

In vitro effects of arsenic trioxide, interferon α and zidovudine in adult T cell leukemia/lymphoma cells

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Abstract. Despite the efficacy of combination chemotherapy with arsenic trioxide (ATO), interferon α (IFN) and zidovudine (AZT) for adult T cell leukemia/lymphoma (ATL), the precise mechanism underlying this combination treatment effect is unknown. In the present study, ATO/IFN/AZT was examined in an ATL leukemic cell line (S1T, non-Tax expressing), a human T-lymphotropic virus 1 (HTLV-1)-infected cell line (MT2, Tax-expressing) and primary ATL cells from patients with acute and chronic ATL. IFN/AZT marginally inhibited MT2 cell proliferation, but substantially inhibited S1T cell proliferation. IFN/AZT increased the cleavage of numerous caspases and PARP in S1T cells, and regulated the signal transducer and activator of transcription 1 and nuclear factor- κ B signaling pathway. These effects represent the potential anti-ATL mechanisms of IFN/AZT *in vitro*. In addition, the combination of ATO and IFN/AZT demonstrated synergistic effects on S1T cells. Therefore, the Tax-independent mechanism underlying the anti-ATL effect of ATO must be further elucidated.

Introduction

Adult T cell leukemia/lymphoma (ATL) is a hematological malignancy derived primarily from cluster of differentiation (CD)4⁺/CD25⁺/C-C Motif Chemokine Receptor 4 (CCR4)⁺ T cells (1). Combination cytotoxic chemotherapy results in 3-year overall survival (OS) rates of ~24% in medically fit patients with ATL (2). Allogeneic stem cell transplantation (allo-SCT) produces long-term remission,

but transplantation-associated toxicity remains a significant concern (3). In addition, allo-SCT confers limited benefits in elderly patients with ATL and those patients with ATL and progressive disease status following inductive chemotherapy (4). Recently, treatment with an anti-CCR4 antibody (mogamulizumab) demonstrated promising results for patients with relapsed/refractory ATL (5); however, studies of long-term outcomes and side effect profiles are required for an accurate assessment of its efficacy and safety.

Human T lymphotropic virus (HTLV)-1 is a known causative agent of ATL; thus, antiviral therapy using interferon α (IFN α) and zidovudine [azidothymidine (AZT)] has been proposed as an alternative therapy for ATL (6). Despite the clinical efficacy of IFN α /AZT, the exact mechanism of IFN α /AZT against ATL is currently unknown (7). Recently, combination therapy using arsenic trioxide (ATO) and IFN α /AZT exhibited promising outcomes in patients with chronic and untreated ATL (8). Furthermore, Dassouki *et al* (9) recently reported that the anti-ATL effect of ATO/IFN α is triggered by Tax, an HTLV-1-derived oncoprotein that is degraded through the small ubiquitin-related modifier 1 (SUMO)/protein PML (PML)/E3 ubiquitin-protein ligase RNF4 (RNF-4) signaling pathway. However, Tax expression at the transcript and protein levels is often undetectable at the leukemic stage (10). In addition, certain ATL cells have Tax mutations (10).

In the present study, the actions of ATO/IFN α /AZT in ATL cell lines and primary ATL cells were assessed. It was identified that IFN α /AZT induces apoptotic cell death via caspase activation in the S1T cell line, a non-Tax-expressing ATL patient-derived cell line, as well as in primary ATL cells obtained from a patient with ATL. Combining ATO with IFN α /AZT produced a synergistic anti-ATL effect. To the best of our knowledge, this is the first *in vitro* evidence to demonstrate the cell growth-inhibition effect of IFN α /AZT in a non-Tax-expressing ATL patient-derived cell line and primary ATL cells. Although not all HTLV-1 derived cell lines and primary ATL cells were killed following treatment with IFN α /AZT, IFN α /AZT was able to produce anti-ATL effects via pro-apoptotic signaling. These results demonstrate that IFN α /AZT exhibits anti-ATL effects *in vitro*; however, this effect may be limited to a subsection of population of patients with ATL.

