Therapeutic Strategies in HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

Key words: HTLV-I, HAM/TSP, Treatment

Tatsufumi Nakamura\textsuperscript{a}\textsuperscript{*}, Yoshihiro Nishiura\textsuperscript{b}, Katsumi Eguchi\textsuperscript{b}

\textsuperscript{a} Department of Molecular Microbiology and Immunology
\textsuperscript{b} First Department of Internal Medicine

Graduate School of Biomedical Sciences, Nagasaki University, Japan

Corresponding author: Tatsufumi Nakamura, M.D.

Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Phone: +81-95-819-7265

Fax: +81-95-849-7270

E-mail: tatsu@net.nagasaki-u.ac.jp
Abstract

Human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is chronic progressive myelopathy characterized by bilateral pyramidal tracts involvement with sphincteric disturbances. HTLV-I infects approximately 10-20 million people worldwide. There are large endemic areas in southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa. Since the primary neuropathological feature of HAM/TSP is chronic inflammation caused by HTLV-I infection in the spinal cord, various treatments focusing on immunomodulatory or anti-viral effects were performed for HAM/TSP patients until now. However, there are still many of problems, such as insufficient effects, side effects and expensive costs in long-term treatments, etc., in these treatments. Therefore, an ideal therapeutic strategy against HAM/TSP is still not established yet. Although only a small proportion of HTLV-I-infected individuals develops HAM/TSP, neurological symptoms are certainly progressive once myelopathy develops, leading to deterioration of the quality of life. Therefore, we now need the therapeutic regimens to protect the development, or be able to commence the treatments as soon as possible after the development safely and inexpensively even in long-term course or lifelong course of treatment. As HTLV-I-infected CD4⁺ T cells are the first responders in the immunopathogenesis of HAM/TSP, the ideal treatment is the elimination of HTLV-I-infected cells from the peripheral blood. In this article, We will review the therapeutic strategies against HAM/TSP up to now and will introduce our
new therapeutic approach focusing on the targeting of HTLV-I-infected cells in HAM/TSP patients.
Introduction

Human T lymphotropic virus type I (HTLV-I) is a member of the exogeneous human retroviruses and the causative agent for both adult T cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1, 2]. Since the discovery of HAM/TSP, it was revealed that HTLV-I has the potentials to not only act as one of the oncoviruses but also cause a chronic inflammation by the immunologic activation. However, the exact mechanisms underlying the entirely different clinical conditions caused by HTLV-I, such as aggressive lymphoproliferative malignancy and chronic inflammation, are still unknown. HTLV-I infects approximately 10-20 million people worldwide [3]. Although there are large endemic areas in southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [4], it is still not clear why only a small proportion of HTLV-I-infected individuals develops either of these HTLV-I-associated diseases.

HAM/TSP is chronic progressive myelopathy characterized by bilateral pyramidal tracts involvement, clinically presented as spastic paraparesis, with sphincteric disturbances [5]. The primary pathological feature of HAM/TSP is chronic myelitis formed by chronic inflammation in the spinal cord, mainly the lower thoracic cord, characterized by perivascular cuffing and parenchymal infiltration of mononuclear cells [6]. Although the bystander mechanisms, such as the destruction of the surrounding tissues by the interaction between HTLV-I-infected CD4+ T cells and HTLV-I-specific CD8+ cytotoxic T cells (CTL), are probably critical as the cause of chronic inflammation in the spinal cord [7, 8] (Fig. 1), the exact molecular
mechanisms of the development of HAM/TSP still remain unresolved. However, it is well known that HTLV-I proviral load in the peripheral blood is significantly higher in HAM/TSP patients than HTLV-I asymptomatic carriers [9, 10]. On the other hand, numerous immunological dysregulations mostly mediated by HTLV-I tax expression are detected in the peripheral blood of HAM/TSP patients [11, 12]. Therefore, it is strongly supposed that the increase of HTLV-I-infected cells possessing immune-activated status, such as Th1 activation, is involved in the immunopathogenesis of HAM/TSP [13] although it is still controversial whether or not HTLV-I exists in the neuronal components of the central nervous system.

When considering the therapeutic strategies in HAM/TSP, they are divided to two ways of the direction as shown in Table 1 and Fig. (1) although both ways connect to each other in some part; 1) immunomodulation therapy, mainly directed to anti-inflammatory effects, 2) anti-viral therapy. Once the myelopathy developed, the neurological symptoms are certainly progressive, leading to the deterioration of the quality of the life. Therefore, we now need the therapeutic regimens to protect the development, or be able to commence the treatments as soon as possible after the development safely and inexpensively even in long-term or lifelong course of treatment. A number of therapeutic approaches for HAM/TSP were carried out until now as shown in Table 2 although almost trials were performed under an open, nonrandomized, uncontrolled studies. Of them, immunomodulation therapies for the suppression of chronic inflammatory status based on immune-activated status as mentioned above were mainly performed for HAM/TSP patients. Indeed, as it is conceivable that the
immune-activated status in the peripheral blood involved in the process of chronic inflammation of the spinal cord is one of the targets for the treatments, almost all treatments produced the good results in their own ways. However, since HTLV-I-infected CD4\(^+\) T cells are the first responders in the immunopathogenesis of HAM/TSP as mentioned above, the primary target for HAM/TSP treatment is HTLV-I-infected cells themselves of the peripheral blood. Therefore, we should focus on the targeting of HTLV-I-infected cells themselves or the suppression of HTLV-I expression and/or replication in the peripheral blood.

In this review, we will refer to the therapeutic strategies, such as the therapies focusing on immunomodulatory effects or anti-viral effects, against HAM/TSP up to now. In addition, we will introduce our new therapeutic approach focusing on the targeting of HTLV-I-infected cells in HAM/TSP patients.

