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MECHANISMS OF CELL DEATH AND RETINOID SIGNALING INDUCED BY THE NOVEL SYNTHETIC RETINOID ST1926 IN HTLV-1 TRANSFORMED AND MALIGNANT T-CELLS

by
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Title: Mechanisms of Cell Death and Retinoid Signaling Induced by the Novel Synthetic Retinoid ST1926 in HTLV-1 Transformed and Malignant T-cells

E-3-(40-Hydroxy-30-adamantylbiphenyl-4-yl)acrylic acid (ST1926) is a novel orally available synthetic retinoid that is currently in phase I clinical trials. Synthetic retinoids are a group of compounds with Vitamin A activity that have promising anti-cancer and anti-leukemic properties, in particular in tumor cells that are resistant to the natural retinoid all-*trans* retinoic acid (ATRA). Adult T-cell leukemia/lymphoma (ATL) is an aggressive neoplasm caused by the human T-cell lymphotropic virus type 1 (HTLV-1) and the oncoprotein Tax plays an important role in disease development. ATL carries a poor prognosis due to chemotherapy resistance resulting in a search for alternative therapies. Therefore, the general aim of our study is to investigate the potential use of ST1926 in ATL and peripheral T-cell lymphoma treatment.

We used several HTLV-1 positive and HTLV-1 negative malignant T-cells, that are mostly ATRA-resistant, primary ATL cells from two patients, and normal lymphocytes from three normal donors. Clinically achievable concentrations of ST1926 induced a dramatic inhibition of cell proliferation and pronounced apoptosis in all tested malignant T-cells, while no effect was observed on resting or activated normal lymphocytes. Interestingly, ST1926 effect on malignant T-cells was five to ten folds stronger than what we observed with other synthetic retinoids such as HPR and CD437.

ST1926 induced apoptosis in all tested malignant T-cells as shown by the accumulation of treated cells in the pre G_1 phase of the cell cycle, TUNEL positivity and loss of mitochondrial membrane potential which was earlier and more pronounced in the HTLV-1 negative cells. Furthermore, ST1926-induced cell death was partially caspase-dependent. We have also shown that total p53 proteins and its phosphorylated form are induced by ST1926 treatment of malignant T-cells and that in Molt-4 cells, ST1926 effect is partially p53-dependent. Interestingly, ST1926 caused an early degradation of Tax and p21 proteins in the HTLV-1 positive cells.

Finally we found that ST1926 works independently of the retinoid signaling pathway in both HTLV-1 positive and negative cells using the pan-RAR inverse agonist BMS493. In addition, ST1926 induces the degradation of the retinoic acid receptors (RARs) alpha and gamma in malignant T-cells.

Our results support a potential therapeutic role of ST1926 in ATL and peripheral T lymphomas, especially those that are resistant to natural retinoids.

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ABBREVIATIONS

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
As	Arsenic
ATF	activating transcription factor
ATL	adult T-cell leukemia/lymphoma
ATRA	all-*trans* retinoic acid
AZT	zidovudine
CBP	CREB binding protein
CD437	6-[3-(1-adamantyl)-4-hydroxyphenyl] -2-naphthalene carboxylic acid
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
cRA	*cis* retinoic acid
CREB	cyclic AMP response element binding protein
DMSO	dimethylsulfoxide
DR5	death receptor
DSB	double strand breaks
EGFR	endothelial growth factor receptor
ELISA	enzyme-linked immuno-sorbent assay
FBS	fetal bovine serum
FACS	fluorescence activated cell sorter
HAT	histone acetylase transferase

HDAC	histone deacetylase
hTert	human telomerase
HTLV-1	human T-cell lymphotropic virus type-1
HPR	N-(4-hydroxyphenyl)retinamide
HPV	human papilloma virus
H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical
IFN	interferon
IκB	inhibitor of kappa b
IL-2	interleukin 2
IKK	inhibitor of IκB
LTR	long terminal repeat
μM	micromolar
MAPK	mitogen activated protein kinase
MPP	mitochondrial permeability potential
NB	neuroblastoma
NES	nuclear export signal
NFκB	nuclear factor kappa b
NK	natural killer
OD	optical density
O_2^-	superoxide anion
PARP	Poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells

PBS	phosphate-buffered saline
PHA	phytohemagglutinin
PI	propidium iodide
PML	promyelocytic leukemia
PS341	proteasome inhibitor
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RB	retinoblastoma
ROS	reactive oxygen species
RRM	retinoid related molecules
RXR	retinoid X receptor
RXRE	retinoid X response element
R123	rhodamine 123
SCID	severe combined immunodeficiency
SD	standard deviation
TNF	tumor necrosis factor
TSP/HAM	tropical spastic paraplegia/HTLV-1 associated myelopathy
VEGF	vascular endothelial growth vactor

CHAPTER I

INTRODUCTION

A. Adult T-cell Leukemia/Lymphoma

1. Overview

Adult T-cell Leukemia/lymphoma (ATL) is an aggressive malignancy of mature activated T-cells. It is the first human disease caused by the human T-cell lymphotropic virus type 1 (HTLV-1). HTLV-1 is an RNA retrovirus composed of two copies of single-stranded RNA which are copied into a double-stranded DNA once integrated into the host cell genome. In addition to causing ATL, HTLV-1 is also the causative agent of a neurological disease called HTLV-1 associated myelopathy/ tropical spastic paraplegia (TSP/HAM). TSP/HAM infects the central nervous system in addition to the blood and is characterized by progressive demyelination of the long motor neuron tracts of the spinal cord.

HTLV-1 is transmitted intravenously, vertically from the mother to her fetus or *via* sexual intercourse. HTLV-1 is mainly endemic in southern Japan, the Caribbean, sub-Saharan Africa, Brazil and northern Iran (Bazarbachi 2004).

Two cases of ATL were diagnosed in Lebanon (Bitar 2009). The first patient of Lebanese origin presented with acute ATL and the second patient of Romanian origin developed early relapse after autologous transplantation for ATL. Both patients had lymphocytosis, severe hypercalcemia, and CD25+ T-cell immunophenotype on peripheral blood. In both patients, HTLV-1 serology was positive by enzyme-linked immunosorbent assay and confirmed by western blot and HTLV-1 oncoprotein Tax expression was documented in the leukemic cells. Upon screening, seven direct family members of the first patient were HTLV-1 positive; four of them were regular blood donors.

2. Clinical Features of ATL

Based on the different clinical features of ATL, four subtypes have been identified: acute (60% of cases), chronic (15%), smoldering (5%) and adult T-cell lymphoma (20%) (Bazarbachi 2004). The most two aggressive forms are the acute and the lymphoma subtypes, characterized with massive lymphadenopathies, hepatosplenomegaly, lytic bone lesions and multiple visceral lesions mainly infiltrating the skin and the lungs. Histological and cytological infiltrations are characterized by malignant activated lymphocytes with convoluted nuclei and basophilic cytoplasm known as "flowers cells". Malignant T-cells express T-cell lymphotropic markers CD2, CD3, CD4 and CD5 on their surface, in addition to markers of lymphocytic activation: HLA-DP, HLA-D4, HLA-DR and interleukin-2 receptor α chain. Moreover, HTLV-1 serology is always positive.

Patients suffering from either one of the two aggressive forms have a poor prognosis due to chemoresistance and severe immunosuppression with a median survival of 6 months for the acute type and 10 months for the lymphoma type with a 5% projected survival (Bazarbachi 2004). The main difference between the acute and the lymphoma types is the massive infiltration of leukemic T-cells into the peripheral blood and malignant hypercalcemia characterizing the acute form, whereas less than 1% of leukemic cells on a blood smear and less frequent hypercalcemia is seen in the lymphoma subtype. The smoldering and chronic forms are less severe and diagnosed after several months of follow up without treatment.

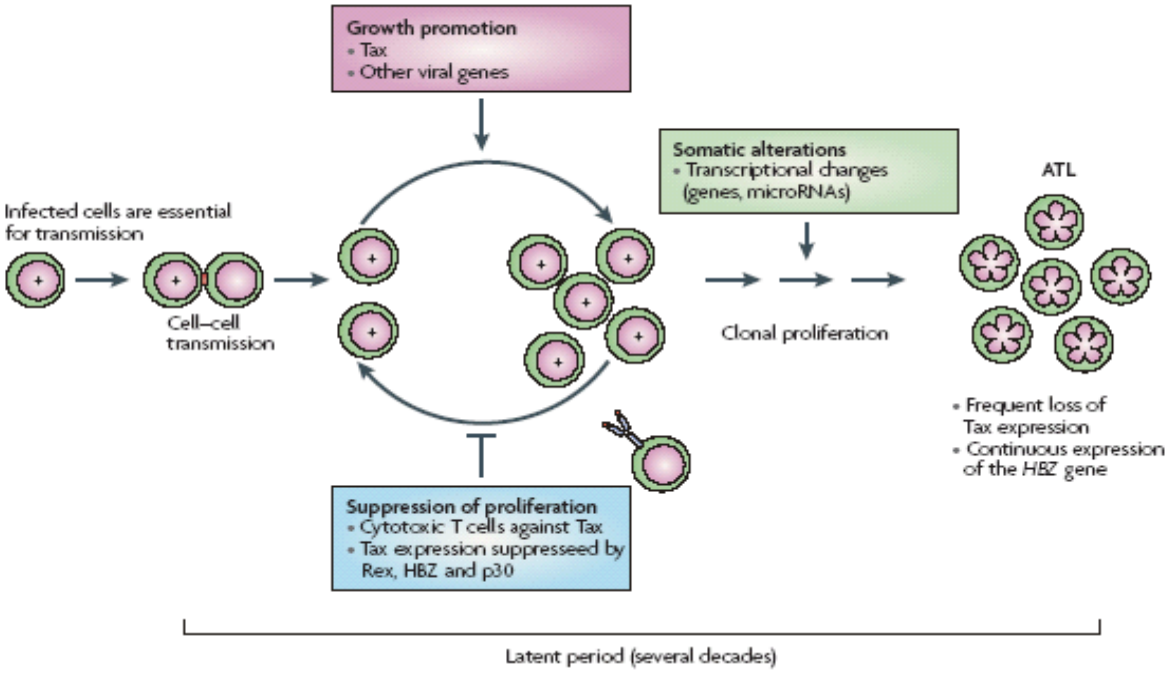


Figure 1. The natural history of HTLV-1 infection.
Source: Matsuoka, M., & Jeang, K. T. 2007. Human T-cell leukemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nature Reviews Cancer* 7(4): 270-280

B. The Oncoprotein Tax

1. Overview

Early after infection with HTLV-1, activated T-cells undergo polyclonal expansion due to virus replication. This is followed by oligoclonal expansion of the infected cells due to the expression of the viral transactivator protein Tax (Morteux 2003). ATL develops after a long latency period (40-60 years) in only 3-5% of HTLV-1 infected individuals. Tax, a 40 KDa phosphoprotein encoded by the PX region of the viral genome, is mainly present in the nucleus of infected cells and is capable of shuttling to the cytoplasm using a nuclear export signal (NES) (Burton 2000). Tax expression in human peripheral blood mononuclear cells (PBMC) induces the death of CD4+ T-cells. In addition, Tax is a target for cytotoxic lymphocytes and Tax expressing cells are continuously eliminated from the circulation (Hanon 2000; Arnulf 2004). Therefore, Tax is hard to detect in circulating leukemic or lymphoma cells.

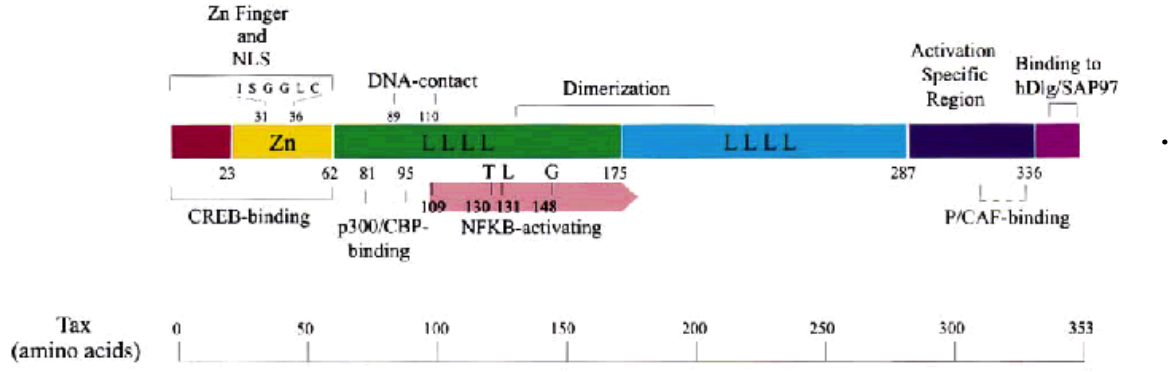


Figure 2. The various functional domains found in Tax.
Source: Jeang KT. 2001. Functional activities of the human T-cell leukemia virus type 1 Tax oncoprotein: cellular signaling through NF kappa B. *Cytokine Growth Factor Reviews* 12: 207-217

2. Cellular Functions of Tax

Tax is a powerful oncogene that alters many cellular pathways including: activation of various transcription factors, inhibition of apoptosis, interference with DNA repair mechanisms and cell cycle regulators (Yoshida 2001). Tax also has a role in influencing the microenvironment *via* inducing angiogenesis, metalloproteinases and gap junction-mediated communication between infected and endothelial cells, eventually causing extravasation and invasiveness of ATL cells (Bazarbachi 2004). Most importantly Tax activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which plays a major role in the transformation and proliferation of T-lymphocytes.

a. Cell Cycle Progression

Tax deregulates the normal cell cycle control in T-cells by targeting different regulators of cell cycle progression. In addition, Tax provides significant mitogenic activity especially at G_1-S-phase transition, by provoking several cellular activities. Although Tax does not interact directly with the G_1 cell cycle regulator protein, Retinoblastoma (Rb), it forms a complex with p16INK4a and p15INK4b, two cell cycle dependent kinase inhibitors (CDK4) rendering them

inactive. Tax-mediated activation of CDK4 results in Rb phosphorylation, which in turn sets the E2F1 transcription factor free and accelerates cell-cycle transition from G_1 to S phase (Yoshida 2001; Waldmann 1993). Tax can also interfere with the cell cycle progression through the transcriptional activation or repression of p18INK4c, cyclins: A, C, D2 and E, CDK2, p21 (CIP1/WAF1), and E2F (Jeang 2004).

Activated by Tax	Consequences
Cell-cycle phase activators (CDK2 and CDK4; cyclin D2; cyclin D3; WAF1; E2F1)	Accelerated G1–S progression and DNA hyper-replication
Growth receptors and proliferative factors (IL2 and IL15; IL2Rα and IL15Rα; telomerase; PCNA)	Increased cellular proliferation and decreased NER DNA repair
Transcription factors (CREB; AP1; SRF)	Increased cellular proliferation
Survival factors (Akt; NFκB)	Suppression of apoptosis and/or senescence; aneuploidy
Centrosome amplification (RANBP1; TAX1BP2)	Aneuploidy
Inactivated by Tax	
Cell-cycle phase inhibitors (p15, p16 and p18; RB; DLG1)	Increased cell-cycle phase transition
DNA repair factors (DNA polymerase β; MMR)	Increased ambient DNA breaks and microsatellite instability
DNA damage response (p53; CHK1; CHK2; telomerase; KU80)	Suppression of apoptosis and/or senescence; abrogation of tumorigenesis barrier
Chromosome instability checkpoint (MAD1; CHK1)	Aneuploidy

Table 1. Cellular activities modulated by Tax
Source: Matsuoka, M., & Jeang, K. T. 2007. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nature Reviews Cancer* 7(4): 270-280

b. DNA Structural Damage

HTLV-1 abrogates cellular checkpoints and DNA repair functions that monitor and edit DNA structural damage in infected cells. The cellular processes attenuated by Tax in HTLV-1 infected cells include the loss of the p53 checkpoint, decreased cellular DNA repair functions

(nucleotide excision repair, base excision repair, or mismatch repair), and the repression of telomerase function.

For example, Tax represses the expression of DNA polymerase, an enzyme involved in base excision repair (Jeang 2004). Furthermore, Tax-mediated inactivation of the p53 checkpoint results in a suppression of the nucleotide excision repair mechanism (Jeang 2004). It is noteworthy that Tax inactivation of p53 is not accomplished by direct binding of Tax to p53, but rather by a unique mechanism that includes constitutive phosphorylation of p53 at S15 and S392 (Pise-Masison 2000) . Analysis of Tax mutants in lymphocytes demonstrates that Tax-induced p53 inhibition correlates with the ability of Tax to activate NF-κB.

