI-positive, double-negative (AV-negative and PI-negative), and double-positive (AV-positive and PI-positive) are shown by circle graphs.*

Conflicts of Interest

Y. Hashimoto and M. Baba are applying for a patent on TMNAA. The other Authors declare that there are no conflicts of interest.

Acknowledgements

We thank Dr. Naomichi Arima (Kagoshima University) for providing ATL cell lines.

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Received January 19, 2014
Revised February 9, 2014
Accepted February 10, 2014