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Anti-adult T-cell leukemia effects of a novel synthetic retinoid, Am80
(Tamibarotene)

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Abbreviations: APL, acute promyelocytic leukemia; ATL, adult T-cell leukemia; ATRA, all-trans retinoic acid; EMSA, electrophoretic mobility shift assay; HTLV-I, human T-cell leukemia virus type I; IL-2Rα, interleukin-2 receptor α chain; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; RT-PCR, reverse transcription-polymerase chain reaction; SCID, severe combined immunodeficient; SDS, sodium dodecyl sulphate; TUNEL, terminal deoxynucleotidyl transferase mediated nick labeling; WST, water-soluble tetrazolium.
Summary

Clinical trials for treatment of adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) using all-trans-retinoic acid (ATRA) have shown satisfactory therapeutic responses, although efficacies were limited. Recently, many synthetic retinoids have been developed, and among them, a novel synthetic retinoid, Am80 (Tamibarotene) is an RARα- and RARβ-specific retinoid expected to overcome ATRA resistance. We examined the inhibitory effects of Am80 on HTLV-I-infected T-cell lines and ATL cells. Am80 had negligible growth inhibition of peripheral blood mononuclear cells but marked growth inhibition of both HTLV-I-infected T-cell lines and ATL cells. Am80 arrested cells in the G1 phase of the cell cycle and induced apoptosis in HTLV-I-infected T-cell lines. It inhibited also the phosphorylation of IκBα and NF-κB-DNA binding, in conjunction with reduction of expression of proteins involved in the G1/S cell cycle transition and apoptosis. Am80 also inhibited the expression of JunD, resulting in suppression of AP-1-DNA binding. Furthermore, severe combined immunodeficient mice with tumors induced by subcutaneous inoculation of HTLV-I-infected T cells, responded to Am80 treatment with partial regression of tumors and no side effects. These findings demonstrate that Am80 is a potential inhibitor of NF-κB and AP-1, and is a potentially useful therapeutic agent against ATL.
Human T-cell leukemia virus type I (HTLV-I) infection causes adult T-cell leukemia (ATL), an aggressive malignancy of CD4$^+$ T cells.($^{1-3}$) ATL develops after a very long latency period and is preceded by oligoclonal expansion of HTLV-I-infected T cells.($^{4}$) Such clonal expansion results from the expression of the viral transcriptional transactivator Tax, which is critical for viral replication, transformation, and gene regulation.($^{5}$) Tax interferes with cell growth control pathways through direct interaction with regulatory proteins and regulation of critical transcription pathways including NF-κB, serum response factor, E2F, and AP-1.($^{5,6}$) Thus, Tax is considered to play a crucial role in several pathways on the transformation of T cells by HTLV-I. On the other hand, Tax expression induces an immune response since it is the major target of cytotoxic T lymphocytes.($^{7}$) Thus, the expression of Tax in HTLV-I-infected T cells provides advantages as well as disadvantages for their survival. To escape from cytotoxic T lymphocytes, ATL cells frequently lose the expression of Tax through several mechanisms, suggesting the requirement for Tax in the initiation but not the maintenance of transformation.($^{8}$) Therefore, Tax may not be a good therapeutic target for ATL. Importantly, fresh ATL cells display exactly the same biochemical phenotype as Tax-expressing cells.

Despite the development of intensive combination chemotherapy regimens, the median survival time of individuals with ATL is less than 13 months.($^{9}$) This extremely grave outcome is mainly due to intrinsic resistance of leukemic cells to conventional or even high doses of chemotherapy and to severe immunosuppression.
Therefore, it is important to find appropriate therapeutic methods to prevent the development of ATL or to prolong survival after its occurrence.

Retinoid is a collective term for compounds that bind to and activate nuclear receptors, retinoic acid receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ), and regulators of cellular proliferation and differentiation. The most important endogenous retinoid is all-trans retinoic acid (ATRA), which is an RARα, β, and γ ligand. ATRA and its mimics are currently used as chemopreventive and therapeutic agents in human cancers, particularly acute promyelocytic leukemia (APL). The clinical usefulness of ATRA in ATL was also reported, although incomplete response was reported. The use of natural retinoids is limited due to their side effects and acquired in vitro and in vivo resistance after prolonged exposure. Therefore, synthetic retinoid analogs that couple increased specific efficacy and reduced toxicity have been developed.

