# Synergistic Inhibition of HTLV-1-infected Cell Proliferation by Combination of Cepharanthine and a Tetramethylnaphthalene Derivative

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**Abstract.** *The tetrahydrotetramethylnaphthalene derivative TMNAA has recently been identified as a selective inhibitor of human T-lymphotropic virus type 1 (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). Although the target molecule of TMNAA is still unknown, it does not inhibit nuclear factor-κB (NF-κB) activity. Therefore, TMNAA was examined for its inhibitory effect on the cell proliferation in combination with the NF-κB inhibitor cepharanthine. Synergism was observed for the combination, in inhibiting the proliferation of HTLV-1-infected T-cell lines. Although TMNAA alone did not induce the apoptosis of HTLV-1-infected T-cell lines, it strongly enhanced their apoptosis induced by cepharanthine. Thus, TMNAA may have potential as a therapeutic agent against ATL either alone or in combination with cepharanthine, which is clinically used as an anti-inflammatory drug in Japan.*

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disorder caused by the infection with the human T-lymphotropic virus type 1 (HTLV-1) (1). HTLV-1 infection is endemic mainly in Japan, the Caribbean islands, in South America, and in South Africa, and the number of HTLV-1 carriers is estimated to be 15 to 20 million worldwide (2). In Japan, the number of HTLV-1 carriers is approximately 1.2 million, so that more than 700 cases of ATL have been diagnosed every year (3). ATL is classified into four subtypes: acute, lymphoma, chronic, and smoldering. The median survival time of patients with chronic and smoldering ATL is more than 2 years, whereas aggressive ATL has a poor prognosis, with a median survival of 13 months (4). Although some combination chemotherapies have improved the clinical outcome of patients with ATL, their mean survival time has not been sufficiently extended (5, 6). Therefore, it is still mandatory to find novel and effective therapeutic agents against ATL.

The viral transcriptional activator protein Tax plays an important role in T-cell immortalization (7). Tax also modulates the activities of various cellular genes, such as nuclear factor κB (NF-κB) and activated protein 1 (AP-1). However, both NF-κB and AP-1 pathways are highly activated in ATL cells without Tax expression. Therefore, the mechanism of ATL cell activation is still unknown (8, 9). In addition, overexpression of P-glycoprotein (P-gp), lung resistance-related protein, and/or multidrug resistance proteins (MRPs) might be correlated with the refractory nature of ATL in patients (10-12). Cepharanthine (CEP) is a biscoclaurine amphipathic alkaloid isolated from the plant *Stephania cepharantha* Hayata. It has antitumor, multidrug resistance-reversing and anti-inflammatory activities. In Japan, biscoclaurine alkaloids, containing CEP as a major component, have been used for the treatment of acute and chronic inflammatory diseases, venomous snakebites, leucopenia induced by radiotherapy, and alopecia areata, without serious side-effects (13, 14). It has been reported that CEP has antiproliferative and apoptosis-inducing effects on a diverse range of tumor cells *in vitro* and *in vivo* (15-19). Furthermore, CEP has been shown to inhibit the activation of NF-κB (20-23).

2-Acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (TMNAA) (Figure 1) was recently identified as a selective inhibitor of the HTLV-1-infected T-cell line S1T but

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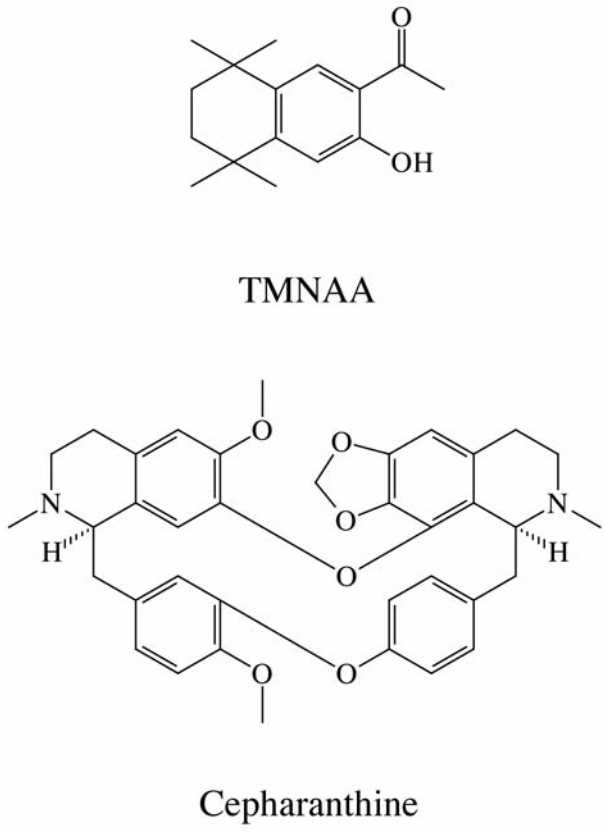