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Materials and methods

Clinical samples. The subjects evaluated in this study included four patients with ATL (two acute-type and two chronic-type; age, 52-66; 2 male and 2 female) who were treated from 2012-2014 at Kagoshima University Hospital (Kagoshima, Japan). The subjects were examined by standard serological testing for the presence of HTLV-1, and by hematological/Southern blotting analysis for the diagnosis of ATL. The classification of ATL was performed according to the Shimoyama criteria (11). All subjects provided written informed consent for participation in this study, their medical records and a sample of peripheral blood for the isolation of peripheral blood mononuclear cells (PBMCs). The study protocol was reviewed and approved by the Medical Ethics Committee of Kagoshima University Hospital. PBMCs were separated from the peripheral blood samples by Ficoll/Hypaque (Pharmacia Biotech; GE Healthcare Life Sciences, Uppsala, Sweden) density gradient centrifugation.

Cell lines. The S1T cell line was derived from a patient with acute-type ATL (12). The integration site of the HTLV-1 provirus in S1T cells was identical to the ATL leukemic clone found in the patient with ATL, and was confirmed by inverse polymerase chain reaction (PCR) (13). Tax protein expression in S1T cells is barely detectable by western blotting, but *Tax* mRNA expression is detectable by reverse transcription-quantitative PCR (12). The MT2 cell line is an HTLV-1-infected T cell line derived from normal human leukocytes transformed by leukemic T cells obtained from a patient with ATL (14). Tax expression is detectable in MT2 cells by western blotting (15). S1T cells, MT2 cells, and freshly isolated ATL cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 50 U/ml recombinant human interleukin-2 (IL-2; PeproTech, Inc., Rocky Hill, NJ, USA).

Reagents. Recombinant IFN α -2b (1,000 U/ml, Intron[®] A; Merck KGaA, Darmstadt, Germany), 5 μ M AZT (GlaxoSmithKline, Brentford, UK) and 1 μ M ATO (Trisenox[®]; Nippon Shinyaku Co., Kyoto, Japan) were used for *in vitro* experiments, unless otherwise specified. Caspase inhibitor Z-VAD-FMK was purchased from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan).

Primary antibodies against Poly (ADP-ribose) polymerase (PARP), cleaved-PARP (#9542; 1:2,000 dilution), caspase-9 (#9502; 1:2,000 dilution), caspase-8 (#4790; 1:2,000 dilution), cleaved caspase-8 (#9496; 1:2,000 dilution), inhibitor of κ light polypeptide gene enhancer in B-cells (I κ B) kinase β (IKK β) (#2370; 1:2,000 dilution), phospho-IKK α / β (#2697; 1:2,000 dilution), inhibitor of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (I κ B α) (#4814; 1:2,000 dilution), phospho-I κ B α (#2859; 1:2,000 dilution), nuclear factor κ B (NF κ B) (#8242; 1:2,000 dilution), NF κ B p65, phospho-NF κ B p65 (#3033; 1:2,000 dilution), signal transducer and activator of transcription 1 (STAT1) (#9172; 1:2,000 dilution), phospho-STAT1 (#8826; 1:2,000 dilution), phospho-p53

(#9284; 1:2,000 dilution), apoptosis-inducing factor (AIF) (#4642; 1:2,000 dilution), and histone H3 (#4499; 1:2,000 dilution) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against p53 (sc-6243; 1:500 dilution) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against cytochrome *c* (S2207; 1:100 dilution) and cytochrome *c* oxidase 4 (COX4; S2050; 1:500 dilution) were obtained from Clontech (Mountain View, CA, USA). Anti- β -actin antibodies (A5441; 1:5,000 dilution) were obtained from Sigma-Aldrich (Merck KGaA).

