Ascorbic Acid Induces Apoptosis in Adult T-cell Leukemia

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Abstract. *Background: Adult T-cell leukemia (ATL) is an acute malignancy of activated T-cells caused by the human T-cell lymphotrophic virus type-1 (HTLV-1). Materials and Methods: The effects of non-cytotoxic concentrations of ascorbic acid (AA) were evaluated against HTLV-1 positive and negative cells. The effect of AA on apoptosis and proliferation was evaluated by cell cycle analysis. The role of p53, p21 Bax and Bcl-2α on cell cycle modulation and apoptosis was also assessed. The anti-proliferative effects were tested by determining the changes in the expression of transforming growth factors (TGF-α, TGF-β1 and TGF-β2). Results: Ascorbic acid was found to reduce the proliferation of cells and induce apoptosis by the modulation of p53, p21, Bcl-2 and Bax. Conclusion: The results of this study show the anti-proliferative effects of AA against leukemic cells.*

Human T-cell lymphotrophic virus type-1 (HTLV-1) is a retrovirus that transforms normal peripheral blood lymphocytes *in vitro* and causes adult T-cell leukemia (ATL), a fatal disease with an average survival time of less than one year. Only 5% of the infected patients develop the disease after a very long latency period of 30-40 years. The site of integration of the virus differs between patients. Therefore, it is unlikely that transformation directly activates certain cellular oncogenes, but may involve the transactivator protein Tax, a nuclear protein encoded by the viral mRNA. The virus is usually transmitted sexually, transplacentally from mother to child, through breast-feeding, or through blood transfusions. No effective cure has yet been achieved to treat HTLV-1. However, a combination of chemotherapy and treatment with the antiretroviral agent zidovudine (AZT), interferon-α (IFN-α) and other agents might prolong the life of patients by down-regulating the transcription factor NF-κB (1, 2).

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Vitamin C (ascorbic acid) has protective effects against the common cold, wound healing, atherosclerosis and in the treatment of cancer (3-5). It has also been reported to be effective against both DNA and RNA viruses (6-8). In their studies, Murata and Kitagawa (6) did not demonstrate an effect on phage protein, in contrast to our results (9), which showed that ascorbic acid caused inactivation of the reverse transcriptase enzyme using HIV as a model upon prolonged incubation of the virus particle *in vitro*.

Investigation on ascorbate effects on viruses (10) using avian retrovirus of chickens reported that while cell-free virus was resistant to inactivation by ascorbate upon short term treatment *in vitro*, exposure of virus infected cells to the vitamin resulted in a slowing of virus replication and a decrease in the infectivity of newly replicated virus. In a follow-up study, it was reported that ascorbate interfered with the replication and cell-transforming potential of Rous Sarcoma Virus by stabilizing the differentiated state of chicken cells (11). In a different study using a lymphocytic cell line latently infected with HTLV-1, ascorbic acid (AA) interfered with virus production triggered by chemical inducers added to the culture medium (12).

The efficacy of AA on HIV have been evaluated using chronically, acutely and latently infected cell lines *in vitro* (9, 13-17). These results indicated that AA produced a 99% reduction in the activity of RT and 90% reduction in the p24 antigen. AA was seen to act on the post-translational level targeting the inactivation of the viral enzymatic acitivity.

It has been shown that in cancerous cells, higher levels of vitamins A, C and E reduce abnormalities in cell kinetics (18). In solid tumor models, ascorbic acid displayed high cytotoxic levels against cancerous cells (19). The objectives of this study are to evaluate the effects of AA on the proliferation and induction of apoptosis using non-cytotoxic concentrations of the test compound against HTLV-1 positive (HuT-102 & C91-PL) and negative (CEM & Jurkat) cells.

Materials and Methods

Preparation of chemicals. Stock solutions of 10 mg/ml of ascorbate were prepared by dissolving ascorbic acid (tissue culture grade

from Sigma, St. Louis, MO, USA) in RPMI 1640 medium and stored at –20˚C. On the day of the experiment, these stock solutions were thawed and used once only.

Cell lines and treatment. Four cell lines were used: HuT-102, C91-PL, CEM and Jurkat. Both HuT-102 and C91-PL cell lines (given as a gift from A. Gessain, Institut Pasteur, Paris, France) are T-cells infected with HTLV-1 derived from ATL cells. The T-cells that are HTLV-1 positive, constitutively express the retrovirus HTLV-1. CEM and Jurkat are uninfected human T-cells obtained from T-cell leukemic patients. The CEM T-lymphoblastoids were obtained from the peripheral blood of a Caucasian female infected with acute lymphoblastic leukemia (ALL). Jurkat cells have the same markers as normal lymphocytes and were also taken from the peripheral blood of a patient with ALL (20). RPMI 1640 media was used to suspend the cell lines, supplemented with 25 mM of Hepes, 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Paisley, Scotland, UK), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco). The cells were allowed to grow in a humidified incubator at 37˚C, 95% air and 5% CO_2.

Preparation and culture of fresh human mononuclear cells. Human peripheral blood mononuclear cells from healthy donors were isolated on a Ficoll-Isopaque gradient (1.077) and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml of streptomycin. β-mercaptoethanol ($5x10^{-5}$) u and PMA (5 μg/ml) were added on the last day of treatment.

Determination of cytotoxicity. Cells ($1x10^5$) were suspended in 1 ml of growth medium and seeded in 24-well microtiter plates. The controls received RPMI medium while test cells were fed daily with appropriate concentrations of AA. At periodic intervals (48 or 96 hours), 0.5 ml of cell suspension (in triplicate) were collected and mixed with 50 μl of trypan blue and counted using a hematocytometer (13).