Moreover, by repressing the expression of human telomerase (hTert), Tax interferes with protective mechanisms used to prevent inappropriate breakages fusion (Jeang 2004). Tax has also been found to bind and thus impair the function of Mad1, a mitotic spindle checkpoint constituent (Gabet 2003).

c. Apoptosis

Tax has the ability to protect HTLV-1 infected cells from both spontaneous and chemotherapy-induced apoptosis. This oncoprotein induces the expression of the anti-apoptotic regulator Bcl-xL through the NF-κB and the cAMP response element-binding (CREB) pathways, and represses the transcription of the pro-apoptotic Bax gene (Mori 2001; Brauweiler 1997). Furthermore, Tax inhibits the caspase cascade *via* the NF-κB pathway which induces the expression of the inhibitors of apoptosis: X-IAP, cIAP-1 and c-IAP-2 (Kawakami 1999). Moreover, Tax-dependent activation of the NF-κB pathway inhibits p53 transactivation

functions, thus, interfering with G_1 arrest and p53-dependent apoptosis. This explains why in ATL-derived cells, p53 is sometimes mutated but most frequently functionally inactive.

d. Tax Induced-Activation of the NK-κB Pathway

NF-κB is a transcription factor that plays an important role in regulating the expression of genes involved in immune response, inflammation, apoptosis and oncogenesis (Sun 2003). Members of the NF-κB family are kept inactive because they are sequestered in the cytoplasm by the inhibitory proteins of the IκB family. NF-κB members can only control gene expression when they are translocated to the nucleus.

Tax acts on different levels to initiate and maintain NF-κB signaling which is in turn responsible for activating most of the cellular genes in HTLV-1 infected cells (Kfoury 2005). For instance, Tax activates IκB kinases, IKKs, the cellular inhibitors of IκBs. This induces the phosphorylation of IκBs, ubiquitination and proteasomal degradation. As a consequence, the nucleus translocation signal of NF- κB dimers will be exposed and, hence, induces NF-κB translocation to the nucleus where it binds to the consensus sequences of several cellular genes and activates their transcription (Sun 1994; Good 1993).

Another way, by which Tax activates NF- κB pathway, is *via* its interaction with the ankyrin motif of the inhibitory proteins: IκBs. Such an interaction disrupts NF-κB/IκB complex, targets IκB to proteasomal degradation and sets NF-κB free to translocate to the nucleus (Kfoury 2005). Hence, being a main route for ATL cells survival, proliferation, escape from apoptosis and resistance development, NK-κB signaling pathway represents an essential therapeutic target.

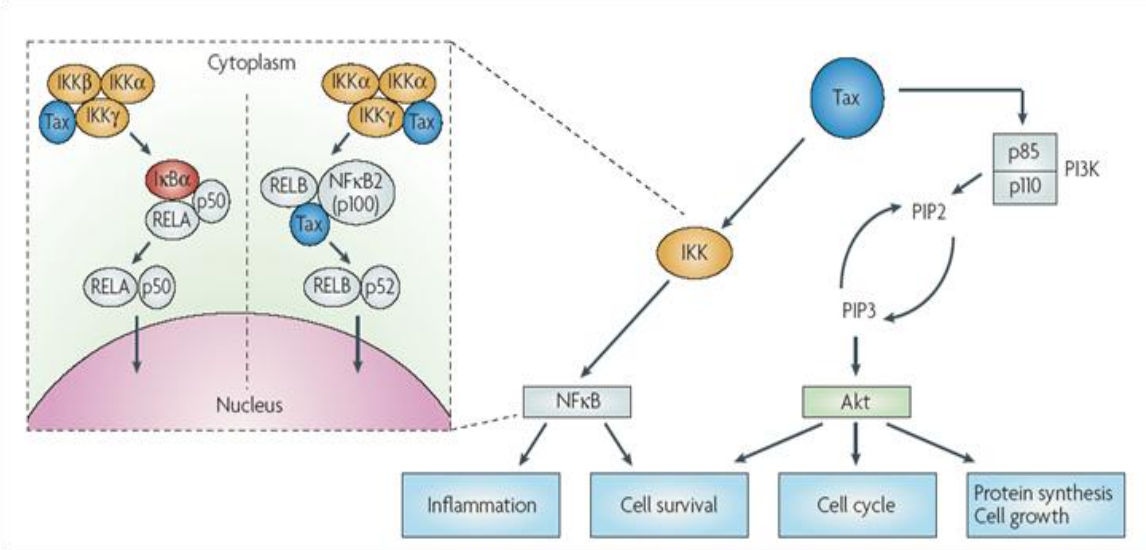


Figure 3. Tax activates two survival pathways, NFκB and Akt, to promote cell survival and proliferation.
Source: Matsuoka, M., & Jeang, K. T. 2007. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nature Reviews Cancer* 7(4): 270-280.

D. Treatment Options of ATL

1. Targeting Tax and NF- κB Pathway

At the molecular level, Arsenic trioxide (AS) and INF-α were able to induce Tax proteasomal degradation with IκB-α and IκB **–ε** upregulation, hence resulting in the cytoplasmic retention of NK-κB and preventing its binding to DNA (Kfoury 2005). The combination therapy of AS/INF-α induces both cell cycle arrest and Tax proteasomal degradation, thus precipitating cell death in the form of apoptosis (Kfoury 2005).

Bay 11-7082, an irreversible inhibitor of IκB-α phosphorylation is another mean of targeting NK-κB pathway and inducing apoptosis of HTLV-1 infected cells. Bay 11-7082 acts by decreasing NK-κB binding to the DNA and down regulating the expression of the anti-apoptotic protein, Bclxl (Mori 2002).

In addition, NK-κB pathway can be shut down by proteasome inhibitors. The proteasome inhibitor PS-341 was shown to block the degradation of IκB-α in ATL cells and to weaken the NK-κB binding to the DNA (Waldmann 2002; Nasr 2005). Moreover, PS-341 stabilizes not only IκB-α, but also IκB-β, IκB-ε, p21, p27 and p53 proteins (Kfoury 2005). PS-341 alone or in combination with chemotherapy inhibits several pathways required for survival of HTLV-1 infected cells (Bazarbachi 2004).

2. Other Treatments Modalities

Adult T-cell leukemia/lymphoma is an aggressive disease characterized with resistance to chemotherapy and poor survival. Importantly, survival is correlated with the disease subtype. For instance, studies have shown that the first treatment option for the acute type should be Zidovudine; an anti-retroviral drug, in combination with INF-α (Gill 1995, Bazarbachi 1996).

Though the mechanism of action of the anti-retroviral therapy is still vague, *in vitro* studies proposed that zidovudine inhibits HTLV-1 replication (Bazarbachi 2000, Isono 1990). In addition, INF-α inhibits the assembly of the virus by preventing the targeting of the viral Gag protein to the lipid micro-domain in the plasma membrane (Feng 2003). An important advantage of the anti-retroviral therapy is the good safety profile that leads to high complete response lasting for several years (Bazarbachi 2004). Yet, consolidative targeted therapies such as arsenic/INF combination or monoclonal antibodies are required after achieving complete response to prevent relapse.

Arsenic trioxide, a very effective treatment for acute promyelocytic leukemia synergizes with INF-α to induce cell-cycle arrest and apoptosis in cells infected with HTLV-1 and in the malignant T-cells of adult T-cell leukemia and lymphoma (Bazarbachi 2004). Arsenic trioxide

in combination with INF-α induces rapid shut-off of the NF-κB pathway and by delayed shut-off of cell-cycle gene after Tax degradation by the proteasome (Bazarbachi 2004).

However, patients with the lymphoma subtype might benefit from initial induction therapy based on aggressive chemotherapy, followed by the anti-retroviral therapy. Targeted therapy is required after achieving complete response to prevent relapse or chemo-resistance (Bazarbachi 2004). In addition, young patients should be considered for allogenic bone marrow transplantation.

Adult T-cell leukemia/lymphoma is invasive in nature and may involve an interaction between HTLV-1 infected cells and endothelial cells. This interaction is achieved when leukemic cells secret high concentrations of the vascular endothelial growth factor (VEGF) and heparin –binding growth factor which together induces angiogenesis (El-Sabban 2002). Kinase inhibitors which act to inhibit angiogenesis provide a new therapeutic approach.

Retinoids, naturally dietary vitamin A (retinol) metabolites, are being tested in different hematological malignancies. HTLV-1 cells are negative for retinoic acid receptor α and thus are resistant to ATRA; the standard induction therapy for acute promyelocytic leukemia (Darwiche 2001). Synthetic retinoids such as HPR was shown to induce cell-cycle arrest and apoptosis in HTLV-1 infected cells both dependently and independently of retinoic acid receptors (Darwiche 2004).

E. Retinoids

1. Overview

"Retinoids" is the generic term that covers naturally dietary vitamin A (retinol) metabolites and active synthetic analogs (Sporn 1976; Chambon 2005). They are important

mediators of a wide variety of essential biological processes such as embryonic morphogenesis and organogenesis, cell growth arrest, differentiation and apoptosis (Sporn 1976; Blomhoff 1994; Sporn 1994; Kastner 1995; Chambon 2005). All *trans*-retinoic acid (ATRA) is an active endogenous metabolite of retinoids and is used in the treatment of patients with acute promyelocytic leukemia (APL) (De The 1990; Degos and Wang 2001; Lin 1999).

2. Mechanism of Retinoid Action

Retinoids bind to and activate the two classes of retinoid receptors: Retinoic acid receptors (RARs) and Retinoid X receptors (RXR). RARs and RXRs are members of the nuclear hormone receptor superfamily (Fontana 2002). Each class has three subtypes: α, β and γ and each subtype have several isoforms that emerge due to differential splicing and promoter usage. RARs are activated by both ATRA and 9-*cis* retinoic acid (9-*cis* RA), while RXRs are activated only by 9-*cis* RA. Activation of RAR or RXR requires the heterodimerization of the two receptors. RARs can only dimerize with RXRs, however, RXRs can homodimerize as well as dimerize with vitamin D, thyroid hormone and with some orphan receptors (Fontana 2002).

The RXR-RAR heterodimers act as a ligand-dependent transcriptional regulators by binding to specific nucleotide sequences termed retinoic acid response element (RARE) or retinoid X response elements (RXRE) in the promoter regions of target genes. In the absence of RAR agonist, RXR-RAR heterodimers recruit co-repressors such as histone decetylases (HDAC) or DNA methyltransferases that condense the chromatin and prevent transcription activation. Once RAR ligands bind, co-repressors are released and co-activator complexes are recruited to activate transcription.

On the other hand, Rexinoids, RXR-selective agonists are not able to autonomously activate RXR-RAR heterodimers in the absence of RAR ligand. This is referred to RXR sub-ordination or silencing (Westin 1998; Germain 2002). A synergistic transcriptional activation requires the simultaneous binding of RAR and RXR agonists. Thus, this indicates that RXR is not a transcriptionally silent partner in the RXR-RAR heterodimer.

3. Retinoids and Leukemia

Several studies demonstrated the effect of ATRA on the growth and differentiation of acute promyelocytic leukemia (APL) cells (Collins 2008). APL cells possess a chromosomal translocation resulting from the fusion of the RARα receptor to the nuclear transcription factor, promyelocytic leukemia gene (PML). RARα nuclear receptor has a vital role in the terminal differentiation of granulocytes only in the presence of its ligand, ATRA. The generation of the PML-RARα fusion product arrests myeloid differentiation at the promyelocyte stage despite the presence of physiological concentration of ATRA (de The 1990). Yet, the role of PML-RARα in the development of leukemia is multi-factorial and is not only through inhibiting RARα function, as PML-RARα represses both RA-dependent and -independent genes in myeloid precursor cells (Fontana 2002). ATRA induces complete remission and increases the survival of more than 90% of APL patients within 40 to 60 days (de The 1990). It is noteworthy that the duration of complete remission induced by ATRA is brief (3-6 months) and relapse is often associated with acquired resistance to ATRA-mediated differentiation, whose etiology appears to be multi-factorial (Freemantle 2003).

Moreover, retinoid administration is associated with severe toxic side effects including teratogenicity, increases in serum triglycerides, mucocutaneous cytotoxicity, head ache and bone

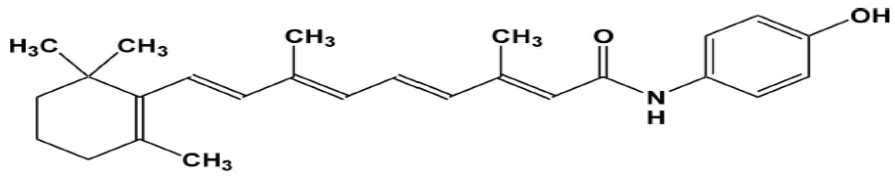
toxicity (Germain 2006). Hence, classical retinoids have a marginal role in the treatment of neoplastic diseases. These facts emphasize the need for indentifying novel strategies to facilitate programmed cell death.

For these reasons, novel series of synthetic retinoid derivatives were synthesized by several pharmaceutical companies. Synthetic retinoids are characterized by their selectivity in binding and trans-activating the retinoid receptor classes and subclasses, but most importantly by working through retinoid-receptor independent pathways (Bernard 1992). These molecules do not show cross-resistance with ATRA, indicating a novel mechanism of action relative to natural retinoids (Holmes 2003; Marchetti 1999). In comparison to classical retinoids, synthetic retinoids are more stable, less toxic and active in cells which are resistant to ATRA and 9-*cis*-RA (Ortiz 2002; Dawson 2004).

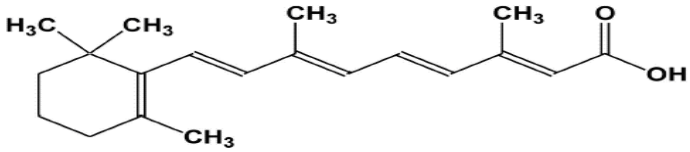
E. Synthetic Retinoids

Atypical retinoids display significant potential in cancer treatment particularly in hematological malignancies (Fontana 2002). Though, the exact mechanism for inducing-apoptosis is still not well defined, yet synthetic retinoids possess unique mechanisms of action. A relevant feature is the ability of retinoid-related molecules to activate p53- independent apoptosis, particularly because mutations in p53, a frequent alteration of tumor cells, lies at the basis of resistance to DNA damaging reagents. Moreover, atypical retinoids do not follow the classical concept of ligand-receptor interaction like natural retinoids, though they can activate ceratin RARs (Cincinelli 2003). However, such compounds have the ability to exert a growth regulatory or apoptogenic activity that is not receptor-mediated. Preclinical and clinical data supported the potential role of synthetic retinoid as cancer therapeutic and chemopreventive

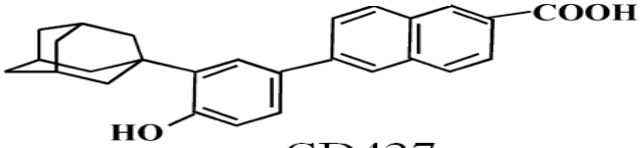
agents. Focusing on developing drugs that induce apoptosis in a RAR-independent manner, researchers synthesized new atypical retinoids such as N-(4-hydroxyphenyl) retinamide (HPR) and -[3-(1-adamantyl)-4-hydroxyl phenyl]-2-naphthalene carboxylic acid (CD437).



HPR



ATRA



CD437

Figure 4. Chemical structures of HPR, ATRA, and CD437

1. N-(4-hydroxyphenyl) Retinamide (HPR)

HPR (Fenritinide) is one of 87 retinoids screened in a series of *in vitro* experiments (Fontana 2002). It was formed to possess significant activity in reversing the keratinization of vitamin A-deficient tracheal organ cultures (Newton 1980). Studies showed that HPR is an extremely active inducer of apoptosis and an important chemopreventive agent against head and neck squamous cell carcinoma, ovarian and breast adenocarcinoma (Fontana 2002). Studies suggested that HPR has multiple pathways to induce apoptosis, some involving the RARs and others are either totally independent of such receptors or involve indirect activation of RARs

(Delia 1993). In most of the cells examined, HPR induced-apoptosis is predominantly RAR-independent.

An initial and important event in HPR-induced apoptosis is through the generation of reactive oxygen species (ROS). This was seen in human cervical carcinoma cell line, prostate carcinoma cell line and HL-60 human leukemia (Sun 1999; Oridate 1997). HPR has also the ability to down-regulate c-Myc expression, telomerase activity and important cell regulatory proteins, such as cyclin D1 and p34/cdc2 (Bednarik 1999; Delia 1995). These events following exposure to HPR appear to be cell type-specific.

2. HPR and ATL

Concerning the clinical relevance of HPR in ATL, studies have shown that clinically achievable concentrations of HPR induce growth inhibition, G_1 arrest and apoptosis in HTLV-1 transformed and HTLV-1 negative lymphoma cells, including ATRA-resistant cell lines (Darwiche 2001; Darwiche 2004). HPR treatment in HLTV-1 negative cell lines resulted in a significant loss of mitochondrial membrane potential, rapid and important ceramide accumulation and activation of the caspase cascade, including caspase-3, 8 and 9 and complete PARP cleavage (Darwiche 2004). On the other hand, in HTLV-1 positive cells, HPR did not induce ceramide accumulation and was associated with a moderate loss of mitochondrial membrane potential. Moreover, no significant caspase activation was observed. Instead, HPR treatment in HTLV-1 positive cells was associated with down regulation of cyclin D1 and up regulation of Bax protein which is consistent with HPR-mediated G_1 arrest and cell death in these cells. Furthermore, in HTLV-1positive cells only, HPR treatment rapidly induced a proteasomal-mediated degradation of p21 whose expression is upregulated at both the transcript

and protein levels in HTLV-1 infected cells. Tax is known to transactivate the p21 promoter which was recently shown to prevent apoptosis and to stimulate the growth of HTLV-1 positive cells (Kawata 2003). Upon Tax degradation by HPR, p21 is downregulated in HTLV-1 positive cells (Darwiche 2004). The induction of p21 during apoptosis and senescence is evident by the secondary increase of p21 at 24 h of treatment with HPR (Darwiche 2004).

