

Selective Inhibition of HTLV-1-infected Cell Proliferation by a Novel Tetramethylnaphthalene Derivative

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Abstract. Adult T-cell leukemia (ATL) is caused by infection with human T-lymphotropic virus type 1 (HTLV-1). A novel tetramethylnaphthalene derivative, TMNAA, selectively inhibited the proliferation of various HTLV-1-infected cells, including ATL cell lines and peripheral blood mononuclear cells (PBMCs) from ATL patients. In contrast, the proliferation of uninfected cell lines and PBMCs from healthy donors was hardly affected by the compound. Cell-cycle analysis revealed that TMNAA increased the population of the G₀/G₁ phase and reduced that of the S phase in HTLV-1-infected cells. TMNAA was found to suppress the phosphorylation of retinoblastoma protein and the expression of cyclin-dependent kinase 4 in HTLV-1-infected cells. Furthermore, the inhibition of cell proliferation was partially annihilated by removing the compound. These results indicate that TMNAA exerts selective inhibition of HTLV-1-infected cells through a novel mechanism, presumably modulating cell cycle regulatory proteins associated with the G₀/G₁ phase.

Human T-lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL) (1-3) and HTLV-1-associated myelopathy/tropical spastic paraparesis (4, 5). The endemic areas of the virus are geographically distributed, and Japan, Africa, Caribbean islands and South America are known as the areas of highest prevalence of the

virus (6). In Japan, the number of HTLV-1 carriers is estimated to be approximately 1.2 million, and more than 700 cases are diagnosed as ATL every year (7). Although the majority of HTLV-1 carriers remain asymptomatic, about 2.5 to 5% of HTLV-1 carriers develop ATL after a long latency period (8, 9), and the median survival time of patients with aggressive ATL is 13 months (10). Conventional anticancer therapies are not effective at prolonging the life of patients with aggressive ATL. Some combination chemotherapies improved the clinical outcome of ATL patients, yet their mean survival time was not sufficiently extended (11, 12).

To develop novel therapy against ATL, compounds targeting nuclear factor κ B (NF- κ B), proteasome, and histone deacetylase (HDAC) are being investigated. The NF- κ B pathway plays an important role in the proliferation of ATL cells, protection of the cells from apoptosis, and induction of drug-resistance (13-15). NF- κ B inhibitors (Bay 11-7082 and dehydroxymethylepoxyquinomicin) directly block the activation of NF- κ B and induce apoptosis of HTLV-1-infected cells and primary ATL cells (16, 17). These compounds inhibited tumor growth in an ATL mouse model (18, 19). Bortezomib, a proteasome inhibitor, interferes with the degradation of the NF- κ B inhibitor I κ B α , resulting in the suppression of NF- κ B followed by the induction of apoptosis in ATL cells (20). HDAC inhibitors are potent inducers of growth arrest and apoptosis in a variety of tumor cells (21, 22). Several HDAC inhibitors (romidepsin, vorinostat, panobinostat, and MS-275) also suppressed the proliferation of HTLV-1-infected cell lines and primary ATL cells *in vitro* (23, 24).

We have examined a number of compounds for their inhibitory effect on the growth of ATL cells and recently found that a novel tetramethylnaphthalene derivative, 2-acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (TMNAA), selectively inhibited the proliferation of the ATL-derived cell line S1T but not the HTLV-1-free T-cell

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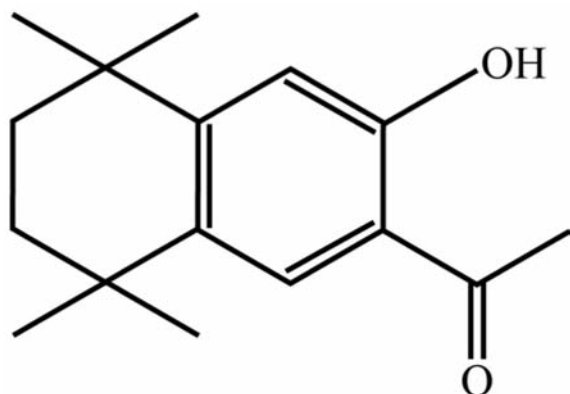


