

**Disulfide-Mediated Apoptosis of HTLV-I-Infected Cells in Patients with  
HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis**

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Short title: Apoptosis of HTLV-I-infected cells by disulfide

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**Abstract**

**Background:** This study was conducted to construct a basis for the therapeutic strategy against human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) using a compound containing the disulfide moiety prosultiamine, which is a homologue of allithiamine originally synthesized by allicin and thiamine-thiol, for the targeting of HTLV-I-infected cells.

**Methods:** 1) Analyses of the apoptotic pathway in allicin or prosultiamine treatment against an HTLV-I-infected T cell line derived from an HAM/TSP patient (HCT-1) by flow cytometry and western blot. 2) Evaluation of the effect of the targeting of HTLV-I-infected cells in a prosultiamine *in vitro* treatment and clinical trial in HAM/TSP patients by quantitative PCR analysis of HTLV-I proviral load.

**Results:** Prosultiamine, like allicin, induced caspase-dependent apoptosis against HCT-1. The fact that the loss of mitochondrial membrane potential was recovered in z-VAD-fmk-pretreated HCT-1 with prosultiamine treatment suggested that prosultiamine can induce caspase-dependent apoptosis through the mitochondrial pathway. Based on the data showing that prosultiamine *in vitro* treatment against peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients induced a significant decrease of HTLV-I proviral copy numbers by apoptosis of HTLV-I-infected cells, we treated 6 HAM/TSP patients with intravenous administration of prosultiamine for 14 days. As a result of this treatment, the copy numbers of HTLV-I provirus in peripheral blood decreased to about 30-50% of their pretreatment levels with some clinical benefits in all patients.

**Conclusions:** Our results suggest that prosultiamine has the potential to be a new therapeutic tool focusing on the targeting of HTLV-I-infected cells in HAM/TSP.

## Introduction

Human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive myelopathy characterized by the involvement of bilateral pyramidal tracts with sphincteric disturbances [1]. The primary pathological feature of HAM/TSP is chronic inflammation of the spinal cord characterized by perivascular cuffing and parenchymal infiltration of lymphocytes [2]. Although the exact mechanism of the pathogenesis of HAM/TSP is still obscure, immunological abnormalities, such as the increased number of activated T cells and Th1 activation arising from a high HTLV-I proviral load, etc., in the peripheral blood play an important role in the pathological process of spinal cord lesions in HAM/TSP patients [3, 4]. Based on this evidence, therefore, immunomodulatory therapy, such as prednisolone [1, 5] and interferon- $\alpha$  [6, 7] treatment, has been the main treatment administered to HAM/TSP patients. Indeed, since it is conceivable that the immune-activated status of the peripheral blood involved in the process of chronic inflammation of the spinal cord is one of the targets of the treatments, these treatments produced good results in their own ways. However, their efficacy is still controversial. In addition, whether or not these treatments are tolerable as a long-term or lifelong treatment is uncertain. In the therapeutic strategy against HAM/TSP, most importantly, the ideal treatment is the elimination of HTLV-I-infected cells themselves from the peripheral blood because HTLV-I-infected CD4<sup>+</sup> T cells are the first responders in the immunopathogenesis of HAM/TSP[4, 8].

Allicin (diallyl thiosulfinate) (Figure 1), a natural organosulfur compound derived from garlic (*Allium sativum*), has diverse biological activities, including anticarcinogenic, antibacterial, and antifungal activity [9, 10]. Although the mechanisms

by which cytotoxic effects are induced by organosulfur compounds are poorly understood, a disulfide moiety in their structures seems to play an important role in triggering cell death [11]. The disruption of the intracellular redox system induced by the chemical reaction of a disulfide moiety with thiol-containing intracellular molecules, such as thioredoxin (Trx), Trx reductase, and glutathione (GSH), might be involved in the cytotoxicity [9, 11]. However, allicin is a very unstable compound and disappears rapidly after intravenous administration [12, 13]. Therefore, its use in therapeutic trials for HAM/TSP is limited.

Prosultiamine (*N*-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-*N*-[4-hydroxy-1-methyl-2-(propyldithio)-1-butenyl]-formamide) (Alinamin<sup>®</sup>), a product of Takeda Pharma Co. Inc. (Osaka, Japan), is a homologue of allithiamine, originally synthesized by thiol-type vitamin B1 and allicin [14]. To enhance stability in the blood and the efficient access of vitamin B1 to the tissues, prosultiamine was developed after allyl disulfide derived from allicin was substituted with propyl disulfide in the structure of allithiamine [15]. Thus, prosultiamine has a disulfide moiety in its structure, like allicin (Figure 1). The fact that prosultiamine is reduced to a part of thiamine and propyl disulfide by the intracellular reducing system after the penetration to the cells might indicate that this compound is expected to have a pharmacological action similar to that of allicin in the cells. Importantly, prosultiamine is pharmacologically stable and is very frequently prescribed for vitamin B1 deficiency in Japan. As such, this drug is potentially available for immediate clinical trials in HAM/TSP patients.

Considering the above, we first investigated the cytotoxic effect of prosultiamine against various HTLV-I-infected T cell lines derived from HAM/TSP patients compared to that of allicin through analyses of apoptotic signaling. Secondly, based on the data

showing that prosultiamine *in vitro* treatment against peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients induced a significant decrease in HTLV-I proviral copy numbers via apoptosis of HTLV-I-infected cells, we treated 6 HAM/TSP patients with intravenous administration of prosultiamine. As a result of this treatment, the copy numbers of HTLV-I provirus in peripheral blood decreased to about 30-50% of their levels at pretreatment with some clinical benefits in all patients. We herein propose prosultiamine treatment as a new therapeutic strategy against HAM/TSP.

## **Subjects and Methods**

### 1) Cell lines and peripheral blood CD4<sup>+</sup> T cells

HTLV-I-infected T cell lines derived from the cerebrospinal fluid of three HAM/TSP patients (HCT-1, HCT-4, and HCT-5) were used [16]. These cell lines were HTLV-I-producing cell lines. Jurkat cells were also used as an HTLV-I-non-infected T cell line. HCT-1, HCT-4, and HCT-5 were interleukin (IL)-2-dependent and were maintained in RPMI 1640 containing 20% fetal bovine serum (FBS) supplemented with 100 units/ml of recombinant human IL-2 (kindly provided by Shionogi, Japan). Jurkat cells were maintained in RPMI 1640 containing 10% FBS. The peripheral blood CD4<sup>+</sup>-enriched T cells of HAM/TSP patients were separated in the negative selection by the depletion of CD8<sup>+</sup> T cells from macrophage/B cell-depleted mononuclear cells using magnetic beads coated with anti-CD8 monoclonal antibody (Dynabeads CD8) (DynaL Biotech, Oslo, Norway) as previously described [17]. CD4<sup>+</sup>-enriched T cells were used as CD4<sup>+</sup> T cells.

### 2) Cytotoxicity assay

Each cell line was cultured at the concentration of  $2 \times 10^5$  /ml for 24 hr in the presence of allicin (LKT Labs Inc., MN) or prosultiamine (kindly provided by Takeda Pharma Co. Inc., Osaka, Japan) at various concentrations in 96-well culture plates. As a control, each cell line was cultured in the presence of vehicle alone. Cultures were studied in quadruplicate. Cell viability was determined by MTS nonradioactive cell proliferation assay (Promega Madison, WI) [17].