It is noteworthy, that HTLV-1 negative cells were more sensitive to HPR than HTLV-1 infected T-cells with a completely different mechanism of action (Darwiche 2004). This might be explained by the fact that Tax-induced activation of NF-κB pathway may protect HTLV-1 transformed cells from caspase-dependent apoptosis and ceramide accumulation (Darwiche 2004). Moreover, HPR induces rapid and progressive ROS generation in malignant T-cells. This increase in intracellular ROS levels results in ceramide accumulation, cell cycle arrest, cytochrome c release, caspase-activation and caspase-dependent apoptosis (Darwiche 2007). In HTLV-1 infected cells, Tax expression inhibits ROS generation and prevents the secondary cellular events and therefore, protects cells from HPR-induced growth inhibition (Darwiche 2007).

3. CD437 Overview

6-[3-(1-adamantyl)-4-hydroxyl phenyl]-2-naphthalene carboxylic acid (CD437) is one of the retinoids synthesized to selectively bind RARγ though it can bind RARβ and minimally RARα (Bernard 1992; Chao 1997). Studies have demonstrated that CD437 may exert its effects through RAR-independent pathways. CD437 is active in retinoid-resistant T-cells and retinoid antagonists cannot block this activity (Fontana 2002). The potential role of the RARs in CD437-mediated apoptosis is cell type dependent. CD437 induces cell-cycle arrest as an initial event

after CD437 treatment (Shao 1995). The stage at which cell-cycle is arrested is cell-type specific. For instance, CD437 induces rapid G_1 cell cycle arrest in breast carcinoma cells, while in other cell-types, CD437 arrests the cell cycle in the S phase (Li XS1996; Zhang 2000).

In addition, the role of the tumor suppressor gene, p53, and the activation of the mitogen-activated protein kinase (MAPK) pathway is implicated in CD437-mediated apoptosis (Sun 1999).

F. ST1926: An Analog of CD437 with Promising Anticancer Activities

1. Discovery and Characteristics

Despite the pre-clinical and clinical data supporting the potential role of CD437 and HPR as antiproliferative and pro-apoptotic agents, these synthetic retinoids have limited potential given their narrow window between therapeutic and toxic doses as well as their relatively unfavorable pharmacokinetic profile. To by-pass these obstacles, researchers synthesized new atypical retinoids, also termed as retinoid related molecules (RRMs), which are analogs of CD437 and maybe more potent, less toxic and more bio-available. The novel chemical series of CD437 congeners, whose prototype is ST1926 was introduced in 2003 by Cincinelli *et al* (Cincinelli 2003).

ST1926 or E-4-(4'-hydroxy-3'-admantyl biphenyl-4-yl)acrylic acid is prepared in a high yielding three-step sequence where CD437 was used as the reference compound, while keeping the pharmacologically important groups (hydroxyl, lipophilic admantyl moiety and carboxylic groups) connected by different linkers and as well keeping the distance between the C1 of adamantyl in CD437 and the carboxylic Carbon at 11.4 Å. However, the naphthalene ring in CD437 was replaced by styrene moiety in ST1926 (Cincinelli 2003).

Studies have shown that ST1926 has almost complete loss of ability to trans-activate RARs and effective apoptosis is mainly *via* genotoxic stress induction in tumor cells. In a large panel of human tumor cell lines, ST1926 growth-inhibitory activity was apparently p53-independent (Cincinelli 2003). In addition, ST1926 indirectly modulates host anti-tumor immune response *via* maintaining T-cell and B-cell functions and increasing their number in blood. ST1926 also increases the percentage of NK-cells in tumor nodules and increases TRAIL concentration in plasma. Moreover, it up-regulates FAS-ligand expression in lymphocytes (Sun 2000).

Based on the fact that ST1926 has a broad spectrum of activity, a unique pharmacological profile and is well tolerated *in vivo* after oral administration, this novel adamantyl retinoid has been tested in cancer clinical trials and has shown promising anticancer effects in solid tumors, leukemia and lymphoma.

2. Effects of ST1926 in Different Malignancies

a. APL

In APL, the molecular mechanism underlying the activity of ST1926 has been explored. Microarray analysis evaluating the expression of around 1200 genes in NB4 cell line treated with ST1926, showed down regulation of mitochondrial, ribosomal and translation-related proteins. Hence, *de-novo* protein synthesis is not required for apoptosis induction by this adamantyl retinoid (Garattini 2004). Interestingly, the data indicated that ST1926 induced an immediate and long- lasting increase in cytosolic calcium in NB4 cell line (Garattini 2004). This increase is dose dependent and correlates with the apoptotic potential of ST1926. Importantly, the effective concentrations inducing apoptosis and cytotoxicity were 6-9 folds lower in ST1926 as compared

to those observed for CD437. Although, elevated cytosolic calcium is necessary for apoptosis, yet it is not sufficient for apoptosis- induction by ST1926, since myeloid cell lines refractory or resistant to ST1926 are still responsive to calcium mobilizing activity of this retinoid related molecule (RRM) (Garattini 2004). Observations made with uncouplers of the oxidative phosphorylation which takes place at the level of the mitochondrial membrane, indicated that ST1926 increases cytosolic calcium because of an inhibition of the energy dependent calcium uptake into the mitochondria. This elevation is immediate and preceeds the opening of the mitochondrial transition pores and release of cytochrome c into the cytosol (Garattini 2004). Moreover, this study shows ST1926 as a promising anti-leukemic agent *in vivo* after oral administration. Interestingly, the combination effect of ST1926 and ATRA in the aggressive model of SCID mouse transplanted with NB4 cells gives insights about the efficiency of such combination therapy in myeloid leukemia.

Another study by Valli *et al* aiming at investigating the mechanism of cell death induced by ST1926 in APL, showed that the adamantyl retinoid produced DNA-double strand breaks (DSB) and chromosomal lesions in the NB4 cell line (Valli 2008). They proposed a direct correlation between the levels of DSBs and the anti-proliferative effect induced by the RRM. Hence, DNA damage was found to be the major mechanism underlying the anti-leukemic activity of ST1926 in NB4 and other AML cell lines (Valli 2008). Specifically, increases in DSBs are maximal during S phase of the cell cycle. Moreover, induction of DSBs by ST1926 was a very early phenomenon occurring minutes after treatment and is not secondary to apoptosis. Although the nature and the exact molecular mechanisms responsible for the DNA damage caused by ST1926 are unknown, it was shown to be associated with the activation of ATM, ATR, DNA-PK and P38.

b. Ovarian Carcinoma

In ovarian carcinoma, several studies revealed the molecular mechanisms mediating apoptosis by ST1926. In 2008, Zuco *et al* showed that p53 activation and early up regulation of p53 target genes were consistent with p53-dependent apoptosis in ovarian carcinoma cell line, IGROV-1 (Zuco 2004). Such a pattern of DNA damage response in ovarian carcinoma supports the hypothesis that genotoxic stress is a critical event mediating drug-induced apoptosis. Moreover, IGROV-1 cells exhibited efficient G_1 cell cycle arrest consistent with p53-mediated apoptosis. This pathway appeared to be regulated by stress-activated protein kinases, JNK and p38.

HDAC inhibitor, a modulator of the chromatin structure, can also sensitize cells to apoptosis and enhanced DNA response following the combination to ST1926 in ovarian carcinoma cell line, IGROV-1 (Zuco 2010). This provides an additional support to the role of genotoxic event in the apoptotic activity of ST1926 in ovarian carcinoma (Zuco 2003).

The activation of survival signaling pathways mediated by the endothelial growth factor receptor (EGFR) is a critical event in determining the susceptibility to ST1926-induced apoptosis (Zuco 2004). ZD1839, an EGFR receptor inhibitor, inhibits protective survival pathways, in particular ERK- mediated processes and cellular pathways controlling DNA repair (Koizumi 2004). Zanchi *et al* showed that ST1926 can also synergize with EGFR receptor inhibitor, ZD1839, to produce an enhanced anti-proliferative and apoptotic effect in the ovarian carcinoma cell lines, IGROV-1 (Zanchi 2005).

c. Lung Carcinoma

In lung carcinoma, H460 cells are sensitive to ST1926-induced apoptosis *via* the activation of caspases 3, 8 and 9, hence implicating that both the intrinsic and extrinsic pathways of apoptosis converge to promote efficient cell-death (Zuco 2005). A genotoxic stress of unknown nature is also detected in ST1926 treated H460 cell line. The pattern of genotoxic stress was evident by p53 and p73 activation, up regulation of p21 and Bax, and cell cycle arrest at S-phase check point in H460-sensitive cells.

d. Teratocarcinoma

ST1926 and CD437 are better RARγ agonists than ATRA (Parrella 2006). In order to evaluate the significance of RARγ in the activity of RRM, teratocarcinoma cell lines with different RARγ expression was used to study the significance of the RARs for the anti-neoplastic activity of ST1926. Only in the presence of RARγ, ST1926 activates cytodifferentiation. However, despite the absence or presence of RARγ *in vitro,* the adamantyl retinoid arrests cells in the G_2/M phase of the cell cycle, up regulates pro-apoptotic genes belonging to both intrinsic and extrinsic pathways of apoptosis and induces the expression of genes involved in DNA repair emphasizing the fact that ST1926 has a DNA damaging action (Parrella 2006).

Experimental data indicated that RARγ and the classical retinoid pathway are not at the basis for the anti-proliferative and apoptotic activity of RRM *in vitro*. In addition, increase in cytosolic calcium was shown to be fundamental for apoptosis in the different teratocarcinoma cell lines. Gene expression profiles associated with ST1926 indicated that this atypical retinoid can work through retinoid receptor independent pathway, in addition to RARγ-dependent pathway (Parrella 2006). Interestingly, RARγ was shown to hinder the anti-tumor activity of

ST1926, where both syngeneic and immune-suppressed SCID mice with tumor lacking RARγ have increased life spans after ST1926 treatment relative to their counterparts bearing RARγ (Parrella 2006). This was explained by the ability of ST1926 to modulate some aspects of the host/tumor interaction *in vivo*, perhaps by inducing the expression of genes involved in cell motility and adhesion, a characteristic not seen *in vitro*.

e. Neuroblastoma

In a neuroblastoma (NB) preclinical model, Di Fransesco *et al* investigated the antitumor activity of ST1926 both *in vitro* and *in vivo.* ST1926 is capable *in vitro* of producing indirect DNA damage due to DNA-drug interaction, cell cycle arrest in late S- G_2 phases, and p53-independent apoptosis (Dri Fransecso 2007). In fact, the data showed that DNA damage was not the result of oxidative stress and was repaired 24 h after drug removal. Moreover, ST1926 was shown to mediate programmed cell death in a caspase-independent manner. *In vivo*, oral administration of ST1926 inhibited tumor growth of NB xenografts with tolerable toxicity (Di Frasnseco 2007). An important observation of this study is the ability of ST1926 to induce cell growth inhibition, DNA damage and apoptosis in cell lines that are refractory to ATRA, especially that ATRA is an important component of treatment for residual disease of stage IV neuroblastoma. And since treatment cytotoxicity and resistance development represent limiting factors for ATRA treatment in NB, ST1926 has promise to be a powerful agent in NB alone or in combination with ATRA. Retinoic acid was shown to enhance the cytostatic and apoptogenic properties of ST1926 in NB cell lines. In combination, ATRA and ST1926 resulted in enhanced G_2/M arrest and accumulation of cells in pre G_0/ G_1 population (Di Fransesco 2011). PARP cleavage, caspase 3, 8 and 9 activation and modulation of DR5 and FAS are evidence for

apoptosis induction upon co-treatment of the two drugs. Although p53 may not be the only upsteam signal for apoptosis induction, it appeared to enhance apoptosis by ST1926 and ATRA combination in NB cell lines (Di Fransesco 2011).

G. Aim of the Study

Using *in vitro* models of ATL/lymphoma and peripheral T lymphomas, we aim at investigating the effect of ST1926 on cell cycle regulators, cell death mechanisms and retinoid signaling. We will use HTLV-1 positive cells (HuT102, MT2 and C81) and HTLV-1 negative cells (CEM, JURKAT and Molt-4).

1- Test the effect of ST1926 on the growth of ATL and malignant T-cell lines, primary ATL and normal resting and activated lymphocytes.
2- Determine the effect of ST1926 on cell cycle and apoptosis.
3- Confirm whether ST1926 induce apoptosis by inducing DNA damage.
4- Investigating whether ST1926-induced apoptosis involves:
    1- Caspase activation
    2- Mitochondrial membrane dissipation
5- Determine whether ST1926 has oxidant properties by measuring reactive oxygen species generation.
6- Evaluate whether ST1926- induced growth inhibition is dependent or independent of RAR/RXR signaling pathway in malignant T-cells.
7- Determine the effect of ST1926 on retinoid signaling pathways.

Understanding the mechanism of action of ST1926 in ATL and malignant T-cells will hopefully support a potential therapeutic role of this synthetic retinoid in ATL/lymphoma and peripheral T-lymphomas.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

1. HTLV-1 Positive and Negative Malignant T-cell Lines

CEM, Jurkat, and Molt-4 are HTLV-1 negative $CD4^+$ malignant T-cell lines, whereas, C8166, HuT-102, and MT2 are HTLV-1 transformed $CD4^+$ T-cell lines. The HTLV-1 transformed ATL cell lines are infected with the HTLV-1 retrovirus and transformed by the oncoprotein and transactivator, Tax, that is produced by the pX region of the HTLV-1 transcriptome. CEM cells are T-lymphoblastoid cell lines derived by Foley *et al,* (Foley 1965) from peripheral blood of a 4-year-old Caucasian female with acute lymphoblastic leukemia (ALL). Jurkat cell lines were originally established by Schneider *et al,* (Schneider 1977), from the peripheral blood of a 14-year-old with ALL in relapse. Molt-4 cells were first identified as rosette-forming human lymphoid thymus-derived T-cell lines. Molt-4 cells were extracted from patients with ALL in relapse and who had received prior multi-drug chemotherapy. Molt-4E6 lacks a functional p53 due to transfection with E6 protein of human papilloma virus. Molt-4LXSN is the control cell line used for Molt-4E6, being transfected with an empty vector. C8166 cells are immortalized, non-producer, human umbilical cord blood lymphocyte cultures developed by cocultivation or fusion of fresh cells with T- cells cultured from leukemia-lymphoma patients. These transformed neonatal leukocytes exhibit morphological, cytochemical, and other phenotypic characteristics similar to those of other HTLV-1 infected cells. However, in contrast to the usual productive infection seen, these cells contain only low amounts of viral proteins and do not release virus particles, thus, they are defective for viral

replication. In addition, viruses in C8166 cells have other defects in their transcriptional machinery. HuT-102 cells were originally derived from the peripheral blood of a 26 year old Black male patient with cutaneous T-cell lymphoma and were found to contain type C retrovirus particles (Poiesz 1980). MT2 cells are similar to HuT-102 cells.

2. Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were collected from healthy HTLV-1 negative donors after obtaining their informed consent (American University Hospital, AUB). Venous blood was collected into heparinized syringes using uniform standards. The heparinized blood was diluted 1:2 with phosphate buffered saline (PBS) (Gibco, Invitrogen, Paisley, UK), pH 7.2, and layered on a Ficoll-Hypaque gradient (Lymphoprep, Nyegaard, Norway). The gradient was centrifuged at 800x*g* for 30 minutes at room temperature and the PBMC band was collected and washed twice in PBS. Before ST1926 addition, resting PBMC were cultured for 24 h at $1x10^5$ cells/ml in RPMI-1640 medium containing 10% heat inactivated FBS) and antibiotics. Activated PBMC were grown in Ham's F10 medium (Gibco) supplemented with 2% PHA (Gibco).

3. Primary ATL Cells

Primary ATL cells were collected from PBMC which were isolated from HTLV-1 positive donors after obtaining their informed consent (American University Hospital, AUB), and centrifuged over Ficoll-Hypaque. Before ST1926 addition, cells were grown for three days in 10% Rec IL-2, 20% heat inactivated FBS, and a ratio of 1:400 PHA. At this stage, the cells

can be frozen for future use. When needed, cells are thawed and cultured in 20% heat inactivated FBS and 10% Rec IL-2.

B. Cell Growth and Treatment

All HTLV-1 positive and negative cells were cultured in RPMI 1640 medium (Gibco) containing 10% heat inactivated FBS, 25 mM Hepes buffer, 1% L-glutamine, 1% penicillin-streptomycin antibiotics, 1% kanamycin solution, and 1% sodium pyruvate (Gibco). All cells were grown in a humidified incubator (95% air, 5% CO_2).

1. Cell Passaging

Cells were passaged or fed for maintenance and expanded before an experiment. To feed or passage the HTLV-1 positive and negative cells, around 75% of the cell suspension volume is discarded, followed by the addition of RPMI 1640 medium three times the volume of the remaining cells. Cells were usually fed every two to three days depending upon the rate of growth of the cell lines. To expand the number of cells (before an experiment), RPMI 1640 medium was added to the cells to increase volume of cells three fold. For experimentation, cell number was calculated using a hemocytometer according to the following formula: cells/ml = average number of cells x dilution factor x volume of suspension x 10^4. Cells were counted using trypan blue dye exclusion using 0.4% trypan blue solution. For most experiments, cells were seeded at a density of 2 x 10^5 cells/ml. Tissue culture flasks were purchased in a variety of sizes from the Falcon division of Becton, Dickinson and Co. (Cockeyesville, MD).