Figure 1. *Chemical structures of 2-Acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetrametylnaphthalene (TMNAA) and cepharanthine (CEP).*

not of the uninfected T-cell line MOLT-4 (24). Although the molecular target of this agent remains unknown, TMNAA did not affect the activity of NF-κB (25). In this study, we have investigated the inhibitory effect of TMNAA in combination with CEP on HTLV-1-infected T-cell lines in cell cultures and found that the combination displays high synergism.

## Materials and Methods

*Compounds*. TMNAA was synthesized by Dr. Hashimoto and his colleagues, according to the method previously described (24). CEP was provided by Kaken Shoyaku (Mitaka, Tokyo, Japan). These compounds were dissolved in dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) at a concentration of 20 mM or higher to exclude the cytotoxicity of DMSO and stored at –20˚C until use.

*Cells*. HTLV-1-infected T-cell lines (S1T and MT-2) and uninfected T cell lines (MOLT-4 and CEM) were used in this study. MT-2 is an HTLV-1-transformed T-cell line (26), and S1T is a leukemia cell line established from patients with ATL (27). S1T and MT-2 cells were kindly provided by Dr. N. Arima (Kagoshima University, Japan), while MOLT-4 and CEM were obtained through American Type Culture Collection (ATCC). All cell lines 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. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers after informed consent was obtained. The cells were isolated with Ficoll-Hypaque gradient density 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 experiments with PBMCs.

*Cell proliferation assay*. The inhibitory effect of test compounds on cell proliferation was determined by a tetrazolium dye method (Tetracolor ONE®; Seikgagaku Biobusiness, Tokyo, Japan). The cells ($1\times10^4$/well for cell lines and $1\times10^5$/well for PBMCs) were cultured in the presence of different 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 the test compounds on cell proliferation was also determined by trypan blue exclusion at 24, 48, 72, and 96 h after incubation with the compounds.

*Apoptosis assay*. S1T cells and PBMCs ($1\times10^5$ cells/ml for S1T cells and $1\times10^6$ cells/ml for PBMCs) were cultured in the absence or presence of 10 μM TMNAA alone, 10 μM CEP alone, or their combination (5 μM TMNAA plus 5 μM CEP). After incubation for 24 and 48 h, the cells were collected and were washed twice with an annexin-binding buffer. The cells ($1\times10^6$) were 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).

*Western blot analysis*. S1T cells ($1\times10^5$ cells/ml) were cultured in the absence or presence of 10 μM TMNAA alone, 10 μM CEP alone, or their combination (5 μM TMNAA plus 5 μM CEP). After incubation for 24 and 48 h, the cells were collected and lysed with RIPA buffer (Nacalai Tesque, Kyoto, Japan). The cell lysate was centrifuged at 14,000 rpm (18,000 ×*g*) at 4˚C for 10 min and stored at –20˚C until analysis. For analysis, the samples (20 μg/lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinyldene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Detection was performed by an enhanced chemiluminescence plus western blotting detection system (ECL plus; GE Healthcare, Buckinghamshire, UK). Anti-cleaved caspase-3 (Asp175) antibody, anti-cleaved caspase-9 (Asp315) antibody, anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) antibody (all from Cell Signaling Technology, Danvers, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (6C5) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for the analysis.

*Synergy calculation and statistical analysis*. The dual-drug effect was evaluated by the median effect principle and the isobologram method (28, 29). The combination index (CI) was obtained by this method. CIs of <1, 1 and >1 indicate synergism, additive effect, and antagonism, respectively. The statistical significance of differences was determined by the Student *t*-test.