### 3) Apoptosis assay

#### a) Flow cytometry

HCT-1 was cultured at the concentration of  $2 \times 10^5$ /ml in the presence of 40  $\mu$ M prosultiamine or vehicle for 1, 3, 5, 8, and 24 hr. After HCT-1 was collected at each time point and stained either by the potential sensitive fluorescent dye DiOC<sub>6</sub> (3) (3,3'-dihexyloxycarbocyanine iodide) (Nacalai Tesque, Kyoto, Japan) as previously described [18] or by FITC-conjugated annexin V (BD Biosciences, San Diego, CA) according to the instructions provided by the manufacturer, the loss of the mitochondrial membrane potential ( $\Delta\Psi$ m) or the percentages of apoptotic cells, respectively, was analyzed by flow cytometry (Epics XL System II, Beckman Coulter, Fullerton, CA).

#### b) Effect of pretreatment with z-VAD-fmk against apoptosis

To examine whether or not apoptosis is dependent on caspase activation, after HCT-1 pretreated with 200  $\mu$ M z-VAD-fmk (the pan-caspase inhibitor) (Calbiochem, La Jolla, CA) for 1 hr was treated with 40  $\mu$ M of either allicin, prosultiamine or vehicle for 24 hr, the cell viability and the loss of  $\Delta\Psi$ m were determined by MTS assay and flow cytometry, respectively.

#### 4) Western blot analysis

HCT-1 was collected at various time points after treatment with 40  $\mu$ M allicin, prosultiamine or vehicle, and lysed by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Sigma, Saint Louis, MO). Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4°C, and the supernatant was used for western blotting. An identical amount of protein for each lysate (10  $\mu$ g) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ATTO, Tokyo). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After overnight blocking with TBS with 0.1% Tween-20 (TBST) containing 5% skim milk (Difco) at 4°C, the PVDF membrane was incubated in the presence of mouse anti-caspase-3 antibody (1:1000 dilution, Cell Signaling, Danvers, MA) at 4°C overnight. After overnight incubation with donkey anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody (Amersham Biosciences, UK) at 4°C overnight, proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

#### 5) Quantification of HTLV-I proviral load

For quantitative analysis of the HTLV-I proviral load, real-time quantitative PCR was performed in a Light-Cycler FastStart DNA Master (Roche Diagnostics, Mannheim, Germany) based on general fluorescence detection with SYBR Green. Briefly, genomic DNA samples from CD4<sup>+</sup> T cells or peripheral blood mononuclear cells (PBMC) from HAM/TSP patients prepared using the Genomic DNA Purification kit (Promega) were subjected to real-time PCR in a LightCycler PCR system using Tax-specific primers,

forward primer (5'- AAACAGCCCTGCAGATACAAAGT -3') and reverse primer (5'- ACTGTAGAGCTGAGCCGATAACG -3'), and  $\beta$ -actin-specific primers, forward primer (5'- GCCCTCATTTCCCTCTCA -3') and reverse primer (5'- GCTCAGGCAGGAAAGACAC -3'). The PCR condition for Tax was 40 cycles of denaturation (95°C, 15 s), annealing (55°C, 5 s), extension (72°C, 10 s), and that for  $\beta$ -actin was 32 cycles of denaturation (95°C, 15 s), annealing (62°C, 5 s), and extension (72°C, 15 s). The HTLV-I proviral load per 10000 cells was calculated according to the following formula: (copy number of Tax)/(copy number of  $\beta$ -actin/2)  $\times$  10000.

6) *In vitro* treatment with prosultiamine against peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients

The peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients were cultured at a concentration of  $1 \times 10^6$  cells/ml in 24-well culture plates in the presence of 5  $\mu$ M prosultiamine for 48 hr. As a control, the cells were cultured in the presence of vehicle alone. After the cells were collected, the dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA) according to the instructions provided by the manufacturer, and viable cells were collected. Total cellular DNA samples prepared from viable cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analysis. In this experiment, to confirm apoptosis of HTLV-I-infected cells, cultured cells of an HAM/TSP patient were stained with anti-HTLV-I (p19, p28 and gag) monoclonal antibody (Chemicon International Inc, Temecula, CA) followed by the incubation with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) supplemented with Hoechst33258 (Sigma) as previously described [19], then analyzed by confocal microscopy.

## 7) Clinical trial with prosultiamine

We enrolled 6 HAM/TSP patients (2 men, 4 women) whose ages ranged from 51 to 73 and who fulfilled the criteria described previously [20]. We show the profiles of the patients in Table 1. Informed written consent was obtained from all patients in the study. This research was approved by the review boards of the Graduate School of Biomedical Sciences, Nagasaki University. Prosultiamine was diluted with 50 ml of physiological saline and administered intravenously at dosages of 40 mg daily for 14 days. The treatments that two patients were already receiving (1 g of vitamin C/day in case 1 and 3 doses of 3 million international units of IFN- $\alpha$  /week with 7.5 and 5.0 mg of oral prednisolone/ alternate days in case 3) were continued on the condition that the dosage was kept constant concomitant with prosultiamine treatment during the study period. None of the patients except these two had received any immunomodulators. The clinical endpoints selected for the assessment of treatment were motor function score and time required to walk 10 or 30 m. The classifications of motor function score were made according to the disability grade [21]. Prior to the administration of prosultiamine, the patients were instructed to walk a fixed distance, and the administration was begun only after the walking time became constant. We monitored the change of both motor function and HTLV-I proviral load in the PBMC.

## 8) Statistical analysis

Student's t test and Wilcoxon single-rank test were used for statistical analysis. Differences were considered significant at  $p < 0.05$ .

## Results

1) The cytotoxic effect of allicin and prosultiamine against HTLV-I-infected T cell lines derived from HAM/TSP patients is based on caspase-dependent apoptosis

The cell viability of the three HTLV-I-infected T cell lines or the Jurkat cell line decreased as a result of allicin treatment for 24 hr in a dose-dependent manner (Figure 2a). Prosultiamine treatment caused an effect similar to that of allicin treatment (Figure 2a). These results indicate that prosultiamine has the potential to induce a cytotoxic effect against these cell lines much like allicin. Of the cell lines treated, the HTLV-I-infected T cell lines seemed to be more sensitive to the treatment with each compound than the Jurkat cell line. As shown in Figure 2b, western blot analysis revealed that the proteolytic cleavage of caspase 3 appeared between 3 and 5 hr after the start of the treatment in allicin- or prosultiamine-treated HCT-1. Next, in order to confirm the involvement of caspases in the cytotoxicity against HCT-1 induced by these compounds, HCT-1 was treated with 40  $\mu$ M of either allicin or prosultiamine after this cell line was pretreated with 200  $\mu$ M z-VAD-fmk for 1 hr. As shown in Figure 2c, the cell viability was recovered almost completely in z-VAD-fmk-pretreated HCT-1. These results strongly suggested that these compounds can induce caspase-dependent apoptosis in HTLV-I-infected cells derived from HAM/TSP patients.

2) Prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HTLV-I-infected cells.

As mentioned above, since allicin is a very unstable compound [12, 13], it is very difficult to use as the therapeutic regimen in the treatment of HAM/TSP patients.