1) The therapies focusing on immunomodulatory effects

This strategy is mainly directed to anti-inflammatory effects as shown in Table 1 and Fig. (1), such as 1) the suppression of immune activation, particularly for activated HTLV-I-infected cells, 2) the inhibition of the transmigration of these cells to the spinal cord, 3) the reduction of chronic inflammation in the spinal cord, through the down-regulation of inflammatory cytokines and/or adhesion molecules expression, etc.. The regimens, of course, exhibit the effects also for the activated HTLV-I-non-infected cells, which are subsequently induced by the activation of HTLV-I-infected cells.

a) Corticosteroid hormone
As well known, this reagent has been shown to be a useful in various inflammatory or autoimmune diseases. Although high-doses of methylprednisolone are sometimes given intravenously, oral administration of prednisolone (PSL) is most popular treatment against HAM/TSP in Japan. Nakagawa et al. recommended that 1 to 2 mg/kg of PSL are given every day or every other day for 1 to 2 months and the dose of PSL, thereafter, is tapered off 5 to 10 mg every other day or is terminated at 6 to 12 months after commencement [14]. They mentioned that 107 (81.7%) of 131 HAM/TSP patients showed improvement of motor function in this therapy. On the other hand, there are some reports that corticosteroid therapy was non-efficacious or the effect of this treatment was transient [15, 16]. Therefore, the efficacy of this treatment for HAM/TSP patients is still controversial although this treatment might show the efficacy in a short-term trial. In addition, there are many adverse events, such as infection, osteoporosis, gastroduodenal ulcer, glucose intolerance, hypertension, and myopathy etc. in a long-term administration of corticosteroid. However, the fact that HTLV-I proviral load in the peripheral blood of HAM/TSP patients was significantly decreased in PSL treatment during 5 years might be noteworthy [17] although its mechanism is unclear.

b) Blood purification

There are two methods, such as plasmapheresis and lymphocytapheresis. We previously treated 18 HAM/TSP patients with plasmapheresis using AP-05H plasma separator or IM-T350 immunoabsorbent column (Asahi Medical Co, Tokyo, Japan) [18]. In 11 of 18 HAM/TSP patients (61.1%) motor, sensory, and/or shincteric disturbance
improved with plasmapheresis (4 to 6 sessions in 2 weeks). Although no adverse events were observed, the effects were transient as maintained only for 2 to 4 weeks. It is supposed that the efficacy of this treatment is based on the elimination of some humoral factors involved in the damage of nervous tissues, such as inflammatory cytokines. However, the exact mechanisms how plasmapheresis treatment induces clinical improvements in HAM/TSP patients are still unclear.

c) Pentoxifylline

Pentoxifylline (PTX) (3,7-dimethyl-1-(5-oxohexyl) xanthine), an inhibitor of phosphodiesterase, is a methylxanthine derivative used in the treatment of vascular diseases [19]. In addition to rheological effects, it is known that PTX has anti-inflammatory or immunomodulatory activity through the increase of intracellular cAMP, such as the suppression of inflammatory cytokines expression including tumor necrosis factor-\(\alpha\), interferon-\(\gamma\) and granulocyte-monocyte colony stimulating factor and the down-regulation of adhesion molecules expression [20, 21]. We previously treated 15 HAM/TSP patients by oral administration of 300 mg/day of PTX for 4 weeks [22]. In 13 of 15 patients, motor disability, especially spasticity, improved concomitant with the suppression of spontaneous peripheral blood lymphocyte (PBL) proliferation, which is one of the major immunological abnormalities observed in vitro in patients with HAM/TSP [23]. No adverse events were observed. The fact that the clinical efficacy was correlated with the elevation of serum Th2 cytokine levels in PTX treatment suggests that PTX has the potentials to regulate the balance of Th1/Th2 activity [24].
Thus, although the exact mechanisms with regard to the efficacy of PTX treatment is still obscure, the correction of the imbalance of Th1/Th2 activity in HAM/TSP patients [25] concomitant with the down-regulation of adhesion molecules, such as integrins expression, might be involved in the efficacy as a one of the mechanisms.

d) Heparin

The main regions in the pathological changes of HAM/TSP are the lower thoracic cord in the spinal cord as mentioned above [6]. These regions are anatomically watershed zones of the spinal cord [26], where the stagnant lymphocytes could easily transmigrate to the the tissues and evoke the immune reactions because of decreased blood flow. Indeed, the efficacies of heparin treatment based on the inhibition of lymphocytes trafficking to the tissues were also reported in another inflammatory diseases, such as multiple sclerosis and experimental autoimmune encephalomyelitis [27, 28]. Therefore, heparin was administered to HAM/TSP patients with the expectation of the clinical improvement by the inhibition of the transmigration of activated T cells to the lower thoracic cord in the spinal cord due to the improvement of the microcirculation. We treated 10 HAM/TSP patients by intravenous administration of 5000 - 10,000 units/day of heparin for 9 - 93 days [29]. In 7 patients, motor dysfunction improved substantially and the effect continued for more than a month after the discontinuation of therapy. No adverse events were observed. Most striking change of the immunological markers in the peripheral blood during treatment was the significant decrease of spontaneous PBL proliferation in vitro. Although the exact mechanisms of
this phenomenon are unclear, it is supposed that heparin can induce not only the
amelioration of the microcirculation but also the down-regulation of immunological
activations based on HTLV-I infection. Considering the efficacy of heparin treatment
together with PTX treatment, they seem to originate in the inhibition of the
transmigration of HTLV-I-infected cells to the spinal cord based on immunomodulatory
effects with rheological effects.

e) High-dose intravenous gammaglobulin

High-dose intravenous gammaglobulin (IVIG) is used in the treatment in various
inflammatory or immune-mediated diseases [30]. Kuroda et al. reported that 10 of 14
HAM/TSP patients had presented the improvement of motor disability within 7 days of
the commencement by administration of 10g/day or 400 mg/kg/day of gammaglobulin
for 5 consecutive day and the effects were sustained for more than 3 weeks in some
patients [31]. Although the exact mechanisms how IVIG exhibits the efficacy, even in
another immune-mediated diseases, are still unclear [30], they mentioned the possibility
of the suppression of perivascular inflammation from the fact that the clinical
improvement was preferentially observed in patients with high anti-HTLV-I antibodies
titer in cerebral spinal fluid (CSF), a high CSF IgG level and a severe white-matter
lesion on brain MRI.

f) Intermittent high-dose vitamin C

Kataoka et al. reported the therapeutic efficacy of intermittent high-dose vitamin
C. They treated 7 HAM/TSP patients by oral administration of 35-40 mg/kg/day of vitamin C in following manner, 3 to 5 successive days followed by a 2-day withdrawal period, for a mean period of 9.7 months [32]. All of patients presented the improvement of motor function with the decrement of serum level of immunosuppressive acidic protein, suggesting the suppression of macrophage activation. However, the mechanisms how the down-regulation of activated macrophages are involved in the efficacy of this treatment are not unclear.