2. Preparation of ST1926 and ATRA

ST1926 was obtained from Sigma TAU (Rome, Italy) and was reconstituted in 0.1% dimethylsulfoxide (DMSO) at a concentration of $1x10^{-2}$ M and $3.3x10^{-2}$ M, respectively under amber light, aliquoted, stored at –80^{o}C, and used for up to six months. For use in experimentation, an aliquot of stock ST1926 ($1x10^{-2}$ M) was either applied directly on cells, or serially diluted under amber light in 0.1% DMSO to produce stock dilutions ranging in concentration from $5x10^{-8}$ M to $5x10^{-6}$ M ST1926. These stocks of diluted ST1926 were used once and then discarded.

ATRA (purchased from Sigma Chemical Co) was prepared as stock solution in DMSO at $3.3x10^{-2}$ and stored in amber tubes at -80°C. The final concentration of DMSO never exceeded 0.1% and this concentration showed no effect on the proliferation of all tested cell lines (data not shown). All experiments using ATRA were performed under yellow light (λ>500 nm) to prevent photoisomerization.

3. Preparation of Pan-Caspase Inhibitor z-VAD and the Pan-RAR Inverse Agonist BMS493

The Z-VAD- FMK (FK109) (MP Biomedicals, LLC) and BMS493 (Santa Cruz Biotechnology) were dissolved in DMSO, stored at -20°C and +4°C, respectively, and used at a final concentration of 100 μM and 1 μM, respectively.

C. Viability Assays

All cell lines were seeded into 96-well plates at a density of $2x\ 10^{5}$ cell/ml. Cells were, then, treated with 0.1% DMSO or varying concentrations of ST1926 ($5x10^{-8}$- $5x10^{-6}$ M). Medium containing the appropriate treatment was kept for three days, and viability at days 1, 2,

or 3 post-ST1926 treatments was assayed using the CellTiter 96® non-radioactive cell proliferation assay kit (Roche, Mannheim, Germany) according to manufacturer's instructions. Relative MTT dye uptake is assessed by optical density (OD) measurement at 595 nm using an ELISA microplate reader. Results are expressed as growth relative to DMSO-treated controls and are derived from the mean of quadruplicate wells.

D. Cell Cycle Analysis

Cells were cultured in 25 cm^2 flasks (Falcon) and collected at 24 or 48 h after ST1926 treatment. Cells were, then, washed twice with cold PBS, fixed in ice cold 100% ethanol, and stored for 24 h at -20°C. Subsequently, they were rinsed with PBS, incubated for one hour in PBS containing 50 units RNaseA (Roche, Mannheim, Germany) and then stained with propidium iodide (PI) (50 μg/ml) (Sigma). Cell cycle analysis was performed using a FACS scan flow cytometer (Becton Dickinson). Each sample was collected as 10,000 ungated events and the corresponding cell cycle distribution was determined using CellQuest software (Becton-Dickinson).

E. TUNEL Assay

The TUNEL assay is a measure of apoptosis through detection of DNA strand breaks (a late apoptotic event). DNA strand breaks contain free 3'-OH termini which may be conjugated to dUTP-fluorescein through the enzymatic action of terminal deoxynucleotidyl transferase. Fluorescein fluorescence is detected by flow cytometry and serves as a measure of apoptosis. In brief, cells are seeded (10 million cells/condition) and treated with 0.1% DMSO for control or $1x10^{-6}$ M ST1926. Two extra control flasks, one for

positive and one for negative controls, are prepared from any cell line used in the experiment. Cells are washed and fixed with 4% formaldehyde solution for 15 min at room temperature, and then the dry pellet is stored at -20 ^{o}C for not more than one week. Cells are then washed with 1X PBS and incubated with 250 µl permeabilization solution (0.1% triton X-100 in 0.1% sodium- citrate) on ice for 4 *min*. Only the positive control cells are then incubate in 200 µl of 1mg/ml DNase (prepared in 50 mM Tris pH 7.5) for 15 min at room temperature, then washed twice with 1X PBS. Other samples are washed once with 1X PBS, and pellets are re-suspended and incubated for 60 min at 37oC in an incubator in the dark in TUNEL reagents: 50 µl Label solution for negative control tube, 50 µl TUNEL reaction mixture for other samples (TUNEL reaction mixture: 50 µl enzyme + 450 µl label solution). Cells are then washed twice with 1X PBS, re-suspended in 1 ml PBS, and transferred into polystyrene falcon round bottom tubes for flow cytometry analysis (with the excitation wavelength set at 470–490 nm and the emission wavelength at 505 nm).

F. Rhodamine 123 Staining

Quantitation of MPT was determined by rhodamine (R123) (Sigma) retention. R123 is a cationic fluorescent dye that accumulates in active mitochondria with high membrane potentials. ST1926 treated cells were washed twice with buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, 1 mM MgCl2, and 25 mM Hepes buffer (pH 7.4), and were then loaded with 5 µM R123 for 30 min in the buffer at 37oC. Subsequently, cells were washed twice with the former buffer. R123 was excited at 488 nm, and fluorescence emission at 525 nm was assessed using flow cytometry. The results were analyzed using CellQuest software (Becton-Dickinson).

G. ROS Measurement

Generation of intracellular ROS was measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) kit (Molecular Probes, Eugene, OR, USA). CM-H2DCFDA is a cell-permeant indicator for ROS that is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell yielding the fluorescent derivative 2'-7'-dichlorofluorescin. Following indicated treatments, cells were spun down and resuspended in 500 μl RPMI containing 2% FBS and 10 μM H2DCFDA for 20 min at 370C. Subsequenly, cells were washed twice with PBS and then analyzed with a FACS scan flow cytometer (Becton Dickinson, CA, USA) with excitation set at 488 nm and emission at 530 nm.

H. Immunoblot Assays

1. Protein Extraction

Total cellular protein extracts were prepared from cultured cells washed twice with ice-cold 1x PBS and lysed with SDS-lysis buffer (0.25 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.002% bromophenol blue, 10% β-mercaptoethanol). Protein concentration was determined using the DC Protein Assay from Bio-Rad (Hercules, CA) according to the manufacturer's instructions.

2. Gel Casting

Denaturing polyacrylamide gels were cast in two layers according to the following procedure. Using the Bio-Rad electrophoresis cell, the gel casting stand was assembled according to manufacturer's instructions. A 12% separating gel was prepared by combining 3 ml

of 30% acrylamide/0.8% N'N'-bis-methylene-acrylamide solution, 2 ml of 4x separating gel buffer (1.6M tris-HCl pH 8.8, 10% (w/v) SDS) and 2.5 ml ddH_2O. A 10% gel was prepared in the same way except 2.5 ml acrylamide solution and 3 ml ddH_2O were used. Before pouring the separating gel, 45 μl 10% APS (freshly prepared) and 12 μl N,N,N',N'-Tetramethylethylenediamine (TEMED) were added. The gel was poured to ~3 cm below the top of the smaller glass plate and water-saturated isobutanol was layered atop the gel to allow for a level upper surface. After polymerization of the separating gel (~10-15 min), the water-saturated isobutanol was rinsed off three times using a 1x separating gel buffer solution. The stacking gel was then prepared by combining 1 ml of 30% acrylamide / 0.8% N'N'-bis-methylene-acrylamide solution, 1.7 ml of 4x stacking gel buffer (0.5 M tris-HCl pH 6.8, 0.4% (w/v) SDS), and 4 ml ddH_2O. Before pouring the gel, 40 μl 10% APS (freshly prepared) and 10.6 μl TEMED were added. The gel solution was poured to the top of the smaller glass plate and a comb was inserted between the plates. After polymerization of the stacking gel (30-60 min), the comb was gently withdrawn and the wells were rinsed with 1x electrophoresis buffer (prepared from a 5x solution containing 120 mM tris base, 1 M glycine, 0.5% (w/v) SDS, pH to 8.3).

3. Gel Running and Protein Transfer

After the gels were cast, cellular protein extracts (25-100 μg) were loaded and run in 1x electrophoresis buffer at 74 Volts for ~1.5 h using the Mini-PROTEAN II electrophoresis cell unit. After the run, gel sandwiches were disassembled and gels were set up for transfer in the Bio-Rad Trans-Blot® Electrophoretic Transfer Cell according to manufacturer's instructions. Proteins were transferred onto nitrocellulose membranes (Biolab) in transfer buffer [(50mM tris

base, 77 mM glycine, 0.04% SDS (w/v), 20% methanol (v/v)] under 30 Volts overnight at 4^{o}C while stirring.

4. Hybridization and Protein Detection

Hybridization conditions for Bax, Bcl2, caspase 3, PARP, p21, Tax, p53, phosphorylated p53, RARα, RARγ, RXRα and GAPDH (Santa Cruz) are similar. Briefly, membranes were incubated in TBS (10 mM tris-HCl pH 8.0, 150 mM NaCl) containing 5% dry milk and 0.05% tween 20 for 1 hr at room temperature while shaking. Membranes were then incubated with the primary antibody for either 2 h at room temperature or overnight at 4^{o}C. Membranes were then washed 3 times (10 min per wash) in TBS containing 0.05% tween 20 and placed in the secondary antibody (Santa Cruz, horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat) at a dilution of 1:5000 for 1 h at room temperature while shaking. Finally, membranes were washed 3 times (10 min per wash) with TBS containing 0.05% tween 20 before protein detection.

Proteins were detected by enhanced chemiluminescence using the ECL system (Santa Cruz) in conjunction with horseradish peroxidase-conjugated secondary antibodies. Membranes were then exposed to x-ray films for varying time periods (10 sec-15 min). Equal protein loading and quality were verified using GAPDH staining and parallel gel staining (20 min) with a filtered comassie blue solution (2.5 g comassie blue, 454 ml methanol, 92 ml glacial acetic acid, 454 ml ddH_2O) followed by 3 washes (10 min per wash) in a high methanol destain solution (45.4% methanol, 7.5% glacial acetic acid) and one overnight wash in a low methanol destain solution (5% methanol, 7.5% glacial acetic acid) at room temperature. Comassie-stained gels were then dried at 70^{o}C for 1 h.

I. Densitometric Analysis

Bands were quantified using Image J software (National Institutes of Health, Maryland, USA).

J. Statistical Analysis

Data presented are the means ± SD of *n* assays as noted in the figure legends. Significant differences were determined using the Independent T-test. Significance was set at indicated p-values = 0.05.

CHAPTER III

RESULTS

A. Effect of ST1926 on the Proliferation of HTLV-1 Positive and Negative Malignant T-cells, Primary ATL Cells and Normal Lymphocytes

We used three HTLV-1 transformed cell lines: HuT102, MT2 and C8166; and three HLTV-1- negative cell lines: CEM, Jurkat and Molt-4. All cell lines with the exception of C8166 ($P<0.01$; Dunnett and ANOVA tests) and to a lesser extent CEM ($P<0.01$; Dunnett test and ANOVA tests), are resistant to pharmacological levels of ATRA up to 5 μM (Darwiche 2001). However, treatment with the synthetic retinoids; HPR and CD437 at concentrations ranging between 0.5 μM and 10 μM induced growth inhibition, G_0/G_1 arrest, and apoptosis in all tested HTLV-1 transformed and malignant T cell lines (Darwiche 2004; unpublished results). Then, we tested the synthetic retinoid ST1926 in order to determine which synthetic retinoid is the most potent in killing ATL and T-lymphoma cells.

1. HTLV-1 Positive Cells are Sensitive to ST1926

We used three HTLV-1 transformed cell lines: HuT-102, MT2, and C8166 to test for the effect of ST1926 on cell growth and viability. We used ST1926 concentrations ranging from from 0.05 to 5 μM as micromolar concentrations are physiologically achievable (Valli 2008). ST1926 treatment at concentrations from 0.05 μM to 5 μM resulted in a dose- and time dependent growth inhibition of the three tested cell lines as measured by the CellTiter 96® non-radioactive cell proliferation assay (Figure 5). Growth inhibition was first observed at 24 h and was almost complete by 72 h in all tested ATL cell lines at 1 μM. After 24 h of treatment, 1 μM ST1926 inhibited growth by 70% of control in HuT102, 60% of control in MT2 and by 50% of

control in C81. Similar trends were observed with the trypan blue dye exclusion and the use of the CellTiter 96® non-radioactive cell proliferation assays (data not shown).

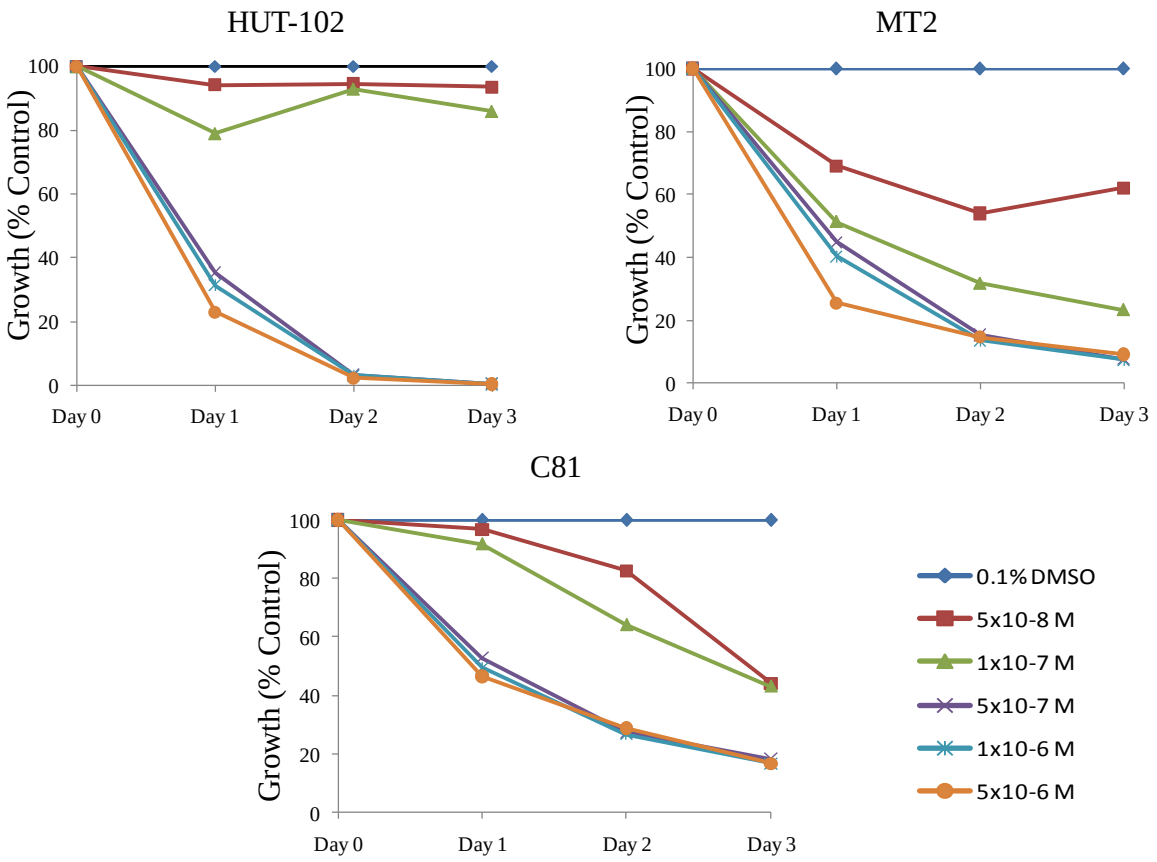


Figure 5. ST1926 treatment of HTLV-1 positive cells causes a concentration and time-dependent growth suppression. HuT-102, C8166, and MT2 were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO or with varying concentrations of ST1926 ranging from $5x10^{-8}$ to $5x10^{-6}$ M up to three days. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of four independent experiments.

2. HTLV-1 Negative Cells are Sensitive to ST1926

We used three HTLV-1 negative cell lines: CEM, Jurkat, and Molt-4 to test for the effect of ST1926 on cell growth and viability. We used concentrations of ST1926 ranging from 0.05 μM to 5 μM. ST1926 treatment at different concentrations resulted in a dose and time-dependent growth inhibition of all tested cell lines (Figure 6). Growth inhibition was first observed at 24 h and was almost complete by 72 h at $5x10^{-6}$ M ST1926. After 24 h of treatment, 1 μM ST1926 inhibited growth by 61% of control in CEM, 70% of control in Jurkat and by 80% of control in Molt-4. Similar trends were observed with the trypan blue dye exclusion and the use of the CellTiter 96® non-radioactive cell proliferation assays (data not shown).

Based on the above results, HTLV-1 negative cells were shown to be as sensitive to ST1926 as HTLV-1 positive cells. Growth inhibition was first observed at 24h and was almost complete by 72h at 1 μM ST1926 in both HTLV-1 positive and HTLV-1 negative malignant T-cells.