However, prosultiamine is pharmacologically stable and is very frequently prescribed as

a safe treatment regimen for thiamine deficiency. Therefore, we had to investigate the mechanisms of the induction of caspase-dependent apoptosis *in vitro* in prosultiamine-treated HCT-1 before performing the clinical trial with prosultiamine treatment. First, we performed a time-course study of the loss of  $\Delta\Psi_m$  concomitant with the appearance of annexin-V positive cells in prosultiamine-treated HCT-1. As shown in Figure 3a, the loss of  $\Delta\Psi_m$  was detected in about 29.4, 78.9, and 95.8% of prosultiamine-treated HCT-1 at 5, 8 and 24 hr, respectively. In parallel with this data, annexin V-positive cells were detected in 9.9 and 97.2% of prosultiamine-treated HCT-1 at 8 and 24 hr, respectively (Figure 3b). These results suggest that prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HCT-1. In order to confirm this finding, we analyzed the effect of pretreatment with z-VAD-fmk on the recovery of the loss of  $\Delta\Psi_m$  in prosultiamine-treated HCT-1. As shown in Figure 3c, the loss of  $\Delta\Psi_m$  in HCT-1 due to treatment with 40  $\mu\text{M}$  prosultiamine was almost completely recovered by pretreatment with 200  $\mu\text{M}$  z-VAD-fmk for 1 hr. Overall, these results strongly suggest that the cytotoxicity of prosultiamine against HTLV-I-infected cells is mainly based on caspase-dependent apoptosis through the mitochondrial pathway.

3) Prosultiamine *in vitro* treatment against peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients induces a significant decrease of HTLV-I proviral copy numbers via apoptosis of HTLV-I-infected cells.

Confocal microscopic study showed that prosultiamine treatment against peripheral blood CD4<sup>+</sup> T cells from a HAM/TSP patient induced the condensation of chromatin in HTLV-I-infected cells only in prosultiamine-treated peripheral blood CD4<sup>+</sup>

T cells, as shown in the double-staining with anti-HTLV-I monoclonal antibody and Hoechst33258 (Figure 4a). Next, we evaluated the effect of prosultiamine treatment on the reduction of HTLV-I-infected cells in peripheral blood CD4<sup>+</sup> T cells from HAM/TSP patients. As shown in Figure 4b, prosultiamine treatment induced a decrease in the numbers of HTLV-I proviral copies ranging from 11.2 – 70.1% (mean; 42.9%) in comparison with the vehicle treatment. Allicin *in vitro* treatment induced the same effect as prosultiamine *in vitro* treatment (data not shown). We also evaluated the cell viability by MTS assay and found that prosultiamine treatment did not affect the viability of total peripheral blood CD4<sup>+</sup> T cells, indicating that the effect did not depend on a non-specific cytotoxic effect (data not shown). These data suggest that prosultiamine *in vitro* treatment can selectively induce apoptosis in HTLV-I-infected peripheral blood CD4<sup>+</sup> T cells from HAM/TSP patients.

#### 4) Clinical trial with prosultiamine

Based on the data mentioned above, we treated 6 HAM/TSP patients by intravenous administration of prosultiamine. As shown in Table 1, although the motor function score did not change among 5 of 6 HAM/TSP patients (cases 1 - 5), some clinical improvement was observed in individuals, including a decrease in the walking time in case 1, the acquisition of an ability to maintain a standing position from a wheelchair-dependent state for a short period in case 2, and the improvement of spasticity in cases 3 - 5. On the other hand, case 6 showed marked improvement of motor function such as a change of motor disability grade from 4 to 2 (Table 1). No adverse events were observed.

We monitored the change in the copy numbers of HTLV-I provirus in PBMC of HAM/TSP patients at pretreatment (day 0), during treatment (days 7 and 14), and 7 days after treatment (day 21). The decrease of the HTLV-I proviral copy number was not statistically significant ( $p=0.056$ ) at day 14 when this point was designated as the primary endpoint of this trial. However, when we analyzed it individually, as shown in Figure 5, the HTLV-I proviral copy number gradually decreased to 33, 55, 44, and 49% of the number at pretreatment in cases 2, 3, 4, and 6, respectively, until 7 days after treatment. Although the HTLV-I proviral copy number at day 21 was almost the same as at pretreatment in cases 1 and 5, the copy number of HTLV-I provirus transiently decreased to 52% and 50%, respectively, of the number at pretreatment at day 7. Overall, this preliminary therapeutic trial with prosultiamine showed the possibility that the clinical improvement was induced by the decrease of HTLV-I-infected cells in the peripheral blood of HAM/TSP patients.

## **Discussion**

We should treat HAM/TSP as an infectious disease. Therefore, in the therapeutic strategy for treating HAM/TSP, we should focus on the suppression of HTLV-I expression and/or replication, the inhibition of the proliferation of HTLV-I-infected cells, or the elimination of HTLV-I-infected cells. To our knowledge, this is the first report to demonstrate that a disulfide moiety in allicin or prosultiamine can induce apoptosis in HTLV-I-infected cells derived from HAM/TSP patients, and that prosultiamine has the potential to be a new therapeutic tool targeting HTLV-I-infected cells in HAM/TSP. We showed that allicin can induce caspase-dependent apoptosis in HTLV-I-infected cells. However, it is not available for clinical use because of its instability. Therefore, we

focused on prosultiamine as a treatment for HAM/TSP patients. In our analysis of the cytotoxic effects of prosultiamine on HTLV-I-infected T cell lines derived from HAM/TSP patients, this compound induced effects on these cell lines that were similar to those of allicin, as expected. Although there are the differences in a cytotoxic effect by both allicin and prosultiamine among three HTLV-I-infected cell lines, they might be based on anti-apoptotic activity that each cell line originally has. The analyses involving HCT-1 showed that prosultiamine could induce caspase-dependent apoptosis through the mitochondrial pathway. Our results from these analyses with the regard to prosultiamine are consistent with the recent report that allicin, in HTLV-I-non-infected cell lines, can induce apoptosis through the mitochondrial pathway [22]. Although the upstream of caspase-dependent apoptosis through the mitochondrial pathway induced by disulfide moiety is still obscure, the disruption of the intracellular redox system by the interaction between the Trx/Trx reductase system and the disulfide moiety leading to the activation of apoptosis signal-regulating kinase 1 (ASK1) might be involved as one of the mechanisms, because the reduced form of Trx is a physiological negative regulator of ASK1 activation [23-25]. Activation of ASK1 signaling, leading to stress-induced apoptosis through the activation of p38 mitogen-activated protein kinase or c-Jun N-terminal kinase, by the oxidation of Trx through a disulfide moiety was supposed. Further investigations of this mechanism are underway.

Based on the data for the differential sensitivities of the cytotoxicity of prosultiamine between HTLV-I-infected and -non-infected cell lines, we treated peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients with prosultiamine *in vitro*. These treatments significantly induced a decrease of HTLV-I proviral copy numbers, selectively showing apoptosis of HTLV-I-infected T cells. This evidence prompted us to

administer the treatment with prosultiamine to HAM/TSP patients because this agent is very frequently prescribed as a safe treatment regimen for thiamine deficiency in Japan. We treated 6 HAM/TSP patients with prosultiamine. As a result, we confirmed the efficacy of prosultiamine treatment in bringing about a decrease of HTLV-I proviral copy numbers in PBMC in all HAM/TSP patients. A finding worthy of mention is that prosultiamine treatment, even when administered for a short term such as 14 days, induced about a 50 - 70% decrease of the copy numbers of HTLV-I provirus in PBMC of HAM/TSP patients. However, the rebound or the fluctuation of HTLV-I proviral copy numbers were observed during the treatment course in 4 of 6 cases. Although the exact reason of these phenomenon is unknown, these findings might suggest the limitations of prosultiamine treatment by the present protocol. Although the degree of clinical improvement was not as great in HAM/TSP patients who had a long-term duration of illness, it is a noteworthy result that prosultiamine treatment induced a marked improvement of motor function in a patient who had a short-term duration of illness of 2 years (case 6).