g) Fosfomycin and Erythromycin

Of 14 HAM/TSP patients treated with intravenous administration of 2 g/day of fosfomycin for 2 weeks followed by oral administration of 2 g/day of fosfomycin for 2 weeks, 11 patients showed the improvement of motor function [14]. Of 25 HAM/TSP patients treated with oral administration of 600 mg/day of erythromycin for 1 to 3 months, 12 patients showed moderate improvements in their motor function [33]. Since these regimens have not only anti-bacterial effects but also immunomodulatory functions [34, 35], the efficacy by these regimens is supposed to be based on the down-regulation of inflammatory cytokines or chemokines expression.

h) Fermented milk drink

*Lactobacillus casei* strain Shirota (LcS) is one of probiotic agents, which have immunomodulatory functions through the interactions with the gastrointestinal mucosal immune system [36]. Matsuzaki et al. reported that oral administration of $4 \times 10^{10}$
viable LcS, twice a day for 4 weeks induced the improvement of motor dysfunction, particularly decrease of spasticity, and of urinary symptoms in all of 10 HAM/TSP patients treated [37]. In addition, the increase of NK cell activity, which was generally decreased in HAM/TSP patients [38, 39], in the peripheral blood was observed in LcS treatment. However, there were no significant changes of not only lymphocytes surface markers but also HTLV-I proviral load in the peripheral blood in the course of LcS treatment. Although the mechanisms how the up-regulation of NK cell activity without the decrement of HTLV-I proviral load in the peripheral blood are involved in clinical improvement is unclear, the benefit which can use safely as the supplement during a long-term might be valuable for the treatment against HAM/TSP patients.

2) The therapies focusing on anti-viral effects

This strategy is mainly directed to anti-viral effects as shown in Table 1 and Fig. (1), such as 1) the suppression of HTLV-I expression and/or replication, 2) the inhibition of the proliferation of HTLV-I-infected cells, 3) the elimination of HTLV-I-infected cells.

a) Interferon-α and -β

Interferon (IFN)-α and IFN-β, which are type I IFNs, have a variety of biological actions including not only anti-viral effects but also cell growth regulation and modulation of the cellular immune response [40, 41, 42]. Therefore, treatment with these regimens might be suitable for HAM/TSP because it can target on the immunological dysregulation based on high HTLV-I proviral load in the peripheral
blood of HAM/TSP.

In various treatments against HAM/TSP, only IFN-α has been proved to be effective in a multicenter, randomized, double-blind, and controlled trial [43] and has been approved as the therapeutic agent against HAM/TSP by the Ministry of Health, Labor and Welfare in Japan. In controlled trial of IFN-α treatment against HAM/TSP as mentioned above, 48 HAM/TSP patients were divided to three groups treated with 0.3 million international units (MU) of natural IFN-α (human lymphoblastoid interferon (HLBI, Sumiferon) (Sumitomo Pharmaceutical Co., Osaka, Japan), 1.0 MU, and 3.0 MU by intramuscular injection, respectively, daily for 4 weeks. In about 70 % of HAM/TSP patients treated with 3.0 MU, motor dysfunction, even urinary disturbances in some cases, improved in significant therapeutic response and its effectiveness continued for 4 weeks after completion of therapy without serious adverse effects. The therapeutic response in the 3.0-MU group was significantly higher than in the 0.3-MU group. We, previously, had also demonstrated a similar efficacy of HLBI treatment against 17 HAM/TSP patients in open trial [44]. In this trial, most striking change of the immunological markers in the peripheral blood was the significant decrease of spontaneous PBL proliferation \textit{in vitro} leading to the recovery of the response to lectin, such as phytohemoagglutinin. Although spontaneous PBL proliferation is one of the major immunological abnormalities observed \textit{in vitro} in patients with HAM/TSP as mentioned above [23], the exact mechanisms of it are still unclear. However, this phenomenon is thought to consist of the proliferation of HTLV-I-infected CD4$^+$ T cells and the expansion of HTLV-I specific CD8$^+$ CTL against virus-expressing cells
concomitant with the involvement of the aberrant signalings of both Interleukin-2 (IL-2) and IL-15 [45, 46]. It was reported that HTLV-I proviral load and HTLV-I tax mRNA expression correlate the frequency of HTLV-I tax specific CD8+ CTL in the peripheral blood of HAM/TSP patients [47, 48]. Therefore, IFN-α treatment might induce the reduction of HTLV-I proviral load or HTLV-I tax mRNA expression in the peripheral blood of HAM/TSP patients. Indeed, Saito et al. recently, reported that HTLV-I proviral loads in the peripheral blood were significantly decreased, concomitant with the reduction of memory T cells in CD8^{high+} T cells, after IFN-α treatment [49]. In addition, CXCR3+ T cells (Th1 cells) were also significantly decreased by this treatment. Thus, IFN-α treatment seems to also induce the correction of Th1/Th2 imbalance, which deviates toward Th1 in HAM/TSP [25]. Indeed, another reports also demonstrated that both the percentage of CCR5+ cells (Th1 cells) in CD4+ T cells and the ratio of intracellular IFN-γ+/IL-4+ T cell in the peripheral blood were significantly decreased by IFN-α treatment [50].

On the other hand, the efficacy of IFN-β, which is another type I IFN, treatment against HAM/TSP was also reported [51]. Twelve patients with HAM/TSP were treated with escalating doses of IFN-β1a, which has already been approved as the treatment against multiple sclerosis [52], over the course of a relatively long-term, 28 weeks. This treatment induced the improvement of motor dysfunction with the reduction of both HTLV-I tax mRNA load and the frequency of HTLV-I-specific CD8+ CTL in the peripheral blood. Although up-regulated expression of HTLV-I tax itself in HTLV-I-infected cells might be one of important factors for the development of
HAM/TSP [48, 53], IFN-β1a treatment might be able to target this point. In addition, the reduction of spontaneous PBL proliferation was also observed as same as it in IFN-α treatment. However, HTLV-I proviral load in the peripheral blood remained unchanged. With regard to the change of HTLV-I proviral load, the reasons of the discrepancy between IFN-α and IFN-β1a treatment are unclear. However, although IFN-α treatment as mentioned above induced the significant reduction of HTLV-I proviral load in total study population, HTLV-I proviral load was rather increased in about 30% out of total study population [49], suggesting that anti-viral effects of IFN-α are different among each individual.