3. Primary ATL Cells are sensitive to ST1926

We also investigated the effect of ST1926 on HTLV-1 infected circulating T-lymphocytes. Peripheral blood mononuclear cells (PBMC) were obtained from two ATL patients (Bitar 2009). ATL cells were treated with $1x10^{-6}$ and $5x10^{-6}$ M ST1926 up to 96 h and cell growth was assayed using the Cell Titer 96® non-radioactive cell proliferation kit. Control cells were treated with 0.1% DMSO. The first patient newly diagnosed and untreated previously, was more sensitive to ST1926, inhibiting growth of primary ATL by 50% at 1 μM concentration after 24 h and growth inhibition was complete after 96 h of treatment. However, the same concentration only inhibited growth of primary ATL cells from patient two by 30%

after 24 h of treatment with ST1926 and growth inhibition reached 60% at 1 μM after 96 h (Figure 7). It is noteworthy that patient two was in a relapse stage when his primary ATL cells were treated with ST1926.

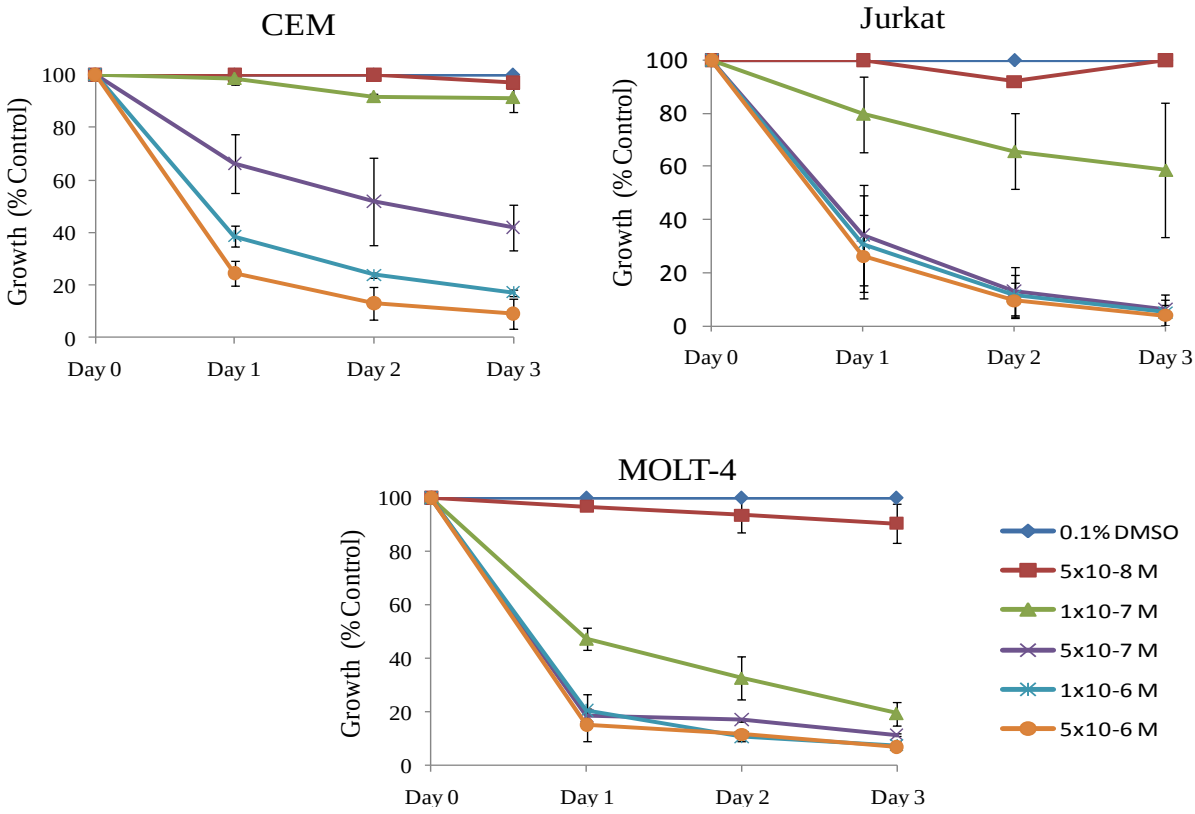


Figure 6. ST1926 treatment of HTLV-1 negative cells causes a concentration - and time-dependent growth suppression. CEM, Jurkat, and Molt-4 were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO or with varying concentrations of ST1926 ranging from $5x10^{-8}$ to $5x10^{-6}$ M up to three days. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of four independent experiments.

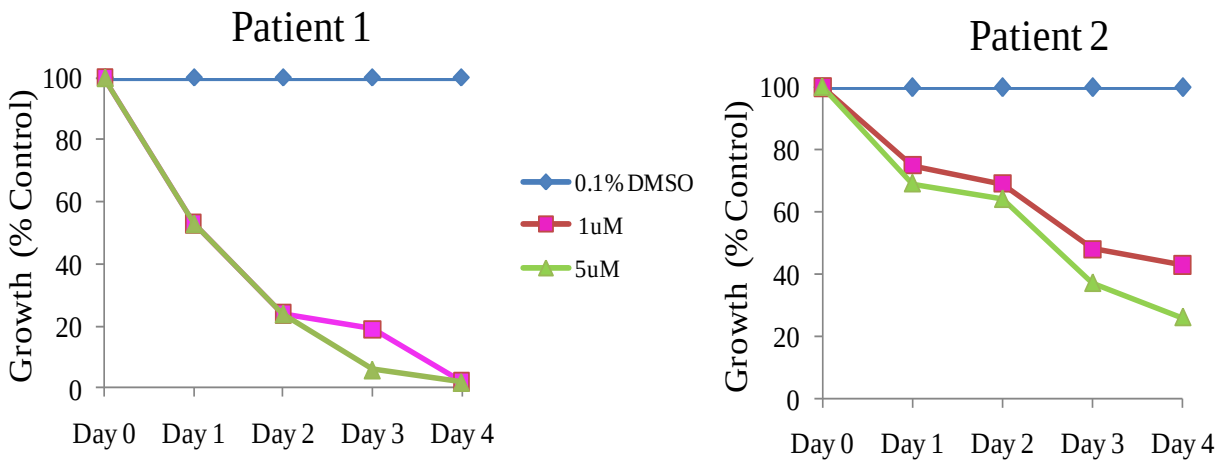


Figure 7. Primary ATL cells are sensitive to ST1926. Before treatment, cells were cultured for a maximum of three days in 10% Rec IL-2, 20% FBS, and 2% PHA. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO) from two ATL patients.

4. Normal T Lymphocytes are Resistant to ST1926

We investigated the effect of ST1926 on normal resting and activated circulating T-Lymphocytes. Peripheral blood mononuclear cells (PBMC) were obtained from three healthy donors. Resting and PHA-activated PBMC were treated with 5 μM and 10 μM ST1926 for 48 h and their viability was assayed by using the Cell Titer 96® non-radioactive cell proliferation assay kit. Control cells were treated with 0.1% DMSO. Most importantly, resting and PHA-stimulated normal T-cells from three healthy donors were completely resistant to ST1926 treatment up to 10 μM pharmacological concentration. We observed less than 10 % decrease in growth of normal resting or activated PBMCs after 48 h of treatment with 10 μM ST1926 (Figure 8).

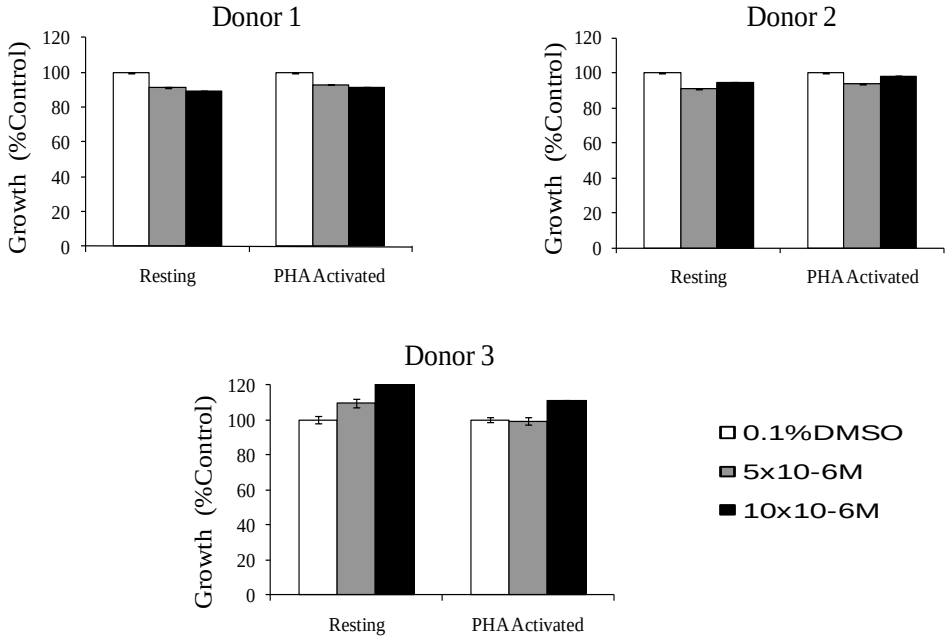


Figure 8. Resting and activated T-lymphocytes are resistant to pharmacological concentrations of ST1926. PBMC were collected from three healthy donors after their informed consent. Activated PBMC were supplemented with 2% PHA. Cells were seeded at a density of $2x10^5$ cell/ml in 24-well plates and treated with 0.1% DMSO or $10x10^{-6}$ M ST1926 up to 48 h. Cell growth was assayed in triplicate wells using the Cell Titer 96® non-radioactive cell proliferation kit. Results are representative of three donors and expressed as percentage of control (0.1% DMSO) (±) SD.

B. Cell Cycle Effects of ST1926 on HTLV-1 Positive and Negative Cells

Four representative cell lines (HuT102, MT2, Molt-4 and Jurkat) were selected for cell cycle analyses in order to investigate the mechanisms involved in ST1926-induced growth inhibition and cell death.

1. ST1926 Treatment Causes an Accumulation of Cells in the Pre G_1 Region

In order to investigate the mechanisms involved in ST1926-induced growth inhibition and cell death, cell cycle analysis was performed after treating the HTLV-1 positive cells (HuT102 and MT2) and the HTLV-1 negative cell lines (Jurkat and Molt-4) with 1 μM ST1926

up to 48 h. 1μM ST1926 concentration caused at least 90% inhibition of growth at 48 h of these four T malignant cell lines.

ST1926-treated and control cells were stained with PI (50 μg/ml) and the cell cycle analysis was performed using a FACScan flow cytometer. No major variation in cell cycle distribution was observed between untreated HuT102, MT2, Jurkat, and Molt-4 cells. ST1926 induced a significant increase in the presumably apoptotic pre G_1 region in all tested cell lines (Figure 9, 10). However, ST1926 effects were more pronounced in Jurkat and Molt-4 cells reaching 54% and 45% of cells in the pre G_1 phase at 24 h of treatment with 1 μM ST1926 (Figure 10), while the same concentration after 24 h of treatment induced pre G_1 accumulation of 24% and 17 % of HuT102 and MT2 cells, respectively (Figure 9).

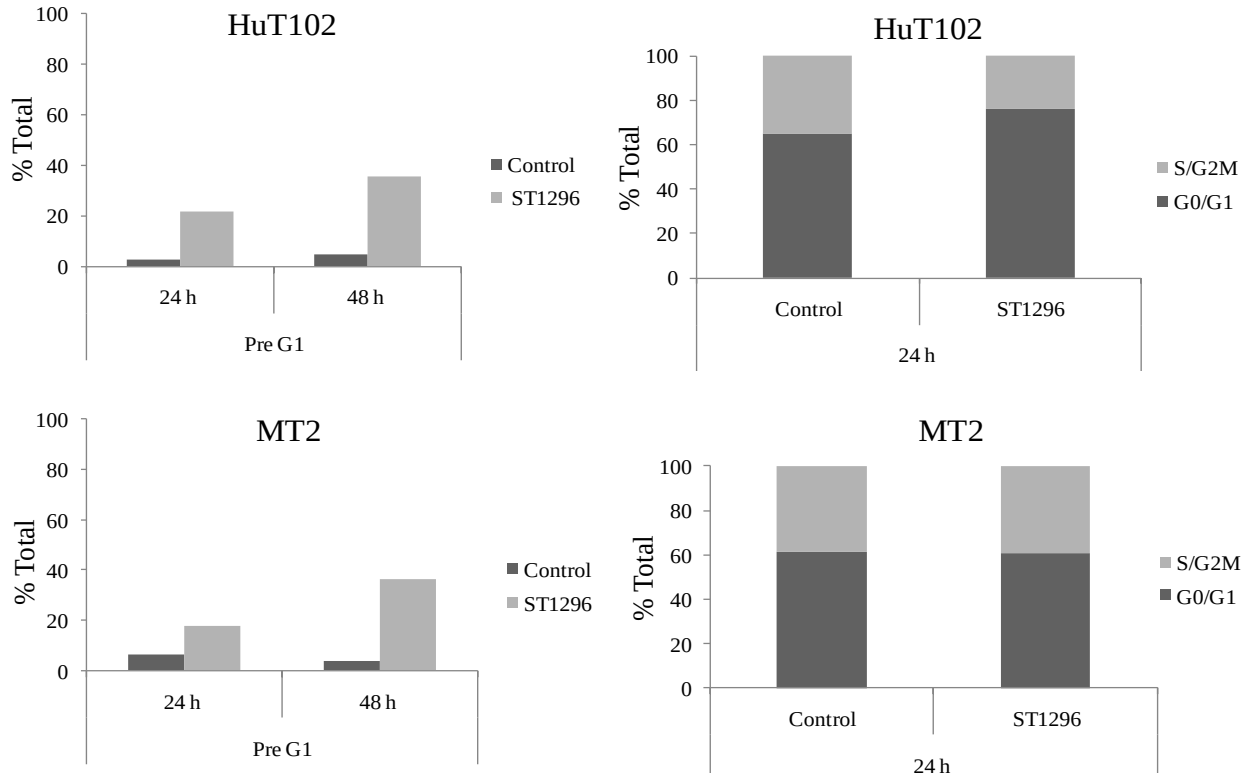


Figure 9. Effects of ST1926 on the cell cycle distribution in HTLV-1 positive cells. HuT102 and MT2 were treated with 1 μM ST1926 up to 48 h while control cells were treated with 0.1% DMSO. The pre G_1 percentage represents apoptotic cells. Cycling cells, the sum of (S+G2/M)

phases, are a percentage of non-apoptotic cells and detected 24 h post treatment. % of cells in G_1 phase is calculated as 100 minus (S+G2/M). The results are representative of two independent experiments.

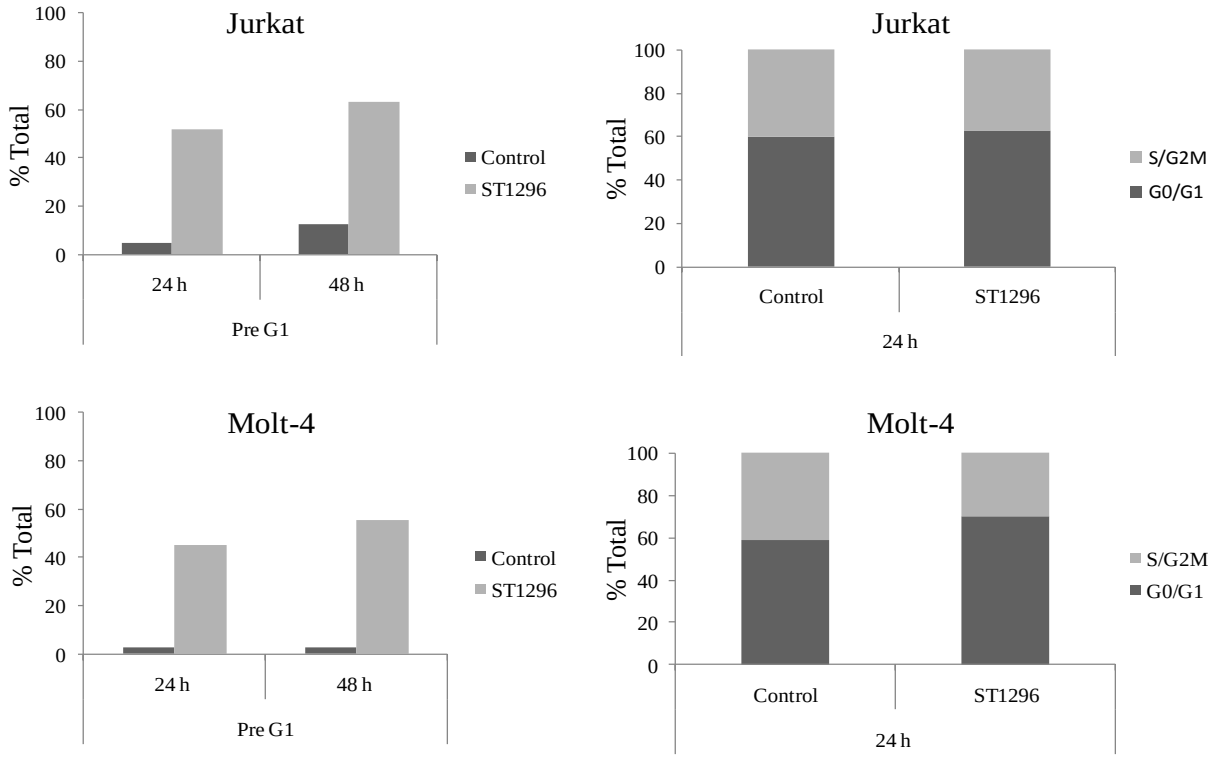


Figure 10. Effects of ST1926 on the cell cycle distribution in HTLV-1 negative cells. Jurkat and Molt-4 were treated with 1 μM ST1926 up to 48 h while control cells were treated with 0.1% DMSO. The pre G_1 percentage represents apoptotic cells. Cycling cells, the sum of (S+G2/M) phases, are a percentage of non-apoptotic cells and detected 24 h post treatment. % of cells in G_1 phase is calculated as 100 minus (S+G2/M). The results are representative of two independent experiments.