In conclusion, we showed that the compounds containing disulfide moiety in their structures, such as allicin and prosultiamine, can induce apoptosis in HTLV-I-infected cells in HAM/TSP patients. Our results suggest that prosultiamine has the potential to be a new therapeutic tool targeting HTLV-I-infected cells by inducing apoptosis in HAM/TSP. Further investigation of long-term treatment for HAM/TSP patients with prosultiamine is needed.

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### **Conflicts of interest**

The authors declare no competing financial interests.

## References

1. Osame M, Matsumoto M, Usuku K, et al. Chronic progressive myelopathy associated with elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemia-like cells. *Ann Neurol* 1987; **21**:117-122.
2. Iwasaki Y. Pathology of chronic myelopathy associated with HTLV-I infection (HAM/TSP). *J Neurol Sci* 1990; **96**:103-123.
3. Nakamura T. Immunopathogenesis of HTLV-I-associated myelopathy/tropical spastic paraparesis. *Ann Med* 2000; **32**:600-607.
4. Osame M. Pathological mechanisms of human T-cell lymphotropic virus type I-associated myelopathy (HAM/TSP). *J Neurovirol* 2002; **8**:359-364.
5. Nakagawa M, Nakahara K, Maruyama Y, et al. Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. *J Neurovirol* 1996; **2**:345-355.
6. Shibayama K, Nakamura T, Nagasato K, Shirabe S, Tsujihata M, Nagataki S. Interferon-alpha treatment in HTLV-I-associated myelopathy: studies of clinical and immunological aspects. *J Neurol Sci* 1991; **106**:186-192.
7. Izumo S, Goto I, Itoyama Y, et al. Interferon-alpha is effective in HTLV-I-associated myelopathy: a multicenter, randomized, double-blind, controlled trials. *Neurology* 1996; **46**:1016-1021.
8. Nakamura T, Nishiura, Y, Fukushima N, Furuya T. The role of HTLV-I-infected CD4<sup>+</sup> T cells as activated Th1 cells in the immunopathogenesis of HTLV-I-associated myelopathy/tropical spastic paraparesis. In: *Neuroimmunology Research Focus*. Broglio PV (editor). New York: Nova Science Publishers; 2007. pp. 43-84.
9. Ankri S, Mirelman D. Antimicrobial properties of allicin from garlic. *Microbes Infect*

- 1999; **2**:125-129.
10. Hirsch K, Danilenko M, Giat J, et al. Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. *Nutr Cancer* 2000; **38**:245-254.
  11. Wong WW, Macdonald S, Langler RF, Penn LZ. Novel synthetic organosulfur compounds induce apoptosis of human leukemic cells. *Anticancer Res* 2000; **20**:1367-1374.
  12. Lawson LD, Wang ZJ. Pre-hepatic fate of the organosulfur compounds derived from garlic (*Allium sativum*). *Planta Med* 1993; **59**(Suppl.):688-689.
  13. Freeman F, Kodera Y. Garlic chemistry – stability of *S*-(2-propenyl) 2-propene-1-sulfinothioate (allicin) in blood, solvents, and simulated physiological fluids. *J Agric Food Chem* 1995; **43**:2332-2338.
  14. Fujiwara M, Watanabe H, Matsui K. “Allithiamine” a newly found derivative of vitamin B1. I. discovery of allithiamine. *J Biochem, Tokyo* 1954; **41**:29-39.
  15. Matsukawa T, Yurugi S. On the structure of allithiamine. *Proc Japan Acad* 1952; **28**:146-149.
  16. Fukushima N, Nakamura T, Nishiura Y, Ida H, Aramaki T, Eguchi K. HTLV-I production based on activation of integrin/ligand signaling in HTLV-I-infected T cell lines derived from HAM/TSP patients. *Intervirology* 2008; **51**:123-129.
  17. Fukushima N, Nishiura Y, Nakamura T, Yamada Y, Kohno S, Eguchi K. Involvement of p38 MAPK signaling pathway in IFN- $\gamma$  and HTLV-I expression in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. *J Neuroimmunol* 2005; **159**:196-202.
  18. Shibatomi K, Ida H, Yamasaki S, et al. A novel role for interleukin-18 in human natural killer cell death: high serum levels and low natural killer cell numbers in

- patients with systemic autoimmune diseases. *Arthritis Rheum* 2001; **44**:884-892.
19. Nakamura H, Kawakami A, Ida H, Koji T, Eguchi K. EGF activates PI3K-Akt and NF- $\kappa$ B via distinct pathways in salivary epithelial cells in Sjögren's syndrome. *Rheumatol Int* 2007; **28**:127-136.
  20. Osame M. Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: *Human Retrovirology: HTLV*. Blattner WA (editor). New York: Raven Press; 1990. pp. 191-197.
  21. Matsuo H, Nakamura T, Tsujihata M, et al. Plasmapheresis in treatment of human T-lymphotropic virus type-I associated myelopathy. *Lancet* 1988; **ii**:1109-1113.
  22. Miron T, Wilchek M, Sharp A, et al. Allicin inhibits cell growth and induces apoptosis through the mitochondrial pathway in HL60 and U937. *J Nutr Biochem* 2008; **19**:524-535.
  23. Saitoh M, Nishitoh H, Fujii M, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 1998; **17**:2596-2606.
  24. Hayakawa T, Matsuzawa A, Noguchi T, Takeda K, Ichijo H. The ASK1-MAP kinase pathways in immune and stress responses. *Microbes Infect* 2006; **8**:1098-1107.
  25. Matsutani H, Ueda S, Yodoi J. The thioredoxin system in retroviral infection and apoptosis. *Cell Death Differ* 2005; **12**:991-998.

**Figure legends**

**Fig. 1.** The structures of both allicin (a) and prosultiamine (b). Prosultiamine has a disulfide moiety in its structure like allicin as shown by [ ] in its molecule.

**Fig. 2.** The cytotoxic effect of allicin and prosultiamine against HTLV-I-infected T cell lines derived from HAM/TSP patients based on caspase-dependent apoptosis. a) Cell viability of three HTLV-I-infected T cell lines or a Jurkat cell line decreased as a result of either allicin or prosultiamine treatment for 24 hr in a dose-dependent manner. HTLV-I-infected T cell lines seem to be more sensitive to the treatment with each compound than the Jurkat cell line. b) Western blot analysis of caspase 3 activation. The proteolytic cleavage of caspase 3 appeared between 3 and 5 hr after the start of the treatment in HCT-1 cells treated with 40  $\mu$ M of either allicin or prosultiamine. c) HCT-1 pretreated with 200  $\mu$ M z-VAD-fmk for 1 hr were treated with 40  $\mu$ M of either allicin or prosultiamine for 24 hr. Cell viabilities were recovered almost completely in z-VAD-fmk-pretreated HCT-1. As a cell viability assay, MTS assay was performed. Cell viability was determined as follows: after each OD titer at a wavelength of 490 nm in quadruplicate cultures in the presence of allicin or prosultiamine / mean of OD titer at a wavelength of 490 nm in quadruplicate cultures under vehicle alone was calculated, and its mean  $\pm$  SD was presented as the cell viability.