High HTLV-I proviral load in the peripheral blood is the most important prerequisite in the development of HAM/TSP [9, 10]. At present time, the increased proliferation of HTLV-I-infected cells is thought to play an important role mainly in the maintenance of high HTLV-I proviral load in the peripheral blood [12, 54, 55]. Either IFN-α or IFN-β treatment does not seem to target this point. However, it is certain that these regimens have somewhat anti-viral activity although its mechanism is obscure. In addition, these regimens also have the activities to correct various immunological dysregulations, such as the imbalance of Th1/Th2 status, in the peripheral blood of HAM/TSP patients. Therefore, these treatments have considerable benefits on therapeutic strategies for HAM/TSP. However, whether these treatments are tolerable as a long-term or lifelong treatment or not is uncertain.

b) Reverse transcriptase inhibitors
Some nucleoside analogues have been shown to block HTLV-I replication by inhibition of reverse transcriptase (RT). Zidovudine (azidothymidine, AZT), which is the thymidine analogue, can inhibit HTLV-I replication \textit{in vitro} although its inhibitory dose for HTLV-I is higher than for human immunodeficiency virus [56]. Zidovudine treatment against 5 HAM/TSP patients during 6 months did not induce the clinical benefits at a first study [57]. However, zidovudine, with higher dose than it in a first study, treatment against 10 HAM/TSP patients for 24 weeks induced the clinical benefits in some patients [58]. However, these studies did not refer to the change of HTLV-I proviral load in each treatment. On the other hand, the clinical trial against HAM/TSP patients with lamivudine, which is the cytosine analogue, was also reported [59]. Although lamivudine treatment against 5 HAM/TSP patients during about 10 months did not induce any symptomatic improvements except one patient, who is a case with recent-onset HAM/TSP, the significant reduction of HTLV-I proviral load in the peripheral blood was observed in all 5 HAM/TSP patients. In addition, the reduction of viral load was associated with the decrease of the frequency of HTLV-I tax specific CTL in one patient who had the clinical efficacy. Thus, RT inhibitors seemed to have some clinical benefits with HTLV-I targeting in the treatment against HAM/TSP.

However, unfortunately, the result of recent clinical trial with combination therapy by zidovudine and lamivudine in a randomized, double blind, placebo controlled study made RT inhibitors pessimistic for the regimen for HTLV-I targeting as the treatment against HAM/TSP [60]. Same group, which reported the efficacy of lamivudine treatment against HAM/TSP, has conducted a controlled study of 6 months
by combination therapy with these two RT inhibitors for 16 HAM/TSP patients. As far as they compared the clinical effects including motor disability score, gait, and bladder function, etc., and the changes of laboratory markers in the peripheral blood including HTLV-I proviral load and T cell subpopulation between each group treated by combined therapy or placebo therapy, no significant changes were seen between two arms although the treatment was well tolerated with no unexpected side effects. This finding strongly suggests that both RT inhibitors have no activities to reduce HTLV-I proviral load, at least, in vivo in HAM/TSP patients. In addition, the reasons of the discrepancy of the results between two studies conducted by same group are unclear. However, if the increased proliferation of HTLV-I-infected cells, rather than new infection through cell-to-cell spread, plays an important role mainly in the maintenance of high HTLV-I proviral load in the peripheral blood of HAM/TSP patients [12, 54, 55], the inefficacy of the treatment with RT inhibitors might be reasonable.

c) Humanized anti-Tac

It is well known that interleukin-2 (IL-2) and IL-2 receptor α (IL-2Rα) are induced by HTLV-I tax transactivation in HTLV-I-infected cells [61, 62]. This dysregulation of cellular genes expression by HTLV-I tax initiates a process of T cell activation and proliferation by autocrine or paracrine loop. Therefore, the blockade of IL-2/IL-2Rα system might lead to the direction toward the decrease of HTLV-I-infected cells in vivo through apoptosis of HTLV-I-infected cells by IL-2 deprivation. The efficacies of humanized anti-Tac antibody (daclizumab), which is the humanized form
of monoclonal antibody against IL-2Rα and blocks the interaction of IL-2 with IL-2Rα, treatments were demonstrated in several immune-mediated like diseases, such as renal allograft rejection, noninfectious uveitis, multiple sclerosis, pure red cell aplasia, aplastic anemia, and psoriasis, and T-cell malignancy [63].

Nine patients with HAM/TSP were treated with administration of five doses (1 mg/kg) of humanized anti-Tac antibody at weeks 0, 2, 6, 10, 14 [64]. This treatment induced mild improvement of motor disability score in only 3 HAM/TSP patients without serious adverse effects. On the other hand, immunological studies, as expected, revealed a selective down-regulation in the number of circulating activated T cells expressing IL-2Rα receptor and a decrease of spontaneous PBL proliferation ex vivo. Furthermore, as most striking finding, HTLV-I proviral load in the peripheral blood was reduced an average of 52 % after this treatment. These findings suggest that humanized anti-Tac treatment have the potential to selectively remove HTLV-I-infected cells expressing IL-2Rα from the peripheral blood of HAM/TSP patients.

d) Histone deacetylase enzyme inhibitor

Histone deacetylase enzyme (HDAC) inhibitor has lately attracted considerable attention as the therapeutic regimens against various diseases such as malignancies, and neurodegenerative diseases etc. [65, 66]. Although acetylated histones are associated with transcriptionally active chromatin and deacetylated histones with inactive chromatin, chromatin acetylation is regulated by the balance between histone acetyltransferases and histone deacetylases (HDACs) as epigenetic control under physiological conditions.
Histone acetylation plays an important role also in the regulation of HTLV-I gene expression [67, 68]. Therefore, Inhibition of HDACs activities leads into histone hyperacetylation followed by increases in HTLV-I gene expression.