Cell proliferation test. Cells were seeded in 96-well microtitre plates (200 μl per well) at a density of $1x10^5$ cells, then incubated for 24 h in RPMI media before being treated with different doses of the test compound. The cell Titer96™ Non-Radioactive Cell Proliferation Assay was used to determine cell proliferation according to the manufacturer's instructions (Promega Corp., Madison, WI, USA). The assay accounting for proliferation is MTT-based and measures the ability of mitochondria in a metabolically active cell to induce the conversion of a tetrazolium salt in a formazan compound (21).

Analysis of TGF-α mRNA using RT-PCR. From the cells grown, total RNA was isolated using the SV Total RNA isolation Kit (Promega). Nucleic acids were precipitated, washed with 75% ethanol, then dried and resuspended in water. Ten U of avian myeloblastosis reverse transcriptase (Promega) transcribed the total RNA into cDNA. In a total volume of 50 μl, oligo(dT)17 primer (50 pM), 0.2 mM each of deoxynucleotide triphophate (dATP, dGTP, dCTP, dTTP) (Promega) and reaction buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol) were added for 60 min at 42˚C. cDNA was amplified with a polymerase chain reaction (PCR) using oligonucleotide primers designed to target TGF-α, TGF-β1 and TGF-β2. The PCR program was performed in 100 μl of 75 mM Tris-HCl, pH 9, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of DNA polymerase, 50 pM of each primer and 2 ml of the cDNA mixture, as follows: 45 sec for denaturation at 94˚C, 45 sec for annealing at 50˚C, 45 sec for elongation at 72˚C and final extension for 5 min. at 72˚C. A preliminary analysis of each primer was performed to appropriately define the cycle range consistent with the exponential increase in amounts of PCR products. This was done by running 10 μl of DNA products on a 2% agarose gel stained with ethidium bromide. The Molecular Analyst/PC image analysis software (BioRad Laboratories, Hercules, CA, USA), was used to determine the intensity of the luminescent bands. The results were expressed in relative densitometric units, normalized to the values of phosphoribosomal protein mRNA which was used as an internal control. In order to investigate the detection of amplified fragments that correspond to the genomic DNA, the control PCR was performed using RNA samples which were not subjected to reverse transcription (21).

Flow cytometry analysis of DNA content. After being treated daily with different doses of AA for 48 and 96 h, cells were fixed in 70% ethanol, treated with 100 μg/ml of RNase for 1 h, stained with propidium iodide and subjected to flow cytometry analysis, as described elsewhere (21).

Cell death by ELISA. Cell death was assessed using an ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany). This kit relies on quantitative detection of DNA fragments related to cytosolic histones. The cells were suspended at a density of $1x10^4$ cells per well, in 96-well culture plates. An aliquot was obtained from the supernatant and used as antigen source to the primary anti-histone monoclonal antibody coated to the streptavidin-coated well in the sandwich ELISA. A second anti-DNA monoclonal antibody, coupled to peroxidases, was then added. The peroxidase activity was photometrically measured at 405 nm with 2, 2'-azino-di (3-ethylbenzthiazolin-sulfonate) (ABTS) as substrate (21).

Protein extraction and Western blot analysis. Total cellular proteins were extracted from cells, quantified and 30 μg of cellular proteins were run on a 12% SDS-polyacrylamide gels. The gels were then blotted onto PVDF membranes and detected by an enhanced chemoluminescence system according to the manufacturer's recommendations, as described elsewhere (21).

Statistical analysis. Data were analyzed using a one-way analysis of variance (ANOVA). A comparison between the mean values of treated and control groups was established to assess for significant differences using Fisher's least significant differences at $p \leq 0.05$ (Fisher PLSD). When the value (+ or –) exceeded the Fisher PLSD in the one-factor ANOVA test, the effect of treatment was considered significant.

Results

Effects of AA on cytotoxicity and cell proliferation. The four cell lines were grown in the presence or absence of different non-cytotoxic concentrations of AA for 48 and 96 h to investigate the anti-proliferative effects of AA. The 50% cytotoxicity dose, IC_{50}, which represents the dose at which 50% of the cells in culture died, was determined for each cell line as compared to cells grown in the absence of AA

(control). At IC_{50}, the reduction of cell proliferation was also measured. The resulting data show a clear decrease in the viable cell counts in a dose-dependant manner, in all tested cell lines of HTLV-1 positive and negative leukemic cells. At 48 h incubation with ascorbic acid (AA), the cytotoxicity on the HTLV-I negative cell lines, CEM and Jurkat, became apparent at an IC_{50} of 253 and 205 μg/ml respectively. In the case of the HTLV-I positive cell line HuT-102, the cytotoxic doses of AA were similar in values to those seen for the HTLV-I negative cell lines, with an IC_{50} of 238 μg/ml. In contrast, the other HTLV-I positive cell line, C91-PL, was much more resistant, with cytotoxicity appearing at an IC_{50} of 427 μg/ml. At 48 h incubation, AA had a similar effect on the four cell lines with an inhibition of proliferation ranging from 45 to 61% (data not shown). However, at 96 h of incubation, the cytotoxic doses of AA in all the four cell lines appeared at IC_{50}'s ranging from 110 to 168 μg/ml with the most inhibition of proliferation in the HTLV-I positive HuT-102 cells reaching 92% (Figure 1). The effect of AA was also tested on healthy human mononuclear cells. Activated cells were plated at a density of $1x10^5$ cells/ml in 24-well plates and tested for viability for 96 h using different concentrations of AA. The IC_{50} was apparent at 450 μg/ml as compared to the control (data not shown). All concentrations of AA chosen to either treat ATL or ALL cells were below 450 μg/ml.