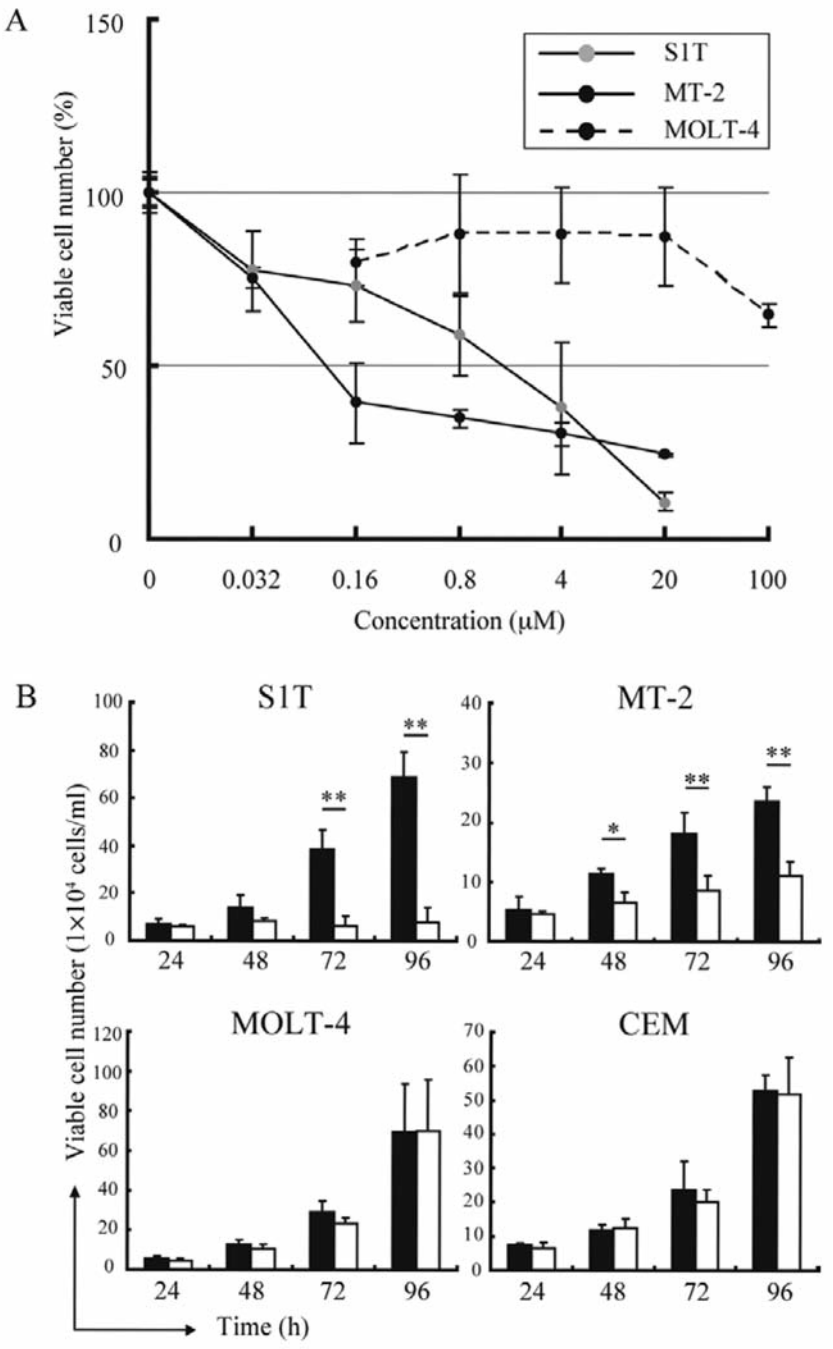


Figure 2. *Inhibitory effect of TMNAA on cell proliferation. A: Cells were incubated in the presence of different concentrations of TMNAA for 96 h. The number of cells was then determined by a tetrazolium dye method. B: Cells were incubated in the absence (closed column) or presence (open column) of TMNAA (20 μM). After incubation for 24, 48, 72 and 96 h, the number of viable cells was determined by trypan blue exclusion. Data represent the mean±SD for three independent experiments. Statistical significance of difference was determined by the Student's t-test (*p<0.05, **p<0.01).*

## Results

*Inhibitory effect of TMNAA on cell proliferation*. When TMNAA was examined for their inhibitory effect on the proliferation of S1T and MOLT-4 cells, it achieved more than 50% inhibition of S1T cell proliferation at a concentration of 4 μM (Figure 2A). TMNAA more potently inhibited the proliferation of MT-2 cells (another HTLV-1-infected cell line) than S1T cells. In contrast, the proliferation of MOLT-4 cells (uninfected T-cell line) was not affected by TMNAA at

