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Inhibition of heat shock protein-90 modulates multiple functions required for survival of human T-cell leukemia virus type I-infected T-cell lines and adult T-cell leukemia cells

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**Inhibition of heat shock protein-90 modulates multiple functions required for survival of human T-cell leukemia virus type I-infected T-cell lines and adult T-cell leukemia cells**

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*Abbreviations:* 17-AAG, 17-allylamino-17-demethoxygeldanamycin; AP 1, activator protein-1; ATL, adult T-cell leukemia; Cdk, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; Hsp90, heat shock protein-90; HTLV-I, human T-cell leukemia virus type I; IC<sub>50</sub>, 50% inhibitory concentration; IKK, IκB kinase; 30 IL-2R, interleukin-2 receptor; LLnL, *N*-acetyl-leucinyI-leucinilnorleucinal-H; NF-κB, nuclear factor-κB; PBMCs, peripheral blood mononuclear cells; PDK1, phosphoinositide dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR; WST-8, water-soluble tetrazolium-8.

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This is the first report to demonstrate the suppressive effect of 17-AAG, an Hsp90 inhibitor, for adult T-cell leukemia and elucidate the molecular mechanism of 17-AAG-induced cell cycle arrest and apoptosis in adult T-cell leukemia cells.

**Abstract**

The molecular chaperone Hsp90 is involved in the stabilization and conformational maturation of many signaling proteins that are deregulated in cancers. The geldanamycin derivative 17-AAG is currently tested in clinical trials and known to inhibit the function of Hsp90 and promote the proteasomal degradation of its misfolded client proteins. ATL is a fatal malignancy of T lymphocytes caused by HTLV-I infection and remains incurable. Since Hsp90 is overexpressed in HTLV-I-infected T-cell lines and primary ATL cells, we analyzed the effects of 17-AAG on cell survival, apoptosis and expression of signal transduction proteins. HTLV-I-infected T-cell lines and primary ATL cells were significantly more sensitive to 17-AAG in cell survival assays than normal PBMCs. 17-AAG induced cell cycle inhibition and apoptosis. These effects could be mediated by inactivation of NF- $\kappa$ B, AP-1 and PI3K/Akt pathways, as well as reduction of expression of proteins involved in the G<sub>1</sub>-S cell cycle transition and apoptosis. Proteasome inhibition interfered with 17-AAG-mediated signaling proteins depletion. Collectively, our results indicate that 17-AAG suppresses ATL cell survival through, at least in part, destabilization of several client proteins and suggest that 17-AAG is a potentially useful chemotherapeutic agent for ATL.

## Introduction

ATL is a severe and fatal lymphoproliferative disease of helper T-cell origin caused by HTLV-I.<sup>1-4</sup> ATL is subclassified into 4 subtypes: acute, lymphoma, chronic and smoldering. In the relatively indolent smoldering and chronic types, the median survival time is  $\geq 2$  years. However, at present, there is no accepted curative therapy for ATL and the condition often progresses to death with a median survival time of 13 months in aggressive ATL.<sup>5</sup> Death is usually due to severe infection or hypercalcemia, often associated with resistance to intensive, combined chemotherapy. Therefore, the establishment of new therapeutic strategies for ATL is very important.

HTLV-I infection is associated with clonal expansion and transformation of mature T lymphocytes. While the mechanisms involved are not completely understood, the viral regulatory protein Tax plays a central role in these processes.<sup>6</sup> Tax functions primarily through protein-protein interactions. The modulation and deregulation of cellular signaling by Tax involves a range of pathways, including both direct and indirect interactions with a range of transcription factors such as cAMP-responsive element binding protein/activating transcription factor, NF- $\kappa$ B, AP-1 and serum response factor, as well as distinct cellular signaling pathways involving PI3K and its downstream target Akt.<sup>7,8</sup> Activation of cAMP-responsive element binding protein/activating transcription factor- and NF- $\kappa$ B-dependent gene expression plays central roles in both viral replication and cellular transformation and proliferation. NF- $\kappa$ B activation by Tax results in the upregulation of expression of a large number of cellular genes involved in cell proliferation and survival. Whereas Tax clearly plays a primary role in HTLV-I-induced cellular signaling pathways,

90 recent studies indicate that NF- $\kappa$ B, AP-1 and Akt are constitutively activated in  
freshly isolated ATL cells despite their lack of detectable Tax expression.<sup>9-11</sup> The need  
for agents that kill HTLV-I-infected T cells is obvious and attempts to induce  
apoptosis by NF- $\kappa$ B inhibitors were successful.<sup>12-15</sup> Anti-ATL drug targets other than  
NF- $\kappa$ B include the AP-1 and PI3K/Akt proteins.<sup>11,16-19</sup>