Cell viability assay. The effects of ATO/IFN α /AZT on cell viability were examined using a cell proliferation reagent and cell counting reagent, according to the manufacturer's instruction (cell count reagent SF; Nacalai Tesque, Inc., Kyoto, Japan). Briefly, 96-well plates containing 1×10^4 cells/well were incubated at 37°C in the absence or presence of ATO/IFN α /AZT for 48 h. WST-8 reagent (Cell counting kit-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to each well and the cells were incubated for 4 h at 37°C in the dark, after which the absorbance at 450 nm (A_{450}) was determined using a Multiskan[™] FC microplate (Thermo Fisher Scientific, Inc.). The viability of the treated cells was expressed relative to that of the untreated control cells, which were considered to be 100% viable. RPMI-1640 medium without phenol-red was used for the WST-8 assay.

Analysis of combination effects. To determine the concentrations of the drugs to be investigated in the combination study, dose-response curves were generated for ATO and IFN α /AZT alone. Experiments with ATO and IFN α /AZT in a fixed ratio combination (a total of 1,000 U/ml IFN α and 5 μ M AZT were defined as 1.0) were performed using a WST-8 assay. Possible drug interactions were calculated using an IC₅₀ plot (16).

Protein extraction and western blot analysis. Whole-cell extracts were lysed in cell lysis buffer, according to the manufacturer's protocol (Cell Signaling Technology, Inc.) with or without a protease inhibitor cocktail (Thermo Fisher Scientific, Inc.) and a Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.). Mitochondrial fractions and nuclear extracts were obtained using an ApoAlert Cell Fraction kit (Clontech Laboratories, Inc.) and a Nuclear/Cytosol Fraction kit (BioVision, Inc., Milpitas, CA, USA), respectively, according to the manufacturer's protocols. The whole-cell, mitochondrial and nuclear extracts were used immediately or stored at -80°C. Western blotting was performed as follows: Cell extracts were subjected to 4-15% SDS-PAGE (Mini-Protean TGX gels; Bio-Rad Laboratories, Inc., Hercules, CA, USA), electroblotted onto Immobilon-P[®] PVDF membranes (Bio-Rad Laboratories, Inc.), and analyzed for immunoreactivity with the appropriate primary and secondary antibodies (anti-rabbit IgG, HRP-linked antibody was used for all the primary antibodies from Cell Signaling Technologies, Inc.; goat anti-rabbit HRP Conjugate was used for anti-p53 and anti-cytochrome *c*; goat anti-mouse IgG-HRP was used for anti-COX4 and anti-beta actin) using Can Get Signal[®] Solution (Toyobo Co., Ltd., Osaka, Japan). The reaction products were visualized using an ECL Advance Western Blotting Detection kit (GE Healthcare BioSciences, Pittsburgh, PA, USA).

NF κ B reporter assay. NF κ B activity was evaluated using a firefly luciferase reporter assay (E1910; Promega Corporation, Madison, WI, USA). Reporter cell lines were established by transducing SIT cells with a Cignal Lenti NF κ B Reporter or a Cignal Lenti TK *Renilla* Control (Puro; Qiagen N.V., Venlo, the Netherlands), according to the manufacturer's protocols. Luciferase assays were performed with firefly luciferase or *Renilla* luciferase assay systems (Promega Corporation) on cell lysates in *Renilla* luciferase lysis buffer (Promega Corporation). Relative NF κ B activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity in each of the samples, according to the manufacturer's protocol (Promega Corporation).

Statistical analysis. Data are expressed as the mean \pm standard deviation. For data analysis, two-tailed Student's t-test and the one-way analysis of variance (ANOVA) were performed using EZR (17). ANOVA was used to determine whether there are any statistically significant differences between the means of three or more independent groups. Dunnett's method was used for multiple comparisons with a control group. Bonferroni's correction was used for an adjustment made to P-values when several dependent or independent statistical tests were being performed simultaneously. In all tests, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ATO/IFN α /AZT inhibit the viability of SIT cells by inducing apoptosis. In the first set of experiments, it was examined whether ATO/IFN α /AZT affected the viability of SIT cells (patient-derived non-Tax-expressing ATL cells) using WST-8 assays. IFN α and ATO significantly inhibited the growth of SIT cells in a dose-dependent manner, with IC₅₀ values of 1,100 U/ml and 1.68 μ M, respectively. Treatment with AZT alone had little observable effect on the proliferation of SIT cells ($P = 0.79$); however, the combination of IFN α and AZT was more effective than IFN α alone ($P < 0.005$). Of the examined treatments, the combination of ATO, IFN α and AZT most effectively inhibited the proliferation of SIT cells. Notably, ATO and IFN α /AZT demonstrated a synergistic effect on SIT cell proliferation (Fig. 1A and B). IFN α /AZT had a marginal effect on MT2 cells (data not presented), whereas ATO inhibited the growth of MT2 cells in a dose-dependent manner.