C. ST1926 Induction of Apoptosis by TUNEL Assay

To confirm the induction of apoptosis in ST1926- treated malignant T-cells, we used the TUNEL assay that reveals DNA cleavage by labeling free 3'-OH ends. We treated HuT102 and MT2 cells with 1 μM ST1926 up to 48 h. At 24 h, the percentage of TUNEL-positive HuT-102 and MT2 cells was 3% and 1% respectively, for the control cells. This percentage did not

increase upon treatment with 1 μM ST1926 for 24 h. However, after 48 h of treatment the percentage of TUNEL-positive HuT-102 increased from 1 % for the control to 18% for cells treated with 1 μM ST1926 and increased in MT2 cells from 7% for the control to 20% (Figure 11)

Interestingly, TUNEL-positive Jurkat cells was 8% for the control cells and 73% for cells treated with 1 μM ST1926 after 24 h of treatment. Molt-4 cells showed a similar trend of TUNEL-positivity where after 24 h of treatment with 1 μM ST1926, the percentage increased from 7% for the control to 78% for treated cells (Figure 12).

These results indicate that ST1926 is more powerful in inducing apoptosis in HLTV-1 negative cells as compared to HTLV-1 transformed cells.

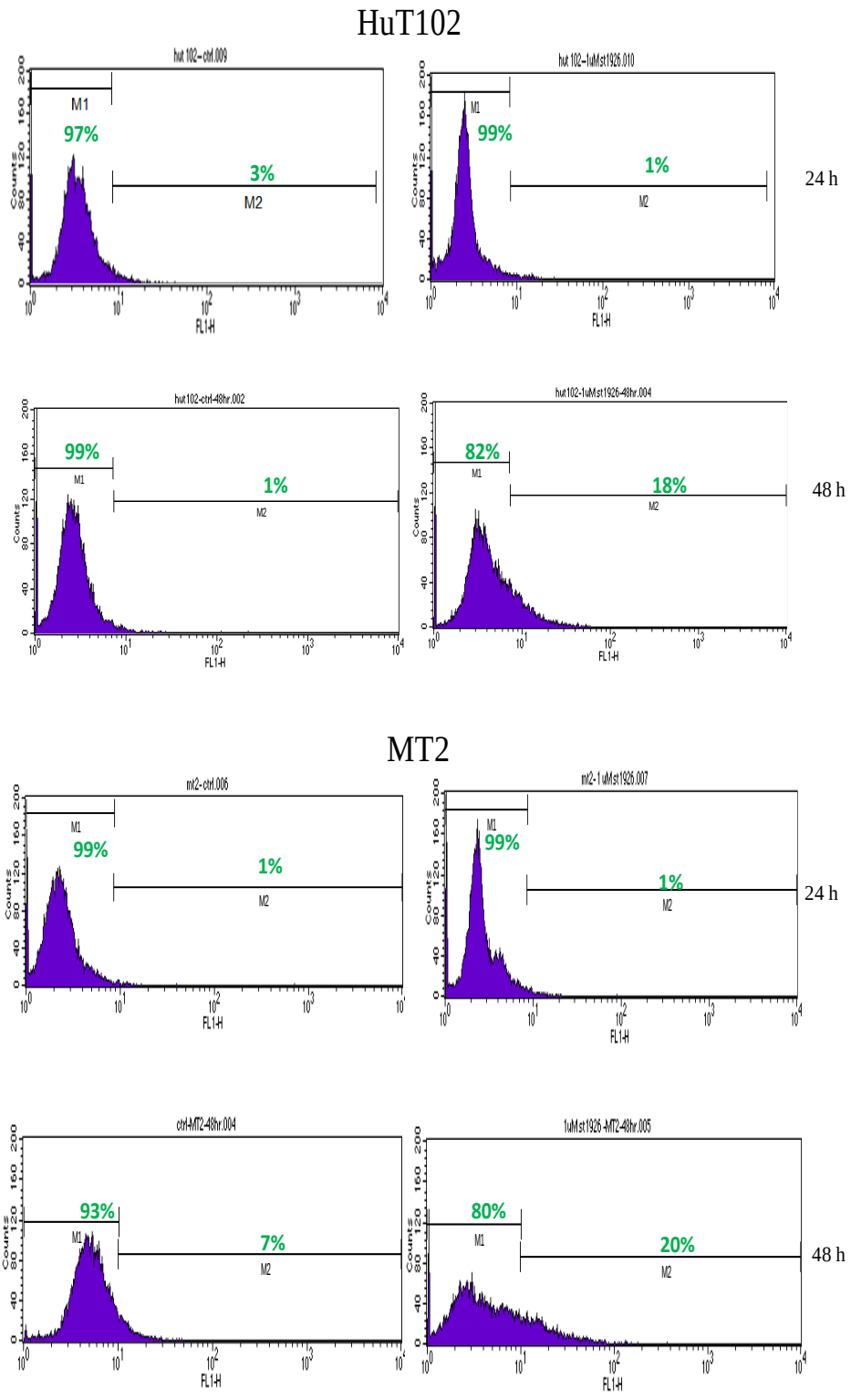


Figure 11. ST1926 induces apoptosis at 48 h by TUNEL positivity in HTLV-1 positive cells. TUNEL analysis of HuT-102 and MT2 cells treated with 1 μM ST1926 for 24 h and 48 h. The results are representative of two independent experiments.

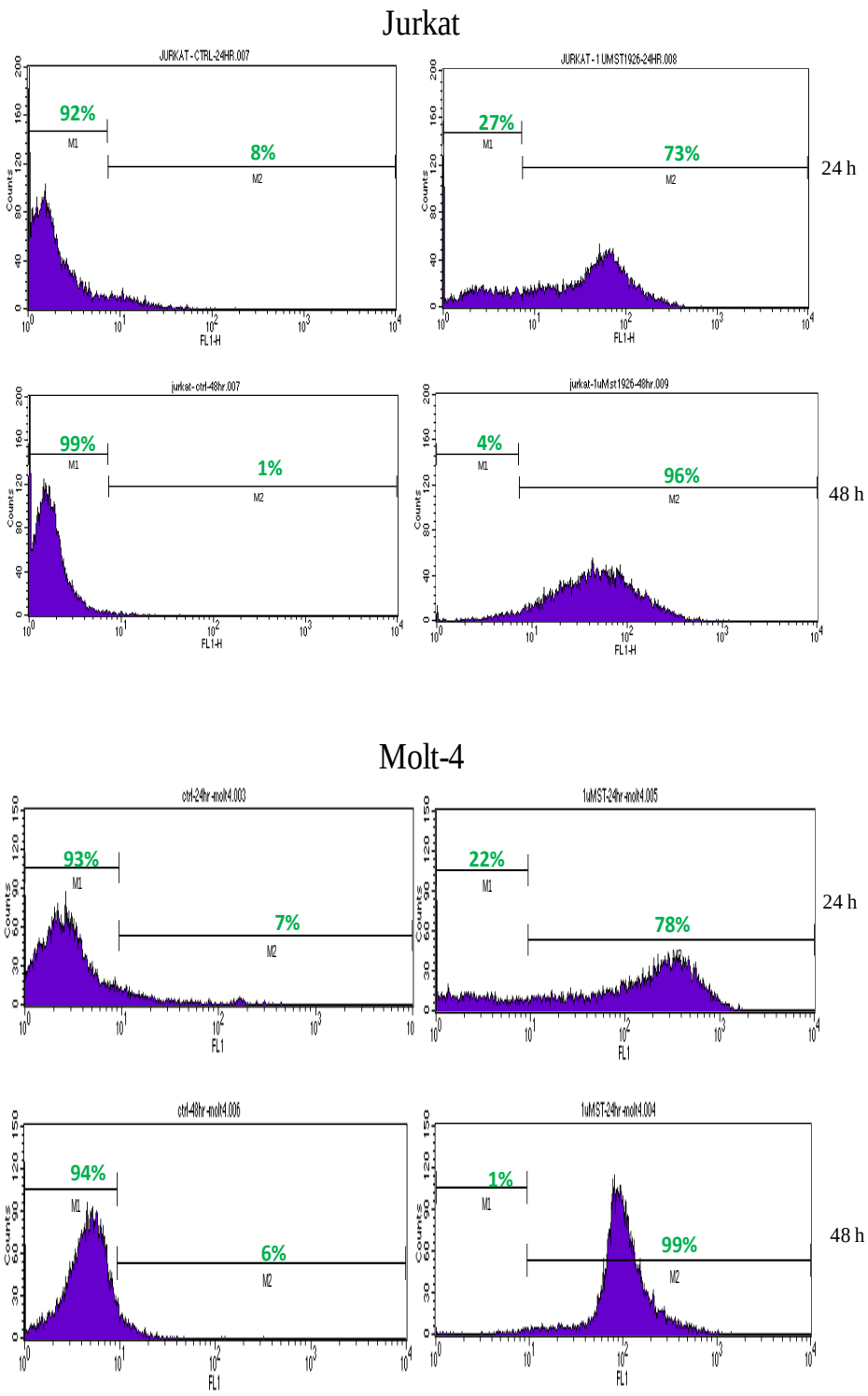


Figure 12. ST1926 induces apoptosis at 24 h by TUNEL positivity in HTLV-1 negative cells. TUNEL analysis of Jurkat and Molt-4 cells treated with 1 μM ST1926 for 24 h and 48 h. The results are representative of two independent experiments.

D. The Effect of ST1926 on Mitochondrial Membrane Potential Dissipation

One of the earliest measurable changes in apoptosis occurs when the outer membrane of the mitochondria becomes permeable and proteins located in the intermembrane space are released into the cytosol. Therefore, we investigated the apoptotic effect of ST1926 on the mitochondrial membrane dissipation in HTLV-1 positive and negative cell lines. We treated HTLV-1 positive cells (HuT-102 and MT2) and HTLV-1 negative cells (Jurkat and Molt-4) with 1 µM ST1926 up to 48 h. The mitochondrial membrane dissipation of ST1926-treated cells was measured by the uptake of the liphophilic green fluorescent dye, rhodamine 123 (R123), by flow cytometric analysis. R123 is a cationic dye that accumulates in the active mitochondria with high membrane potential and decreases upon mitochondrial membrane potential dissipation. HTLV-1 positive HuT-102 and MT2 cells showed minor mitochondrial membrane dissipation at 1 µM ST1926 concentrations after 24 h of treatment. The percentage of HuT-102 cells with low rhodamine fluorescence slightly increased from 13% in control cells to 19 % in 1 µM ST1926-treated cells and from 9% to 17% in MT2 at 1 µM ST1926-treated cells (Figures 13). Jurkat and Molt-4 cells showed a more pronounced decrease of mitochondrial membrane potential after 24 h of treatment at the same ST1926 concentration. Jurkat cells, treated with 1 µM ST1926 for 24 h, resulted in 38% of cells with low rhodamine fluorescence as compared to 8% of control cells (Figure 14). Similar results were obtained with ST1926-treated Molt-4 cells, where percentage of cells with low rhodamine fluorescence after 24 h of treatment with 1 µM ST1926 increased from 7% in control cells to 56% in treated cells (Figure 14). These results clearly indicate that ST1926 treatment causes a major loss of mitochondrial membrane potential in HTLV-1 negative cells while a minor loss is observed in HTLV-1 positive cells.

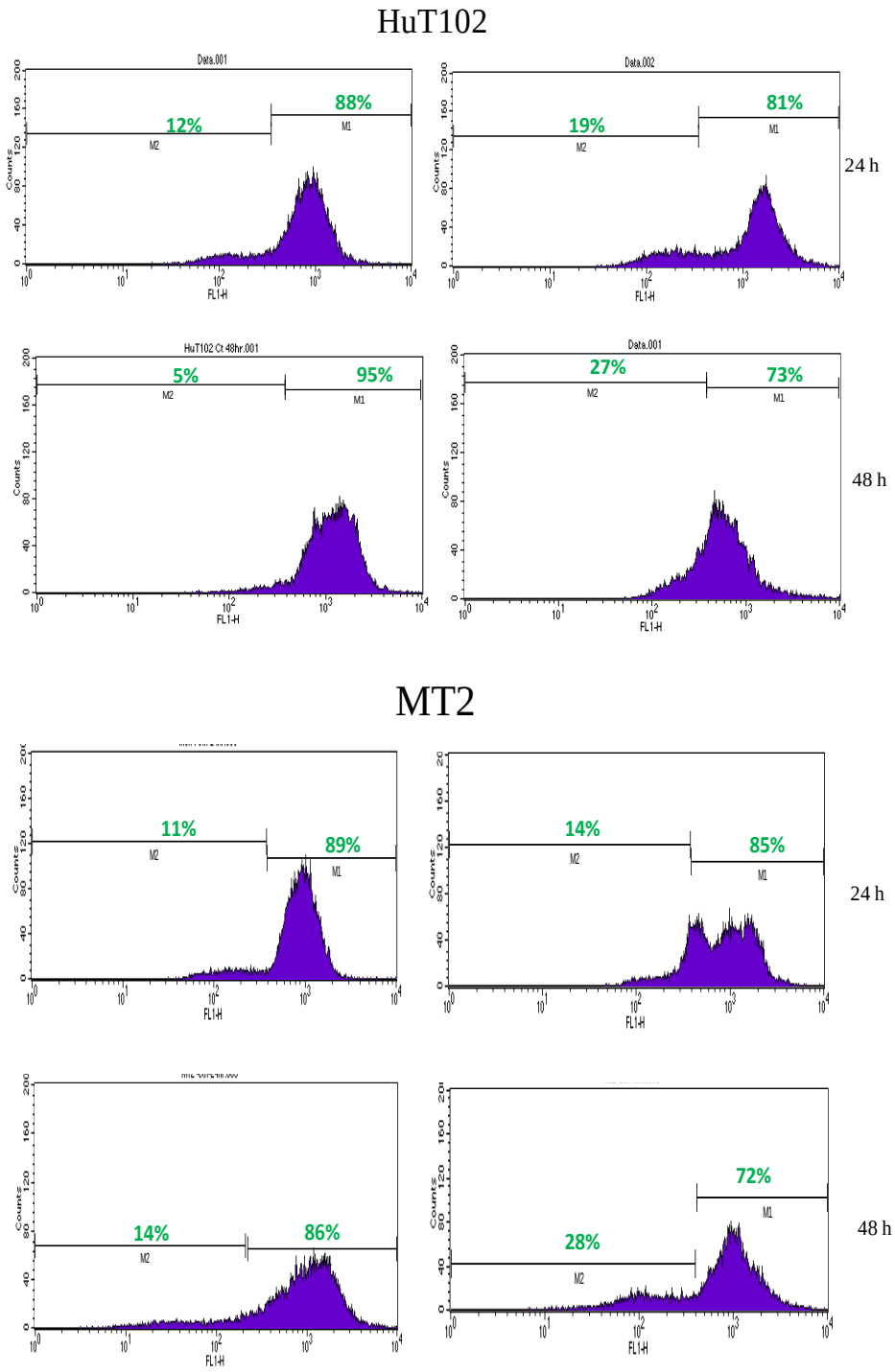


Figure 13. ST1926 treatment causes minor dissipation of mitochondrial membrane potential in HTLV-1 positive cells. HuT102 and MT2 were seeded at a density of $2x10^5$ cells/ml and treated with either 0.1% DMSO, $1x10^{-6}$ M ST1926 up to 48 h. Cells were then stained with R123 as described in "Materials and Methods". Fluorescence emission at 525 nm after excitation at 488 nm was quantified using CellQuest software. Results are representative of two independent experiments.