95 The molecular chaperone protein Hsp90 is involved in the folding, activation  
and assembly of a variety of proteins.<sup>20,21</sup> These Hsp90 client proteins are crucial in  
oncogenesis, including transmembrane tyrosine kinases (HER-2/new, epidermal  
growth factor receptor, Met and insulin-like growth factor 1 receptor), metastable  
signaling proteins (Akt, Raf-1 and IKK), mutated signaling proteins (p53, Kit, Flt-3  
100 and v-Src), chimeric signaling proteins (NPM-ALK and BCR-ABL), steroid receptors  
(androgen, estrogen and progesterone receptors) and cell cycle regulators (Cdk4 and  
Cdk6). Under unstressed conditions, these proteins form complexes with Hsp90 and  
the cochaperones to attain their active conformations or enhance stability. Upon ATP  
binding and hydrolysis, Hsp90 forms a mature complex that catalyzes the  
105 conformational maturation of Hsp90 client proteins.<sup>21</sup> Inhibition of Hsp90 function  
disrupts the complex and leads to degradation of client proteins in a  
proteasome-dependent manner. This results in simultaneous interruption of many  
signal transduction pathways pivotal to tumor progression and survival. The naturally  
occurring ansamycin antibiotic geldanamycin binds to a conserved binding pocket in  
110 the NH<sub>2</sub>-terminal ATP-binding domain of Hsp90, inhibiting ATP binding and  
ATP-dependent Hsp90 chaperone activity. The less toxic geldanamycin derivative  
17-AAG also binds to Hsp90, exerts a potent antitumor activity in preclinical models

and is currently tested in clinical trials. Recent data have revealed that the therapeutic selectivity of Hsp90 inhibitors results from the presence of a high-affinity activated form of Hsp90 in tumors, which is in a multi-chaperone complex with high ATP activity, whereas the Hsp90 in normal tissues is in an inactive, uncomplexed form with low affinity.<sup>22</sup>

The aim of the present *in vitro* study was to determine the effects of 17-AAG and the molecular mechanisms of any such effect, on ATL cell survival, and hence its potential chemotherapeutic use for ATL. The results showed overexpression of Hsp90 in HTLV-I-infected T-cell lines and primary ATL cells and that inhibition of Hsp90 by 17-AAG resulted in induction of cell cycle arrest and apoptosis through inactivation of NF- $\kappa$ B, AP-1 and Akt signaling.

## Material and methods

### *Cell lines*

HTLV-I-infected T-cell lines, MT-2,<sup>23</sup> MT-4,<sup>24</sup> SLB-1,<sup>25</sup> HUT-102<sup>1</sup> and ED-40515(-)<sup>26</sup> and an uninfected T-cell line, Jurkat were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. MT-2, MT-4 and SLB-1 are HTLV-I-transformed T-cell lines and constitutively express viral genes including Tax. ED-40515(-) is a T-cell line of leukemic cell origin established from a patient with ATL and does not express viral genes. HUT-102 was established from a patient with ATL and constitutively expresses viral genes, but it is unclear whether HUT-102 cells represent the tumor clone from the donor ATL patient.

### *Clinical samples*

The diagnosis of ATL was based on clinical features, hematological findings and the presence of anti-HTLV-I antibodies in the sera. Monoclonal HTLV-I provirus  
140 integration into the DNA of leukemic cells was confirmed in all patients by Southern blot hybridization (data not shown). PBMCs from healthy volunteers and patients with acute type ATL were analyzed. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Piscataway, NJ) and washed with PBS. Each sample was collected after obtaining an informed consent.

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### *Reagents*

17-AAG was purchased from Alomone Labs (Jerusalem, Israel). The proteasome inhibitor LLnL was obtained from Calbiochem (La Jolla, CA). Rabbit polyclonal antibodies to cIAP2, survivin, I $\kappa$ B $\alpha$  and JunD were purchased from Santa Cruz  
150 Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to Bcl-x<sub>L</sub> and mouse monoclonal antibody to Hsp90 were purchased from BD Transduction Laboratories (San Jose, CA). Rabbit polyclonal antibodies to Hsp70 and Hsp27, and mouse monoclonal antibodies to XIAP and cyclin D1 were purchased from Medical & Biological Laboratories (Nagoya, Japan). Mouse monoclonal antibodies to Bcl-2,  
155 Cdk4, Cdk6 and actin were purchased from NeoMarkers (Fremont, CA). Mouse monoclonal antibody to phospho-I $\kappa$ B $\alpha$  (Ser32/36) and rabbit polyclonal antibodies to IKK $\alpha$ , IKK $\beta$ , Akt, PDK1 and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody to IKK $\gamma$  was

160 purchased from Sigma-Aldrich Inc. (Saint Louis, MO). Mouse monoclonal antibody  
to Tax, Lt-4, was described previously.<sup>27</sup>

#### *Cell viability and apoptosis assays*

The effect of 17-AAG on cell survival was examined by using the cell viability  
reagent, WST-8 (Wako Chemicals, Osaka Japan). Briefly, cell lines ( $1 \times 10^5$  cells/ml)  
165 or PBMCs ( $1 \times 10^6$  cells/ml) were incubated in a 96-well microculture plate in the  
absence or presence of various concentrations of 17-AAG. After 48-hr culture, WST-8  
(5  $\mu$ l) was added for the last 4 hr of incubation and absorbance at 450 nm was  
measured using an automated microplate reader. Measurement of mitochondrial  
dehydrogenase cleavage of WST-8 to formazan dye provides an indication of the level  
170 of cell viability. The  $IC_{50}$  was extrapolated from trend line data. For detection of  
apoptosis, the Annexin V-binding capacity of the treated cells was examined by flow  
cytometry (FACSCaliber, Becton Dickinson, San Jose, CA) using Annexin V-Fluos  
(Roche Diagnostics, Mannheim, Germany).