To clarify the molecular mechanisms underlying the ATO/IFN α /AZT-induced inhibition of SIT cell proliferation, the expression levels of various intracellular regulators of apoptosis were examined using western blotting. IFN α /AZT treatment increased the expression levels of cleaved PARP and cleaved caspase-3, caspase-7, caspase-8 and caspase-9, indicating that these cysteine proteases are involved in the regulation of apoptosis in SIT cells (Fig. 1C). The effect of IFN α /AZT treatment on PARP and caspase cleavage was attenuated by inhibitors of caspase-3, caspase-8 and caspase-9, as well as by a pan-caspase inhibitor (Fig. 1D). Treatment with ATO alone had a less marked effect on cleaved PARP expression than treatment with IFN α /AZT, indicating that the inhibition of SIT cell proliferation by ATO is not primarily due to pro-apoptotic signaling.

To further dissect the mechanism of ATO/IFN α /AZT induced apoptosis in SIT cells, the mitochondrial apoptosis signaling pathway was examined. In this process, mitochondrial

protein AIF is released as a result of mitochondrial outer membrane permeabilization, which leads to the release of pro-apoptotic proteins from the mitochondrial intermembrane space and the promotion of caspase-independent cell death (18). Cytosolic cytochrome *c* and cytosolic AIF expression levels were markedly increased following treatment with ATO/IFN α /AZT (Fig. 1E). The potential of ATO/IFN α /AZT to treat ATL was then analyzed *in vitro* using cells from two patients with acute-type ATL and two patients with chronic-type ATL. Increased levels of cleaved PARP following treatment with IFN α /AZT were observed only in ATL cells from a single patient (acute, patient 2; Fig. 1F); the WST assay revealed the anti-ATL effect of IFN α /AZT on cells obtained from this patient ($P < 0.05$, Dunnett contrast over untreated group). The ATL cells collected from the other patients in the current study were only identified to be sensitive to ATO.

Mediation of the anti-ATL effect of ATO/IFN α /AZT by the NF κ B pathway. The HTLV-1 derived protein Tax is a powerful activator of the NF κ B signaling pathway. ATO is recognized to promote Tax degradation (19); however, little is known about the effect of IFN α /AZT on non-Tax-expressing cells such as the SIT line. In the present experiment, IFN α /AZT treatment decreased phospho-IKK and phospho-I κ B α levels in the cytoplasm in the SIT cells (Fig. 2A). Concomitantly, nuclear NF κ B protein levels were decreased, indicating that the translocation of NF κ B from the cytoplasm to the nucleus was inhibited under the conditions of IFN α /AZT-induced cell death. Regulation of nuclear NF κ B, phospho-IKK and phospho-I κ B α expression was not observed when MT2 cells were treated with IFN α /AZT. Furthermore, IFN α /AZT decreased NF κ B activity in SIT cells, as determined by a luciferase reporter assay ($P < 0.001$; Fig. 2B).

ATO/IFN α /AZT induces STAT1 activation. The STAT family of transcription factors regulates cell fate and can promote apoptosis via interactions with p53 (20). STAT1 is activated by phosphorylation, upon which it inhibits the transcription of anti-apoptotic B-cell lymphoma 2 (Bcl-2) family genes, whereas p53, when activated by DNA damage, is able to activate downstream cysteine proteases, specifically caspase-8, which then enters the mitochondria, triggering cytochrome *c* release and an apoptotic signaling cascade via caspase-9 (21). STAT1 acts in conjunction with p53 via protein-protein interactions (20). The combined activity of phospho-STAT1 and p53 more efficiently promotes apoptosis, as compared with the activity of each protein alone. Ectopic treatment with IFN α activates STAT1 signaling (22); therefore, STAT1 and phospho-STAT1 expression was examined in the IFN α treated SIT cells. Marked induction of STAT1 and phospho-STAT1 expression was observed in IFN α -treated SIT cells (Fig. 3). Phospho-p53 was also upregulated in IFN α -treated SIT cells, whereas anti-apoptotic Bcl-extra large (xL) expression was downregulated, which may have led to apoptosis.