As mentioned above, the relationship between HTLV-I proviral load and/or expression and the host immune system, such as HTLV-I specific CTL, is at equilibrium in the peripheral blood [12]. Therefore, if HTLV-I proviral load is increased based on up-regulation of HTLV-I expression, e.g. in cells infected with latent or silent form, HTLV-specific CTL are more activated and number of HTLV-I-infected cells might be reduced in the peripheral blood. That is, the tilting of host-pathogen balance might lead to the elimination of HTLV-I-infected cells from HAM/TSP patients. Based on this new concept such as “gene activation therapy”, very recently, clinical trial by oral administration of 20 mg/kg/day valproate (VPA), which is one of HDAC inhibitors, during 3 months was performed in 16 HAM/TSP patients [69]. Although HTLV-I proviral loads in the peripheral blood were transiently increased in early stages after administration as expected, they significantly decreased in all patients by 2.3- to 89.3-fold (mean; 24-fold) at the end point. Although authors did not describe the changes of clinical status in detail, they mentioned that VPA treatment induced the reduction of spasticity in all patients. This result is very intriguing because there are no reports such a significant drop of HTLV-I proviral load in the treatments against HAM/TSP until now.

However, there is one report that HDAC inhibitors including VPA, sodium butyrate, and trichostatin A, unexpectedly, decrease the activity of HTLV-I specific CTL
against the increased HTLV-I expression in HTLV-I-infected cell ex vivo [70]. This finding suggests that HDAC inhibitors reduce the efficiency of CTL surveillance of HTLV-I and is contrary to the concept leading to the treatment with HDAC inhibitors as mentioned above. Indeed, VPA induced apoptosis in not only CD4+ but also CD8+ cells at relatively high frequency in short-term in vitro treatment [69]. Then, authors recommend caution in the use of HDAC inhibitors in nonmalignant cases of HTLV-I infection such as HAM/TSP.

Overall, anyway, VPA is the anti-epileptic drug which has the good safety profiles as long-term therapy and is easily available. Since this drug might be expected as one of new anti-HTLV-I agents, we now need to perform the case controlled study by VPA treatment against HAM/TSP patients.

3) New therapeutic approach focusing on anti-viral effect

As another therapeutic strategy for the elimination of HTLV-I-infected cells, we can focus on the targeting of HTLV-I-infected cells themselves from the peripheral blood. If HTLV-I-infected cells is selectively removed, for example-by apoptosis, from the peripheral blood, its strategy must become one of the ideal therapeutic tools against HAM/TSP patients. With this regard, the strategy requires the regimen, which is well tolerated, additionally inexpensive, even in long-term treatment. 

Allicin (diallyl thiosulfinate), which is one of natural organosulfur compounds derived from garlic (Allium sativum), has diverse biological activities, such as anticarcinogenic activity, antibacterial activity, antifungal activity, etc. [71, 72].
Although the exact mechanisms how cytotoxic effects described above are induced by organosulfur compounds, such as allicin, are still obscure, a disulfide moiety in their structures seems to have an important role for the trigger of cell death [73]. 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), etc., might be involved in cytotoxic effects [71]. However, allicin is very unstable compound as reported that this compound rapidly disappears after the injection into the blood [74, 75]. Therefore, it is difficult to use this compound in the therapeutic trial against HAM/TSP patients. Prosultiamine (\(N\)-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-\(N\)-[4-hydroxy-1-methyl-2-(propyldithio)-1-butenyl]-formamide) (Alinamin®), which is the product of Takeda Pharma. Co. Inc., Osaka, Japan, is a homologue of allithiamine originally synthesized by thiol type vitamin B1 and allicin (Fig. (2)) [76]. For the 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 to propyl disulfide in the structure of allithiamine (Fig. (2)) [77]. Thus, Prosultiamine has a disulfide moiety in its structure as same as allicin (Fig. (2)). Therefore, it is expected that prosultiamine has the same activity as allicin. Importantly, Prosultiamine is pharmacologically stable and is very frequently available as the regimen of vitamin B1 deficiency with the safety in Japan. Therefore, this drug has the potential to be able to immediately conduct the clinical trial against HAM/TSP patients. We showed the structure of allicin and the generation of prosultiamine (Fig. (2)).
a) **Prosultiamine has the cytotoxic activity against HTLV-I-infected T cell lines derived from HAM/TSP patients as same as allicin.**

It is revealed, by MTS assay (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) nonradioactive cell proliferation assay), that cell viability of two HTLV-I-infected T cell lines derived from HAM/TSP patients (HCT-1 and HCT-4) decreased by allicin treatment in dose-dependent manner (Fig. (3a)). Prosultiamine (kindly provided by Takeda Pharma Co. Inc., Osaka, Japan) treatment against these cell lines also caused the similar effect to allicin treatment as expected (Fig. (3b)). As shown in Fig. (3ab), HTLV-I-infected T cell lines were more sensitive for the treatment with each compound than HTLV-I-non-infected T cell line, Jurkat cell line. These results suggest that the cytotoxic effect against HTLV-I-infected T cell lines is based on the disulfide moiety as the common structure in these compounds (Fig. (2)).

b) **The cytotoxic effect of prosultiamine is based on caspase-dependent apoptosis.**

As shown in Fig. (4a), prosultiamine treatment against HCT-1 induced the loss of mitochondrial membrane potential with the appearance of annexin V-positive cells, suggesting that the cytotoxicity by prosultiamine was based on the cell death induced by apoptosis through the mitochondrial pathway. Indeed, as shown in Fig. (4b), the loss of mitochondrial membrane potential was recovered in z-VAD-fmk, which is pan-caspase inhibitor, -pretreated HCT-1. In addition, immunoblot analysis revealed that the treatment with prosultiamine resulted in the proteolytic cleavage of caspase 3, which is the effector molecule in the final process of apoptosis (Fig. (4c)). Overall, these results
suggested that prosultiamine can induce caspase-dependent apoptosis through the mitochondrial pathway for HTLV-I-infected T cells.

c) **Involvement of activation of apoptosis signal-regulating kinase (ASK) 1 signaling in the induction of caspase-dependent apoptosis in prosultiamine-treated HTLV-I-infected cells.**