**Fig. 3.** Prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HCT-1 cells. The time-course study of the loss of  $\Delta\Psi_m$  (a) and the appearance of annexin-V positive cells (b) in prosultiamine-treated HCT-1 cells by flow cytometric analysis. Prosultiamine treatment of HCT-1 cells induced the loss of

mitochondrial membrane potential with the appearance of annexin V-positive cells. c) The effect of pretreatment with z-VAD-fmk on the recovery of the loss of  $\Delta\Psi_m$  in prosultiamine-treated HCT-1 cells. The loss of  $\Delta\Psi_m$  in HCT-1 cells resulting from treatment with 40  $\mu\text{M}$  prosultiamine was almost completely recovered by pretreatment with 200  $\mu\text{M}$  z-VAD-fmk for 1 hr.

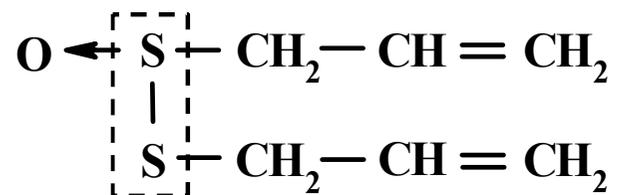
**Fig. 4.** Effect of *in vitro* treatment with prosultiamine on HTLV-I-infected cells of HAM/TSP patients. The peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients were cultured in the presence of 5  $\mu\text{M}$  prosultiamine or vehicle for 48 hr. a) Confocal microscopic study in prosultiamine- or vehicle-treated peripheral blood CD4<sup>+</sup> T cells of a HAM/TSP patient. The condensation of chromatin in HTLV-I-infected cells was shown in only prosultiamine-treated peripheral blood CD4<sup>+</sup> T cells by double-staining with anti-HTLV-I antibody and Hoechst33258. b) Decrease of HTLV-I proviral copy numbers in peripheral blood CD4<sup>+</sup> T cells of HAM/TSP patients as a result of the *in vitro* treatment with prosultiamine. After the dead cells induced by prosultiamine *in vitro* treatment were removed using Dead Cell Removal Kit, total cellular DNA samples prepared from viable cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analysis. Prosultiamine treatment induced a significant decrease of HTLV-I proviral copies compared with vehicle treatment.

**Fig. 5.** Decrease of HTLV-I proviral copy numbers in PBMC of HAM/TSP patients as a result of clinical trial with prosultiamine. We treated 6 HAM/TSP patients with intravenous prosultiamine at a dose of 40 mg daily for 14 days. Total cellular DNA prepared from peripheral blood mononuclear cells was subjected to measurement of

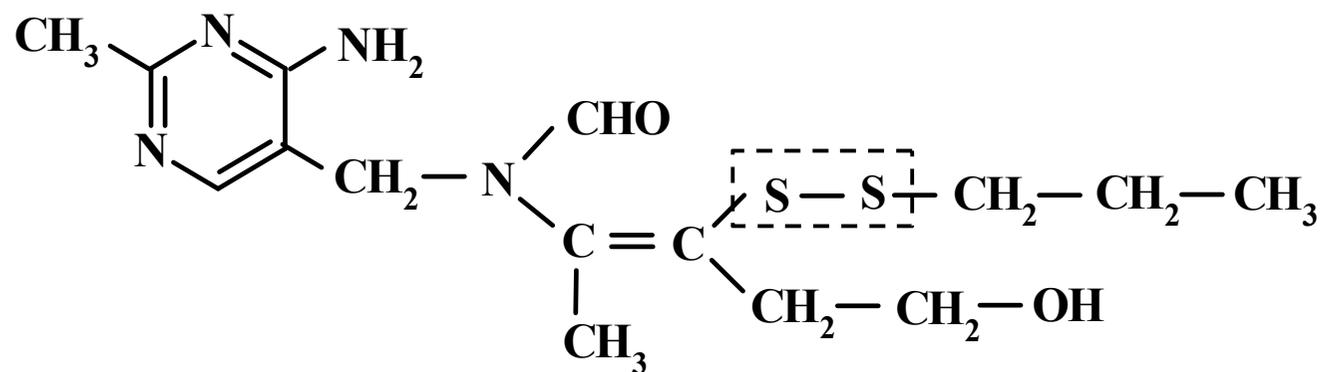
HTLV-I proviral copies by quantitative PCR analysis. We monitored the copy numbers of HTLV-I provirus in the peripheral blood at pretreatment (day 0), during treatment (days 7 and 14), and 7 days after treatment (day 21), and they were observed to decrease to 33-55 % of the numbers at pretreatment at their nadir in all HAM/TSP patients.

**Figure 1**

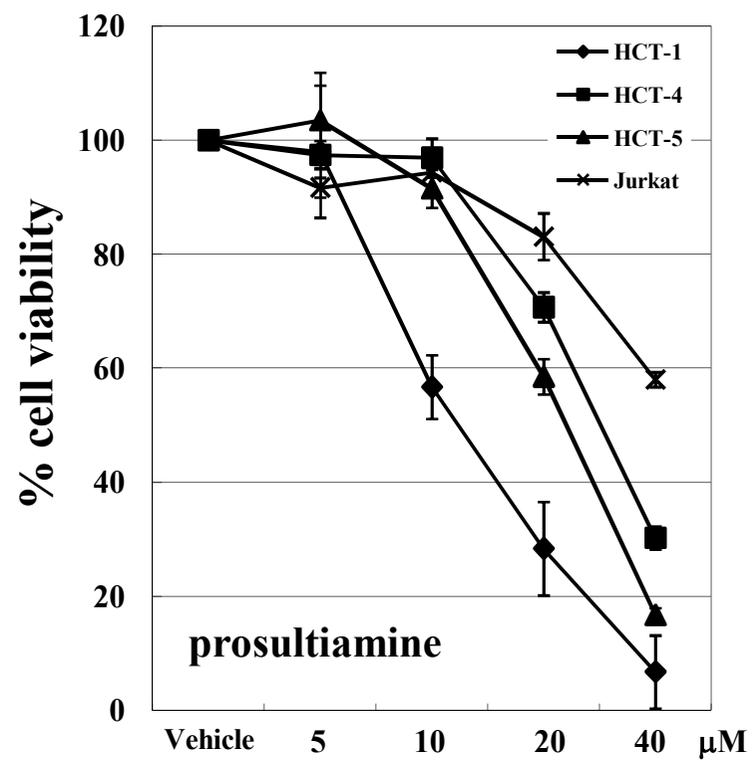
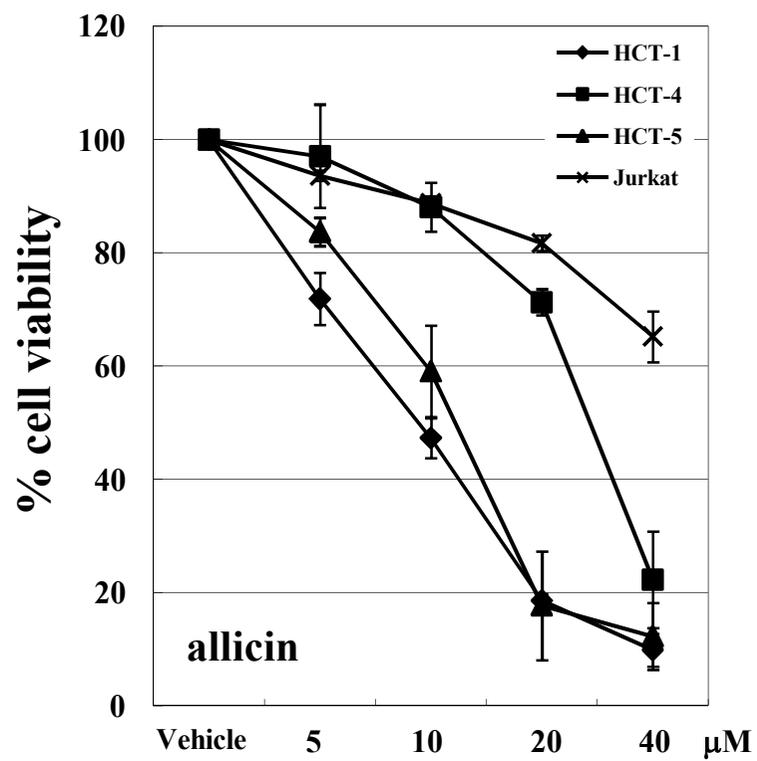
**a) Allicin**