Figure 1. Chemical Structure of TMNAA.

line MOLT-4 (25). In this study, we have extended the previous study and investigated the inhibitory effect of TMNAA on the viability and proliferation of various HTLV-1-infected cell lines, ATL-derived cell lines, and primary peripheral blood mononuclear cells (PBMCs) obtained from ATL patients. We have also investigated its mechanism of action.

Materials and Methods

Reagents. TMNAA (Figure 1) was synthesized, according to the procedure previously described (25). The compound was dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) at a concentration of 20 mM and stored at -20°C . A water-soluble tetrazolium, Tetracolor One[®], was purchased from Seikagaku Corporation (Tokyo, Japan). Anti-phosphorylated retinoblastoma protein (Rb) (S807/811) rabbit antibody, anti-cyclin-dependent kinase (CDK) 4 mouse IgG1 antibody, and anti-cyclin D3 mouse IgG1 antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Rb mouse IgG1 antibody, anti-cyclin B1 rabbit antibody, anti-CDK1 mouse IgG2a antibody, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse IgG1 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cells. HTLV-1-infected cell lines, MT-2 and MT-4, are derived from umbilical cord blood lymphocytes after co-cultivation with leukemia cells from ATL patients (2). MT-2 cells were reported to integrate at least eight copies, including defective types, of HTLV-1 proviral DNA into their chromosomes. ATL cell lines, F6T, K3T, S1T, and Su9T01, were established from PBMCs of ATL patients (26). F6T and K3T cells express TAX protein, whereas S1T and Su9T01 cells do not (Table I). The HTLV-1-free cell lines, MOLT-4, CEM, and Jurkat, were also used for experiments. All cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin. PBMCs were donated under informed consent from healthy volunteers and patients with smoldering-type and acute-type ATL. The cells were isolated from heparinized blood with Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden). Diagnosis of ATL was based on clinical features, hematological characterizations,

Table I. Inhibitory effect of TMNAA on cell proliferation.

Cell	HTLV-1		IC ₅₀ (μM)
	Proviral DNA	Tax	
MT-2	+	+	2.4 \pm 2.4
MT-4	+	+	31.9 \pm 3.4
S1T	+	–	5.4 \pm 2.6
F6T	+	+	30.9 \pm 10.9
K3T	+	+	16.8 \pm 19.6
Su9T01	+	–	21.4 \pm 22.5
CEM	–	–	>100
Jurkat	–	–	>100
MOLT-4	–	–	>100
Acute_1	+	ND	52.9
Smoldering_1	+	ND	56.1
Healthy_1	–	–	>100
Healthy_2	–	–	>100

IC₅₀: 50% Inhibitory concentration, based on the reduction of viable cells. ND: Not determined.

the presence of serum antibodies against HTLV-1, and the insertion of proviral DNA into leukemia cells.

Cell proliferation assay. The inhibitory effect of TMNAA on cell proliferation was determined by a tetrazolium dye method using Tetracolor One[®]. Briefly, the cells (1×10^4 cells/well) were incubated in a microtiter plate in the absence or presence of different concentrations of TMNAA at 37°C . To examine the effect of caspase inhibition on cell proliferation, Z-VAD-FMK (20 μM) (Promega, Madison, WI, USA) was added to the above cultures. After incubation for 3 days, 10 μl of the dye solution was added to each well, and the cells were further incubated at 37°C . After 4 hours, the absorbance of each well was measured at 450 nm with a microplate reader (Bio-Rad, Richmond, CA, USA).