Effects of AA on the expression of Transforming Growth Factor (TGF). TGFs are proteins that belong to the family of cytokines that promote regulation of normal cell function such as proliferation, differentiation and apoptosis. Of those TGFs, we assessed the differential patterns of expression of TGF-α, TGF-β1 and TGF-β2 upon exposure of the cells to non-cytotoxic concentrations of AA at 96 h. The mRNA expression of TGF-α, TGF-β1 and TGF-β2 was evaluated using RT-PCR. The RT-PCR results illustrated the effects of AA on the mRNA of ATL and ALL cells according to the dose administered. It was shown that in all cell lines, AA induced a decrease in cell proliferation by down-regulating TGF-α and up-regulating TGF-β2. However TGF-β1 levels of expression were not altered by AA addition. This confirms that AA inhibits cell proliferation in both HTLV-1 positive and negative leukemic cells (Figure 2).

Effects of AA on cell cycle progression and apoptosis. Flow cytometry and an ELISA-based apoptosis assay were the two techniques used to account for the effects of AA on cell cycle progression and/or induction of apoptotic responses: i) Flow cytometry. A FACScan Flow Cytometer was used to determine the DNA content of the tested leukemic cells. Variations in the cell cycle distribution were observed in the cell lines after exposure to non-cytotoxic concentrations of AA. An increase in the number of cells found to be in the $preG_1$-phase would indicate an increase in apoptosis. Therefore, the apoptotic effects of AA on all tested HTLV-1 positive and negative cells were evaluated according to the significant increase of $preG_1$-phase paralleled with a decrease of cells found in the S-phase. The flow cytometric results were assessed at 48 and 96 h. An increase in the number of cells found in the $preG_1$-phase was found with AA, revealing apoptotic effects of this compound. Increasing doses of AA resulted in a significant shifting in the cell distribution to the $preG_1$-phase in all tested cell lines. The most striking data was seen in the HTLV-I positive cell line, HuT-102, with a 21-fold increase as compared to the control at 48 h (data not shown) and an approximately 30-fold increase at 96 h with 150 μg/ml AA. At 96 h, AA also had an apoptotic effect on the other HTLV-I positive cell line, C91-PL, where the distribution of cells in the $preG_1$-phase increased 32.4-fold as compared to the control (Figure 3). In addition, the induction of apoptosis was also observed on HTLV-I negative cell lines especially on CEM cells treated with 150 μg/ml of AA. At 48 h incubation, a 9-fold increase in $preG_1$-phase cells was detected (data not shown) whereas at 96 h the increase was almost 56-fold. The increase in cell numbers in the PreG1-phase and the decrease in the S-phase of the cell cycle in all tested cell lines indicate the apoptotic effect of AA (Figure 3).

ii) Cell Death ELISA. The fragmentation of DNA forming the DNA ladder is a hallmark of apoptosis. Cell Death ELISA measures the cytosolic histone fragments and apoptotic cells quantitatively, based on optical density. This experiment was used to further confirm the results obtained by flow cytometry. For all the cell lines tested, the fragmentation of DNA illustrated the apoptotic effects of AA on both HTLV-1 positive and negative leukemic cells in a dose-dependant manner. An approximately 4-fold increase was detected in both HuT-102 and CEM cells tested at 96 h treatment at the highest non-cytotoxic concentration of AA (150 μg/ml). All results were expressed as compared to the control (Figure 4).

Effects of AA on cell cycle arrest and apoptotic related proteins. In view of the obtained confirmations of the effects of AA on the induction of apoptosis, further testing was performed to focus more precisely on AA effects on the expression levels of four regulator proteins involved in cell cycle regulation and apoptosis by Western-blotting using β-actin as a control to ensure equal loading. The four proteins tested were: Bcl-2α (an anti-apoptotic protein), Bax (a pro-apoptotic protein of the same family as Bcl-2α), p53 (a tumor suppressor protein known as the 'guardian of the genome') and p21 (an important cyclin-dependant kinase inhibitor which causes arrest of the cells at the G_1-phase). Treatment of

