

# Efficiency of AUY922 in mice with adult T-cell leukemia/lymphoma

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**Abstract.** Adult T-cell leukemia/lymphoma (ATLL) is an aggressive malignancy caused by human T-cell leukemia virus type 1 (HTLV-1). ATLL is associated with poor prognosis mainly due to resistance to chemotherapy, which highlights the requirement for alternative therapies. The chaperone heat shock protein (HSP) 90 assist proteins involved in the onset and progression of ATLL. In the present study, the efficacy of a second generation HSP90 inhibitor termed AUY922 was investigated in ATLL. *In vitro*, AUY922 induced marked inhibition of cell viability in the HTLV-1-infected T-cell lines HUT-102 and MT-4. In immunodeficient mice bearing HUT-102 xenotransplants, AUY922 markedly retarded tumor growth, compared with the control group. Apoptosis was evident in hematoxylin and eosin stained- and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling-labeled tissue sections from AUY922-treated mice. In addition, AUY922 significantly reduced the serum levels of the surrogate tumor markers soluble interleukin-2 receptor and soluble cluster of differentiation 30. Overall, the present results demonstrate that AUY922 has potent anti-ATLL activity, thus providing a rationale for continuing the clinical development of HSP90 inhibitors in clinical trials for the treatment of patients with ATLL.

## Introduction

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive lymphoid proliferative malignancy associated with the oncoretrovirus human T-cell leukemia virus type 1 (HTLV-1) (1-3). ATLL develops upon a long latency period following viral infection, and carries poor prognosis due to intrinsic resistance to chemotherapy and profound immunosuppression (4). Recent advances in the treatment of ATLL include the introduction of therapies that target C-C chemokine receptor 4, which is abundantly expressed on the majority of ATLL cells, and the use of allogeneic hematopoietic stem cell transplantation for aggressive ATLL (5). However, patients suffer relapse following the above treatments; thus, alternative or complementary therapies for the treatment of ATLL are required.

Heat shock protein (HSP) 90 is a molecular chaperone that enables the correct folding and function of a broad range of substrate proteins called clients (6). HSP90 client proteins participate in various ATLL processes, including oncogenic signal transduction [nuclear factor- $\kappa$ B (NF- $\kappa$ B), Janus kinase/signal transducer and activator of transcription and Akt], resistance to cell death (survivin), cell cycle progression [cyclin-dependent kinase (CDK)4 and CDK6] and promotion of cell invasion (matrix metalloproteinases) (7). These client proteins may be depleted through the ubiquitin proteasome pathway using HSP90 inhibitors. Since HSP90 interacts with a multitude of client proteins, it is assumed that inhibition of HSP90 could potentiate a greater antitumor effect than that of therapies based on individual protein targeting. In preclinical studies, HSP90 inhibition alone produced promising results in the treatment of ATLL (8-10). However, first generation HSP90 inhibitors demonstrated borderline efficacy in clinical trials (11,12). The borderline therapeutic effect of the first generation HSP90 inhibitors [which are known as geldanamycin analogs and include 17-allylamino-17-demethoxygeldanamycin and 17-(dimethylaminoethyl-amino)-17-demethoxygeldanamycin] is considered to be due to poor solubility and pharmacokinetics, hepatotoxicity, susceptibility to P-glycoprotein efflux and failure of metabolism by nicotinamide adenine dinucleotide phosphate dehydrogenase, quinone 1/deoxythymidine-diaphorase enzymes (13).

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AUY922 is a resorcylic isoxazole amide and a potent inhibitor of HSP90 (14). Since AUY922 is a non-geldanamycin analog, it does not exhibit the aforementioned limitations of the geldanamycin analogs (14), thus being a more promising agent for clinical testing. AUY922 has demonstrated antitumor activity against a variety of solid tumors in preclinical mouse models (14,15). Furthermore, ongoing phase I and II clinical trials are currently testing the effects of AUY922 in hematologic malignancies and solid tumors (7). The objective of the present study was to test the therapeutic efficacy of AUY922 in ATLL.