Discussion

In the present study, the anti-ATL effect of IFN- α /AZT treatment was demonstrated in an ATL patient-derived cell line and freshly isolated ATL cells via numerous apoptosis

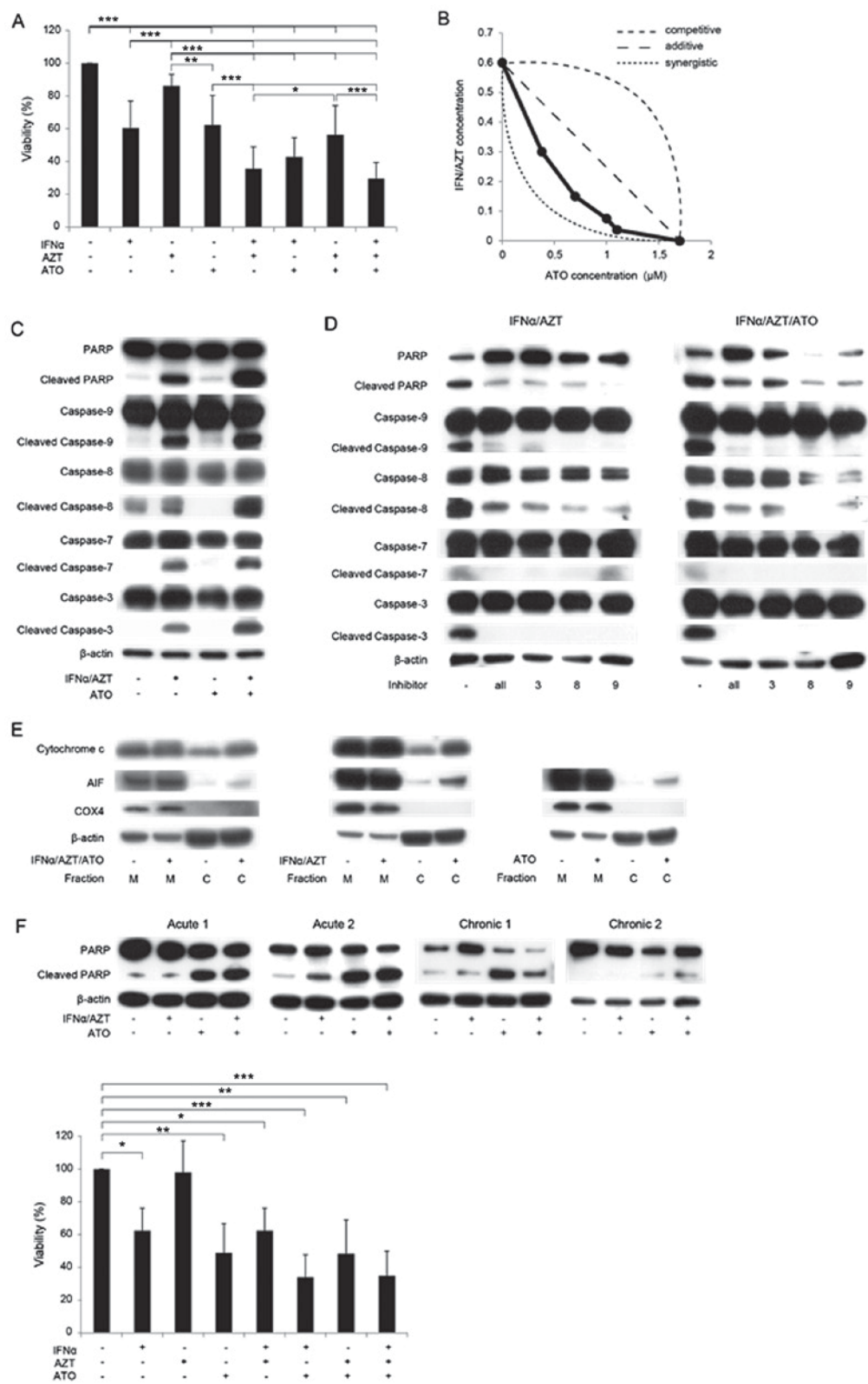


Figure 1. *In vitro* inhibition of cell proliferation by ATO/IFN α /AZT. (A) ATO/IFN α /AZT inhibits the proliferation of S1T cells as determined by an WST-8 assay. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. * P <0.05, ** P <0.01, *** P <0.005. (B) Effects of combination treatment with ATO/IFN α /AZT. ATO/IFN α /AZT exhibited a synergistic inhibitory effect on proliferation in S1T cells. Cells were treated with ATO, IFN α or AZT for 48 h. A total of 1,000 U/ml IFN α and 5 μ M AZT were defined as 1.0 on their respective axes in this experiment. (C) The expression levels of numerous intracellular regulators of apoptosis were measured by western blotting. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. (D) IFN α /AZT-induced apoptosis was reversed by caspase inhibitors (a pan-caspase inhibitor, a caspase-3 inhibitor, a caspase-8 inhibitor and a caspase-9 inhibitor; 50 μ M each). Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. (E) Increased cytosolic cytochrome *c* and AIF expression following ATO/IFN α /AZT treatment. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. (F) The *in vitro* anti-ATL effect of ATO/IFN α /AZT in primary ATL cells. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. In ATL cells derived from one patient (acute 2), IFN α /AZT treatment increased cleaved PARP expression levels. IFN α /AZT inhibited the proliferation of primary ATL cells from patient acute 2, as determined by a WST-8 assay. * P <0.05, ** P <0.01, *** P <0.005. M, mitochondrial fraction; C, cytosolic fraction; ATO, arsenic trioxide; IFN α , interferon α ; AZT, zidovudine/azidothymidine; ATL adult T cell leukemia/lymphoma; AIF, apoptosis inducing factor; PARP, poly (ADP-ribose) polymerase 1.