Table I. *Inhibitory effect of 2-Acetyl-3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetrametylnaphthalene (TMNAA) alone, cepharanthine (CEP) alone, and their combination on cell proliferation.*

| Cell | HTLV-1 infection | Combination (molar ratio) | $IC_{50}$ (μM) | $IC_{90}$ (μM) | CI | | |
|---|---|---|---|---|---|---|---|
| | | | | | 50% | 70% | 90% |
| S1T | + | TMNAA alone | 1.65±0.03 | 11.4±0.57 | | | |
| | | CEP alone | 1.97±0.29 | 13.4±1.27 | | | |
| | | TMNAA+CEP (1:1) | 0.93±0.13 | 2.85±0.40 | 0.52±0.05 | 0.37±0.03 | 0.24±0.06 |
| | | TMNAA+CEP (3.162:1) | 1.62±0.18 | 3.40±0.22 | 0.92±0.08 | 0.58±0.04 | 0.30±0.07 |
| | | TMNAA+CEP (10:1) | 1.33±0.10 | 5.15±1.24 | 0.77±0.04 | 0.63±0.06 | 0.46±0.08 |
| MT-2 | + | TMNAA alone | 1.17±0.25 | 12.8±4.0 | | | |
| | | CEP alone | 3.42±1.02 | 7.11±0.58 | | | |
| | | TMNAA+CEP (1:1) | 1.08±0.07 | 2.00±0.17 | 0.65±0.09 | 0.40±0.08 | 0.23±0.05 |
| | | TMNAA+CEP (3.162:1) | 0.65±0.11 | 2.07±0.47 | 0.48±0.11 | 0.32±0.04 | 0.20±0.05 |
| | | TMNAA+CEP (10:1) | 0.63±0.08 | 1.61±0.36 | 0.52±0.11 | 0.31±0.06 | 0.15±0.06 |
| MOLT-4 | – | TMNAA alone | >10 | ND | | | |
| | | CEP alone | 4.60±0.83 | ND | | | |
| | | TMNAA+CEP (1:1) | 8.78±1.34 | ND | ND | ND | ND |
| CEM | – | TMNAA alone | >10 | ND | | | |
| | | CEP alone | 3.89±0.78 | ND | | | |
| | | TMNAA+CEP (1:1) | 6.29±2.30 | ND | ND | ND | ND |
| PBMCs | – | TMNAA alone | >100 | ND | | | |
| | | CEP alone | 0.84 | ND | | | |
| | | TMNAA+CEP (1:1) | 1.17 | ND | ND | ND | ND |

CI: Combination index at the indicated percentage inhibition; ND: not determined; PBMCs: peripheral blood mononuclear cells; $IC_{50}$: 50% inhibitory concentration. Except for PBMCs, all data represent the mean±SD for three independent experiments.

concentrations up to 20 and 4 μM, respectively. These results suggest that TMNAA is a potent and selective inhibitor of the proliferation of HTLV-1-infected cells. To confirm the selective inhibition of HTLV-1-infected cells by TMNAA, the viable cell number of the HTLV-1-infected cell line S1T and MT-2 and of the uninfected T-cell line MOLT-4 and CEM was also examined by trypan blue exclusion every 24 h (up to 96 h) after drug exposure. As shown in Figure 2B, TMNAA strongly suppressed the proliferation of S1T and MT-2 cells in a time-dependent fashion at a concentration of 20 μM. Again, the compound did not affect the growth of MOLT-4 and CEM cells at these concentrations.

*Inhibitory effect of TMNAA and CEP combination on cell proliferation.* Prior to the combination experiments with TMNAA and CEP, the optimal molar ratio of the two compounds had to be determined on the basis of the $IC_{50}$ of each compound alone. From the results of preliminary experiments at various molar ratios, TMNAA/CEP ratios of 1:1, 3.162:1, and 10:1 were chosen for the experiment (data not shown). As shown in Table I, the resulting CIs were always less than 1.0, irrespective of the TMNAA/CEP molar ratios, indicating that the combination of TMNAA and CEP was synergistic for inhibition of S1T and MT-2 cell proliferation. The highest synergism was achieved by TMNAA/CEP at a molar ratio of 1:1 and 3.162:1 in S1T and MT-2 cells, respectively. Furthermore, TMNAA did not affect the activity of CEP towards MOLT-4 cells, CEM cells, or PBMCs.