#### 175 *Cell cycle analysis*

Cell cycle was analyzed with the CycleTEST PLUS DNA reagent kit (Becton  
Dickinson). In brief,  $1 \times 10^6$  cells were washed with a buffer solution containing  
sodium citrate, sucrose and dimethyl sulfoxide suspended in a solution containing  
RNase A and stained with 125  $\mu$ g/ml propidium iodide for 10 min. After passing the  
180 cells through a nylon mesh, cell suspensions were analyzed on a FACSCaliber using

CellQuest. The population of cells in each cell cycle phase was determined with ModFit software.

#### *Western blot analysis*

185 Cells were lysed in a buffer containing 62.5 mM TRIS-HCl (pH 6.8), 2% SDS, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20  $\mu$ g) were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing sequentially with the specific antibodies. The bands were visualized with the  
190 enhanced chemiluminescence kit (Amersham Biosciences). The displayed results are representative of 3 independent experiments. Similar results are obtained.

#### *RT-PCR*

Total cellular RNA was extracted with Trizol (Invitrogen Corp., Carlsbad, CA)  
195 according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1  $\mu$ g total cellular RNA using an RNA PCR kit (Takara Shuzo, Kyoto, Japan) with random primers. Thereafter, cDNA was amplified for 30 cycles for Hsp90 and 28 cycles for  $\beta$ -actin. The oligonucleotide primers used were as followed: for Hsp90  $\alpha$  , sense, 5'-AAAAGTTGAAAAGGTGGTTG-3' and antisense,  
200 5'-TATCACAGCATCACTTAGTA-3'; for Hsp90  $\beta$ , sense, 5'-AGAAGGTTGAGAAGGTAAGCC-3' and antisense, 5'-AAGAGTAGAGAGGGAATGGG-3'; and for  $\beta$ -actin, sense, 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense,

5'-CTCCTTAATGTCACGCACGATTTC-3'. Product sizes were 624 bp for Hsp90  $\alpha$ ,  
205 641 bp for Hsp90  $\beta$  and 548 bp for  $\beta$ -actin. Cycling conditions were as follows:  
denaturing at 94°C for 45 sec (for Hsp90) or 30 sec (for  $\beta$ -actin), annealing at 58°C  
(for Hsp90) or 60°C (for  $\beta$ -actin) for 30 sec and extension at 72°C for 90 sec. The  
PCR products were fractionated on 2% agarose gels and visualized by ethidium  
bromide staining.

210

#### *Preparation of nuclear extracts and EMSA*

Cells were placed in culture and examined for inhibition of NF- $\kappa$ B and AP-1 after  
exposure to 17-AAG for 24 hr. Nuclear proteins were extracted, and NF- $\kappa$ B and AP-1  
DNA-binding activities were examined by EMSA as described previously.<sup>9,10</sup> In brief,  
215 5  $\mu$ g of nuclear extracts were preincubated in a binding buffer containing 1  $\mu$ g  
poly-deoxy-inosinic-deoxy-cytidylic acid (Amersham Biosciences), followed by  
addition of [ $\alpha$ -<sup>32</sup>P]-labeled oligonucleotide probe containing NF- $\kappa$ B or AP-1 element  
(approximately 50,000 cpm). These mixtures were incubated for 15 min at room  
temperature. The DNA-protein complexes were separated on 4% polyacrylamide gel  
220 and visualized by autoradiography. To examine the specificity of the each element  
probe, unlabeled competitor oligonucleotides were preincubated with nuclear extracts  
for 15 min before incubation with probes. The probes or competitors used were  
prepared by annealing the sense and antisense synthetic oligonucleotides; a typical  
NF- $\kappa$ B element from the *IL-2R  $\alpha$  chain* gene  
225 (5'-gatcCGGCAGGGGAATCTCCCTCTC-3') and an AP-1 element of the *IL-8* gene  
(5'-gatcGTGATGACTCAGGTT-3'). The oligonucleotide

5'-gacTGTCGAATGCAAATCACTAGAA-3', containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. This transcription factor regulates transcription of a number of so-called housekeeping genes. Underlined sequences represent the NF- $\kappa$ B, AP-1 or Oct-1 binding site. To identify nuclear proteins in the DNA-protein complex revealed by EMSA, we used antibodies specific for various NF- $\kappa$ B family proteins, including p65, p50, c-Rel and p52 and various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD (Santa Cruz Biotechnology), to elicit a supershift DNA-protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probes.

## Results

*Overexpression of Hsp90 protein in HTLV-I-infected T-cell lines and PBMCs from patients with ATL*

We first examined the levels of Hsp90, Hsp70 and Hsp27 proteins in 5 HTLV-I-infected T-cell lines and PBMCs from 7 patients with ATL, and compared the levels with the 2 from normal PBMCs. The level of Hsp90 in primary ATL cells and HTLV-I-infected T-cell lines was higher than in normal PBMCs (Fig. 1a). However, Hsp27 was absent in MT-4 and PBMCs from 2 patients with ATL (ATL 6 and 7) and was weakly expressed in PBMCs from 3 patients with ATL (ATL 1, 3 and 5) compared with normal PBMCs. PBMCs from the residual patients with ATL (ATL 2 and 4) expressed the same levels of Hsp27 protein as normal PBMCs. Hsp70 was abundant in normal PBMCs, primary ATL cells and HTLV-I-infected T-cell lines. An

250 uninfected T-cell line, Jurkat expressed Hsp90 and Hsp70, but not Hsp27. To determine whether the expression of Hsp90 is deregulated in T-cell lines at transcription level, we examined mRNA levels of Hsp90  $\alpha$  and  $\beta$  in normal PBMCs and T-cell lines. As shown in Figure 1*b*, the expression of Hsp90  $\alpha$  mRNA was enhanced in all T-cell lines as compared with that of normal PBMCs. In contrast, the  
255 expression of Hsp90  $\beta$  mRNA could hardly be recognized in either T-cell lines or normal PBMCs (data not shown).