Cell cycle analysis. The cells (1×10^5 cells/ml) were incubated in the absence or presence of TMNAA (20 μM) at 37°C . After incubation for 24, 48 and 72 hours, the cells were washed with ice-cold phosphate-buffered saline (PBS), fixed with 70% ice-cold ethanol, and treated with a propidium iodide (PI) staining buffer containing RNase (BD Biosciences Pharmingen, San Diego, CA) for 30 minutes at 25°C . The cells were analyzed for their DNA content by FACSscan[™] (Becton Dickinson, San Jose, CA). The software Modfit (Verity Software House, Topsham, ME) was used for cell cycle analysis.

Western blot analysis. The cells (1×10^5 cells/ml) were incubated in the absence or presence of TMNAA (20 μM) at 37°C . After incubation for 48 hours, the cells were washed and lysed with a radioimmunoprecipitation assay buffer (Nacalai Tesque). The cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% gel. The separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) using a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% nonfat dry milk (Becton Dickinson, Sparks, NJ, USA) in Tris-buffered saline

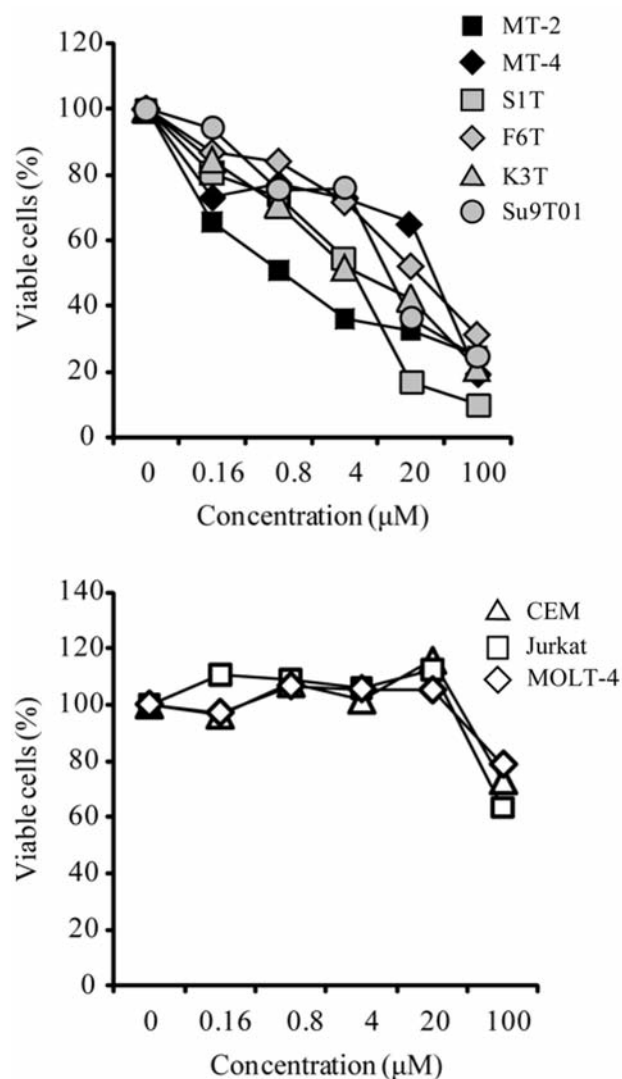


Figure 2. Effect of TMNAA on cell proliferation. The inhibitory effect of TMNAA on cell proliferation was examined in HTLV-1-infected cell lines (MT-2 and MT-4), ATL cell lines (S1T, F6T, K3T, and Su9T01), and HTLV-1-free cell lines (CEM, Jurkat, and MOLT-4). The cells were incubated in the presence of different concentrations of TMNAA. After 3 days, cell viability was measured by a tetrazolium dye method. All data represent means for three independent experiments.

containing Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) (T-TBS) and incubated with the antibodies indicated above. After washing three times with T-TBS, the membrane was further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The membrane was washed three times with T-TBS, and blots were analyzed for their chemiluminescence by Chemi-Lumi One (Nacalai Tesque).