*Enhancement of CEP-induced apoptosis by TMNAA.* The inhibitory effect of the combination on cell proliferation was further investigated by flow cytometry and western blot analysis. S1T cells and PBMCs were treated with either 10 μM TMNAA alone, 10 μM CEP alone, or their combination (5 μM TMNAA plus 5 μM CEP) with incubation for 24 and 48 h. When annexin V-positive and PI-negative cells, indicating early apoptosis, were analyzed by flow cytometry, treatment of S1T cells with CEP, but not with TMNAA, significantly increased the proportion of apoptotic cells after 48 h (Figure 3A and 3B). Apoptotic cells were further increased, when treated with the combination of TMNAA and CEP, indicating that TMNAA enhanced CEP-induced apoptosis in S1T cells. This observation was further confirmed by western blot analysis for cleaved products of the caspase-3-specific substrate PARP, caspase-3, and caspase-9. After incubation for 24 and 48 h, CEP alone, but not TMNAA alone, increased the levels of the cleaved products in S1T cells as compared to those in untreated cells (Figure 3C). The combination treatment further increased their levels. In PBMCs, CEP strongly induced cell death (PI-positive cells), yet TMNAA did not affect the activity of CEP (Figure 3D).

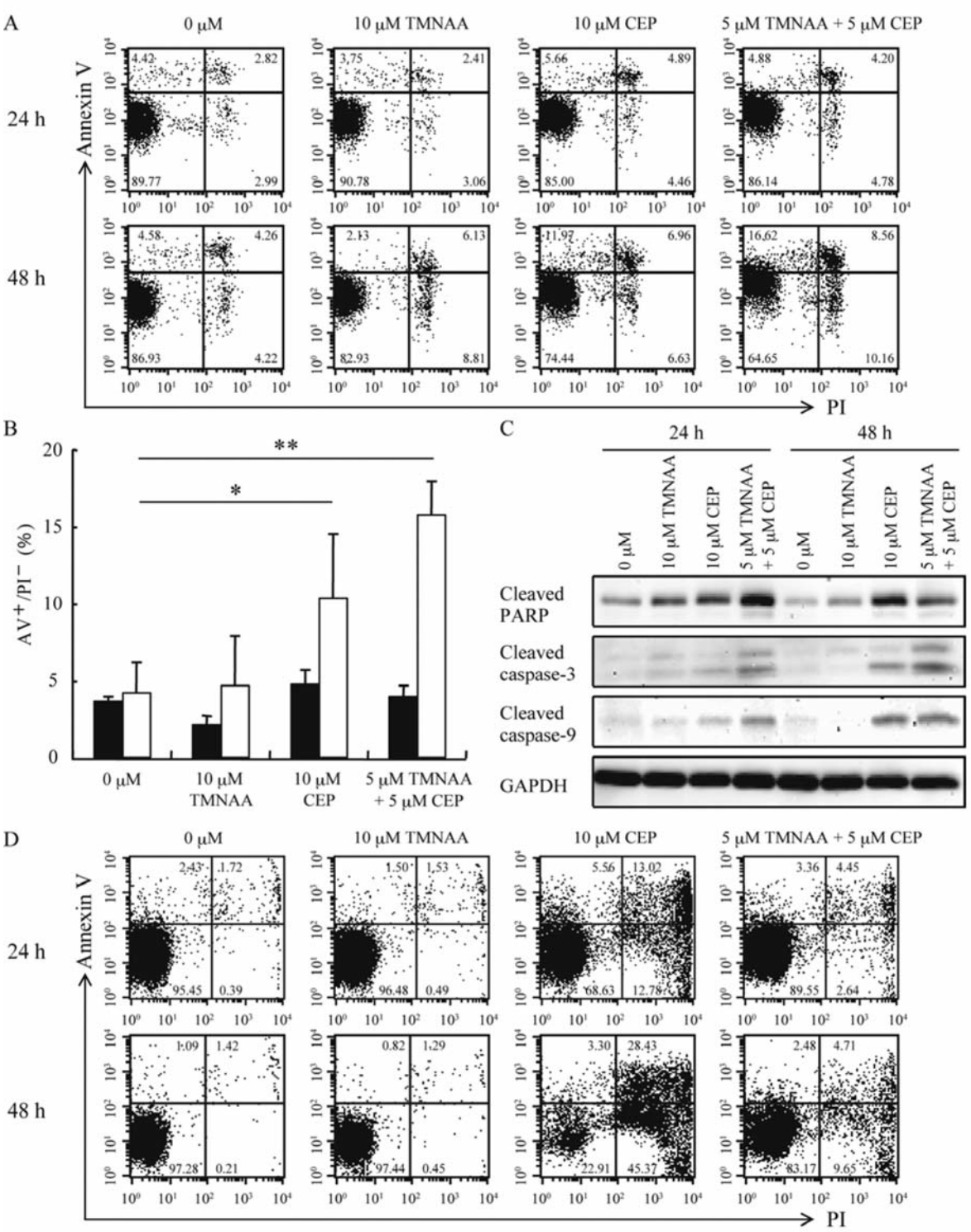


Figure 3. *Enhancement of cepharanthine (CEP)-induced apoptosis by TMNAA. S1T cells and peripheral blood mononuclear cells (PBMCs) were incubated with either TMNAA alone (10 μM), CEP alone (10 μM), or their combination (5 μM TMNAA plus 5 μM CEP) for 24 and 48 h. After incubation, the cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) and analyzed by flow cytometry. The percentage of early apoptotic cells (annexin V-positive and PI-negative cells) as shown by dot-plot graphs (A and D) and bar graphs (B). Closed and open columns indicate the percentage of early apoptotic cells after incubation for 24 h (closed column) and 48 h (open column). Data represent the mean±SD for three independent experiments. The cell lysates were subjected to western blot analysis using antibodies to the indicated proteins (C). Statistical significance of difference was determined by the Student's t-test (*p<0.05, **p<0.01).*

## Discussion

TMNAA is structurally related to tamibarotene, which was reported to inhibit the proliferation of HTLV-1-infected cell lines and primary ATL cells but not of uninfected PBMCs (30). Its mechanism of action against HTLV-1-infected cells is the induction of cell cycle arrest in the G1 phase and cell death by interrupting NF-κB and AP-1 pathways. Since TMNAA does not inhibit NF-κB (25), its mechanism of action is clearly different from that of tamibarotene. Furthermore, the molecular mechanism (target) of TMNAA for its selective inhibition of HTLV-1-infected cells also remains to be elucidated.