*17-AAG inhibits cell survival of HTLV-I-infected T-cell lines and primary ATL cells but not normal PBMCs*

260 Since Hsp90 was overexpressed in all HTLV-I-infected T-cell lines and primary ATL cells, we next analyzed the effect of Hsp90 inhibitor 17-AAG on the survival of HTLV-I-infected T-cell lines. Tax protein was detected by immunoblot analysis in the 4 HTLV-I-infected T-cell lines (MT-2, MT-4, SLB-1 and HUT-102) but not in the one ATL-derived T-cell line [ED-40515(-)].<sup>28</sup> Culture of cells with various  
265 concentrations (0 to 5,000 nM) of 17-AAG for 48 hr resulted in cell survival suppression in a dose-dependent manner in all 5 lines tested as assessed by the WST-8 assay (Fig. 2*a*). Cell survival of an uninfected T-cell line, Jurkat was also inhibited, but Jurkat was less susceptible to 17-AAG than HTLV-I-infected T-cell lines. The concentrations of 17-AAG required to inhibit cell survival of MT-2, MT-4, SLB-1,  
270 HUT-102, ED-40515(-) and Jurkat by 50% (IC<sub>50</sub>) were 461, 85, 990, 316, 140 and 2,414 nM, respectively. We further assessed the 17-AAG-induced cell survival inhibition of PBMCs from normal individuals and patients with ATL. Tax was not

present in patient cells that were freshly isolated and lysed by immunoblot analysis (data not shown). Figure 2*b* shows that 17-AAG induced a clearer cell survival suppression of primary ATL cells than normal PBMCs.

#### *17-AAG induces G<sub>1</sub> phase arrest of HTLV-I-infected T-cell lines*

We investigated the effect of 17-AAG on the cell cycle progression in cell lines. The cells were incubated with 17-AAG for 24 hr and analyzed for cell cycle distribution by flow cytometry (Fig. 3*a*). 17-AAG inhibited cell cycle progression, as evidenced by increasing the proportion of cells in G<sub>1</sub> phase and reducing cells in the S phase, indicating G<sub>1</sub> arrest in all cell lines.

#### *17-AAG induces apoptosis of HTLV-I-infected T-cell lines*

To examine whether induction of apoptosis accounts for the cell survival inhibition observed in HTLV-I-infected T-cell lines, cells treated with 17-AAG for 48 hr were examined by the Annexin V method. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic finding in cells entering apoptosis. 17-AAG increased the proportion of cells positive for Annexin V in all cell lines (Fig. 3*b*, right panel) and such effect was observed in a dose-dependent fashion in MT-2 cells (Fig. 3*b*, left panel), indicating increased incidence of apoptosis of 17-AAG-treated cells.

#### *17-AAG decreases the level of intracellular regulators of cell cycle and apoptosis*

295 To clarify the molecular mechanisms of 17-AAG-induced cell survival inhibition and apoptosis in HTLV-I-infected T-cell lines, we examined the expression of several intracellular regulators of cell cycle and apoptosis, including cyclin D1, Cdk4, Cdk6, Bcl-2, Bcl-x<sub>L</sub>, cIAP2, XIAP and survivin in MT-2 cells by Western blot analysis. As shown in Figures 4a and 4b, 17-AAG did not alter Bcl-2, Bcl-x<sub>L</sub> and cIAP2 levels. In contrast, it significantly decreased the expression of cyclin D1, Cdk4, Cdk6, XIAP and survivin in MT-2 cells in time- and dose-dependent manners. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin.

We also examined the effect of 17-AAG in other HTLV-I-infected T-cell lines (Fig. 4c). We did not find altered Bcl-2 expression following treatment with 17-AAG in all cell lines. In contrast, in all cell lines, we found diminished expression of cyclin D1, Cdk6, XIAP and survivin following treatment with 17-AAG. Although SLB-1 cells expressed undetectable level of Cdk4, the expression of Cdk4 was also reduced in 17-AAG-treated MT-4, HUT-102 and ED-40515(-) cells. Because cyclin D1, Cdk4, Cdk6, XIAP and survivin are Tax-responsive genes,<sup>29-32</sup> we also examined the level of Tax expression. 17-AAG did not change Tax protein level in MT-2 cells (Figs. 5a and 5b). 17-AAG treatment also did not influence Hsp90 level (Fig. 5a).