Many synthetic retinoids have been developed and attempts to improve their medicinal properties have been made. Among them, tamibarotene (Am80) is an RARα- and RARβ-specific (but RARγ- and RXRs-nonbinding) synthetic retinoid that is effective in the treatment of relapsed APL. With the objective of finding newer agents for the treatment of ATL, the present study was designed to investigate whether Am80 is a pharmacologically safe and effective inhibitor of cell growth of HTLV-I-infected T cells.

Materials and methods
Reagents. Am80 was synthesized by Toko Pharmaceutical Industrial Co. (Tokyo, Japan). Antibodies to cyclin D2, c-IAP2, IκBα, JunD, NF-κB subunits p65, p50, c-Rel, p52, and RelB, and AP-1 subunits c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to Bax, Bcl-2, Cdk6, c-FLIP, and actin were purchased from NeoMarkers (Fremont, CA, USA). Antibodies to XIAP and cyclin D1 were purchased from Medical & Biological Laboratories, Nagoya, Japan. Antibodies to phospho-IκBα (Ser32 and Ser36), cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), and Bcl-x<sub>L</sub> were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to Tax, Lt-4, was described previously.\(^\text{(19)}\)

Cells. The HTLV-I-infected T-cell lines, MT-2,\(^\text{(20)}\) MT-4,\(^\text{(21)}\) C5/MJ,\(^\text{(22)}\) SLB-1,\(^\text{(23)}\) HUT-102,\(^\text{(1)}\) MT-1,\(^\text{(24)}\) and ED-40515(-),\(^\text{(25)}\) and human myeloid leukemia cell line, HL-60 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. MT-2, MT-4, C5/MJ, and SLB-1 are HTLV-I-transformed T-cell lines, established by an \textit{in vitro} coculture protocol. MT-1 and ED-40515(-) are T-cell lines of leukemic cell origin established from ATL patients. Peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients with ATL were also analyzed. Activated PBMC were grown in RPMI 1640 medium supplemented with phytohemagglutinin (PHA) (10 μg/mL) for 48 h. All samples were obtained after informed consent.
**Cell viability and apoptosis assays.** 1 × 10^5/mL (cell lines) or 1 × 10^6/mL (PBMC) were cultured with various concentrations of Am80 in 96-well plates. After 48 h, cell viability was evaluated by measuring the mitochondrial-dependent conversion of the water-soluble tetrazolium (WST)-8 (Wako Chemicals, Osaka, Japan) to a colored formazan product. For detection of apoptosis, the Annexin V binding capacity of the treated cells was examined by flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA, USA) using Annexin V-Fluos (Roche Diagnostics, Mannheim, Germany).

**Cell cycle analysis.** Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton-Dickinson, Mountain View, CA, USA). Cell suspensions were analyzed on an Epics XL flow cytometer. The population of cells in each cell cycle phase was determined.

**In vitro measurement of caspase activity.** Caspase activity was measured using the colorimetric caspase assay kits (Medical & Biological Laboratories). Cell extracts were recovered using the Cell Lysis buffer and assessed for caspase-3, -8, and -9 activities using colorimetric probes. The colorimetric caspase assay kits are based on detection of chromophore p-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader.

**Western blot analysis.** Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to
electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene
difluoride membrane and probing with the specific antibodies. The bands were
visualized with the enhanced chemiluminescence kit (Amersham Biosciences,
Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was reverse
transcribed using an RNA PCR kit (Takara Bio Inc, Otsu, Japan). Thereafter, cDNA
was amplified for 40 cycles for RARs, RXRs, and peroxisome proliferator-activated
receptor (PPAR) γ, 22 cycles for cyclin D2 and c-IAP2, and 28 cycles for β-actin. The
oligonucleotide primers for c-IAP2 were as follows: sense,