It has been reported that CEP induces apoptosis through the caspase-dependent pathway in a variety of cell lines (15-19, 21-23). Our results are in accordance with these previous observations. CEP alone and in combination with TMNAA induced apoptosis through the caspase-dependent pathway in S1T cells (Figure 3). Since NF-κB activation is an important pathway for the proliferation of HTLV-1-infected cells, it is possible that NF-κB inhibition by CEP induces their apoptosis. On the other hand, the suppression of HTLV-1-infected cells by TMNAA was cytostatic (25). It is assumed that the cytostatic cells were more susceptible to apoptotic stimuli, including the inhibition of NF-κB inhibition by CEP.

TMNAA alone or in combination with CEP suppressed the p19 antigen production in the culture supernatants of MT-2 cells along with their proliferation (data not shown), suggesting that the inhibition of NF-κB activation was not correlated with viral production. Furthermore, S1T cells do not express HTLV-1 Env or Tax (31), indicating that Tax is not a target molecule of TMNAA for inhibition of S1T cells. Cell-cycle analysis revealed that TMNAA increased the cell population of the $G_0/G_1$ phase and reduced that of the S phase in S1T and MT-2 cells but not in MOLT-4 cells (25). TMNAA was found to reduce the expression of cyclin-dependent kinase 4, down-regulation of which induces cell cycle arrest in the $G_0/G_1$ phase. Although we previously reported that TMNAA induced $G_2$ arrest in S1T cells (24), a subsequent study revealed that TMNAA also induced $G_0/G_1$ arrest in addition to $G_2$ arrest (25).

In general, combination chemotherapy with different classes of inhibitors may have increased efficacy through additive or synergistic effects, reduced levels of side-effects through the use of each compound at lower doses, and reduced possibility of the emergence of drug resistance. Our results clearly demonstrated that the combination of TMNAA and CEP generated synergistic antiproliferative activity, so that the doses of both compounds could be reduced to obtain the same efficacy as the one achieved by each compound alone. In addition, CEP is known to reverse the resistance of various tumor cells to anticancer agents (32-34). CEP interacts with P-gp and inhibits its transport activity (35). CEP also inhibits MRP1 and MRP7 (34, 36). Although the transporter involved in the efflux of TMNAA from ATL cells is unknown, co-administration of CEP may also be beneficial in circumventing the emergence of drug-resistant ATL cells during chemotherapy with TMNAA.

In conclusion, TMNAA is a novel compound with unique biological activities. Considering the potent and selective inhibition of HTLV-1-infected cells when TMNAA is used in combination with CEP, further studies should be conducted to determine whether such a chemotherapeutic intervention is promising for treatment of patients with ATL.

## Conflict of Interest Statement

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

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