#### *17-AAG modulates activated NF-κB*

315 Several reports have suggested that NF-κB can act as a survival factor and is required for the proliferation of a variety of tumor cell types.<sup>33</sup> Because NF-κB is constitutively active in Tax-expressing and HTLV-I-infected T-cell lines as well as primary ATL

cells,<sup>9</sup> and the expression levels of cyclin D1, Cdk4, Cdk6, XIAP and survivin are known to be regulated by NF- $\kappa$ B,<sup>29-32,34,35</sup> we examined whether 17-AAG inhibits the NF- $\kappa$ B pathway. To study the DNA-binding activity of NF- $\kappa$ B, we did EMSA with radiolabeled double-stranded NF- $\kappa$ B oligonucleotides and nuclear extracts from untreated or 17-AAG-treated HTLV-I-infected T-cell lines. NF- $\kappa$ B oligonucleotide probe with nuclear extracts from untreated HTLV-I-infected T-cell lines generated DNA-protein gel shift complexes (Figs. 6*a* and 6*b*). These complexes were due to specific bindings of nuclear proteins to the NF- $\kappa$ B sequence since these binding activities diminished following the addition of cold probe but not irrespective sequence (Fig. 6*b*, *left panel*, *lanes 2,3*). We also showed that NF- $\kappa$ B complexes contain p50, p65 and c-Rel (Fig. 6*b*, *left panel*, *lanes 4-6*). As shown in Figure 6*a*, nuclear extracts prepared from HTLV-I-infected T-cell lines treated with 17-AAG for 24 hr exhibited a decrease in the intensity of the NF- $\kappa$ B-containing gel shift complexes, suggesting that 17-AAG downregulates the DNA-binding activities of NF- $\kappa$ B. Of note, no differences in the absence or presence of 17-AAG in binding to the octamer motif on DNA were found.

Degradation of I $\kappa$ B $\alpha$  and subsequent release of NF- $\kappa$ B requires prior phosphorylation at Ser32 and Ser36 residues.<sup>36</sup> To investigate whether the inhibitory effect of 17-AAG is mediated through alteration of phosphorylation of I $\kappa$ B $\alpha$ , MT-2 cells were treated with 17-AAG and their protein extracts were checked for phosphorylated I $\kappa$ B $\alpha$  expression. Untreated MT-2 cells constitutively expressed Ser32/36-phosphorylated I $\kappa$ B $\alpha$ , while 17-AAG treatment decreased the phosphorylated I $\kappa$ B $\alpha$  in a time-dependent manner (Fig. 5*a*). 17-AAG treatment also

increased total I $\kappa$ B $\alpha$  protein in a time-dependent manner (Fig. 5a), suggesting that inhibition of phosphorylation of I $\kappa$ B $\alpha$  leads to stabilization of I $\kappa$ B $\alpha$  by blocking degradation of I $\kappa$ B $\alpha$  protein.

I $\kappa$ B $\alpha$  is phosphorylated by an IKK complex, which invokes subsequent I $\kappa$ B $\alpha$  polyubiquitination by the SCF <sup>$\beta$ TrCP</sup> complex and degradation by the 26S proteasome.<sup>37</sup>  
The IKK complex is composed of 2 related kinase molecules, IKK $\alpha$  and IKK $\beta$ , and a regulatory molecule IKK $\gamma$ . A recent study indicated that IKK $\alpha$  and IKK $\beta$  are clients of Hsp90.<sup>38</sup> Treating MT-2 cells with 17-AAG reduced the amounts of IKK $\alpha$  and IKK $\beta$  proteins but not of IKK $\gamma$  in dose- and time-dependent manners (Figs. 5a and 5b). Reduction of IKK $\alpha$  and IKK $\beta$  protein levels was also observed in other cell lines (Fig. 5c). These results suggest that 17-AAG-mediated Hsp90 inhibition depletes IKK $\alpha$  and IKK $\beta$ , resulting in inactivation of NF- $\kappa$ B.

#### *17-AAG modulates activated AP-1*

AP-1 is a group of dimeric transcription factor complexes composed of members of the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB and JunD) families which play a central role in the proliferation and transformation of T cells, prevention of apoptosis and cytokine production.<sup>39</sup> Since constitutive activation of AP-1 is observed in HTLV-I-infected T-cell lines and primary ATL cells,<sup>10</sup> we examined the effect of 17-AAG on the constitutive AP-1 activity in HTLV-I-infected T-cell lines (Fig. 6a). Constitutive AP-1 activity was blocked by 17-AAG in all HTLV-I-infected T-cell lines. The increased DNA-binding AP-1 protein was composed of JunD (Fig. 6b, right panel, lane 10)<sup>10</sup> and JunD protein was decreased by 17-AAG in time- and

dose-dependent manners (Figs. 5a and 5b). Similar results were observed in other cell  
365 lines (Fig. 5c). These findings suggest that 17-AAG-mediated Hsp90 inhibition  
depletes JunD, resulting in inactivation of AP-1.

#### *17-AAG modulates activated Akt*

PI3K and its downstream target Akt are activated in response to cytokine receptors  
370 and the T-cell receptor activation; this pathway provides growth stimulatory and  
antiapoptotic signals.<sup>40</sup> PI3K/Akt was found to be activated in HTLV-I-infected T-cell  
lines and primary ATL cells, and to be involved in cell transformation and  
survival.<sup>11,17-19</sup> A recent study showed that Akt relies on Hsp90 for its stability and  
activity.<sup>41</sup> 17-AAG inhibits Akt phosphorylation prior to the loss of Akt expression,  
375 probably due to more rapid-acting Hsp90-dependent effects on the upstream regulator  
PDK1.<sup>42</sup> We thus examined the expression and phosphorylation of Akt after treatment  
with 17-AAG. After 24-hr incubation, 17-AAG (1,000 nM) downregulated the  
expression of Akt protein itself in MT-2 cells (Fig. 7a). A short period (8 hr) of  
17-AAG treatment did not reduce the amounts of Akt protein, but phosphorylation of  
380 Akt was diminished in 17-AAG-treated cells. Complete downregulation of Akt  
protein expression was found when MT-2 cells were treated with 500 nM of 17-AAG  
for 24 hr, but the minimum concentration of 17-AAG that could downregulate the  
phospho-Akt level was 250 nM (Fig. 7b). Because treatment of 17-AAG  
downregulates the phospho-Akt level without the loss of Akt expression, 17-AAG  
385 might interfere with the kinase activity of the upstream Akt kinase PDK1. We then  
examined the change in PDK1 protein expression after drug treatment. As shown in