5' - GTTTCCAAGGTGTGAGTACTT-3' and antisense,
5' - TCTGCATTGAGTAAGTCTAACA-3'. The other oligonucleotide primers used
were described previously. Product sizes were 400 bp for RARα, 435 bp for
RARβ, 428 bp for RARγ, 460 bp for RXRα, 462 bp for RXRβ, 440 bp for RXRγ, 250
bp for PPARγ, 268 bp for cyclin D2, 328 bp for c-IAP2, and 548 bp for β-actin.
Cycling conditions were as follows: denaturing at 94°C for 30 s (for RARs, RXRs,
c-IAP2, and β-actin) or for 60 s (for PPARγ and cyclin D2), annealing at 57°C (for
RARs), 55°C (for RXRs), 64°C (for c-IAP2), or 60°C (for β-actin) for 30 s, or 57°C
(for PPARγ) or 55°C (for cyclin D2) for 60 s, and extension at 72 °C for 60 s (for
RARs, RXRs, and c-IAP2), for 90 s (for PPARγ and β-actin), or for 120 s (for cyclin
D2).
Electrophoretic mobility shift assay (EMSA). EMSA was done as described previously. Briefly, 5 μg of nuclear extract was incubated with [\(^{32}\)P]-labeled probes. The DNA-protein complex was separated from the free oligonucleotides on a 4% polyacrylamide gel.

Reporter assay. For reporter assays, an NF-κB site-dependent luciferase vector, κB-LUC, and an expression plasmid for Tax, were used. Transfections were performed in Jurkat cells by electroporation using 5 × 10^6 cells and 2 μg of κB-LUC with 0.1 μg of Tax expression plasmid or empty vector. In all cases, the reference plasmid, 2 μg of phRL-TK, which contains the Renilla luciferase gene under the control of the herpes simplex virus thymidine kinase promoter, was cotransfected to correct for transfection efficiency. After 16 h, the cells were treated with Am80 for 12 h, and then collected by centrifugation, washed with phosphate-buffered saline, and lysed in reporter lysis buffer (Promega, Madison, WI, USA). Luciferase assays were performed by using the Dual-Luciferase Reporter System (Promega), in which the relative luciferase activity was calculated by normalizing transfection efficiency according to the Renilla luciferase activities.

In vivo therapeutic effect of Am80. Five-week-old female C.B-17/Icr-SCID mice were obtained from Ryukyu Biotec Co. (Urasoe, Japan). Mice were engrafted with 1 × 10^7 HUT-102 cells by subcutaneous injection in the postauricular region and then randomly placed into two groups of four mice each, one received vehicle only, while the other was treated with Am80. Treatment was initiated on the next day of cell
inoculation. Am80 was dissolved in soybean oil at a concentration of 1 mg/mL, and 15 mg/kg body weight of Am80 was administered by oral gavage every day for 28 days. Control mice received the same volume of the vehicle (soybean oil) only for 28 days. Tumor size was monitored once a week. All mice were sacrificed on day 28, and then the tumors were dissected out and their weight was physically measured. Thereafter, tumors were fixed for paraffin embedding and tissue sectioning. Analysis of DNA fragmentation by fluorescent terminal deoxynucleotidyl transferase mediated nick labeling (TUNEL) was performed using a commercial kit (Takara Bio Inc) as described in the instructions provided by the manufacturer. Treatment efficacy was determined by measuring the serum levels of soluble interleukin-2 receptor α chain (IL-2Rα) by ELISA (BioSource, Camarillo, CA, USA) 28 days after inoculation. This experiment was performed according to the Guidelines for the Animal Experimentation of the University of the Ryukyus and was approved by the Animal Care and Use Committee of the University of the Ryukyus.

**Statistical analysis.** Statistical analyses were carried out by the Mann-Whitney U-test.

**Results**

**Am80 induces growth inhibition of HTLV-I-infected T-cell lines and primary ATL cells.** First, we determined whether Am80 affects the growth of HTLV-I-infected T-cell lines and PBMC from normal healthy controls. Tax protein
was detected by immunoblot analysis in the five HTLV-I-infected T-cell lines (MT-2, MT-4, C5/MJ, SLB-1, and HUT-102) but not in the two ATL-derived T-cell lines [MT-1 and ED-40515(-)]. Cell growth was assessed by the WST-8 assay.