Results

Selective inhibition of cell proliferation by TMNAA. When TMNAA was examined for its inhibitory effect on the

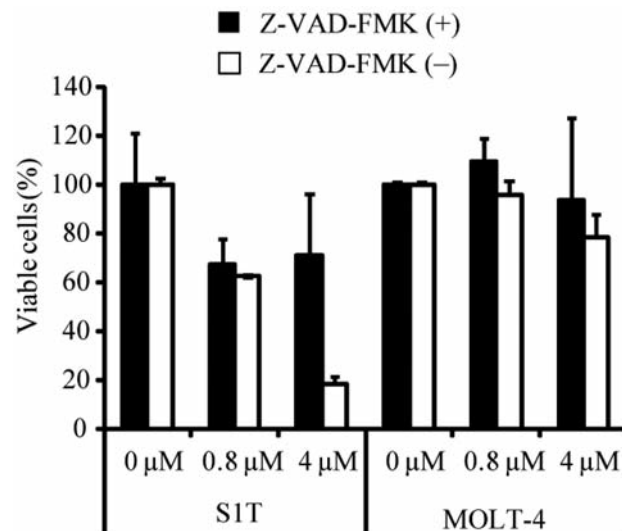


Figure 3. Effect of caspase inhibitor on TMNAA-induced cell death. S1T and MOLT-4 cells were incubated in the absence or presence of TMNAA (0.8 and 4 μM) with or without the caspase inhibitor Z-VAD-FMK (4 μM). After 3 days, cell viability was measured by a tetrazolium dye method. All data represent means±ranges for two independent experiments.

proliferation of various HTLV-1-infected and -free cells, the viable cell number of HTLV-1-infected cell lines (MT-2 and MT-4) and ATL cell lines (S1T, F6T, K3T, and Su9T01) decreased with increasing concentration of TMNAA (Figure 2A). In contrast, the viable cell number of HTLV-1-free cell lines (CEM, Jurkat, and MOLT-4) was not affected by the compound up to 20 μM (Figure 2B). TMNAA slightly reduced the viable cell number of the uninfected cell lines at a concentration of 100 μM. Table I shows the 50% inhibitory concentrations (IC₅₀s) of TMNAA for these cell lines and PBMCs tested in this study, as well as their harboring HTLV-1 proviral DNA and TAX. Among the cells, S1T and MT-2 were highly sensitive to TMNAA, with IC₅₀s of 2.4 and 5.4 μM, respectively. Furthermore, TMNAA also inhibited the proliferation of primary PBMCs from patients with acute-type and smoldering-type ATL (Table I). However, the proliferation of primary PBMCs from healthy donors was not affected by the compound up to 100 μM. These results indicate that TMNAA may be a selective inhibitor of HTLV-1-infected cells including primary ATL cells.

Effect of caspase inhibitor on TMNAA-induced cell death. To determine whether the drug-induced cell death is associated with the activation of caspase, S1T cells were exposed to TMNAA and incubated in the absence or presence of the caspase inhibitor Z-VAD-FMK (20 μM). Again, TMNAA inhibited the proliferation of S1T cells in a dose-dependent fashion, and this inhibition was partially reversed by the