## Materials and methods

**Cell lines and inhibitor.** The HTLV-1-infected T-cell lines, HUT-102 (Fujisaki Cell Center, Hayashibara Biomedical Laboratories, Okayama, Japan) and MT-4 (provided by Professor Naoki Yamamoto, Tokyo Medical and Dental University, Tokyo, Japan), were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) medium containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (Nacalai Tesque, Inc., Kyoto, Japan). AUY922 was kindly provided by Novartis Institutes for BioMedical Research (Basel, Switzerland) for *in vivo* study and purchased from Shanghai Biochempartner Co., Ltd. (Shanghai, China) for *in vitro* study.

**Water-soluble tetrazolium (WST)-8 assay.** The effect of AUY922 on cell viability was assayed by WST-8 assay. Mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided a measure of cell viability. WST-8 assays were conducted according to the manufacturer's protocol (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly,  $1 \times 10^4$  HUT-102 and MT-4 cells/well were incubated in 96-well plates at 37°C for 24 h in the presence of different concentrations of AUY922. A total of 10  $\mu$ l WST-8/well was added, and cells were incubated for 4 h. Absorbance was measured at 450 nm using an iMark™ microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Xenograft tumor model.** Five-week-old female C.B-17/Icr-severe combined immune deficiency (SCID) mice were obtained from Kyudo, Co., Ltd. (Tosu, Japan). The mice were kept in specific pathogen-free conditions. Animal cages were maintained at a temperature of 24°C and a humidity of 60%, with a 12 h light/dark cycle. Mice were fed a standard rodent diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. To induce malignancy,  $1 \times 10^7$  HUT-102 cells suspended in 200  $\mu$ l sterile RPMI-1640 medium were inoculated subcutaneously into the postauricular region of the SCID mice, which were then divided randomly into four treatment groups (n=6/group). AUY922 was solubilized in water containing 5% glucose (Nacalai Tesque, Inc., Kyoto, Japan) and administered intraperitoneally every day for 27 days, beginning on the day subsequent to cell inoculation. The control group received vehicle (5% glucose solution) only, while the treated groups received AUY922 at doses of 12.5, 18 or 30 mg/kg. The highest dose of AUY922 (30 mg/kg) was administered for 5-6 days/week with 1-2 days rest, and the treatment was continued for 4 weeks, while the 12.5 and 18 mg/

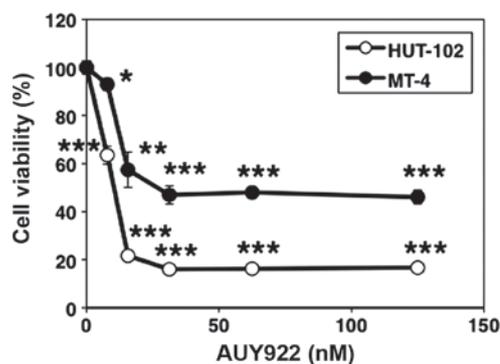


Figure 1. AUY922 reduces cell viability of human T-cell leukemia virus type 1-infected T-cell lines. Cell lines were incubated with or without increasing concentrations of AUY922 for 24 h. The viability of the cultured cells was determined by water-soluble tetrazolium-8 assay. A relative viability of 100% was considered as the total number of cells that survived following 24-h incubation in the absence of AUY922. \* $P < 0.05$ , \*\* $P < 0.001$  and \*\*\* $P < 0.0005$  vs. vehicle-treated control. The relative viability of the cultured cells is presented as the mean  $\pm$  standard deviation of triplicate cultures.

kg doses, were administered daily for 4 weeks. Tumor diameter was measured weekly with a shifting caliper, and tumor volume was calculated. Mice were weighed daily, beginning on day 4. All mice were sacrificed on day 28, before tumors were able to reach the ethically allowed maximal size. Subsequently, tumors were excised and their weight was measured. The present study was performed according to the Guidelines for Animal Experimentation of the University of the Ryukyus (Nishihara, Japan), and was approved by the Animal Care and Use Committee of the University of the Ryukyus.

**Morphological analysis of tumor tissues and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay.** Tumor specimens were collected from the control group and the 30 mg/kg AUY922-treated group, fixed in formalin solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), dehydrated through graded ethanol series (Japan Alcohol Trading Co., Ltd., Tokyo, Japan) and embedded in paraffin (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The paraffin-embedded specimens of ATLL tumors were stained with hematoxylin and eosin (H&E; Merck Millipore, Darmstadt, Germany). Cells were examined under a light microscope (Axioskop 2 Plus) with an Achroplan x40/0.65 lens (both from Carl Zeiss AG, Oberkochen, Germany). Images were captured with an AxioCam MRC camera and AxioVision 4.7 software (Carl Zeiss AG). Analysis of DNA fragmentation by TUNEL assay was performed using a commercial kit (Roche Applied Science, Penzberg, Germany).