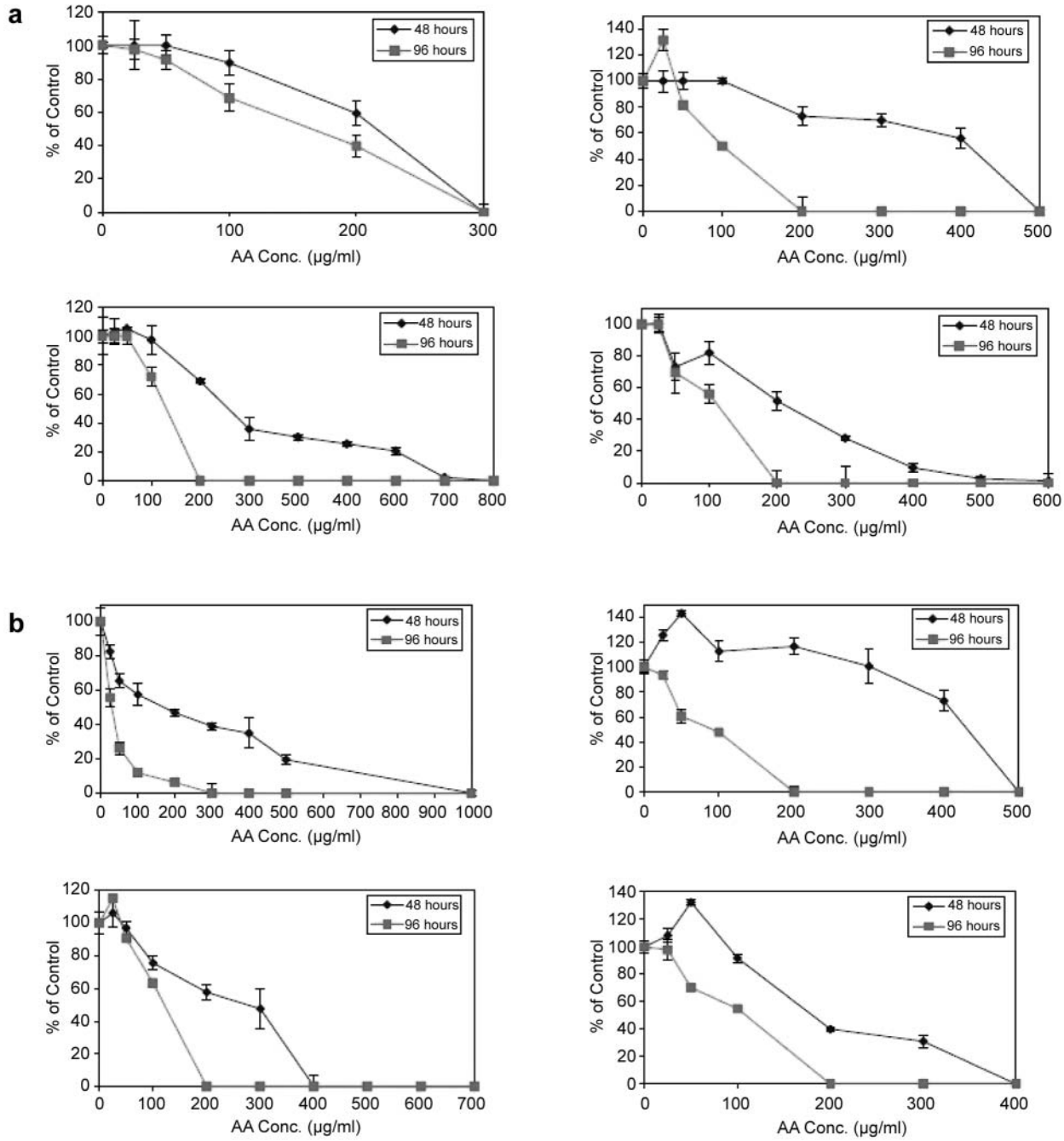

Figure 1. *a) Toxicity of AA to both HTLV-1-positive (HuT-102 and C91-PL) and HTLV-1-negative (CEM and Jurkat) cells at 48 h and 96 h of exposure. Each value is the mean±S.D. of three separate experiments done in triplicate. b) Inhibition of proliferation of HTLV-1 negative (CEM and Jurkat) and HTLV-1 positive (HuT-102 and C91-PL) cells using non-cytotoxic concentrations of AA at 48 and 96 h. Each value is the mean±S.D. of three separate experiments done in triplicate.*

the cells with non-cytotoxic doses of AA induced an up-regulation of Bax, p53 and p21, which all lead to cell cycle arrest and eventual apoptosis. AA also caused a down-regulation of the Bcl-2α protein expression levels, in all of the cell lines tested, in a dose-dependent manner (Figures 5 and 6). The variation of Bax and Bcl-2α was significant in both HTLV-I positive (HuT-102 and C91-PL) and negative cell lines (CEM and Jurkat) suggesting the high involvement of AA on the intrinsic apoptotic pathway in leukemic cell lines.