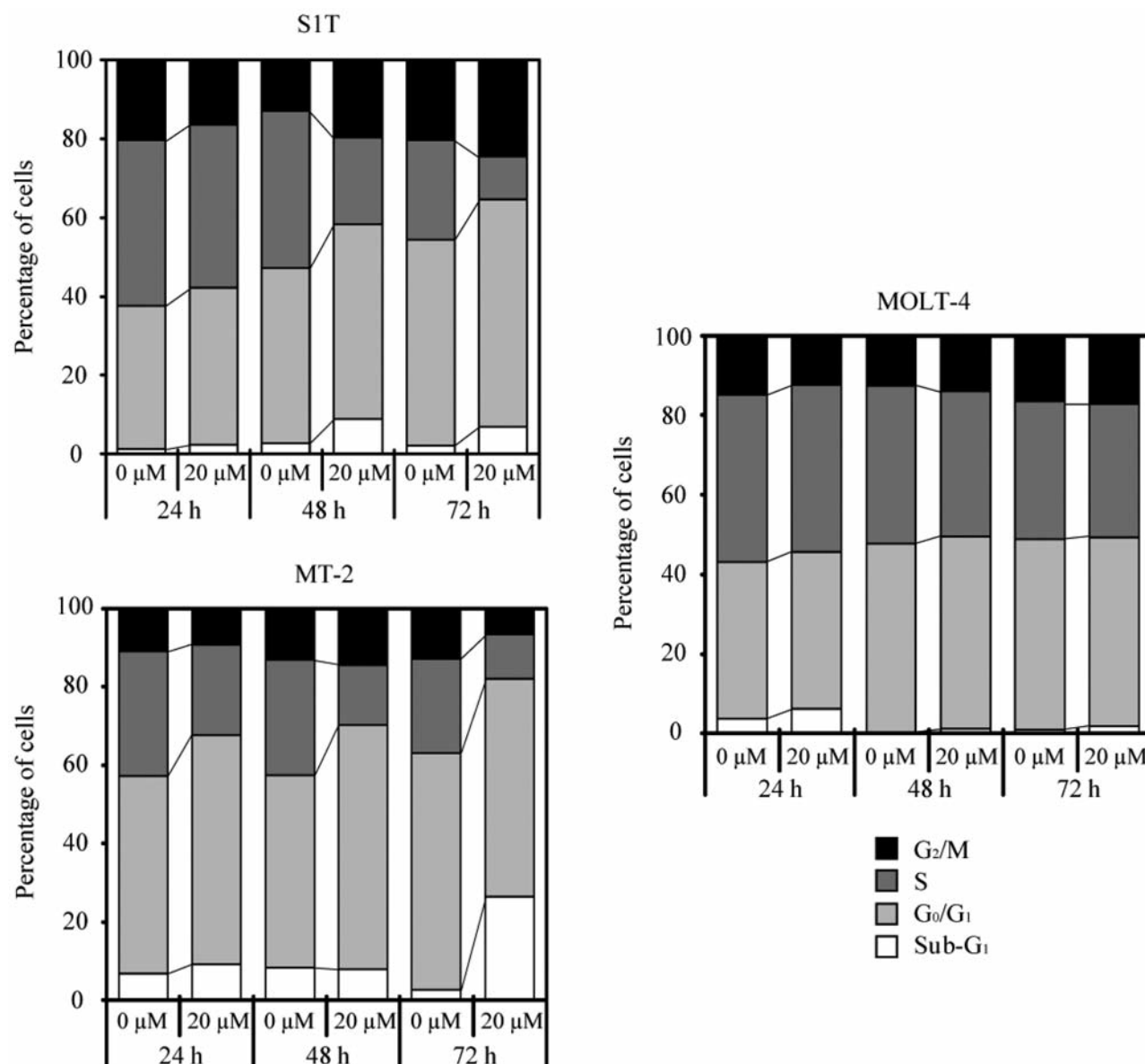


Figure 4. Effect of TMNAA on cell cycle. S1T, MT-2, and MOLT-4 were incubated in the absence or presence of TMNAA (20 μ M) and stained with PI at 24, 48, and 72 hours. The cells were analyzed for their cell cycle by flow cytometry. Experiments were performed three times and representative data are shown.

caspase inhibitor (Figure 3), suggesting that activation of caspase is at least in part involved in the inhibitory effect of TMNAA on the proliferation of HTLV-1-infected cells.