Figure 7a, treatment of MT-2 cells with 1,000 nM of 17-AAG for 8 hr decreased the amount of PDK1 protein and the phosphorylation of Akt was also decreased. PDK1 protein expression was downregulated following treatment of MT-2 cells with  
390 17-AAG from the concentration of 250 nM for 24 hr and the phosphorylation of Akt was also decreased (Fig. 7b). Reductions of Akt, phospho-Akt and PDK1 levels were also found in other HTLV-I-infected T-cell lines treated with 17-AAG, suggesting that these effects of 17-AAG are not restricted to 1 particular cell line (Fig 7c). These results suggest that 17-AAG-mediated Hsp90 inhibition depletes PDK1 and Akt,  
395 resulting in inactivation of Akt.

#### *Effects of 17-AAG on fresh primary ATL cells*

We then examined the effects of 17-AAG on freshly isolated ATL cells. Treatment of ATL cells from 4 independent patients with 17-AAG significantly downregulated the  
400 expression and activity of client proteins including survivin, IKK $\alpha$ , IKK $\beta$  and Akt (Fig. 8). In contrast, 17-AAG treatment did not affect IKK $\gamma$  protein level.

#### *Hsp90 protects client proteins from proteasomal degradation*

Several Hsp90 client proteins are degraded by the proteasome following Hsp90  
405 inhibitors.<sup>20,21</sup> To examine whether proteasomal degradation was responsible for decreased levels of client proteins after 17-AAG treatment, MT-2 cells were cultured in a medium containing 17-AAG and proteasome inhibitor LLnL. 17-AAG-mediated degradation of client proteins (Cdk4, Cdk6, survivin, IKK $\alpha$ , IKK $\beta$ , Akt, PDK1 and JunD) was partially blocked by LLnL (Fig. 9). In contrast, IKK $\gamma$  was not destabilized

410 by 17-AAG and LLnL did not change the protein level of IKK $\gamma$ . The reversal of  
17-AAG-induced degradation of client proteins by LLnL suggests that these proteins  
are subject to ubiquitin-dependent turnover.

## Discussion

415 Hsp90, one of the most abundant molecular chaperones in eukaryotes, plays a part in  
the folding and conformational maturation of proteins required for cell survival. Since  
higher levels of Hsp90 protein have been observed in HTLV-I-infected T-cell lines  
and primary ATL cells, the effect of Hsp90 inhibitor, 17-AAG, on the survival of  
these cells was examined. We found that HTLV-I-infected T-cell lines and ATL cells  
420 from different patients are more susceptible to survival inhibition induced by  
treatment with 17-AAG than normal PBMCs. In contrast, we did not observe  
17-AAG-mediated inhibition of cell survival in normal PBMCs, even when higher  
concentrations of 17-AAG were used. As such, 17-AAG may selectively target ATL  
cells at concentrations that do not affect normal PBMCs. Therefore, we investigated  
425 the basis for the selective antitumor activity of 17-AAG in HTLV-I-infected T-cell  
lines and ATL cells.

The cell survival-inhibitory effect of 17-AAG on HTLV-I-infected T-cell lines  
was mainly due to the induction of cell cycle arrest and apoptosis, based on the  
observation that a significant population of cells remained in the G<sub>1</sub> phase of cell cycle  
and underwent to apoptosis after treatment with 17-AAG in a dose-dependent manner.  
430 Our results showed that 17-AAG induced G<sub>1</sub> arrest concomitant with decreased  
protein levels of cyclin D1, Cdk4 and Cdk6. The latter 2 kinases have been identified

as Hsp90 clients.<sup>20</sup> Indeed, treatment with 17-AAG, induced degradation of Cdk4 and Cdk6, and LLnL, a proteasome inhibitor, prevented such degradation. Furthermore, we showed that 17-AAG induced apoptosis was associated with reduction of survivin and XIAP in HTLV-I-infected T-cell lines. Survivin has also been identified as an Hsp90 client<sup>20</sup> and treatment with 17-AAG induced degradation of survivin by proteasome pathway in HTLV-I-infected T-cell lines. Together, these data indicate that Cdk4, Cdk6 and survivin are themselves Hsp90 client proteins susceptible to specific degradation induced by an Hsp90 inhibitor. Cyclin D1 and XIAP have not been identified as Hsp90 clients, but the expression of these proteins is regulated by NF- $\kappa$ B.<sup>29-31,34,35</sup> Hsp90 is a regulator of NF- $\kappa$ B signaling through its general involvement in IKK activation.<sup>38</sup> Treatment with 17-AAG decreased IKK complex proteins, IKK $\alpha$  and IKK $\beta$ . The loss of IKK inhibited I $\kappa$ B $\alpha$  phosphorylation and reduced NF- $\kappa$ B DNA-binding, resulting in reduced cyclin D1 and XIAP. Therefore, it is possible that 17-AAG affects the expression of Cdk4, Cdk6 and survivin through NF- $\kappa$ B inhibition. Bcl-x<sub>L</sub>, Bcl-2 and cIAP2 are also known as NF- $\kappa$ B targets, but not repressed by 17-AAG. NF- $\kappa$ B participates in the transcription of over 150 target genes, but not all are activated when NF- $\kappa$ B is induced. Several different mechanisms confer selectivity on the NF- $\kappa$ B inhibitory response to 17-AAG. Reduced NF- $\kappa$ B activity may be responsible, at least in part, for the induction of cell cycle arrest and apoptosis of HTLV-I-infected T-cell lines.