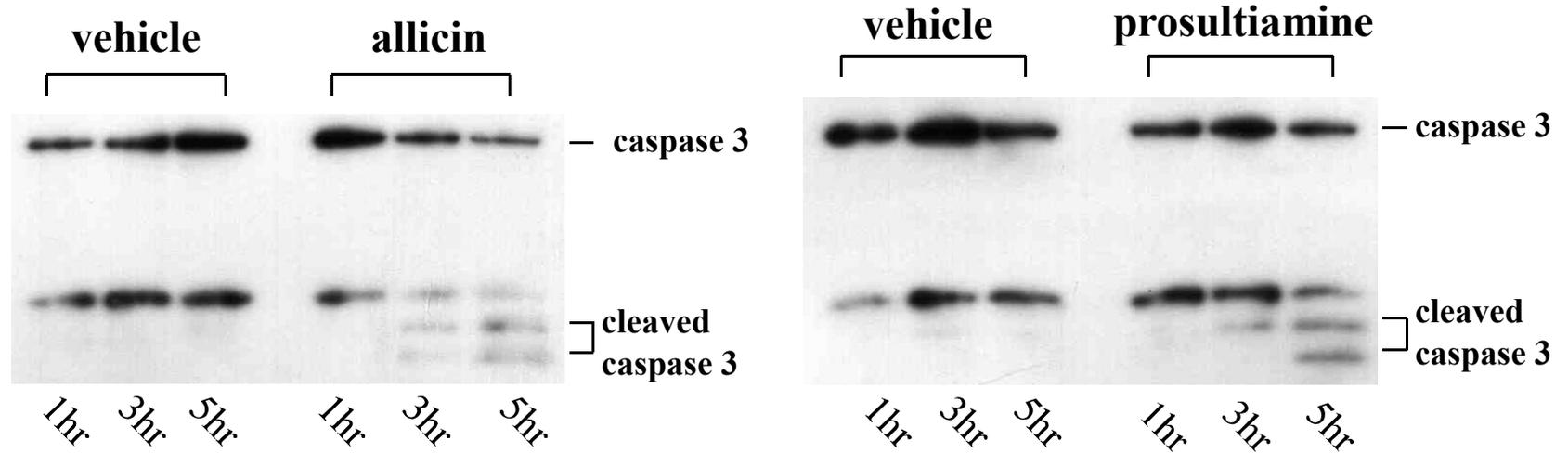
**b) Prosultiamine**



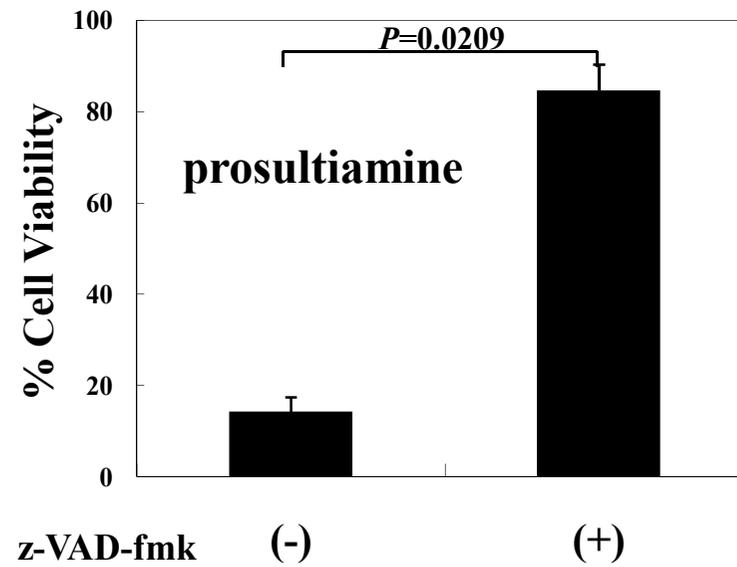
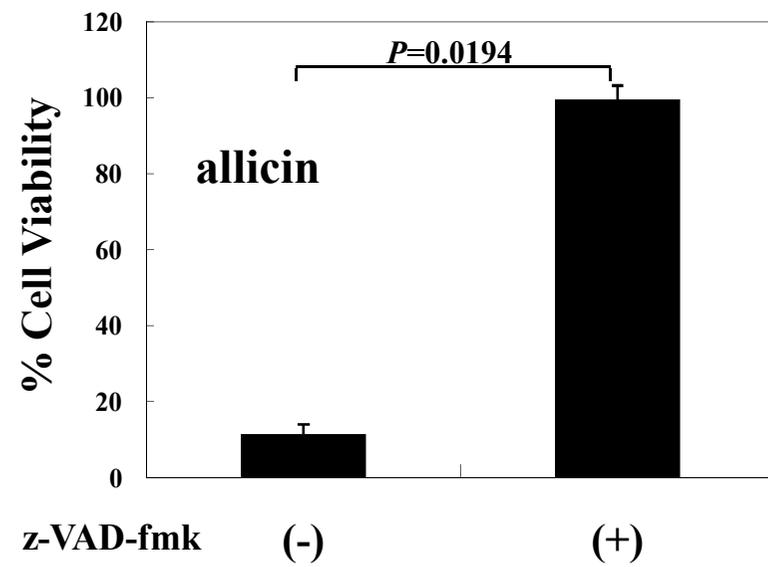
**Figure 2a)**