Effect of TMNAA on cell cycle. To determine whether TMNAA affects the cell cycle of HTLV-1-infected cells, S1T and MT-2 cells were incubated in the absence or presence of the compound and analyzed for their cell cycle distribution by flow cytometry. TMNAA treatment (20 μ M) slightly increased the number of S1T cells in the G₀/G₁ phase at 24,

48, and 72 hours and that in the G₂/M phase at 48 and 72 hours after exposure of the compound (Figure 4A). On the other hand, the cell number in the S phase significantly decreased at 48 and 72 hours. TMNAA treatment increased the number of MT-2 cells in the G₀/G₁ phase at 24, 48, and 72 hours and decreased that in the S phase at 24, 48, and 72 hours (Figure 4B). Furthermore, the treatment significantly increased the number of MT-2 cells in the sub-G₁ phase at 72 hours. In contrast, the cell cycle of MOLT-4 cells was not affected by the drug treatment at any time during the

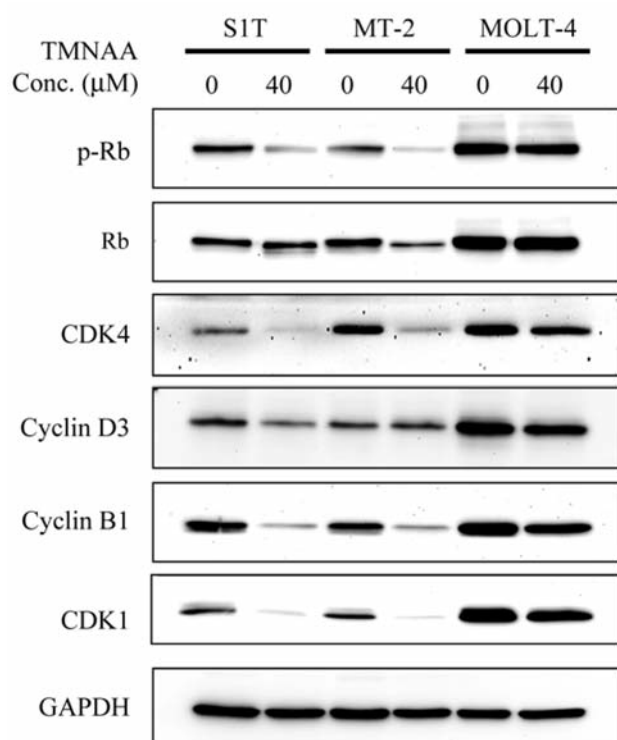


Figure 5. Effect of TMNAA on proteins related to cell cycle. S1T, MT-2, and MOLT-4 cells were incubated in the absence or presence of TMNAA (40 μ M). After 48 hours, cell lysates were prepared and subjected to Western blot analysis using antibodies specific to p-Rb (S807/811), Rb, CDK4, Cyclin D3, Cyclin B1, CDK1, and GAPDH.

incubation period (Figure 4C). Although the shapes of the cell cycle histograms were slightly different in S1T cells from MT-2 cells (data not shown), TMNAA treatment increased the number of HTLV-1-infected cells in the G₀/G₁ phase and decreased that in the S phase. These results suggest that the compound induces cell cycle arrest at the G₀/G₁ phase in HTLV-1-infected cells.

Modulation of CDK4 expression and Rb phosphorylation by TMNAA. To examine whether TMNAA modulates the expression of proteins related to the regulation of cell cycle, Western blot analysis for the lysates of cells treated with the compound was conducted. The expression of CDK4, cyclin B1, and CDK1 decreased in both S1T and MT-2 cells but not in MOLT-4 cells (Figure 5). The phosphorylated form of Rb was highly reduced in both S1T and MT-2 cells treated with TMNAA. Although the total Rb was also reduced by TMNAA treatment, the level of reduction was not comparable to that of phosphorylated Rb. It has been shown that the unphosphorylated form of Rb suppresses the process from the G₀/G₁ phase to the S phase, and that the phosphorylation of Rb is conducted by CDK4 (27, 28). Thus,