Treatment with Am80 for 48 h resulted in a dose-dependent growth inhibition of all tested HTLV-I-infected T-cell lines (Fig. 1a). The concentrations of Am80 required to inhibit growth of HTLV-I-infected T-cell lines by 50% (IC50 values) ranged from 25.3 to 42.9 μM. Although the sensitivity to Am80 varied among the cell lines studied, Tax did not influence the susceptibility to Am80 among the HTLV-I-infected T-cell lines. On the other hand, HTLV-I-uninfected myeloid leukemia cell line, HL-60, was less susceptible to Am80 than HTLV-I-infected T-cell lines (with an IC50 of 68.1 μM). Importantly, normal PBMC were resistant to Am80. In contrast, PHA-activated PBMC proliferation was inhibited by Am80 in a dose-dependent manner (Fig. 1b).

We also examined the effects of Am80 on freshly isolated ATL cells from eight acute type patients. Tax protein was not detected by immunoblot analysis in all patients (data not shown). ATL cells treated with Am80 showed reduced cell survival compared with normal healthy controls (Fig. 1b).

**Expression patterns of RARs, RXRs, and PPARγ.** Two subfamilies of nuclear receptors, RARs and RXRs, mediate the biological effects of various retinoids. These receptors are also thought to bind a variety of synthetic retinoids. Using RT-PCR, we evaluated the expression of RARs α, β, and γ, RXRs α, β, and γ, and also PPARγ, which is known to heterodimerize with RXRα. Fig. 1c shows RXRα and RXRβ transcripts obtained in all HTLV-I-infected T-cell lines, while PPARγ was
detected in MT-2 cells only. The degree and pattern of RARs did not explain the sensitivity to Am80 in HTLV-I-infected T-cell lines.

**Am80 induces apoptosis in HTLV-I-infected T-cell lines.** To investigate the mechanisms by which Am80 inhibited the growth of HTLV-I-infected T-cell lines, we analyzed apoptosis by the Annexin V method. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic of cells entering apoptosis. A significant increase in the proportion of cells positive for Annexin V was detected in all cell lines after treatment with 100 μM Am80 for 48 h (Fig. 2a). Consistent with these results, apoptosis was also confirmed by immunostaining with Apo2.7, which specifically detects the 38 kDa mitochondrial membrane antigen 7A6, which is expressed on the mitochondrial outer membrane during apoptosis (data not shown).\(^{35,36}\)

**Am80-induced apoptosis is caspase-dependent.** We then investigated whether the observed apoptosis was due or not to caspase activation. Cell extracts were obtained after various treatments and processed for Western blot analysis. Indeed, in both HUT-102 and ED-40515(-) cells, Am80-induced apoptosis was associated with caspase activation, as shown by PARP cleavage (Fig. 2b). Furthermore, Am80 treatment resulted in cleavage of procaspase-3. In addition, we assessed caspase-3, -8, and -9 activities using colorimetric probes. Am80 resulted in activation of caspases-3, -8, and -9 in both cell lines (Fig. 2c). These results indicate that Am80-induced apoptosis of HTLV-I-infected T-cell lines is mediated through caspase activation.
Am80 causes G1 cell cycle arrest. We then examined the cellular DNA contents distribution by flow cytometric analysis on cell treatment. ED-40515(-) cells were incubated with 100 μM Am80 for various periods (Fig. 2d). Cultivation with Am80 for 6 h increased the population of the cells in the G1 phase, with a marked reduction of the cells in the S phase, relative to untreated cells. At 24 h after treatment, the percentage of cells in sub-G1 markedly increased from 2.8 in untreated cells to 41.5%, suggesting that cell cycle arrest is the cause of apoptosis. Cultivation with Am80 for 6 h increased the proportion of cells in the G1 phase, with a reduction of cells in the S phase in all cell lines (Fig. 2e). These results indicate that, together with induction of apoptosis, Am80 treatment induces a G1 cell cycle arrest in HTLV-I-infected T-cell lines, which likely contributes to the growth inhibitory effects.