**Figure 2b)**



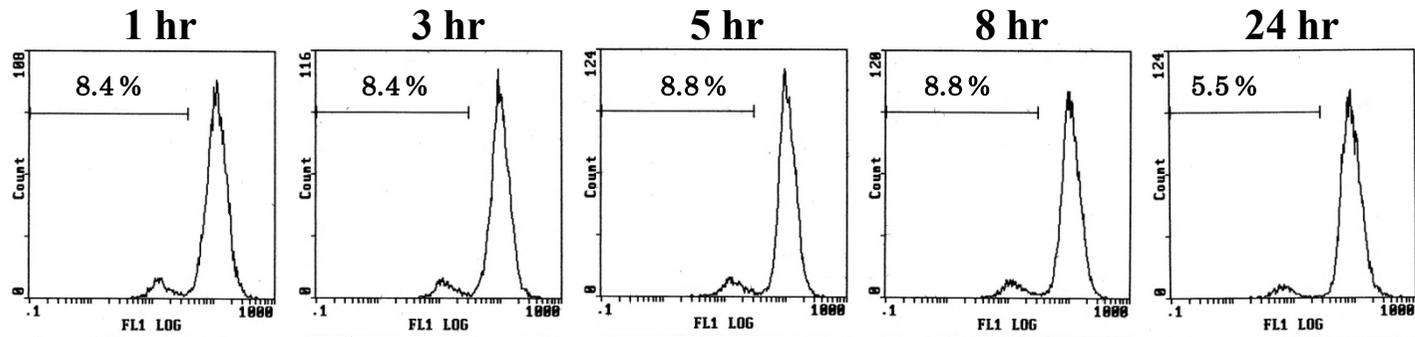
**Figure 2c)**



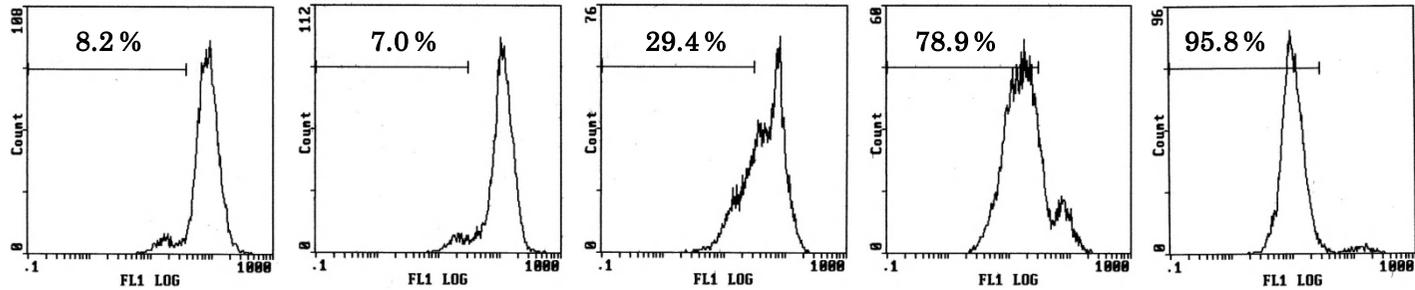
**Figure 3a)b)**

**a)**

**vehicle**

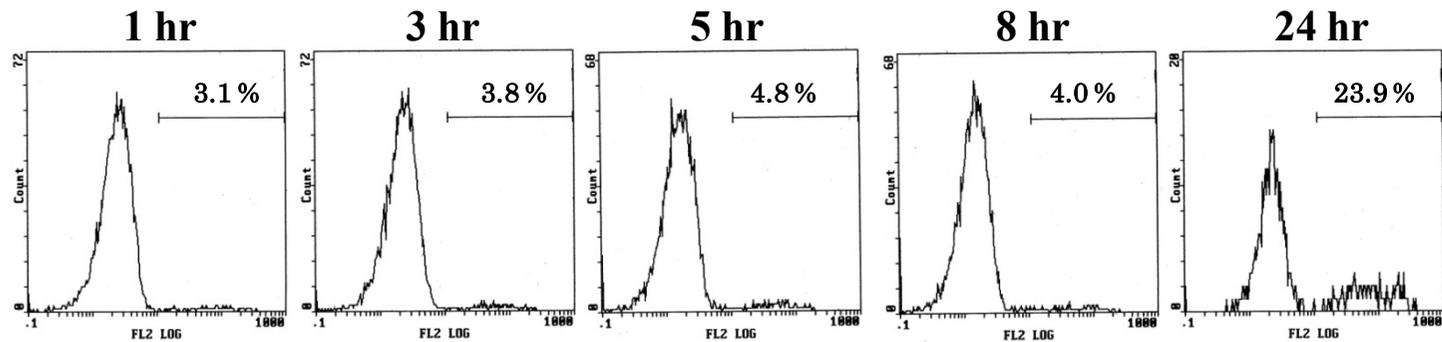


**prosultiamine**



**b)**

**vehicle**



**prosultiamine**

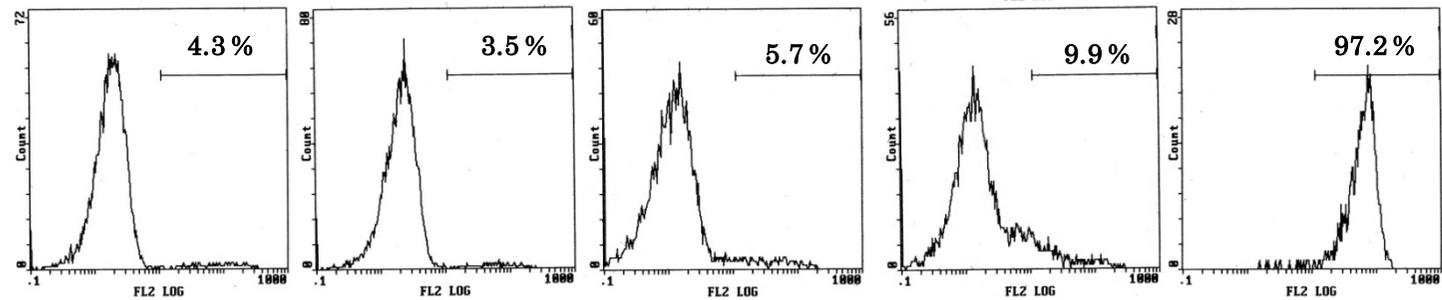
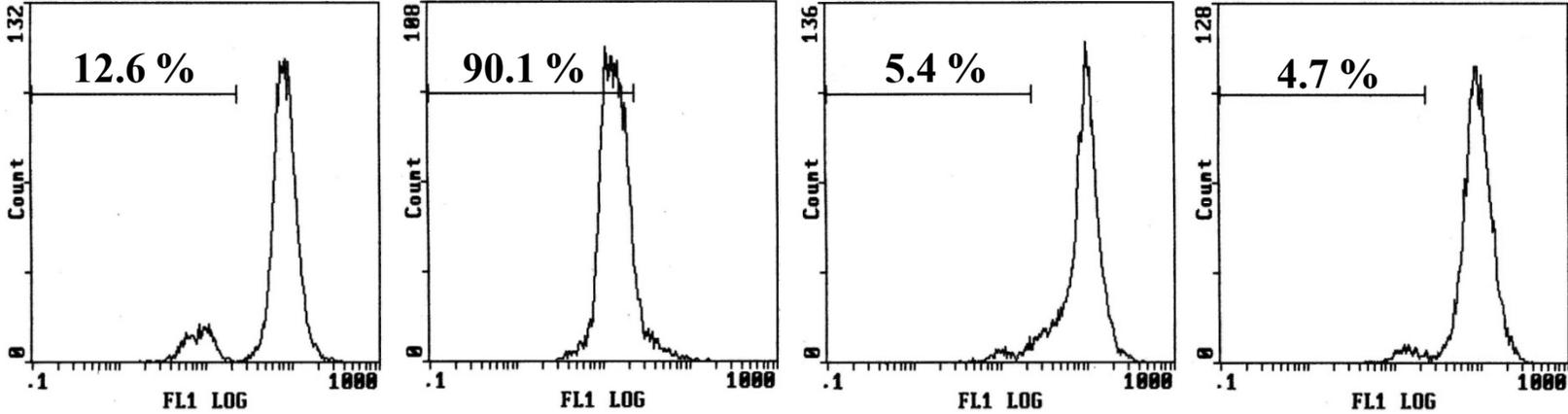