As mentioned above, Trx plays an important role in the cellular reducing system, interacting with GSH [78]. Although Trx is ubiquitously expressed in many cell types of mammalians [79], human Trx is a homologue of adult T cell leukemia-derived factor (ADF), which was originally defined as an IL-2 receptor a-chain inducer produced by HTLV-I-transformed T cells [80, 81]. Trx is a small protein (12 kDa) with two redox-active cysteine residues in an active center (-Cys-Gly-Pro-Cys-) and operates together with NADPH and Trx reductase as a protein disulfide-reducing system [79, 82]. Apoptosis signal-regulating kinase (ASK) 1 is a mitogen-activated protein (MAP) kinase kinase kinase, which is located in the upstream of p38 MAP kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) leading to cytokine- and stress-induced apoptosis [83, 84]. Trx is a redox-sensitive physiological inhibitor of ASK1 activity [85]. Reduced form of Trx binds to ASK1 and inhibits kinase activity of ASK1. However, upon the change to oxidized form of Trx such as formation of disulfides through the oxidation of cysteine residues, ASK1 dissociates from Trx and a free ASK1 is autophosphorylated in Threonine residue, subsequently activates the cascade of apoptosis signaling [86]. Therefore, prosultiamine might have the potentials to oxidize Trx in the interaction between Trx/Trx reductase system and a disulfide moiety and induce ASK1 activation.
leading to the induction of apoptosis of HTLV-I-infected cells. We showed the hypothetical pathway in allicin or prosultiamine-induced apoptosis of HTLV-I-infected cells (Fig. (5)).

As shown in Fig. (6), immunoblot analysis revealed that ASK1 is activated within a short period after prosultiamine treatment in HCT-1. It is reported that activation of p38 MAPK, which is located in the downstream of ASK1 signaling, connects to the mitochondrial apoptotic pathway through caspase 8 [87]. Therefore, as mentioned above, the fact that the loss of mitochondrial membrane potential was recovered in z-VAD-fmk-pretreated HCT-1 suggests that the activation of ASK1 signaling is involved in apoptosis of HTLV-I-infected cells by the treatment with prosultiamine.

d) The decrease of numbers of HTLV-I provirus in the peripheral blood CD4\(^+\) T cells of HAM/TSP patients by the \textit{in vitro} treatment with prosultiamine and clinical trial with prosultiamine against HAM/TSP patients.

In next, we studied whether prosultiamine \textit{in vitro} treatment can selectively target HTLV-I-infected cells of peripheral blood CD4\(^+\) T cells of HAM/TSP patients or not. After the peripheral blood CD4\(^+\) T cells of HAM/TSP patients were cultured in the presence of 5 \(\mu\)M prosultiamine or vehicle for 48 hr, total cellular DNA samples prepared from the viable cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analysis. As shown in Fig. (7a), prosultiamine \textit{in vitro} treatment against the peripheral blood CD4\(^+\) T cells of 7 HAM/TSP patients induced the decrease of numbers of HTLV-I proviral copies, ranged from 29.9 - 80.2% (mean; 60.7%), compared with vehicle treatment. As far as we evaluated the cell viability by
MTS assay, prosultiamine treatment did not affect the viability of total peripheral blood CD4\(^+\) T cells. These data suggest that prosultiamine \textit{in vitro} treatment can selectively induce apoptosis of HTLV-I-infected cells of the peripheral blood CD4\(^+\) T cells of HAM/TSP patients.

These evidences prompted us to the treatment with prosultiamine against HAM/TSP patients because this agent is frequently and easily available as the regimen of vitamin B1 deficiency with the safety in Japan. We treated 5 HAM/TSP patients with intravenous administration of prosultiamine at the dosages of 40 mg daily for 14 days. We showed the profiles of the patients in Table 3. As shown in Table 3, although motor disability grade was not changed among 4 of 5 HAM/TSP patients (case 1-4), some clinical improvements, such as the reduction of spasticity etc., were individually observed. On the other hand, case 5, who had the short duration of illness, showed marked improvement of motor function such as the change of motor disability grade (Table 3). No adverse events were observed. As far as we monitored the copy numbers of HTLV-I provirus in the peripheral blood mononuclear cells before treatment (day 0), during treatment (day 7 and 14), and 7 days after treatment (day 21), copy numbers of HTLV-I provirus decreased to 33-55 % of them at day 0 at nadir in all HAM/TSP patients (Fig. (7b)).

Overall, our results indicated that prosultiamine treatment against HAM/TSP patients have the potential to be able to induce the clinical improvement based on the decrease of HTLV-I-infected cells by apoptosis in the peripheral blood, suggesting that prosultiamine can work as the new anti-viral agent against HTLV-I.
Conclusion

Since the discovery of HAM/TSP, over 20 years have passed. During that period, numerous findings have been presented in the research field of HAM/TSP. Unfortunately, these findings have not translated into an optimal therapeutic strategy against HAM/TSP. Although the pathophysiology of HAM/TSP is, in a word, a chronic inflammatory status triggered by HTLV-I infection, we should treat HAM/TSP as one of the infectious diseases because HTLV-I-infected cells are the first responders in the development of HAM/TSP. Therefore, the therapeutic strategy that manages to decrease or eliminate HTLV-I-infected cells seems to be critical in considering the ideal treatment for HAM/TSP. With this regard, either VPA or prosultiamine might function as a new anti-viral agent against HTLV-I. However, the trial for the targeting of HTLV-I-infected cells toward the depletion of HTLV-I, as a new therapeutic strategy against HAM/TSP, has just opened now.

Acknowledgements

This work was supported in part by the Grant-in-Aid for Research on Brain Science and the Health and Labour Sciences Research Grant on Intractable Diseases (Neuroimmunological Diseases) from the Ministry of Health, Labour and Welfare of Japan.

References


[9] Jeffery, K.J.; Usuku, K.; Hall, S.E.; Matsumoto, W.; Taylor, G.P.; Procter, J.; Bunce,
Kodama, D.; Izumo, S.; Osame, M.; Bangham, C.R. HLA alleles determine human
T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated

Hashiguchi, S.; Ichinose, M.; Bangham, C.RM.; Izumo, S.; Osame, M. Analysis of
HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I
carriers: high proviral load strongly predisposes to HAM/TSP. J. Neurovirol., 1998, 4,
586-593.


[12] Bangham, C.RM; Osame, M. Cellular immune response to HTLV-I. Oncogene,
2005, 24, 6035-6046.

43-84.

[14] Nakagawa, M.; Nakahara, K.; Maruyama, Y.; Kawabata, M.; Higuchi, I.; Kubota,
H.; Izumo, S.; Arimura, K.; Osame, M. Therapeutic trials in 200 patients with
HTLV-I-associated myelopathy/tropical spastic paraparesis. J. Neurovirol., 1996, 2,
345-355.