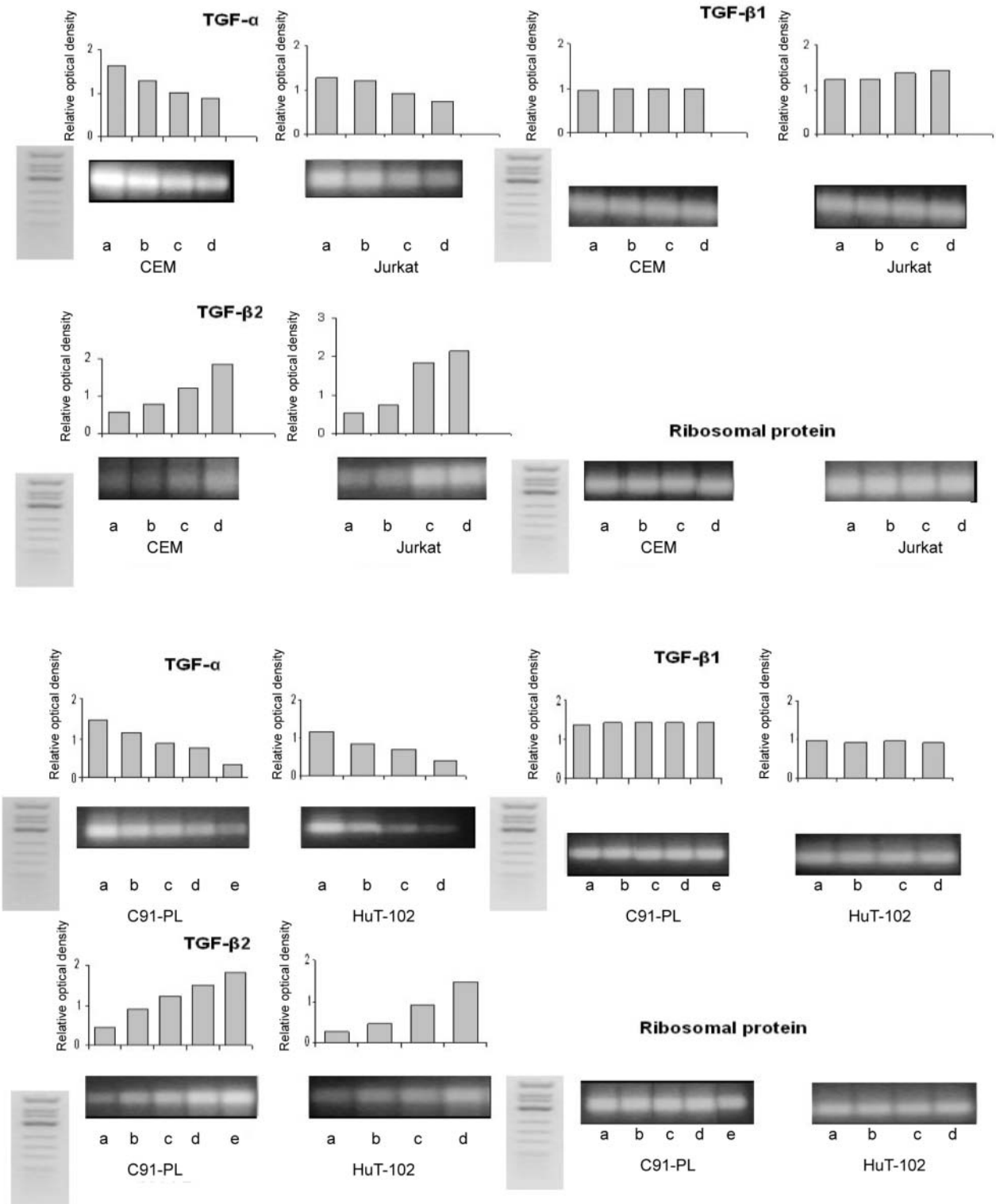

Figure 2. *The effects of AA on TGF-α, TGF-β2 and TGF-β1 mRNA levels in the four cell lines at 96 h exposure to non-cytotoxic concentrations of AA. Panel A shows the effects on HTLV-1 negative cells. For CEM: a=control, b=50 μg/ml, c=100 μg/ml and d=150 μg/ml. For Jurkat: a=control, b=25 μg/ml, c=50 μg/ml and d=100 μg/ml. Panel B shows the effects on HTLV-1 positive cells. For HuT-102: a=control, b=50 μg/ml, c=100 μg/ml and d=150 μg/ml. For C91-PL: a=control, b=10 μg/ml, c=25 μg/ml, d=50 μg/ml and e=100 μg/ml. Results are expressed as relative densitometric units normalized to the values of the phosphoribosomal protein mRNA used as an internal control.*