Effects of Am80 on cell cycle and apoptosis regulatory proteins. To clarify the molecular mechanisms of Am80-induced inhibition of cell growth and apoptosis of HTLV-I-infected T-cell lines, we investigated the effect of Am80 on the expression of several intracellular regulators of cell cycle and apoptosis including cyclin D1, cyclin D2, Cdk6, XIAP, c-IAP2, Bcl-xL, Bcl-2, Bax, and c-FLIP by Western blot analysis. As shown in Fig. 3a,b, Am80 did not alter cyclin D1, Bcl-2, and Bax expression levels. In contrast, Am80 significantly decreased the expression of cyclin D2, Cdk6, XIAP, c-IAP2, Bcl-xL, and c-FLIP in HUT-102 cells in a dose-dependent manner. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin (Fig. 3a,b). Because cyclin D2, Cdk6, XIAP, c-IAP2,
Bcl-x\textsubscript{L}, and c-FLIP are Tax-responsive genes,\textsuperscript{(37-42)} we also examined the level of Tax expression. Am80 did not change the protein level of Tax in HUT-102 cells (Fig. 3a). We also explored the effects of Am80 on the expression of these proteins in ATL-derived ED-40515(-) cells (Fig. 3a,b) and freshly isolated ATL cells (Fig. 3c), without detectable Tax expression. A similar inhibition of the expression was observed in the analysis of ED-40515(-) cells. XIAP and cyclin D2 were detectable in primary ATL cells from ATL 7 and ATL 6, respectively. Am80 also decreased both expression. Am80 decreased Bcl-x\textsubscript{L} expression in ATL cells from two cases. Cdk6 and c-IAP2 were not detectable in both cases. Consistent with the decreased expression of XIAP and Bcl-x\textsubscript{L}, Western blot analysis of ATL cell extracts demonstrated that cleaved PARP increased dramatically. These results indicate that the altered expression levels of cyclin D2, Cdk6, XIAP, c-IAP2, Bcl-x\textsubscript{L}, and c-FLIP proteins did not result from Tax downregulation. To investigate the change in expression levels of intracellular regulators at transcriptional level, we examined the expression of cyclin D2 and c-IAP2 mRNA by RT-PCR. The results demonstrated downregulation of both genes by Am80 (Fig. 3d). Thus, Am80 downregulates the expression of intracellular regulators at transcriptional level.

**Inhibitory effects of Am80 on NF-\kappa B activation.** Several reports have suggested that NF-\kappa B can prevent apoptosis and caspase activation as a survival factor and is required for the proliferation of various tumor cell types.\textsuperscript{(43)} Because NF-\kappa B is constitutively active in Tax-expressing and HTLV-I-infected T-cell lines as well as primary ATL cells,\textsuperscript{(30)} and Tax stimulates the expression of cyclin D2, Cdk6, XIAP,
c-IAP2, Bcl-xL, and c-FLIP through the NF-κB pathway,\(^{(37-42)}\) we examined whether Am80 inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we performed EMSA with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from untreated or Am80-treated HTLV-I-infected T-cell lines. NF-κB oligonucleotide probe with nuclear extracts from untreated HTLV-I-infected T-cell lines generated DNA-protein gel shift complexes (Fig. 4a). These complexes were due to specific bindings of nuclear proteins to the NF-κB sequences because the binding activities were reduced by the addition of cold probe but not by an irrespective sequence (Fig. 4a, left, lanes 2,3). We also showed that NF-κB complexes contain p50, p65, and c-Rel in HUT-102 cells and p50 and p65 in ED-40515(-) cells, respectively (Fig. 4a, left, lanes 4-6). As shown in Fig. 4b,c, nuclear extracts prepared from HTLV-I-infected T-cell lines treated with Am80 exhibited a decrease in the intensity of the NF-κB-containing gel shift complexes in dose- and time-dependent manners, suggesting that Am80 downregulates the DNA-binding activities of NF-κB. Inhibition appeared specific to NF-κB and not due to cell death because no significant change in binding activity of Oct-1 was observed after treatment of cells with Am80 (Fig. 4b).

Degradation of IκBα and subsequent release of NF-κB require prior phosphorylation at Ser32 and Ser36 residues.\(^{(44)}\) To investigate whether the inhibitory effects of Am80 are mediated through alteration of phosphorylation of IκBα, HUT-102 and ED-40515(-) cells were treated with Am80 and their protein extracts were checked for phosho-IκBα expression. Untreated cells constitutively expressed Ser32- and Ser36-phosphorylated IκBα (Fig. 4d), while Am80 decreased the
phosphorylated IκBα in a dose-dependent manner, with a concomitant accumulation of IκBα, suggesting that Am80 inhibited phosphorylation of IκBα, followed by accumulation of this protein.