HTLV-I-associated myelopathy/tropical spastic paraparesis: MRI analysis and a 2 year


[74] Lawson, L.D.; Wang, Z.J. Pre-hepatic fate of the organosulfur compounds derived


[83] Ichijo, H.; Nishida, E.; Irie, K.; Ten Dijke, P.; Saitoh, M.; Moriguchi, T.; Takagi,


**Figure legends**

**Fig. 1.** The immunopathogenesis of and the therapeutic strategies in HAM/TSP. The bystander mechanism, such as the destruction of the surrounding tissues by inflammatory cytokines expression, etc., during the interaction between HTLV-I-infected CD4\(^+\) T cells and HTLV-I-specific CD8\(^+\) CTL, plays an important role as the cause of chronic myelitis in the spinal cord. The therapeutic strategies in HAM/TSP are divided to two ways of the direction as shown by \( \Longrightarrow \); 1) immunomodulation therapy, such as a) the suppression of immune activation, particularly for activated HTLV-I-infected cells, b) the inhibition of the transmigration of activated HTLV-I-infected cells to the spinal cord, c) the reduction of chronic inflammation in the spinal cord  2) anti-viral therapy, such as a) the suppression of HTLV-I expression and/or replication, b) the inhibition of the proliferation of HTLV-I-infected cells, c) the elimination of HTLV-I-infected cells.

**Fig. 2.** The structure of allicin (a) and the generation of prosultiamine (b). Allithiamine was originally synthesized by thiol type vitamin B\(1\) and allicin. Prosultiamine was developed after allyl disulfide derived from allicin was substituted to propyl disulfide in the structure of allithiamine as shown by \( \square \). Either prosultiamine or allicin have a disulfide moiety in its structure as shown by \( \bigcirc \bigcirc \bigcirc \).

**Fig. 3.** The cytotoxic effect of allicin or prosultiamine against HTLV-I-infected T cell lines derived from HAM/TSP patients (HCT-1 and HCT-4) or non-infected T
cell line (Jurkat). a) Cell viability of all cell lines decreased by the treatment with allicin for 24 hr in dose-dependent manner. b) Prosultiamine treatment against these cell lines also caused the similar effect to allicin treatment. Both HCT-1 and HCT-4 were more sensitive for the treatment with each compound than Jurkat cell line.

HCT-1; —■— , HCT-4; —□— , Jurkat; —▲— . For cell viability assay, MTS assay was performed. Cell viability was determined as follows: after each OD titer at wavelength of 490 nm in triplicate cultures in the presence of allicin, or prosultiamine or vehicle / mean of OD titer at wavelength of 490 nm in triplicate cultures under medium alone was calculated, its mean ± SD was presented as the cell viability.

**Fig. 4.** The cytotoxic effect of prosultiamine is based on caspase-dependent apoptosis.

a) The loss of mitochondrial membrane potential and the frequency of annexin V-positive cells in prosultiamine-treated HCT-1. After HCT-1 was cultured for 24 hr in the presence of 40 μM prosultiamine or vehicle, these cells was analyzed by staining the cells with the potential sensitive fluorescent dye DiOC₆(3) or FITC-conjugated annexin V to evaluate ΔΨₘ or the frequency of apoptotic cells, respectively. Either the loss of ΔΨₘ or the frequency of apoptotic cells was measured by flow cytometry. b) The recovery of the loss of mitochondrial membrane potential in prosultiamine treatment against HCT-1 pretreated with z-VAD-fmk. Before the treatment with 40 μM prosultiamine for 24 hr, HCT-1 was pretreated by 200 μM z-VAD-fmk for 1 hr. c) Immunoblot analysis of 40 μM prosultiamine-treated HCT-1 for the proteolytic cleavage of caspase 3. After HCT-1 was cultured for 1, 3, or 5 hr in the presence of 40
µM prosultiamine, the cells were collected and lysed for immunoblot analysis. The

treatment of HCT-1 with prosultiamine resulted in the proteolytic cleavage of caspase 3.

**Fig. 5.** Hypothetical pathway in allicin or prosultiamine-induced apoptosis of
HTLV-I-infected cells. Trx has two redox-active cysteine residues in an active center
(-Cys-Gly-Pro-Cys-) and operates together with NADPH and Trx reductase as a protein
disulfide-reducing system. Trx is a redox-sensitive physiological inhibitor of ASK1
activity. Reduced form of Trx binds to ASK1 and inhibits kinase activity of ASK1.
Upon the change to oxidized form of Trx such as formation of disulfides through the
oxidation of cysteine residues, ASK1 dissociates from Trx and a free ASK1 is
autophosphorylated in Threonine residue, subsequently activates the cascade of
apoptosis signaling through p38 MAPK and JNK activation. Both allicin and
prosultiamine might have the potentials to oxidize Trx in the interaction between
Trx/Trx reductase system and a disulfide moiety in their structures and induce ASK1
activation leading to the induction of apoptosis of HTLV-I-infected cells through
mitochondrial pathway. Trx; Thioredoxin, ASK1; Apoptosis signal-regulating kinase1,
p38 MAPK/JNK; p38 mitogen-activated protein kinase/c-Jun N-terminal kinase.

**Fig. 6.** Immunoblot analysis of 40 µM prosultiamine-treated HCT-1 for ASK1
activation. After HCT-1 was cultured for 1, 5, 15, or 30 min in the presence of 40 µM
prosultiamine, the cells were collected and lysed for immunoblot analysis. The
treatment of HCT-1 with prosultiamine resulted in phosphorylation of ASK1. P-ASK1;
phosphorylated ASK1.