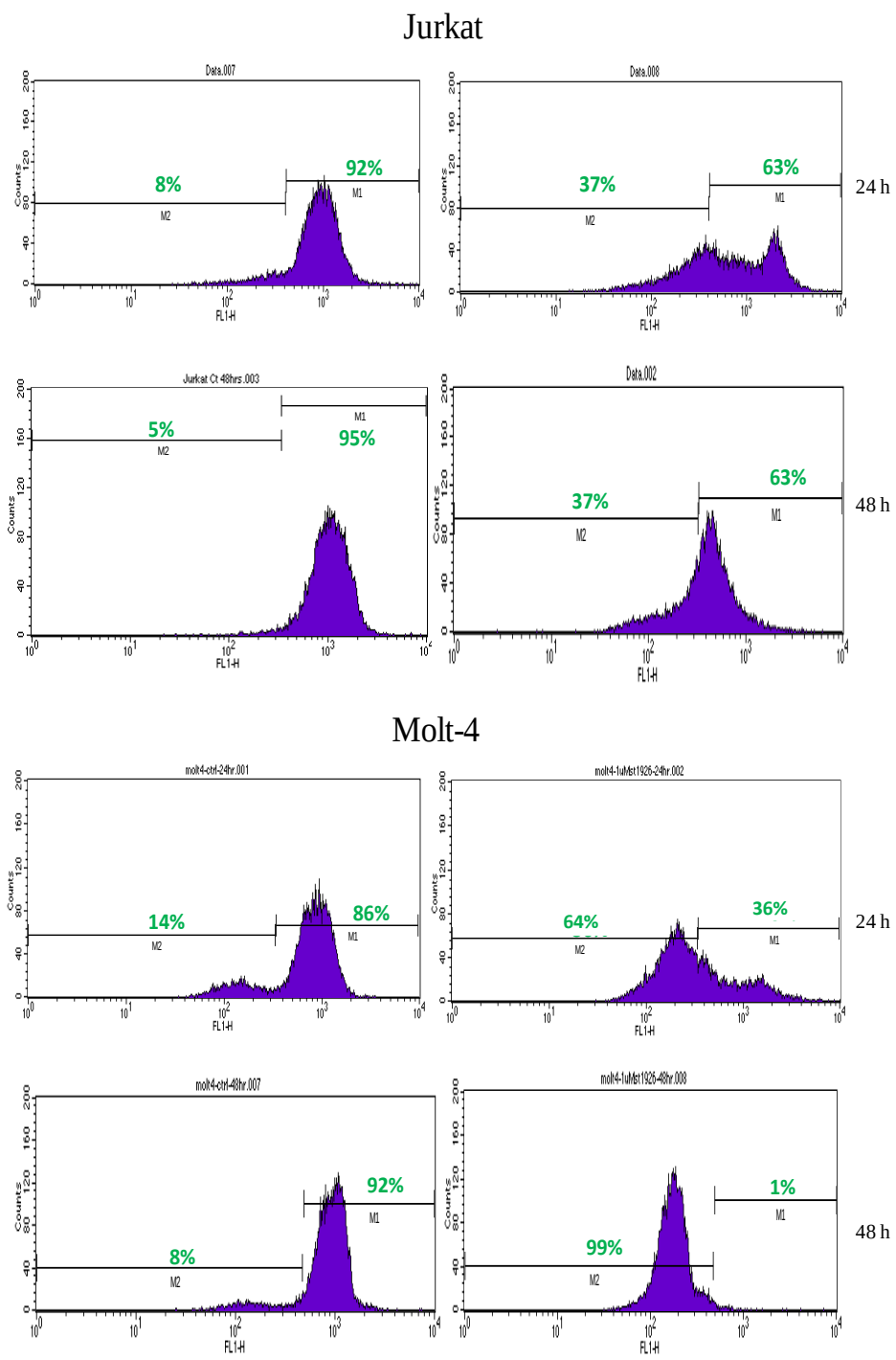


Figure 14. ST1926 treatment causes major dissipation of mitochondrial membrane potential in HTLV-1 negative cells. Jurkat and Molt-4 were seeded at a density of $2x10^5$ cells/ml and treated with either 0.1% DMSO, $1x10^{-6}$ M ST1926 up to 48 h. Cells were then stained with R123 as described in "Materials and Methods". Fluorescence emission at 525 nm after excitation at 488 nm was quantified using CellQuest software. Results are representative of two independent experiments.

E. Effects of ST1926 on Several Apoptotic Regulators in HTLV-1 Positive and Negative Malignant T-cells

Moreover, we studied the effects of ST1926 on several apoptotic regulators to further understand ST1926-induced growth inhibition and apoptosis in malignant T-cells. We tested the effect of 1 µM ST1926 after 2, 12 and 24 h on the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins in the HTLV-1 positive cells (HuT-102 and MT2) and HTLV-1 negative cells (Jurkat and Molt-4). We did not observe any decrease in Bcl-2 neither an increase in Bax protein levels up to 24 h post-treatment (Figure 15). Similar results were observed in HTLV-1 negative cells (Jurkat and Molt-4) treated with 1 µM ST1926 regarding Bax and Bcl2 proteins (Figure 16).

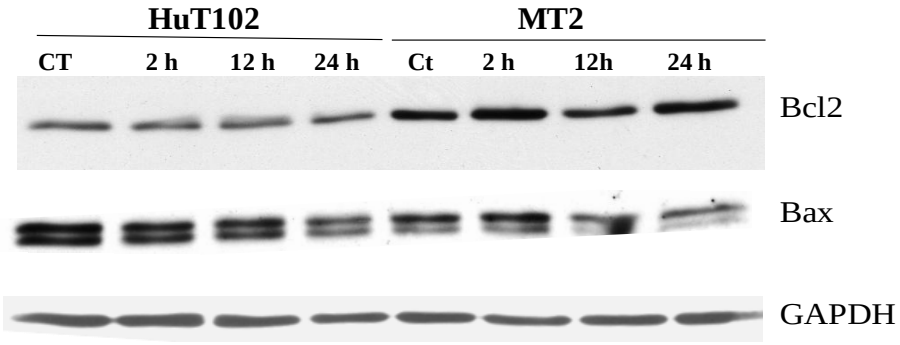


Figure 15. Effects of ST1926 on Bcl2 and Bax proteins in HTLV-1 positive cells. HuT102 and MT2 were seeded at a density of $2x10^5$cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 µg/lane) were prepared and immunoblotted against Bcl2 and Bax antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in at two independent experiments.

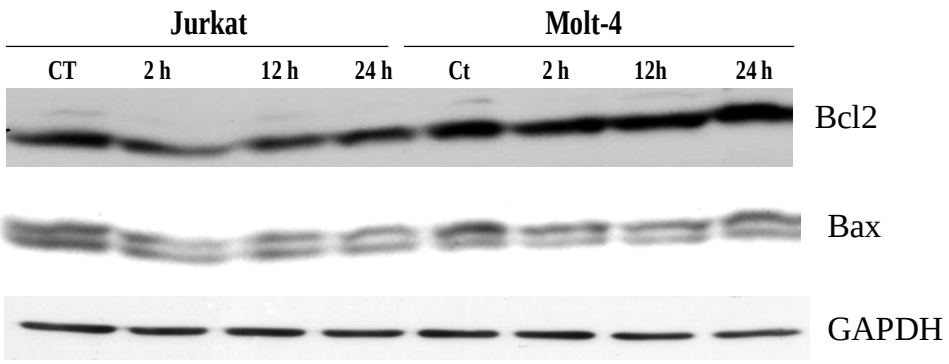


Figure 16. Effects of ST1926 on Bcl2 and Bax proteins in HTLV-1 negative cells. Jurkat and Molt-4 were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against Bcl2 and Bax antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in at two independent experiments.

F. Modulation of Caspase Activity by ST1926 in HTLV-1 Positive and Negative Malignant T-cells

Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation. There are two types of apoptotic caspases: initiator caspases and effector caspases. Initiator caspases (2, 8 and 9) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (3, 6 and 7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. ST1926-induced loss of mitochondrial membrane potential may result in the activation of the caspase cascade. Therefore, we investigated whether the apoptotic effect of ST1926 on the HTLV-1 positive cells (HuT-102 and MT2) and HTLV-1 negative cells (Jurkat and Molt-4) is caspase-dependent. We studied the process of caspase activation and cleavage by immunoblotting techniques. The HTLV-1 positive and negative cells were treated for 2, 12 and 24 h with 1 μM ST1926. ST1926-induced apoptosis was associated

with caspase activation, as shown by cleavage of its substrate PARP (113 kDa) into its death-associated fragment (89 kDa). Caspase 3 activation as detected by the cleavage of caspase 3 into a 35 kDa and 17 kDa fragments was observed in all cell lines after 24 h of ST1926 treatment (Figure 17). PARP cleavage was observed earlier at 12 h in HuT102 and MT2 and after 2 h in Jurkat and Molt-4, and was more pronounced in the HTLV-1 negative cells (Figure 17).

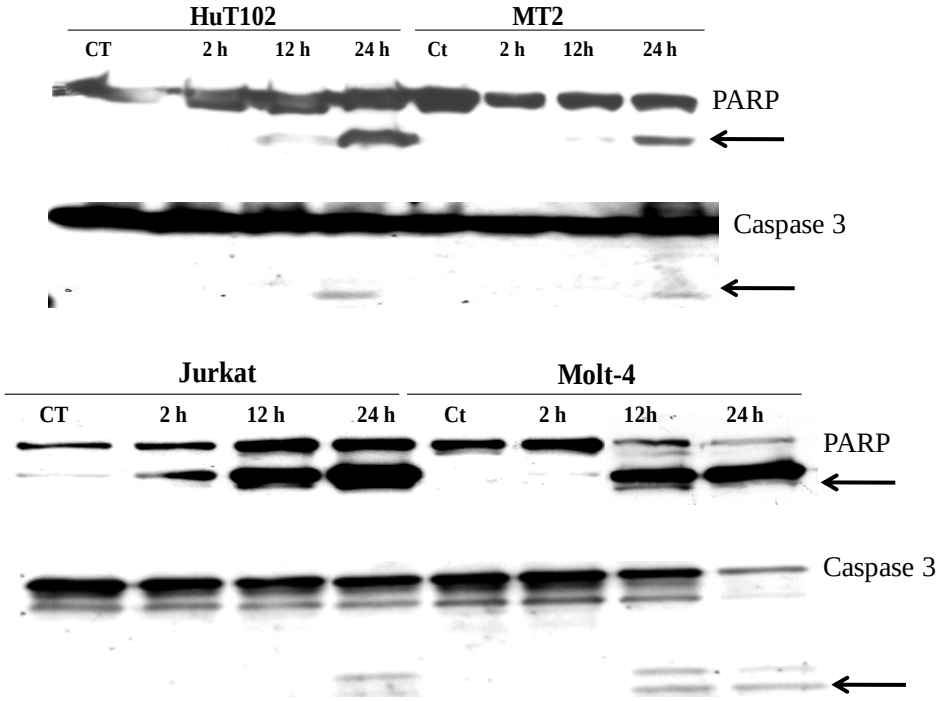


Figure 17. Effects of ST1926 on PARP and caspase 3 in malignant T-cells. Cells were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against PARP and caspase 3 antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Arrows indicate cleaved PARP or caspase 3. Similar trends in protein levels were observed in two independent experiments.

G. ST1926-Induced Apoptosis is Partially Caspase-Dependent

To further investigate the involvement of caspases in ST1926-induced apoptosis, we treated HuT102, as a representative of the HTLV-1 positive cells, and Jurkat, as a representative of the HTLV-1 negative cells, with the general caspase inhibitor z-VAD for 2 h and then ST1926

was added at 1 µM up to 24 h. In HuT102, 1 µM ST1926 induced 71% growth inhibition after 24 h of treatment and in the presence of z-VAD, growth inhibition was reduced to 55 %. Similarly, 1 µM ST1926 induced 82% growth inhibition by 24 h, and this percentage decreased to 66 % in the presence of z-VAD (Figure 18). This indicates that ST1926 induced-apoptosis is partially caspase-dependent.

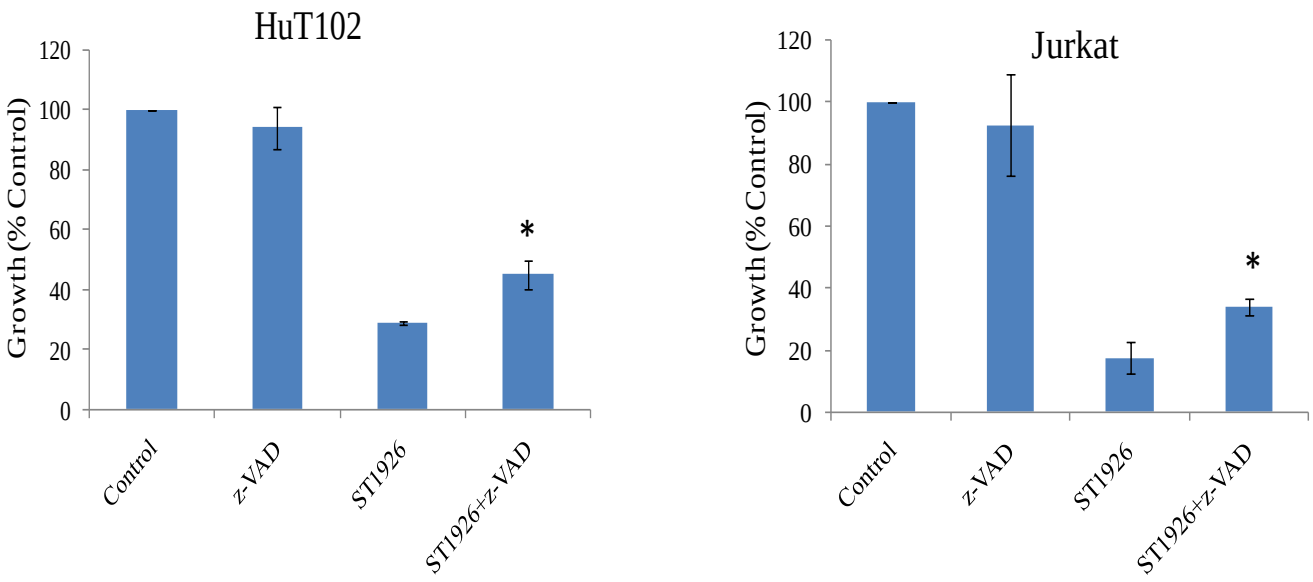


Figure 18. Effects of the general caspase inhibitor z-VAD on ST1926-induced growth inhibition of HuT-102 and Jurkat cells. HuT-102 and Jurkat cells were pretreated for 2 h with 100 µM z-VAD followed by 24 h treatment with 1 µM ST1926. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of two independent experiments ± SD. Asterisks indicate statistically significant ($P<0.05$) differences (Independent T-test).

H. ST1926 Induces Tax and p21 Degradation in HTLV-1 Positive Cells

To investigate the effect of ST1926 on the Tax oncoprotein, HuT102 and MT2 were treated with 1 µM ST1926 for 2, 12 and 24 h. Tax protein levels were detected by western blot analysis and showed that Tax degradation started after 12 h of treatment and disappeared

completely by 24 h in HuT102 treated cells. However, we could not detect Tax protein in MT2 cells (Figure 19).

Tax is known to transactivate the p21 promoter, thus p21 expression is upregulated at both the transcript and protein levels in HTLV-1 infected cells (Grassmann 2005). Hence, we investigated the effect of ST1926 on p21 protein levels in HuT102 and MT2 cells treated with 1 μM ST1926 for 2, 12 and 24 h. p21 protein levels started to be degraded after 12 h of treatment in both cell lines (Figure 19).

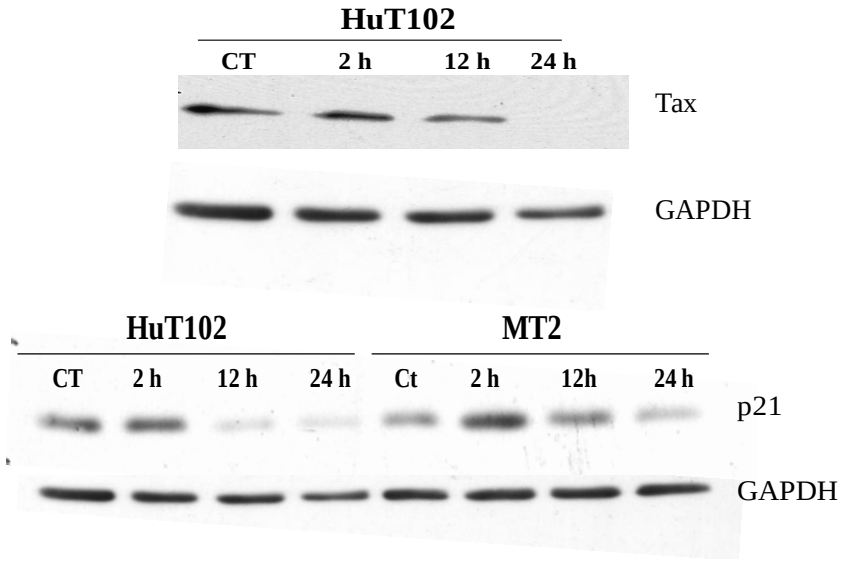


Figure 19. ST1926 degrades Tax and p21 proteins in treated HuT102 and MT2. Cells were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against p21 and Tax antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in at two independent experiments.

I. ST1926 Effect of p21 in HTLV-1 Negative Cells

The HTLV-1 negative cell line, Jurkat possesses a mutant p53. The expression of p21 increased slightly after 24 h of treatment with 1 μM ST1926. The increase in p21 in Jurkat cells might be p53-independent. On the other hand, the other HTLV-1 negative cell line, Molt-4,

which has a functional p53, showed no change in the expression of p21 protein after 24 h of treatment (Figure 20).

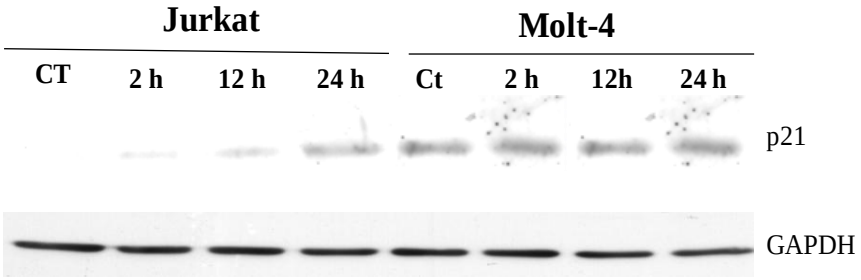


Figure 20. Effect of ST1926 on p21 protein level in treated HTLV-1 negative cells. Jurkat and Molt-4 were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against p21 antibody. All blots were re-probed with GAPDH antibody to ensure equal protein loading.