**Biomarker analysis.** Serum concentrations of human soluble interleukin-2 receptor (sIL-2R; R&D Systems, Inc., Minneapolis, MN, USA) and human soluble cluster of differentiation 30 (sCD30; BioVendor Inc., Brno, Czech Republic) were measured by enzyme-linked immunosorbent assay, according to the manufacturer's protocol.

**Statistical analysis.** All values are expressed as the mean  $\pm$  standard deviation. Differences between groups and between treatments were tested for statistical significance by the

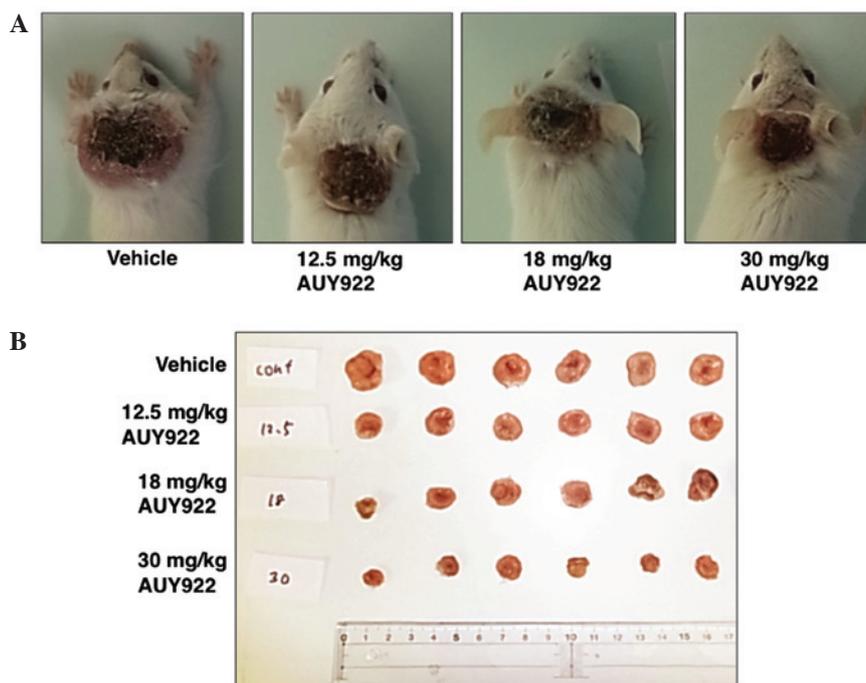


Figure 2. Effect of AUY922 on tumor cell growth. (A) Photographs of SCID mice at 4 weeks following subcutaneous inoculation of HUT-102 cells in the postauricular region. HUT-102 cells were inoculated subcutaneously in female SCID mice, and drug treatment commenced the following day (n=6/group). AUY922 was administered intraperitoneally at 12.5 or 18 mg/kg/day for 4 weeks, or at 30 mg/kg for 5-6 days/week for 4 weeks. (B) Photographs of subcutaneously formed HUT-102 tumors excised from the mice that had been treated with placebo (vehicle) or AUY922. SCID, severe combined immune deficiency.

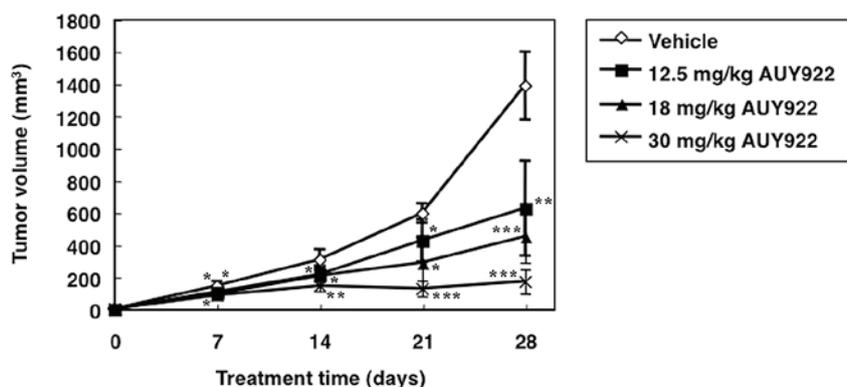


Figure 3. Antitumor efficacy of AUY922 in the HUT-102 adult T-cell leukemia/lymphoma xenograft model. Tumor volume was monitored at regular intervals. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.005 vs. vehicle-treated control. Data are expressed as the mean  $\pm$  standard deviation.