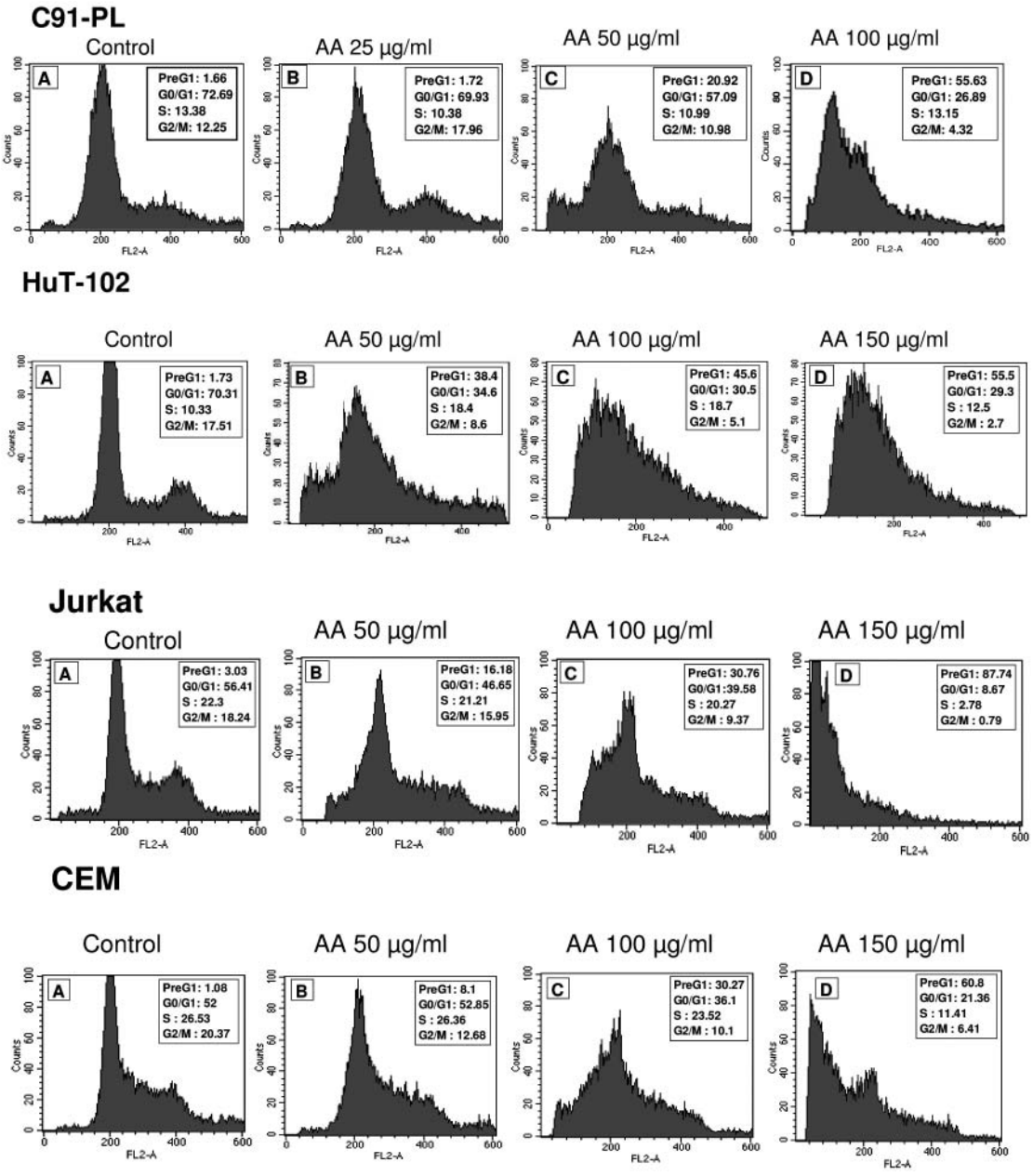

Figure 3. *Flow cytometry data showing the effects of non-cytotoxic doses of AA on cell cycle distribution at 96 h using a FACScan flow cytometer and analysed using Cell Quest program.*

Discussion

No treatments have yet been found to completely cure patients infected with HTLV-1, rather chemotherapy or a combination of therapies have helped in improving the patients' conditions and increasing their life span. AA is receiving much attention with respect to cancer therapy; it appears to have a preventive effect and may improve the quality of life and the longevity in cancer patients (22). AA is a potent water-soluble antioxidant, capable of scavenging reactive oxygen species (ROS) at certain concentrations (23). It reduces the spontaneous mutation rate in mismatching repair-deficient human colon cancer cells. In addition, lymphocytes from colorectal carcinoma patients contain higher levels of 8-oxo-2'-deoxyguanosine, a marker for alterations caused by ROS, associated with lower levels of vitamins in plasma (22). AA also caused a decrease in the incidence of kidney tumors in hamsters possibly caused by a

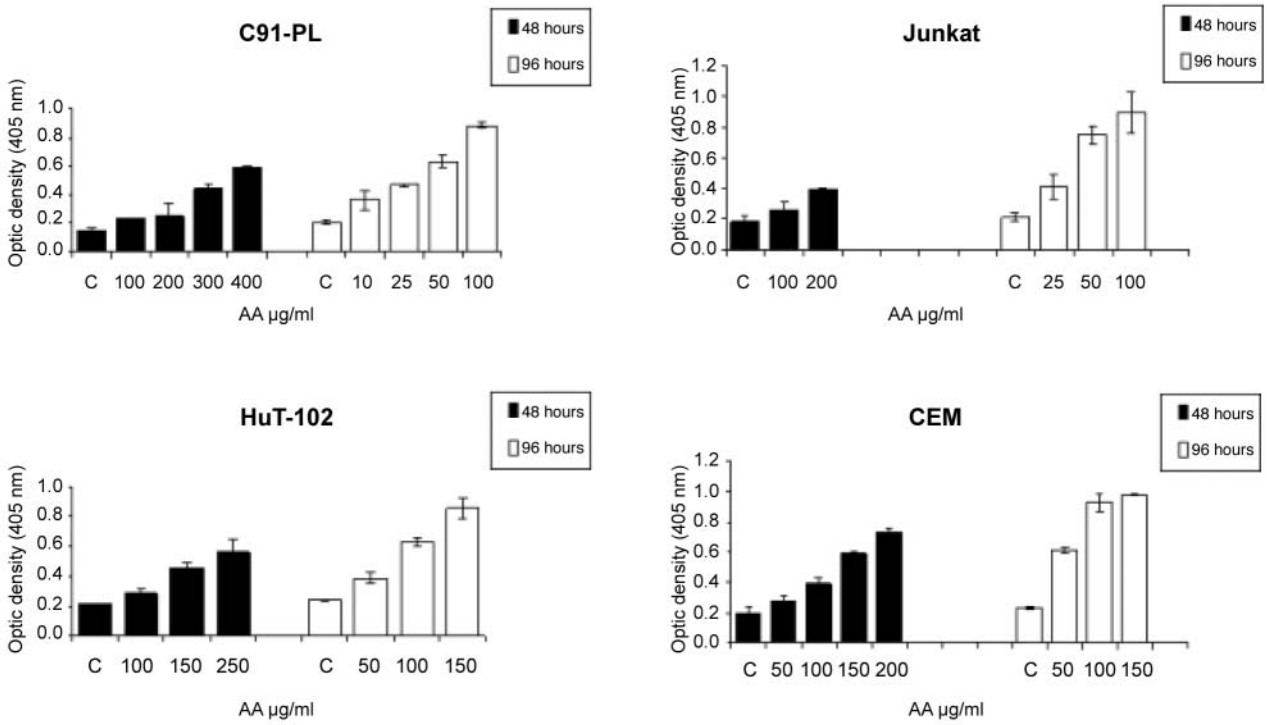

Figure 4. *The induction of apoptosis by AA in ATL and ALL cells using ELISA. Each value is the mean±S.D. of three separate experiments done in triplicate.*