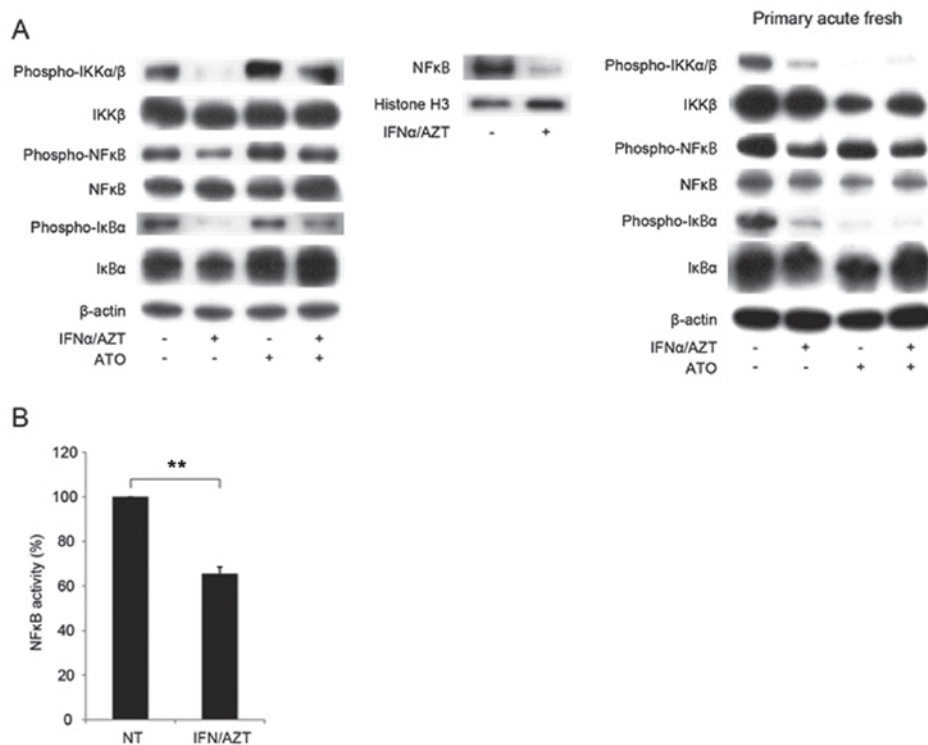


Figure 2. IFN α /AZT induced cell death via the NF κ B signaling pathway. (A) Translocation of NF κ B into the nucleus was inhibited by IFN α /AZT through decreased levels of phospho-IKK and phospho-I κ B. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. (B) The presence of histone H3 indicates the nuclear fraction. The NF κ B promoter assay demonstrated that NF κ B activity was decreased by IFN α /AZT treatment. Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. **P<0.01. ATO, arsenic trioxide; IFN α , interferon α ; AZT, zidovudine/azidothymidine; NF κ B, nuclear factor κ B; I κ B, inhibitor of κ light polypeptide gene enhancer in B-cells; IKK, I κ B kinase β .

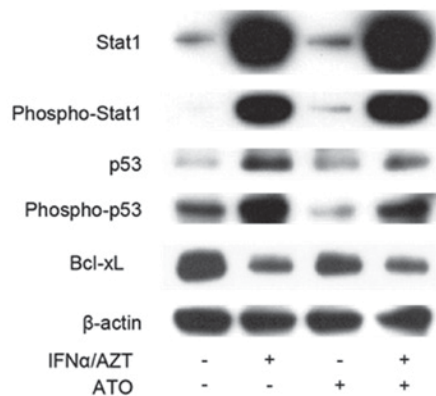


Figure 3. Induction of STAT1 by IFN α . Cells were treated with 1 μ M ATO, 1,000 U/ml IFN α or 5 μ M AZT for 48 h. STAT1, phospho-STAT1 and phospho-p53 were upregulated by IFN α /AZT treatment. Bcl-xL expression was suppressed by IFN α /AZT treatment. ATO, arsenic trioxide; IFN α , interferon α ; AZT, zidovudine/azidothymidine; STAT1, signal transducer and activator of transcription 1; BCL-xL, B-cell lymphoma extra-large.

signaling pathways, in addition to the synergistic effect of the combination of ATO and IFN α /AZT. Bazarbachi *et al* (7) previously reported that the combination of AZT and IFN α did not exhibit cytotoxic effects on HuT-102, MT2 or fresh ATL cells, despite inducing complete remission *in vivo* in one patient treated with IFN- α /AZT. HuT-102 and MT2 cells constitutively express Tax, and the authors examined only the anti-ATL effect of AZT/IFN α in PBMCs derived from one patient with ATL who was treated with 100 U/ml IFN α and 5 μ M AZT in the presence of phytohemagglutinin (PHA) and