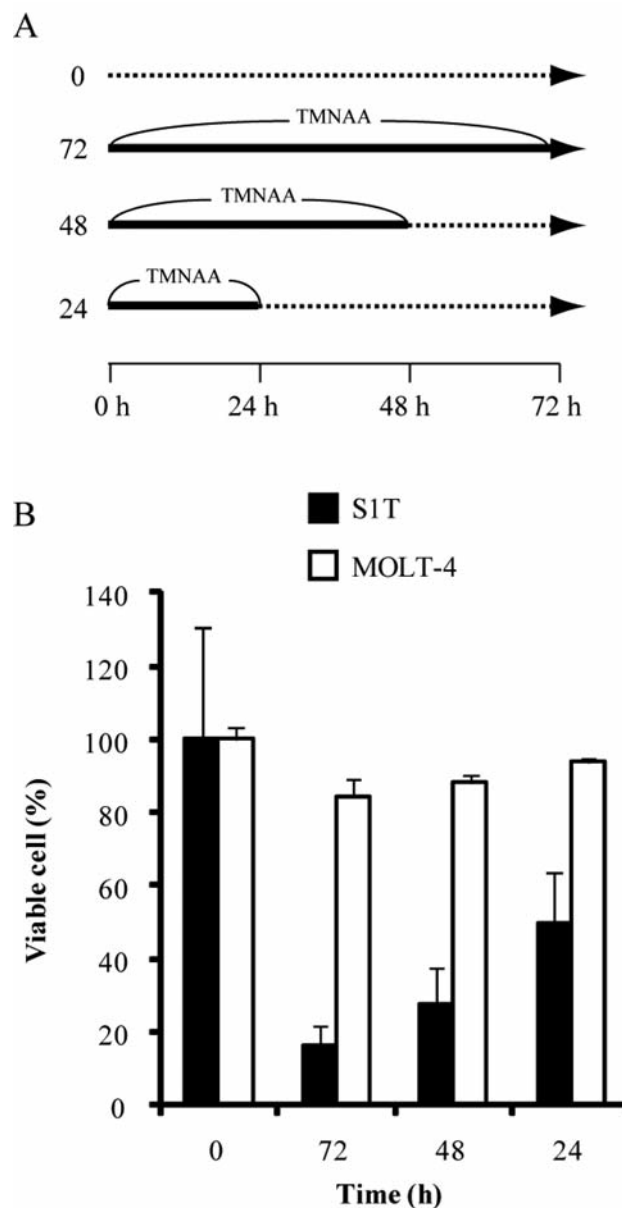


Figure 6. Effect of drug removal on cell proliferation. S1T and MOLT-4 cells were incubated in the absence or presence of TMNAA (40 μ M). The cells were exposed to the compound from 0 to 72, 0 to 48, or 0 to 24 hours (A). After 72 hours, cell viability was determined by a tetrazolium dye method (B). All data represent means \pm ranges for two independent experiments.

these results suggest that in HTLV-1-infected cells, TMNAA induces cell cycle arrest in the G₀/G₁ phase by down-regulating CDK4 expression, resulting in an increase in the unphosphorylated form of Rb.

Cytostatic inhibition of cell proliferation by TMNAA. To gain further insight into the mechanism of TMNAA, the influence

of drug removal on the proliferation of HTLV-1-infected cells was examined. S1T and MT-2 cells were cultured in the presence of TMNAA (40 μ M), and the compound was removed from culture medium at 24 and 48 hours. At 72 hours after the initial exposure to the compound, cell viability was determined by the tetrazolium dye method. As shown in Figure 6, the viability of S1T cells was partially restored by removing TMNAA from the culture medium, suggesting that the suppression of HTLV-1-infected cells by TMNAA is cytostatic.