**Fig. 7.** The effect of *in vitro* and *in vivo* treatment with prosultiamine for the reduction of HTLV-I-infected cells. a) The decrease of numbers of HTLV-I provirus in peripheral blood CD4⁺ T cells of HAM/TSP patients by the *in vitro* treatment with prosultiamine. The peripheral blood CD4⁺ T cells of HAM/TSP patients were cultured in the presence of 5 µM prosultiamine or vehicle for 48 hr. After the dead cells induced by prosultiamine *in vitro* treatment were removed using annexin V microbead 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 the decrease of HTLV-I proviral copies, ranged from 29.9 - 80.2% (mean; 60.7%) (*p* < 0.05), compared with vehicle treatment. b) The decrease of numbers of HTLV-I provirus in the peripheral blood mononuclear cells of HAM/TSP patients by the *in vivo* treatment with prosultiamine. We treated 5 HAM/TSP patients with intravenous administration of prosultiamine at the dosages of 40 mg daily for 14 days. Total cellular DNA samples prepared from the peripheral blood mononuclear cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analysis. As far as we monitored the copy numbers of HTLV-I provirus in the peripheral blood before treatment (day 0) (Pre), during treatment (day 7 and 14), and 7 days after treatment (day 21), copy numbers of HTLV-I provirus decreased to 33-55 % of them at day 0 at nadir in all HAM/TSP patients.

**The list of abbreviations:**
HTLV-I; human T lymphotropic virus type I

HAM/TSP; HTLV-I-associated myelopathy/tropical spastic paraparesis

CTL; cytotoxic T cells

PSL; predonisolone

PTX; pentoxifylline

IFN-α and -β; interferon-α and -β

RT inhibitor; reverse transcriptase inhibitor

IL-2; interleukin-2

HDAC inhibitor; histone deacetylase enzyme inhibitor

VPA; valproate

Trx; thioredoxin

Trx reductase; thioredoxin reductase

GSH; glutathione

MTS assay; (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-
[4-sulfophenyl]-2H tetrazolium) nonradioactive cell proliferation assay

ASK1; apoptosis signal-regulating kinase 1
Table 1. Therapeutic strategies in HAM/TSP

1) The therapies focusing on immunomodulatory effects:
   Mainly directed to anti-inflammatory effects;
   a) the suppression of immune activation, particularly for activated HTLV-I-infected cells
   b) the inhibition of the transmigration of activated HTLV-I-infected cells to the spinal cord
   c) the reduction of chronic inflammation in the spinal cord

2) The therapies focusing on anti-viral effects:
   a) the suppression of HTLV-I expression and/or replication
   b) the inhibition of the proliferation of HTLV-I-infected cells
   c) the elimination of HTLV-I-infected cells
Table 2. Therapeutic trials against HAM/TSP patients

<table>
<thead>
<tr>
<th>1) The therapies focusing on immunomodulatory effects:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Corticosteroid hormone</td>
<td>[14], [17]</td>
</tr>
<tr>
<td>b) Blood purification</td>
<td>[18]</td>
</tr>
<tr>
<td>c) Pentoxifylline</td>
<td>[21], [22], [24]</td>
</tr>
<tr>
<td>d) Heparin</td>
<td>[29]</td>
</tr>
<tr>
<td>e) High dose-intravenous gammaglobulin</td>
<td>[31]</td>
</tr>
<tr>
<td>f) Intermittent high-dose vitamin C</td>
<td>[32]</td>
</tr>
<tr>
<td>g) Fosfomycin and Erythromycin</td>
<td>[14], [33], [34]</td>
</tr>
<tr>
<td>h) Fermented milk drink</td>
<td>[37]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2) The therapies focusing on anti-viral effects:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Interferon-α and -β</td>
<td>[43], [44], [49], [51]</td>
</tr>
<tr>
<td>b) Reverse transcriptase inhibitors</td>
<td>[57], [58], [59], [60]</td>
</tr>
<tr>
<td>c) Humanized anti-Tac</td>
<td>[64]</td>
</tr>
<tr>
<td>d) Histone deacetylase enzyme inhibitor</td>
<td>[69], [70]</td>
</tr>
</tbody>
</table>


Table 3. The profiles of patients and the clinical efficacy of prosultiamine treatment

<table>
<thead>
<tr>
<th>Case</th>
<th>Age / Gender</th>
<th>Duration of illness (years)</th>
<th>The changes of motor disability score a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>1</td>
<td>58 / female</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>51 / male</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>52 / male</td>
<td>46</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>73 / female</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>53 / female</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

a) Motor disability score was rated from 0 to 10 according to the scale of reference [18].
a) Allicin

\[ \text{Thiol type vitamin B1} \]

\[ \text{Allicin} \]

b) Prosultiamine

\[ \text{Allithiamine} \]

\[ \text{Substitution} \]

\[ \text{Prosultiamine} \]
Fig. 3

a) Allicin

b) Prosultiamine
Fig. 4

1) Loss of mitochondrial membrane potential

a.

<table>
<thead>
<tr>
<th></th>
<th>FL1 Log</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td>5.5%</td>
</tr>
<tr>
<td><strong>Prosultiamine</strong></td>
<td>95.8%</td>
<td></td>
</tr>
</tbody>
</table>

2) Frequency of annexin V-positive cells

a.

<table>
<thead>
<tr>
<th></th>
<th>FL1 Log</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td>23.9%</td>
</tr>
<tr>
<td><strong>Prosultiamine</strong></td>
<td>97.2%</td>
<td></td>
</tr>
</tbody>
</table>

b.

<table>
<thead>
<tr>
<th></th>
<th>FL1 Log</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>z-VAD-fmk</strong></td>
<td>12.6%</td>
<td></td>
</tr>
<tr>
<td><strong>Prosultiamine</strong></td>
<td>90.1%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FL1 Log</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>z-VAD-fmk</strong></td>
<td>5.4%</td>
<td></td>
</tr>
<tr>
<td><strong>Prosultiamine</strong></td>
<td>4.7%</td>
<td></td>
</tr>
</tbody>
</table>

c.

- **Vehicle**
- **Prosultiamine**

- caspase 3
- cleaved caspase 3

CNSA-MC-166870, Nakamura, et al.
Fig. 5

NADPH → Trx → ASK1 activation → p38MAPK/JNK ↑ → Caspase 3 ↑ → Apoptosis

Trx reductase → NADP+ → Trx → S-S → ASK1 → p38MAPK/JNK ↑ → Caspase 3 ↑ → Apoptosis

Allicin or Prosultiamine → Trx → S-S → Mitochondria

CNSA-MC-166870, Nakamura, et al.
Fig. 6

Vehicle

Prosultiamine

P-ASK1

ASK1

1  5  15  30  1  5  15  30  min
Fig. 7

a) [Graph showing copies/10000 cells for Vehicle and Prosaliramine with p < 0.05]

b) [Graph showing copies/10000 cells for Pre and Nadir with cases 1 to 5]