IL-2. By contrast, in the current study, an ATL patient-derived Tax-non-expressing ATL cell line (S1T) and freshly isolated ATL cells from numerous patients, which were cultured with a 10-fold higher concentration of IFN α in the absence of PHA, were tested; however, little cytotoxic effect was observed on MT2 cells. An *in vitro* anti-ATL effect of IFN α /AZT was demonstrated for only one patient with ATL; the cells from the other three patients with ATL were not identified to be sensitive to IFN α /AZT exposure, similar to the results of previous studies (7). Kinpara *et al* (23) recently reported an *in vitro* anti-ATL effect of IFN α /AZT in IL-2-dependent HTLV-1-infected T cells derived from patients with ATL. In concordance with the data from the current study, they demonstrated that IFN α activates the p53 signaling pathway in cooperation with AZT and the suppression of NF κ B activity. Kinpara *et al* (23) used IL-2-dependent HTLV-1-infected T cells and did not specify whether the cells were ATL clone-derived. IL-2-dependent HTLV-1-infected T cells express Tax protein that is detectable using flow cytometry, though at a level markedly lower than that present in HuT102 cells (19). In addition, the authors did not demonstrate an *in vitro* anti-ATL effect of IFN α /AZT on primary ATL cells. The results of the current study demonstrated the following: The *in vitro* anti-ATL effects of IFN α /AZT in a non-Tax-expressing ATL patient-derived cell line and in ATL patient-derived primary ATL cells; and the apoptosis of ATL cells that was associated with increased levels of phospho-p53, possibly due to STAT1 activity directly induced by IFN α stimulation, decreased expression levels of Bcl-xL and the suppression of NF κ B. The *in vitro* anti-ATL effect of IFN α was observed to be enhanced