In addition to NF-κB DNA-binding activity, we investigated the effect of Am80 on the transcriptional activity of NF-κB. We examined the effect of Am80 on Tax-induced NF-κB transcriptional activity. Tax expression plasmid, together with luciferase reporter plasmid regulated by NF-κB elements (κB-LUC) were transfected into Jurkat cells. Then the cells were treated with Am80 for 12 h. Am80 suppressed Tax-induced NF-κB transcriptional activation in a dose-dependent manner (Fig. 4e).

**Inhibitory effects of Am80 on AP-1 activation.** AP-1 is also a crucial mediator of both cell cycle promoting and cell-death inhibiting pathways in HTLV-I-infected T cells. Therefore, we examined the effect of Am80 treatment on AP-1. High constitutive AP-1 DNA-binding activities were detected in HUT-102 and ED-40515(-) cells (Fig. 4a, right). Supershift analysis with antibodies indicated that the AP-1 complex in both cell lines contained JunD. As shown in Fig. 4b, AP-1 DNA-binding activity diminished in the presence of Am80 in dose- and time-dependent manners. In addition, Am80 also dose-dependently decreased the expression of JunD (Fig. 4d). These findings suggest that Am80 depletes JunD, resulting in inactivation of AP-1.

**In vivo effects of Am80 on SCID mice inoculated with HTLV-I-infected T-cell line.** Because the Am80-induced growth inhibition of ATL cells suggests that Am80
can be an effective agent to treat ATL, we finally examined the in vivo effects of Am80 in a SCID mouse model. After 28-day treatment, the mean tumor volume (Fig. 5a) and weight (Fig. 5b) were significantly lower than those of vehicle-treated mice ($P < 0.05$). The efficacy of the treatment was reflected by a decrease in serum levels of the surrogate marker soluble IL-2Rα; the soluble IL-2Rα levels tended to be lower in mice treated with Am80 than with the vehicle, albeit statistically insignificant ($P = 0.15$; Fig. 5c). On the other hand, TUNEL assay showed few apoptotic cells in tumors from untreated mice, while abundant apoptotic cells were noted in tumors from Am80-treated mice (Fig. 5d). There was no significant difference in body weight gain found during the period from day 0 to day 28 among the vehicle group and the group treated with Am80 (data not shown). During this period, mice treated with Am80 appeared generally healthy. These results suggest that Am80 also has in vivo anti-ATL effect.

**Discussion**

The results of the present study indicate that Am80 induces growth inhibition in all tested HTLV-I-transformed and ATL-derived cell lines as well as primary ATL cells. Importantly, Am80 had negligible effect on normal PBMC. Here we describe a novel mechanism of action in HTLV-I-infected T cells, where Am80-induced growth inhibition was associated with NF-κB inhibition. It is possible that this NF-κB inhibition is associated with the exquisite sensitivity of HTLV-I-infected T cells to Am80, because NF-κB is inactivated in uninfected PBMC. Consistent with this,
Am80 inhibited the proliferation of PHA-activated PBMC, where NF-κB is activated (data not shown).

The HTLV-I encodes the oncoprotein Tax from its pX gene, which plays a central role in leukemogenesis of ATL. Tax activates not only viral replication but also induces the expression of cellular genes through NF-κB activation. The Tax/NF-κB signaling pathway was implicated as a potential target for ATRA therapy because transfection of tax gene into a T-cell line conferred ATRA sensitivity with decreased NF-κB transcriptional activity. We examined the level of Tax expression by Western blot analysis in HTLV-I-infected T-cell line, HUT-102, but Tax was not a molecular target of Am80 treatment. Furthermore, Am80 induced growth inhibition in Tax-negative HTLV-I-infected T-cell lines, MT-1 and ED-40515(-), and primary ATL cells. Therefore, the effects of Am80 on HTLV-I-infected T-cell lines and primary ATL cells appear to be mediated through a Tax-independent pathway.