Mann-Whitney U test and Student's *t* test, as appropriate. P<0.05 was considered to indicate a statistical significant difference.

## Results

**AUY922 reduces cell viability of HTLV-1-infected T-cell lines *in vitro*.** Two HTLV-1-infected T-cell lines (HUT-102 and MT-4) were used to determine the efficacy of AUY922 against ATLL. The two cell lines were treated with various concentrations of AUY922 for 24 h, and cell viability was measured using the WST-8 assay. AUY922 at 0-31 nM concentration resulted in a dose-dependent decrease in cell viability of both tested cell lines. However, cell viability reached a plateau level at concentrations >31 nM (Fig. 1).

**AUY922 delays ATLL tumor growth *in vivo*.** Treatment of mice harboring HUT-102 ATLL tumors with AUY922 resulted in significant reduction in tumor volume, compared with vehicle-treated mice. All three doses of AUY922 used in the present study (12.5, 18 and 30 mg/kg) resulted in significant suppression of the growth rate of tumors, compared with vehicle-treated mice (Figs. 2A and 3). The most efficient antitumor effect was observed in mice treated with 30 mg/kg AUY922, with almost complete stasis. This reduction in tumor growth was also reflected in the weight of the excised tumor harvested on day 28, which was significantly lower in the AUY922-treated groups than in the control groups (Figs. 2B and 4A). The tumor inhibition rates for 12.5, 18 and 30 mg/kg AUY922 were 45.21, 52.26 and 77.94%, respectively (Fig. 4A).