by AZT. The mechanism underlying the synergistic effect of the combination of AZT and IFN α remains to be elucidated, and the SIT cell line may be a good model for this purpose.

The synergistic anti-ATL effect of ATO/IFN α has been extensively examined *in vitro* (9,19,24–26). ATO synergizes with IFN α to induce cell cycle arrest and apoptosis, whereas ATO/IFN α leads to Tax degradation, suggesting that the apoptosis of HTLV-1-derived cells may reflect targeting of the Tax oncoprotein (19). In the present study, a synergistic anti-ATL effect of ATO/IFN α /AZT in a non-Tax-expressing cell line was demonstrated (Fig. 1B). In addition, the ATO/IFN α /AZT combination was not synergistic, but was instead competitive in the Tax-expressing MT2 cell line (data not presented). Primary ATL cells from three patients with ATL were only sensitive to ATO treatment *in vitro* (Fig. 1F), and were not observed to be sensitive to IFN α /AZT, as was previously reported (7). Dassouki *et al* (9) recently demonstrated that the ATL response to ATO/IFN α therapy is triggered by SUMO/PML/RNF4-dependent Tax degradation. In this study, Tax expression was only detectable at the mRNA level in the MT-1 cell line, which was sensitive to ATO/IFN α /AZT treatment.

In MT-1 cells, silencing of Tax by short hairpin (sh)RNA impairs cell survival (9). This result suggests that even significantly low Tax expression levels are sufficient to sustain HTLV-1 transformed cell survival. Kinpara *et al* (23) also demonstrated the anti-ATL effect of IFN α on low-Tax-expressing cells with a notable further reduction in Tax expression. As significantly low levels of Tax mRNA were identified in SIT cells in the current study, we cannot exclude the possibility that IFN α /AZT induces the degradation of Tax in SIT cells. The silencing of Tax in SIT cells by Tax shRNA may facilitate the elucidation of this mechanism. Takeda *et al* (10) reported that Tax mRNA could be detected in only 14/41 freshly isolated ATL cases (34%) using RT-PCR. In addition, Tax is significantly upregulated following overnight cell culture, even without any stimulation. Even with this induction of Tax expression, the *in vitro* treatment of cells with IFN α /AZT did not previously demonstrate an anti-ATL effect, and thus further studies were warranted in order to elucidate the mechanism underlying cell sensitivity to IFN α /AZT *in vitro*. Notably, SIT cells were observed to be sensitive to ATO/IFN α /AZT, possibly due to a mechanism independent of Tax degradation. Therefore, the Tax-independent mechanism underlying the anti-ATL effect of ATO must also be clarified. These findings provide a foundation for further studies aimed at revealing the underlying molecular mechanisms of IFN α /AZT, and identifying the subgroup of patients with ATL for whom this treatment could be effective.

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Availability of data and materials

All the datasets generated during the present study are included in this manuscript.

Authors' contributions

MH and MY designed and performed the experiments, analyzed the data and wrote the manuscript. CE and AK performed the experiments; TK designed and performed the experiments. NA designed and supervised the project and wrote the manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Medical Ethics Committee of Kagoshima University Hospital (Kagoshima, Japan). All subjects provided written informed consent for participation in the present study.

Consent for publication

Study participants provided their consent for the publication of this data and any associated images.

Competing interests

The authors declare that they have no competing interests.

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