Discussion

TMNAA treatment resulted in dose-dependent inhibition of cell proliferation in a variety of HTLV-1-infected and ATL-derived cell lines, as well as primary PBMCs isolated from ATL patients. In contrast, it did not affect cell proliferation in HTLV-1-free T-cell lines and mitogen-stimulated PBMCs from healthy volunteers at concentrations up to 20 and 100 μ M, respectively (Figure 2 and Table I). These results indicate that the inhibitory effect of TMNAA is selective in the leukemia cells infected with HTLV-1. Although the activity of TMNAA was less pronounced in primary PBMCs from ATL patients than in ATL cell lines, this may be due to the fact that PBMCs from the patients consist not only of leukemia cells but also normal lymphocytes and monocytes.

Addition of the caspase inhibitor Z-VAD-FMK partially reversed the inhibitory effect of TMNAA on the proliferation of S1T cells (Figure 3). However, we did not observe that TMNAA increased the number of annexin V-positive/PI-negative cells or induce the activation of caspase 3 or caspase 9 (data not shown). These results suggest that although the caspase pathway is at least in part involved in TMNAA-induced cell death, TMNAA does not directly induce apoptosis of ATL cells. In fact, the inhibitory effect of TMNAA on S1T cells seemed to be cytostatic, since the cells started to proliferate again after removal of the compound (Figure 6).

Cell cycle analysis clearly showed that TMNAA increased the proportion of HTLV-1-infected cells in the G_0/G_1 phase and reduced that in the S phase (Figure 4). However, the compound did not affect the cell cycle of MOLT-4 cells. The G_0/G_1 phase of the mammalian cell cycle is regulated by the phosphorylation of Rb, which is conducted by CDK4. The unphosphorylated form of Rb protein family binds to an E2F transcription factor family and blocks the E2F-dependent gene expression, which controls the G_1 to S phase transition and subsequent DNA synthesis (29). The phosphorylation of Rb disrupts its association with E2F, resulting in the increase of gene expression targeted by E2F. Inhibition of Rb phosphorylation and down-regulation of CDK4 induces cell cycle arrest in the G_0/G_1 phase. According to the result of the cell cycle analysis, TMNAA reduced the expression of

CDK4 in S1T and MT-2 cells but not in MOLT-4 cells (Figure 5). Although TMNAA treatment slightly reduced the amount of total Rb in MT-2 cells, the phosphorylated form of Rb markedly decreased in both S1T and MT-2 cells, indicating the relative increase of the unphosphorylated form of Rb. Since the unphosphorylated form of Rb inhibits E2F-dependent gene expression, TMNAA may bring about cell cycle arrest in the G_0/G_1 phase through an E2F-dependent mechanism. Interestingly, TMNAA also significantly reduced the expression of G_2/M regulatory proteins, including cyclin B1 and CDK1 in HTLV-1-infected cells (Figure 5). Although the effect of TMNAA on the G_2/M phase was not apparent in MT-2 cells, the compound increased the number of S1T cells in the G_2/M phase (Figure 4).

TMNAA is structurally related to tamibarotene, which was reported to inhibit the proliferation of HTLV-1-infected and ATL-derived cell lines and primary ATL cells but not of PBMCs from healthy donors (30). Its mechanism of action against HTLV-1-infected cells is the induction of cell cycle arrest in the G_1 phase and cell death by interrupting the NF- κ B and activator protein 1 pathways. NF- κ B activation is an important pathway for the proliferation of HTLV-1-infected cells, thus NF- κ B inhibition induces their apoptosis. However, we did not find inhibition of NF- κ B by TMNAA (data not shown), suggesting that TMNAA attenuated the growth of HTLV-1-infected cells through a mechanism different from that of tamibarotene.

In conclusion, TMNAA is a novel compound with a unique biological activity. The compound selectively inhibits HTLV-1-infected cells, including primary ATL cells, without affecting the viability and proliferation of HTLV-1-free T-cells. Further studies are in progress to determine whether TMNAA is a promising candidate drug for treatment of ATL patients in combination with existing anti-leukemia agents.

Conflict of Interest Statement

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

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