decrease in genotoxic metabolites, as well as an inhibition of the growth of some malignant and non-malignant tumors, both *in vitro* and *in vivo* (24). Other effects on human tumor cells have been reported, such as its effect against neuroblastoma (25), osteosarcoma and retinoblastoma (26). The present study aimed at evaluating the apoptotic effects of AA on HTLV-1 positive and negative cell lines of malignant T-cell leukemia. The results of this study suggest that AA expresses anti-proliferative effects on ATL cells in a dose and time-dependent manner. This is consistent with other studies that suggested that vitamin C has a role in down-regulating E6, a viral oncoprotein of the human papillomavirus (HPV), as function of the doses administered; this resulted in the up-regulation of pro-apoptotic proteins, such as p53 and Bax, paralleled with the down-regulation of the anti-apoptotic Bcl-2 protein, hence inducing cell death (27). It has been shown that ascorbyl stearate caused a decrease in cell proliferation through induction of apoptosis and interferance with the cell cycle of human ovarian and pancreatic cancer cells in a dose-dependent manner (28). The arrest was observed in the S/G_2M-phase of the cell cycle with an increased fraction of apoptotic cells. The alteration of the cell cycle was found to be linked with the reduced expression and phosphorylation of the insulin-like growth factor 1-receptor (IGF-1 receptor) by ascorbyl stearate, leading to apoptosis. AA was found to inhibit apoptosis induced by vincristine. Vincristine is known to induce cell death through the mitochondria by the way of production of ROS. By scavenging ROS, AA inhibited cell death by blocking the upstream pathway of caspase-9 and -3 (29). It was found that the pretreatment of human lung fibroblasts (HLF cells) with AA did not significantly protect against apoptosis induced by sodium chromate (30).

This study provides evidence that AA induces apoptosis and inhibits proliferation in the cells tested. AA caused a down-regulation in the proliferation enhancer, TGF-α and an up-regulation in the cell proliferation inhibitor, TGF-β2, but did not affect TGF-β1 mRNA levels. These data confirm that AA induces growth inhibition in HTLV-1 positive and negative malignant T-cells. TGF-β and TGF-α have contradicting functions: TGF-β is a multifunctional protein, potentially responsible for inhibition of cell growth, as well as induction of apoptotic response in many types of cells (31). TGF-α on the other hand, is a key mediator for stimulation of cell proliferation and cell growth, with direct effects on mitosis (32).

TGF-α is a crucial mediator of cell growth stimulation, involved in anti-apoptotic activities *via* the NF-κB-dependant pathway (33). These findings were further confirmed by the involvement of AA in the up-regulation of TGF-β2, a pro-apoptotic multifunctional protein with IL-2

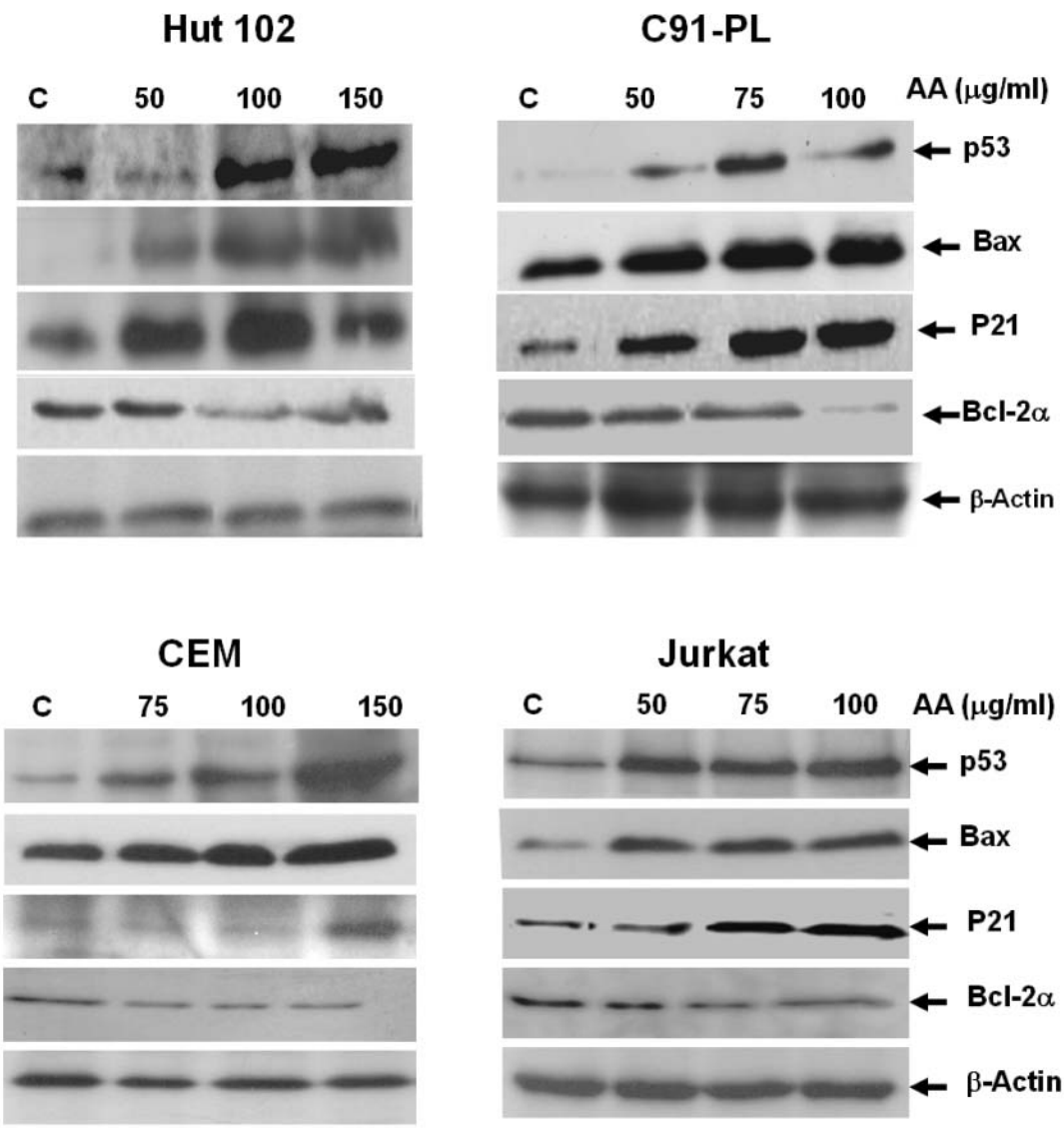

Figure 5. *Immunoblots showing the influence of AA on the levels of p53, p21, Bax and Bcl-2a proteins in all tested cell lines.*

dependant T-cell growth inhibition activities (34), as well as induction of apoptosis by controlling the expression of anti-apoptotic proteins such as Bcl-xL and bcl-2α *in vivo* (35). On the other hand, no changes were observed regarding TGF-β1 expression, although it is known that TGF-β1 can promote apoptosis in certain cell types and that over expression of this protein prevents proliferation of T and B cells (36).