AP-1 is also known to regulate cell proliferation, differentiation, and apoptosis in various cell lines, and required for proliferation of HTLV-I-infected T cells. This study also shows that Am80 inhibited JunD expression, resulting in the suppression of AP-1 DNA-binding in HTLV-I-infected T-cell lines. The cyclin D2 promoter contains NF-κB and AP-1 sites. It is therefore likely that NF-κB and AP-1, in concert, support proliferation of HTLV-I-infected T cells by activating cyclin D2. We speculate that Am80 inhibits cyclin D2 expression through the suppression of both NF-κB and AP-1, resulting in the induction of G1 cell cycle arrest. Based on these findings, it is likely that Am80 exerts its anti-ATL effects through the suppression of HTLV-I-induced activation of NF-κB and AP-1. Previously, we have
shown that the blockade of NF-κB by the novel synthetic retinoid NIK-333 effectively induces apoptosis of HTLV-I-infected T cells.\textsuperscript{(48)} However, NIK-333 did not alter JunD expression. Thus, the blockade of AP-1 may be a feature characteristic of Am80.

The effects of retinoic acid are thought to be mediated by the nuclear retinoid receptors; however, not all the effects of retinoic acid can be explained by the nuclear receptor pathway. Indeed, the degree and pattern of RARs did not explain the sensitivity to Am80 in HTLV-I-infected T-cell lines. Mechanisms other than those involving nuclear receptors may be involved in the biological effects of Am80.

Retinoylation (acetylation of proteins by retinoic acid) is another mechanism underlying retinoic acid action.\textsuperscript{(49)} The retinoylation mechanism involves the formation of a retinoyl-CoA intermediate\textsuperscript{(50)} and the subsequent transfer and covalent binding of the retinoyl moiety to protein(s).\textsuperscript{(51)} Further investigation of retinoylation may help to reveal the exact mechanism(s) underlying the effects of Am80 in HTLV-I-infected T cells.

In SCID mice bearing an HTLV-I-infected T-cell line, 15 mg/kg body weight of Am80 inhibited the growth of tumors derived from HUT-102 cells, suggesting that Am80 can exert a growth-inhibitory effect on ATL cells \textit{in vivo}. Non-specific drug toxicity is one of the major problems in drug development. IC\textsubscript{50} of Am80 and ATRA ranged from 25.3 to 42.9 μM and 8.0 to 22.7 μM, respectively. Importantly, normal PBMC were resistant to Am80 up to 100 μM but sensitive to ATRA up to 50 μM.\textsuperscript{(48)} Am80 is chemically more stable to light, heat, and oxidation than ATRA; has a low affinity for cellular retinoic acid binding protein; and does not bind to RARγ.\textsuperscript{(52,53)} Therefore, Am80 would be expected to have therapeutic effectiveness in patients with
ATRA-resistant APL with increased cellular retinoic acid binding protein, and to have fewer adverse drug reactions related to RARγ, which is the major RAR in the dermal epithelium.\(^{(54)}\) Although IC50 of NIK-333 was low compared with that of Am80,\(^{(48)}\) we used 100 mg/kg of NIK-333 in our model. This dose is far high than that of administered Am80. Am80 may be a feasible and less toxic candidate as a novel anti-ATL agent, although the pharmacokinetics is not yet elucidated.

In conclusion, we have demonstrated the efficiency of Am80 in inducing cell growth arrest and death through inactivation of NF-κB and AP-1 pathways in HTLV-I-infected T cells, and anti-ATL effect in SCID mice bearing an HTLV-I-infected T-cell line. We are now conducting a clinical trial of this Am80 in patients with ATL.

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References


Figure legends

**Fig. 1.** Am80 inhibits growth of HTLV-I-infected T-cell lines and PBMC from ATL patients. HTLV-I-infected T-cell lines (a), PBMC from ATL patients (b) and healthy controls (a,b), and PHA (10 μg/mL) activated PBMC (b) were cultured with the indicated concentrations of Am80 for 48 h, and cell growth was determined in triplicate cultures by WST-8 assay. The results are expressed as percentage of control and represent the mean ± SD of the results obtained. (c) Expression of nuclear receptor mRNA in HTLV-I-infected T-cell lines. β-actin was used as an internal control. Representative results of three experiments with similar findings.