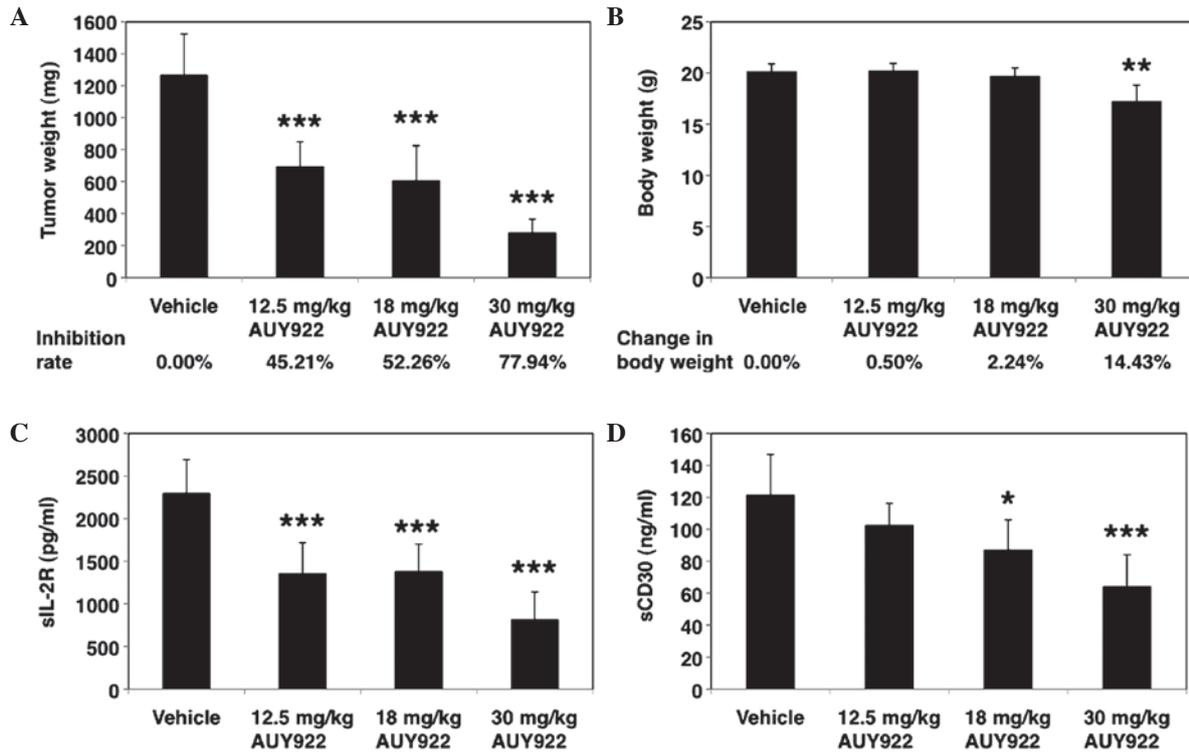


Figure 4. AUY922 inhibits growth of adult T-cell leukemia/lymphoma cells in SCID mice bearing HUT-102 tumors. Mice were inoculated subcutaneously with HUT-102 cells, and divided into control and AUY922 treatment groups (12.5 or 18 mg/kg/day, or 30 mg/kg for 5-6 days/week; n=6 mice/group). Following 4 weeks of treatment, (B) all mice were weighed and sacrificed, and (A) each tumor was excised and weighed. Serum samples from AUY922-treated and untreated SCID mice bearing HUT-102 tumors were assayed for (C) soluble interleukin-2 receptor and (D) soluble cluster of differentiation 30 by enzyme-linked immunosorbent assay. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.005 vs. vehicle-treated control. SCID, severe combined immune deficiency; sIL-2R, soluble interleukin-2 receptor; sCD30, soluble cluster of differentiation 30.

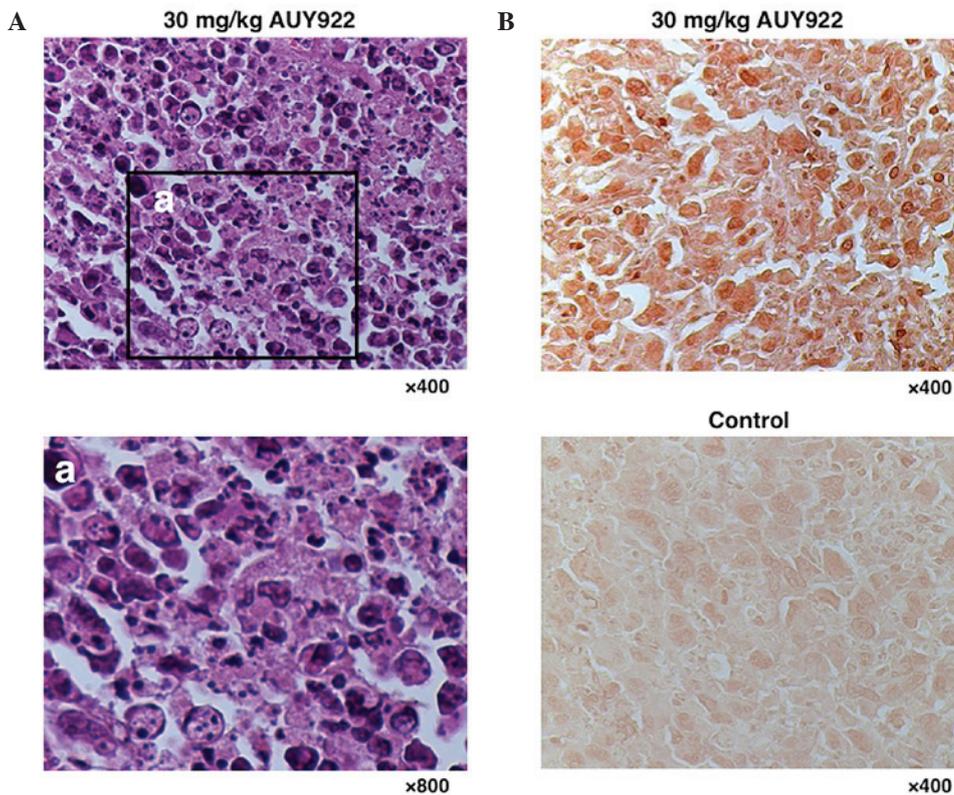


Figure 5. Apoptotic tumor cells in AUY922-treated mice. (A) Morphological changes in tumors of HUT-102-inoculated mice treated with 30 mg/kg AUY922 (hematoxylin and eosin staining; magnification, x400). The bottom panel shows the squared area (marked with a) at a higher magnification (x800). (B) Apoptotic changes in tumor cells were detected by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling assay. Top panel, 30 mg/kg AUY922 group. Bottom panel, control group. Magnification, x400.