Figure 3c)



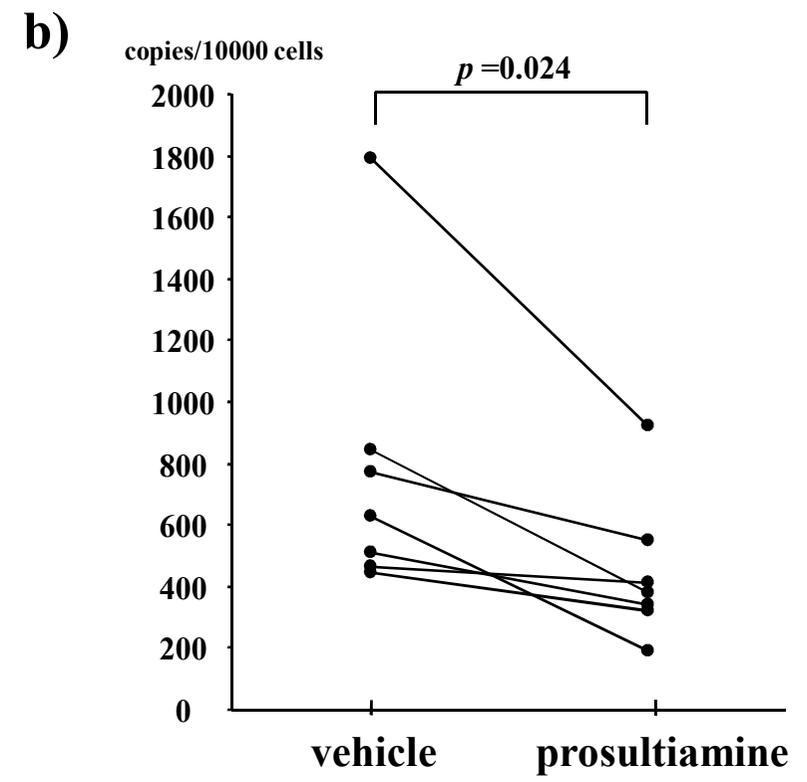
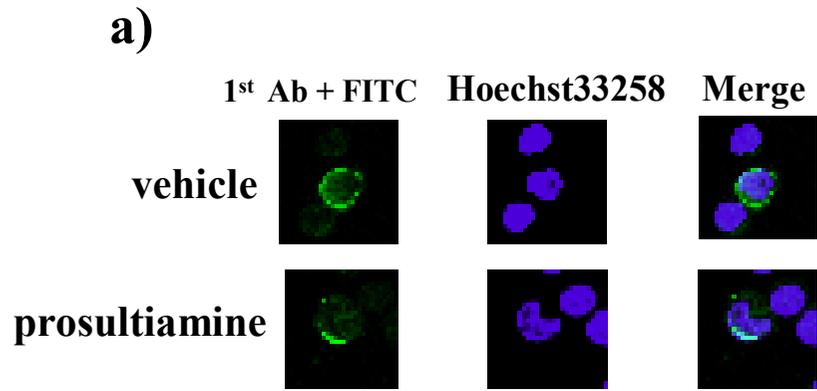
**z-VAD-fmk** (-)  
**prosultiamine** (-)

(-)  
(+)

(+)  
(-)

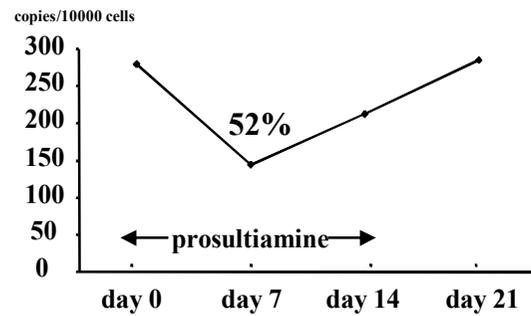
(+)  
(+)

# Figure 4

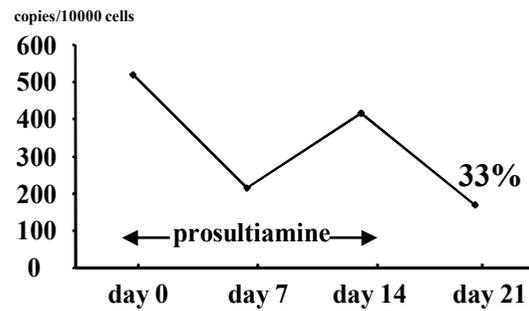


# Figure 5

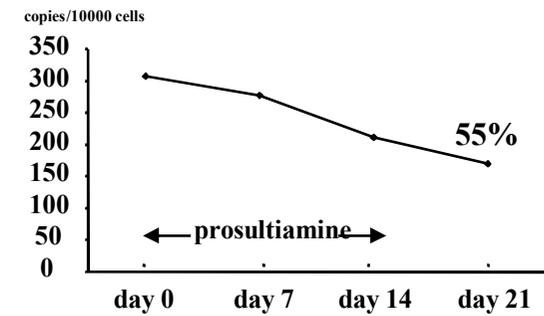
case 1



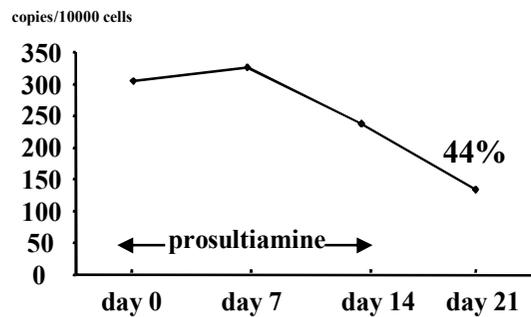
case 2



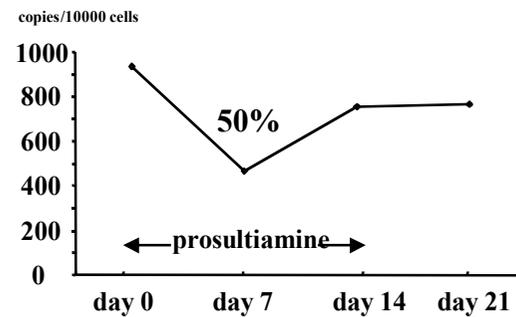
case 3



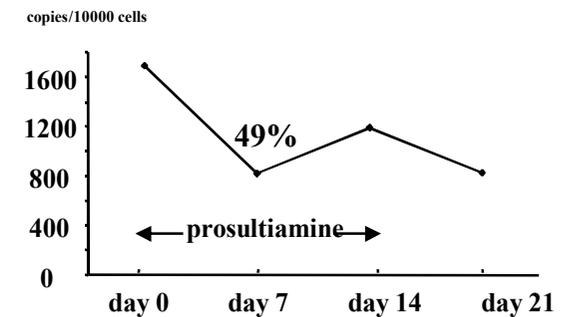
case 4



case 5



case 6



**Table 1. The profiles of patients and the clinical efficacy of prosultiamine treatment**

Case	Age / Gender	Duration of illness (years)	The changes of motor function score <sup>a)</sup>	
			Before	After
1	58 / female	24	4	4
2	51 / male	13	8	8
3	52 / male	46	4	4
4	73 / female	13	4	4
5	59/ female	12	4	4
6	53 / female	2	4	2

a) Motor function score was rated from 0 to 10 according to the scale of reference [21].