**Fig. 2.** Am80 induces G1 cell cycle arrest and apoptosis in HTLV-I-infected T-cell lines. (a) HTLV-I-infected T-cell lines were cultured in the absence or presence of Am80 (100 μM). After 48 h, Annexin V staining was analyzed by flow cytometry. Data represent mean ± SD percentages of apoptotic cells for both untreated and Am80-treated cells. (b) Cells were treated with the indicated concentrations of Am80 for 24 h and subjected to immunoblotting analyses with the indicated antibodies. Representative data of three experiments with similar results. (c) The indicated cell lines were treated with or without Am80 (100 μM) for 24 h. Caspase activity was assayed as described in *Materials and methods* and expressed relative to untreated cells, which were assigned a value of 1. Values represent mean ± SD of three experiments. (d) ED-40515(-) cells were incubated with Am80 (100 μM) for the indicated time periods. (e) The indicated cell lines were incubated in the absence or
presence of Am80 (100 μM) for 6 h. Cell cycle distribution was analyzed by flow
cytometry by staining with propidium iodide. Three independent experiments per cell
line were performed and results are presented as mean percentage.

Fig. 3. Effect of Am80 on cell cycle and apoptosis regulatory proteins. Cells were
treated with various concentrations of Am80 for 24 h (a,b). PBMC from ATL patients
were also treated with Am80 (100 μM) for 24 h (c). Whole cell extracts were prepared
and immunoblotted with specific antibodies (a,b,c). (d) Total RNA was extracted from
ED-40515(-) cells following treatment with various concentrations of Am80 for 24 h.
The mRNA expression of cyclin D2 and c-IAP2 was analyzed by RT-PCR analysis.
β-actin served as an internal control in the RT-PCR procedure. Representative data of
three experiments each with similar results.

Fig. 4. Am80 inhibits NF-κB activity in HTLV-I-infected T-cell lines. (a) EMSA
using untreated HUT-102 and ED-40515(-) nuclear extracts and radiolabeled NF-κB
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-κB and
AP-1 probes, untreated nuclear extracts, and the indicated antibodies to NF-κB and
AP-1 components showed that the NF-κB and AP-1 bands consisted of p50, p65, and
c-Rel subunits and JunD subunit, respectively. (b,c) Effects of Am80 on activation of
NF-κB, AP-1, and Oct-1 in HTLV-I-infected T-cell lines assessed by EMSA using
oligonucleotide probes for NF-κB, AP-1, and Oct-1, respectively. Cells were treated
with the indicated concentrations of Am80 for 24 h (b) or with Am80 (100 μM) for the indicated time periods (c). (d) Effects of Am80 on the levels of IκBα and phosphorylated IκBα and JunD by Western blot analysis. Cells were treated with various concentrations of Am80 for 24 h, followed by protein extraction. Whole cell extracts of treated cells were immunoblotted with specific antibodies. Representative data of three experiments with similar results. (e) Inhibition of Tax-induced NF-κB transcriptional activation by Am80. κB-LUC was transfected into Jurkat cells with Tax expression plasmid or empty vector. Luciferase activity is expressed relative to the basal level measured in cells transfected with the reporter plasmid and Tax without further treatment, which was defined as 100. Data are expressed as mean ± SD of three separate transfections.

**Fig. 5.** Inhibition of growth of HUT-102 cells in SCID mice. (a) Growth of the tumors after inoculation of HUT-102 cells subcutaneously. The mice were monitored for tumor volumes. (b) Weight of tumors removed from Am80-treated mice and untreated mice on day 28 after cell inoculation. (c) Serum levels of soluble IL-2Rα in Am80-treated and -untreated mice. Serum soluble IL-2Rα levels tended to be lower in mice treated with Am80 than in those treated with the vehicle control, albeit statistically insignificant. Data are expressed as mean ± SD of four mice in each group. (d) TUNEL assays show apoptotic cells in tumors from mice treated with vehicle control or Am80. Note the presence of only few apoptotic cells in tumors from the control mice (left), compared with the abundant apoptotic cells in tumors from the Am80-treated mice. Magnification, ×100.