Flow cytometry data showed that AA caused cell cycle arrest in the preG_1-phase in all the cells tested, providing evidence for the pro-apoptotic effects of AA. Similar data has been reported using human bladder tumor cells (T24) where cell lines revealed growth arrest upon treatment with vitamin C in the G_1-phase (37). Flow cytometry and Western blot analysis of important apoptotic proteins, were the two verification techniques used for further confirmation of the previous results. It was observed that treatment with AA led to the up-regulation of p53, p21 and Bax proteins and the down-regulation of Bcl-2α protein in a dose-dependant manner. When the Bcl-2α anti-apoptotic protein is up-regulated, the cells become immortal by preventing cytochrome-c release from the mitochondria leading to malignancy (38). The activities of Bax pro-apoptotic protein oppose that of Bcl-2α, as they allow the release of cytochrome-c from the mitochondria. The balance between the two antagonist proteins Bcl-2α and Bax

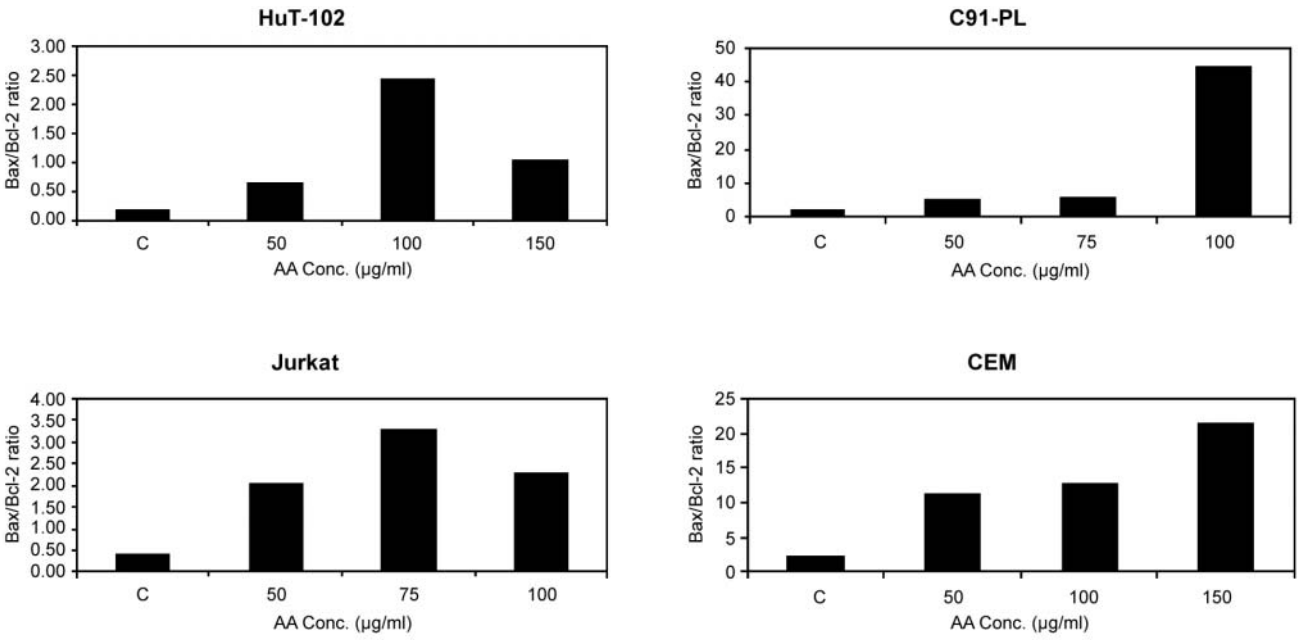

Figure 6. *Densitometric analysis was performed to measure the ratio of Bax/Bcl-2a in all tested cell lines.*

is crucial for normal cell function. The tumor suppressor p53 protein protects the cell by leading it to apoptosis when DNA damage occurs in the cell without possibility of reparation. The up-regulation of p53 is stabilized by a post-transcriptional mechanism (39). The cyclin-dependant kinase inhibitor p21 protein causes the cell to arrest at the preG_1-phase. Its expression is dependant on the accumulation of activated p53 in the nucleus when responding to oxidative stress and genotoxic damage. The expression of p21, along with other proteins, inhibits cell proliferation by arresting the cells in the G_1- or G_2-phases and inducing apoptosis. Any mutation or inactivation of p53 protein leads to a potential pathway for tumor growth and cancer development. Furthermore, cancerous cells often show an over-expression of Bcl-2α protein which prevents the release of cytochrome-c from the mitochondria and provides the cell with resistance to chemotherapy and avoidance of apoptosis. Since the increased expression of p53 associated with the increased ratio of Bax/Bcl-2α is involved in AA-induced apoptosis, AA might have an important role in potential future cancer treatment and might constitute a possible chemotherapeutic agent for treatment of ATL patients.

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