PI3K/Akt has been reported to play a role in the activation of prosurvival pathways in HTLV-I-infected T-cell lines and primary ATL cells.<sup>11,17-19</sup> Based on these studies, Akt could be considered as a molecular target in ATL. Akt and PDK1

have been shown previously to be sensitive to Hsp90 inhibitors.<sup>41,42</sup> Translation of D-type cyclins is mediated *via* a PI3K-dependent pathway.<sup>43</sup> Therefore, we hypothesized that the effect of 17-AAG on Akt and PDK1 might explain the cell survival inhibitory effect of 17-AAG. In this study, we showed that 17-AAG induced degradation of Akt and PDK1, which resulted in inactivation of Akt.

The increased AP-1 DNA-binding activity in HTLV-I-infected T-cell lines was composed of JunD, which was not known previously as an Hsp90 client.<sup>10</sup> The increase of AP-1 binding activity in HTLV-I-infected T-cell lines was decreased by 17-AAG. 17-AAG treatment resulted in significant depletion of JunD, which is likely mediated by the 26S proteasome as shown with a specific inhibitor. Although it is not determined whether JunD interacts with Hsp90, JunD is suggested to be an Hsp90 client.

In HTLV-I-expressing cells, the virus-encoded regulatory protein, Tax, plays a critical role in the growth and survival of infected T cells by perturbing normal regulatory mechanisms including transcription, signal transduction and cell cycle progression, resulting in uncontrolled cell growth.<sup>8</sup> Tax activates NF- $\kappa$ B by stimulating the activity of the IKK, which in turn leads to phosphorylation and degradation of I $\kappa$ B $\alpha$ .<sup>44</sup> Tax also activates AP-1 and Akt.<sup>7,10,17-19</sup> However, primary ATL cells with high activities of NF- $\kappa$ B, AP-1 and Akt are known to have very low or no expression of Tax<sup>9-11</sup> and therefore, growth of ATL cells *in vivo* is believed to be Tax-independent. Elevated CD30 expression<sup>45</sup> and decreased expression of inositol phosphatases, phosphatase and tensin homolog deleted on chromosome 10 tumor suppressor and Src homology 2 domain containing inositol polyphosphate

phosphatase,<sup>11</sup> are considered the causes of constitutive NF- $\kappa$ B and Akt activation in  
480 ATL cells, respectively. 17-AAG could inhibit NF- $\kappa$ B, AP-1 and Akt, resulting in  
induction of cell death in Tax-negative HTLV-I-infected T-cell line, ED-40515(-). In  
addition, 17-AAG did not inhibit the level of Tax expression in HTLV-I-infected  
T-cell lines. Therefore, the survival inhibition of HTLV-I-infected T-cell lines induced  
by 17-AAG appears to be mediated by Tax-independent pathway.

485 17-AAG also inhibited cell survival of an uninfected T-cell line, Jurkat in  
which Hsp90 was overexpressed. However, Jurkat was less susceptible to 17-AAG  
than HTLV-I-infected T-cell lines. Although Akt is similarly activated in Jurkat  
cells,<sup>11</sup> the activities of NF- $\kappa$ B and AP-1 in these cells are markedly weaker than those  
in HTLV-I-infected T-cell lines.<sup>9,10</sup> Thus, the difference of susceptibility to 17-AAG  
490 may be due to the level of NF- $\kappa$ B and AP-1 activities. 17-AAG may function by  
affecting multiple signaling pathways in different cell types.

In the present study, we showed that Cdk4, Cdk6, survivin, IKK $\alpha$ , IKK $\beta$ , Akt  
and PDK1 are Hsp90 client proteins in HTLV-I-infected T-cell lines and that  
degradation of client proteins *via* inhibition of Hsp90 leads to impaired signaling and  
495 cell apoptosis. The phase I trial of 17-AAG has demonstrated that plasma 17-AAG  
concentrations exceeded 10  $\mu$ M at the highest dose level, 450 mg/m<sup>2</sup>/week.<sup>46</sup> The  
concentrations of drug remained above 120 nM, which is the IC<sub>50</sub> in MT-4 cell line  
and primary ATL cells from some patients, for periods in excess of 24 hr. These  
results suggest that 17-AAG treatment is feasible to deliver a dose resulting in a  
500 potentially therapeutic plasma concentration, target inhibition in ATL cells and  
possible antitumor activity. We conclude that 17-AAG could be potentially useful as a

chemotherapeutic or chemopreventive agent in ATL, but further clinical studies will be necessary to assess its effect on primary fresh ATL cells.

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**Figure legends**

**FIGURE 1** - Overexpression of Hsp90 in HTLV-I-infected and uninfected T-cell lines and PBMCs from patients with ATL. (a) Western blot analysis of 5 HTLV-I-infected T-cell lines, 1 uninfected T-cell line, PBMCs from 7 patients with ATL and PBMCs from 2 normal individuals was performed using anti-Hsp90, Hsp70 and Hsp27 antibodies. (b) Expression of Hsp90  $\alpha$  mRNA in 5 HTLV-I-infected T-cell lines, 1 uninfected T-cell line and PBMCs from 2 normal individuals as assessed by RT-PCR.