To test whether apoptosis is the major process underlying HSP90 inhibition by AUY922, H&E-stained and TUNEL-labeled slides were examined. As represented in Fig. 5A, apoptotic HUT-102 cells were observed in the AUY922 treatment group (30 mg/kg), which were characterized by cytoplasmic condensation, chromatin hyperchromatism and condensation, and nuclear fragmentation. TUNEL staining revealed abundant apoptotic cells in the tumors of the AUY922-treated group, compared with only a few apoptotic cells in the tumors of the control group (Fig. 5B).

Importantly, the treatment regimens were well tolerated, and the mean body weight of the animals increased during the study in the 12.5 and 18 mg/kg treatment groups (Fig. 4B). However, the body weight was significantly different between the AUY922 (30 mg/kg) and the control groups (Fig. 4B). AUY922 at 30 mg/kg caused considerable loss of body weight (14.43%) due to diarrhea, whereas at 12.5 and 18 mg/kg, it was well tolerated, with a final mean change in body weight of 0.50 and 2.24%, respectively (Fig. 4B).

The serum levels of the surrogate tumor markers sIL-2R (16) and sCD30 (17) were also measured in order to determine the therapeutic efficacy of AUY922. Compared with the vehicle control group, there was a significant reduction in serum sIL-2R and sCD30 levels in the animals of the AUY922 groups (Fig. 4C and D). These results are consistent with the dose-dependent antitumor efficacy observed in the HUT-102 ATLL *in vivo* model. Taken together, the above results indicate that treatment with AUY922 results in substantial inhibition of *in vivo* growth of ATLL cells through its direct effects on tumor cells.

## Discussion

Novel therapies aimed at simultaneous targeting of multiple signaling pathways have been considered for the treatment of ATLL, since such approaches could prevent the development of molecular escape mechanisms towards selective targeted therapy and aid to overcome chemoresistance (18). In this context, the use of HSP90 inhibitors, which is based on interference with a broad range of oncogenic signaling components in ATLL cells, has gained momentum (8-10). In the present study, the anti-ATLL efficacy of AUY922, a second generation synthetic HSP90 inhibitor, was demonstrated. Previous studies have reported that AUY922 induces cell-cycle arrest and apoptosis in ATLL cell lines and primary ATLL cells *in vitro* (19). HSP90 blockade resulted in the inhibition of NF- $\kappa$ B, Akt and proviral integration site for Moloney murine leukemia virus family (19). The present study investigated the effects of AUY922 on mice harboring ATLL tumor cells. The results demonstrated that AUY922 has significant anti-ATLL properties. Compared with the control group, AUY922 significantly decreased tumor volume and weight, and increased tumor inhibition rate, as demonstrated by morphological changes indicative of apoptosis and increased tumor cell apoptosis. Furthermore, the present results revealed the dose-dependent effects of AUY922, whose maximum effect was noted at a dose of 30 mg/day. These results suggest that HSP90 blockade with the novel inhibitor AUY922 represents an efficacious approach for the treatment of ATLL.

In contrast to previous studies that investigated the chemotherapeutic effects of AUY922 in other malignancies, this agent was used at doses of 12.5 and 18 mg/kg/day, or 30 mg/kg for 5-6 days/week in the present study, which is below the maximum tolerated dose for AUY922, instead of daily injections of 50 mg/kg reported in other studies (14). This aspect is important in minimizing potential side-effects of HSP90-targeted therapy. In fact, diarrhea occurred in mice injected with AUY922 at 30 mg/kg for 5-6 days/week, and it has been reported that the most common AUY922-related toxicity was diarrhea in phase I dose-escalation studies involving patients with advanced solid tumors (20,21). Taken together, the present data support the selection of AUY922 as a novel anti-ATLL candidate for clinical evaluation.

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