**FIGURE 2** - Inhibitory effects of 17-AAG on cell survival of HTLV-I-infected and uninfected T-cell lines and primary ATL cells. Cells were incubated in the presence of various concentrations of 17-AAG for 48 hr and *in vitro* survival of the cultured cells was measured by WST-8 assay. Relative viability of the cultured cells is presented as the mean determined on T-cell lines (a) and PBMCs from healthy control and ATL patients (b) from triplicate cultures. A relative viability of 100% was designated as the total number of cells that survive in the 48-hr cultures in the absence of 17-AAG.

**FIGURE 3** - Effects of 17-AAG on the cell cycle status and apoptosis of HTLV-I-infected T-cell lines. (a) HTLV-I-infected T-cell lines were incubated in the absence or presence of 17-AAG (1,000 nM) for 24 hr and stained with propidium iodide, and the DNA content was assayed by flow cytometry. The percentage of cells in the various phases of cell cycle was determined. (b) Cells were treated with or without 17-AAG (1,000 nM) for 48 hr, harvested, then stained with Annexin V, and

analyzed by flow cytometry. Data represent the percentages of apoptotic cells for both  
685 untreated (open bars) and 17-AAG-treated (solid bars) cells (*right panel*). MT-2 cells  
were also incubated with various concentrations of 17-AAG for 48 hr. The  
proapoptotic activity of 17-AAG was assessed by Annexin V staining (*left panel*).  
Data are mean  $\pm$  SD values of 3 independent experiments.

690 **FIGURE 4** - Western blot analysis of the cell cycle- and apoptosis-associated proteins.  
MT-2 cells were treated with 1,000 nM 17-AAG for the indicated time periods (*a*) or  
for 24 hr with the indicated concentrations of 17-AAG (*b*). (*c*) The indicated cell lines  
were treated with (+) or without (-) 1,000 nM 17-AAG for 24 hr. Total cellular  
proteins (20  $\mu$ g/lane) were separated on SDS-polyacrylamide gels and transferred to  
695 the membrane. Protein levels were detected by Western blotting with antibodies  
directed against each protein.

**FIGURE 5** - Western blot analysis of IKK, I $\kappa$ B $\alpha$ , AP-1 protein JunD, Hsp90 and  
viral protein Tax. MT-2 cells were treated with 1,000 nM 17-AAG for the indicated  
700 time periods (*a*) and for 24 hr with the indicated concentrations of 17-AAG (*b*),  
followed by protein extraction. (*c*) The indicated cell lines were treated with (+) or  
without (-) 1,000 nM 17-AAG for 24 hr, followed by protein extraction. Whole cell  
extracts (20  $\mu$ g/lane) of treated cells were immunoblotted with specific antibodies  
against each protein.

**FIGURE 6** - Inhibition of constitutive NF- $\kappa$ B and AP-1 DNA-binding activities in HTLV-I-infected T-cell lines treated with 17-AAG. (a) HTLV-I-infected T-cell lines were treated with (+) or without (-) 1,000 nM 17-AAG and assessed for NF- $\kappa$ B, AP-1 and Oct-1 DNA-binding. After 24 hr, nuclear proteins were extracted and EMSA was performed using oligonucleotide probes for NF- $\kappa$ B, AP-1 and Oct-1. (b) EMSA using untreated MT-2 nuclear extracts and radiolabeled NF- $\kappa$ B and AP-1 probes generated DNA-protein complexes (arrows), which were eliminated by 100-fold molar excess of self-competitors but not by the same molar excess of the irrespective oligonucleotides. Supershift assays using the radiolabeled NF- $\kappa$ B and AP-1 probes, untreated nuclear extracts and the indicated polyclonal antibodies to NF- $\kappa$ B and AP-1 components showed that the NF- $\kappa$ B and AP-1 bands consisted of p50, p65 and c-Rel subunits and JunD subunit, respectively (arrowheads).

**FIGURE 7** - 17-AAG induces Akt and PDK1 degradation, and Akt inactivation. MT-2 cells were treated with 1,000 nM 17-AAG for the indicated time periods (a) or for 24 hr with the indicated concentrations of 17-AAG (b), followed by protein extraction. (c) The indicated cell lines were treated with (+) or without (-) 1,000 nM 17-AAG for 24 hr, followed by protein extraction. Whole cell extracts (20  $\mu$ g/lane) of treated cells were immunoblotted with specific antibodies against each protein.

**FIGURE 8** - Effects of 17-AAG on fresh primary ATL cells. PBMCs from patients with ATL were treated with 250 or 1,000 nM 17-AAG. After culture for 24 hr, Western blot analysis was performed using specific antibodies against each protein.

730 **FIGURE 9** - Hsp90 protects Cdk4, Cdk6, survivin, IKK $\alpha$ , IKK $\beta$ , Akt, PDK1 and JunD. MT-2 cells were either pretreated with the proteasomal inhibitor LLnL (5  $\mu$ M) for 1 hr, followed or not by the addition of 17-AAG (1,000 nM) for 24 hr or were treated with 17-AAG for 24 hr or left untreated, as indicated. Samples were analyzed for each protein by Western blotting.