J. Effects of ST1926 on the Inhibitor IκBα of NF-κB pathway

NK-κB signaling is upregulated in HTLV-1 positive cells. To investigate the involvement of the NF-κB pathway in ST1926 induced-apoptosis in malignant T-cells, we studied the regulation of the inhibitor of NF-κB, particularly the IκBα. IκBα, prevents NF-κB binding to the DNA and consequently abrogates NF-κB activation. For this purpose, we treated the HTLV-1 positive cell lines (HuT102 and MT2) with 1 μM ST1926 for 2, 12 and 24 h. Western blot analysis revealed no changes in the expression of IκBα protein in both ATL cell lines (Figure 21). However, these results open the door to investigate the regulation of other players in the NF-κB signaling as Tax interfers with this pathway at multiple levels. Also, we need to investigate whether ST1926 regulates NF-κB DNA signaling or activities in treated cells.

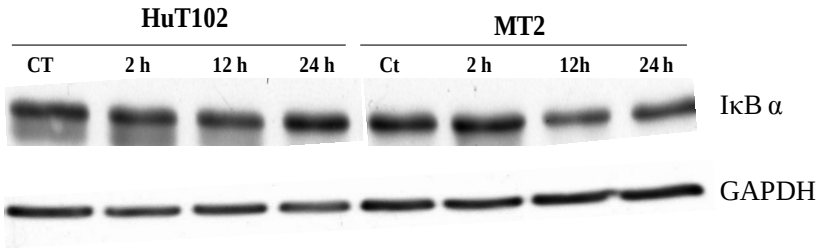


Figure 21. ST1926 does not regulate IκBα proteins in HTLV-1 positive cells. HuT102 and MT2 were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against IκBα antibody. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in two independent experiments.

K. ST1926 Upregulates Total and Phosphorylated p53 Protein Expression in Most Tested Malignant T-cells

The tumor suppressor gene, p53, which plays important roles in cell cycle arrest and apoptosis, is mutated in various human tumors (Levine, 1997). Many DNA viral oncoproteins including SV40 T antigen, human papillomavirus (HPV) E6, adeno-virus E1B and adenovirus E4orf6 oncoproteins inhibit p53 function through direct binding (Bargonetti et al., 1992). Mutated p53 genes have been found in only quarter of ATL cases (Nagai, 1991; Yamato 1993).

p53 protein expression was evaluated in HTLV-1 positive cells (HuT102 and MT2) treated with 1 μM ST1926 for 2, 12 and 24 h. Total p53 protein levels started to increase after 2 and 12 h of treatment in both HuT102 and MT2 cells, respectively. Furthermore, the expression of the active p53 in its phosphorylated form started to increase after 2 h of treatment with ST1926 in both cell lines (Figure 22). This increase in p53 protein expression trend was also detected in the HTLV-1 negative cell line Molt-4 (Figure 23). On the other hand, p53 expression did not change in the HTLV-1 negative cell line, Jurkat, which possesses a mutant p53 (Figure 23).

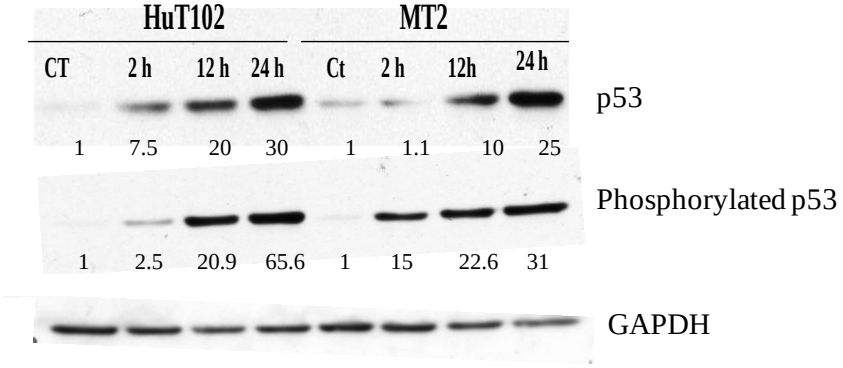


Figure 22. ST1926 upregulates p53 and its phosphorylated form in HTLV-1 positive cells. HuT102 and MT2 were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12 and 24 h. Whole SDS protein lysates (50 µg/lane) were prepared and immunoblotted against p53 and phosphorylated p53 antibodies. All blots were re-probed against GAPDH to ensure equal protein loading. Similar trends in protein levels were observed in two independent experiments. p53 and phosphorylated p53 bands were quantified and results are expressed relative to control cells or 2 h time point, respectively.

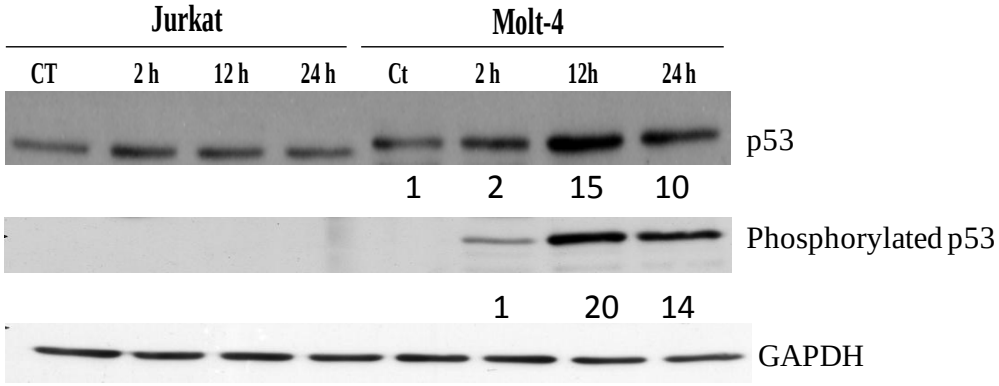


Figure 23. ST1926 upregulates total p53 and its phosphorylated form Molt-4. Jurkat and Molt-4 cells were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12, 24 h. Whole SDS protein lysates (50 µg/lane) were prepared and immunoblotted against p53 and phosphorylated p53 antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in at two independent experiments. p53 and phosphorylated p53 bands were quantified and results are expressed relative to control cells or 2 h time point, respectively.

L. p53 Sensitizes the Effect of ST1926 in Molt-4 Cells

To further investigate the involvement of the tumor suppressor protein p53 in ST1926-induced apoptosis in Molt-4 cells, we used the Molt-4E6 cell line transfected with the human papillomavirus (HPV) E6 which inhibits p53 function through direct binding. The Molt-4LXSN

which is transfected with an empty vector was used as our control cell line. Both Molt-4LSXN and Molt-4E6 were treated with ST1926 concentrations ranging from 0.05 µM to 5 µM up to 72 h. A 0.1 µM ST1926 concentration was not able to inhibit the growth of Molt-4E6, while in Molt-4LXSN with functional p53, the same concentration inhibited growth by around 50% of control at 24 h (Figure 24). It is noted that concentrations higher than 0.1 µM ST1926 showed similar growth suppressive effects in both cell lines. These results indicate that p53 sensitizes Molt-4 cells to ST1926 especially at low concentrations.

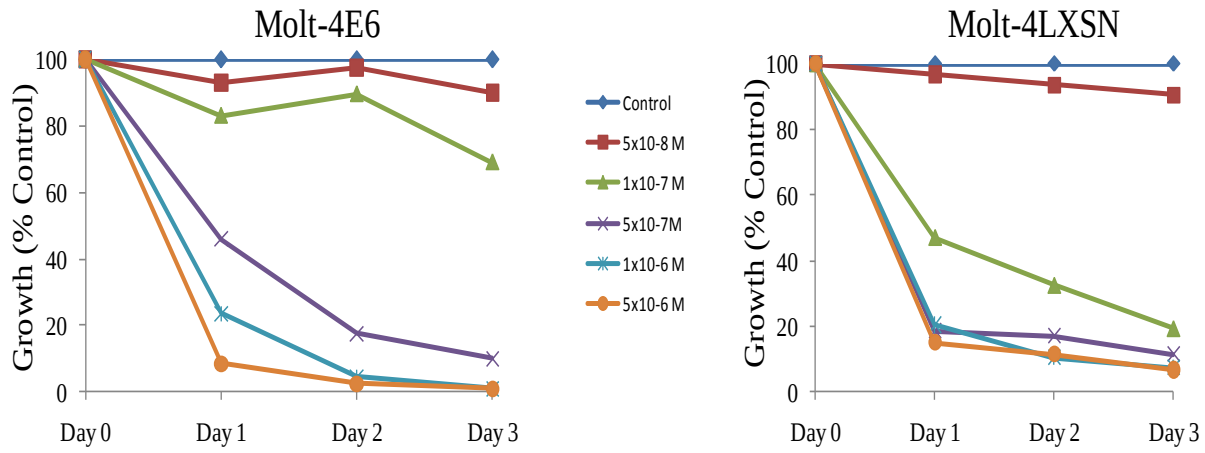


Figure 24. Role of p53 in ST1926-induced growth inhibition in Molt-4 cells. Molt-4LXSN and Molt-4E6 cells were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO or with varying concentrations of ST1926 ranging from $5x10^{-8}$ to $5x10^{-6}$ M for 24 hrs. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of two independent experiments.

M. ST1926- Induced Growth Inhibition is not Associated with ROS Accumulation

We next investigated whether ST1926 acts as an oxidant, we measured ROS accumulation in treated cells. ROS describes the group of chemical species that are formed upon incomplete reduction of oxygen which includes the superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO•) (D'Autreaux 2007). ROS is an important mediator of

cell cycle arrest and apoptotic cell death (Boonstra 2004). Intracellular levels of ROS are induced by a variety of apoptotic stimuli such as tumor necrosis factor (TNF), irradiation, and chemicals such as etoposide. For this purpose, we measured ROS levels at different time points following treatment of the HTLV-1 negative Jurkat and the HTLV-1 positive cells (HuT102, MT2). Previously, we have shown that, ROS levels increased only in 5 μM HPR-treated HTLV-1 negative and not HTLV-1 positive cells, as early as 30 min, by 25–30% of control and reached 60–75% by 6 h of treatment (Darwiche 2004; 2007). This concentration inhibits growth of HTLV-1 negative cells (CEM and Jurkat) by around 70% (Darwiche 2004). These elevated levels of ROS rapidly returned to baseline levels by 12 h (Darwiche 2007). Therefore, CEM treated with 5 μM HPR for 6 h was used as our positive control. Jurkat cells were treated with 1 μM ST1926 for 6 h and no increase in ROS generation was detected as compared to our positive control (Figure 25).

In HTLV-1 positive cells, minimal and delayed increase in ROS levels was observed upon HPR treatment of HuT-102 and MT-2 cells, only increasing to a maximum of 10 and 20% of control, respectively, 12 h post-HPR treatment (Darwiche 2007). MT2 treated with 1 μM ST1926 for 6 h showed no ROS generation detected. Similarly, HuT102 did not show any ROS production after 24 h of treatment with 1 μM ST1926 (Figure 26).

Altogether, these results indicate that ST1926-induced growth inhibition is not associated with ROS accumulation in both HTLV-1 positive and HTLV-1 negative cell lines (Figure 25, 26).

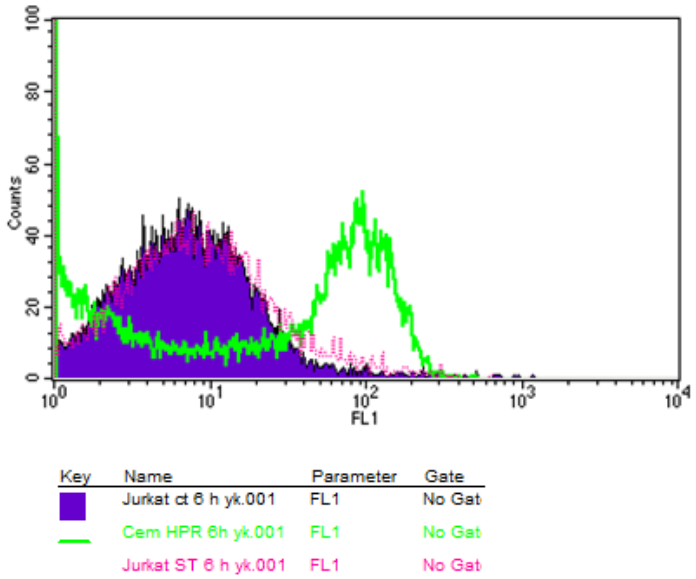


Figure 25. ST1926 treatment of Jurkat does not result in ROS generation. Jurkat cells were exposed to $1x10^{-6}$ M ST1926 and ROS levels were determined by the H_2DCFDA assay at 6 h.

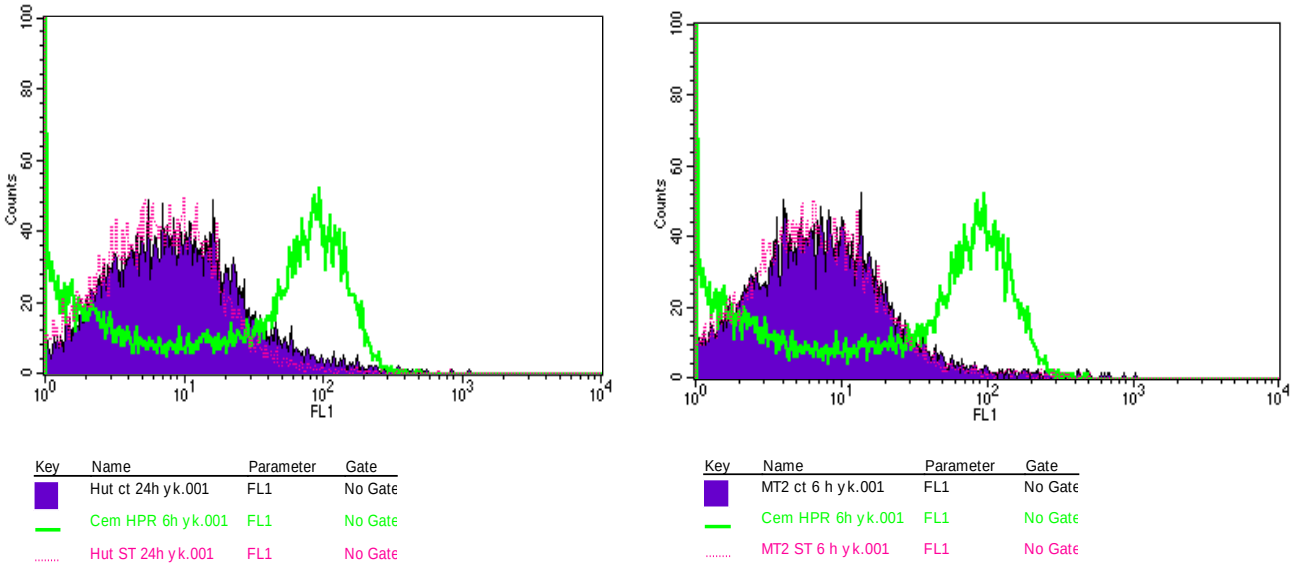


Figure 26. ST1926 treatment of HTLV-1 positive cells does not result in ROS generation. HuT-102 and MT2 cells were continuously exposed to $1x10^{-6}$ M ST1926 and ROS levels were determined by the H_2DCFDA assay at the indicated time points.

N. ST1926 Functions through Retinoid-Receptor Independent Pathway in Malignant T Cells

The synthetic retinoids have the ability to selectively activate RARs but most importantly they function through retinoid-receptor independent pathways. To investigate whether ST1926 acts *via* retinoid receptors, we used a pan-RAR inverse agonist BMS493. It is note-worthy to mention that RARs heterodimerize with RXRs and that RXRs are not able to transactivate cellular genes in the absence of ligands binding to RARs. Therefore, by blocking the RARs, we are abrogating the whole RAR/RXR signaling pathway. In addition, RXR homodimers do not seem to play a role in the physiological setting.

We treated HTLV-1 positive (HuT102, MT2 and C8166) and HTLV-1 negative (Jurkat and Molt-4) cells with 1μM ST1926 for 24 h, and assessed growth inhibition in the absence or presence of BMS493. BMS493 was added 20 min at 1 μM concentration prior to ST1926 addition. In all tested cell lines, the presence of the antagonist did not alter the percentage of growth inhibition induced by ST1926. ST1926-induced growth inhibition in all tested malignant T-cells was not changed upon addition of the pan-RAR antagonist (Figures 27 and 28). This percentage was the same detected by the MTT assay. To confirm that the inverse agonist BMS493 is active and functionally blocking RARs, we used the HTLV-1 positive cell line C8166, the only malignant T-cells that we tested and found to be sensitive to ATRA (Darwiche 2001; Darwiche 2004). And since ATRA mainly works through RARs and inhibits the growth of C8166 cells by 70% at 3 μM after 72 h of treatment, we used this particular cell line as our positive control for evaluating BMS493 effect. In the presence of the inverse agonist BMS493, ATRA induced-growth inhibition was reduced to only 13% after 72 h of treatment with 3 μM ATRA (Figure 29). These results clearly indicate that ATRA induced-growth inhibition is RAR-dependent in C8166 cells and hence, validated that BMS493 is truly blocking the action of RARs

and consequently the retinoid receptors signaling pathway. Our results show that ST1926 mode of action in malignant T-cells is retinoid-receptor independent.

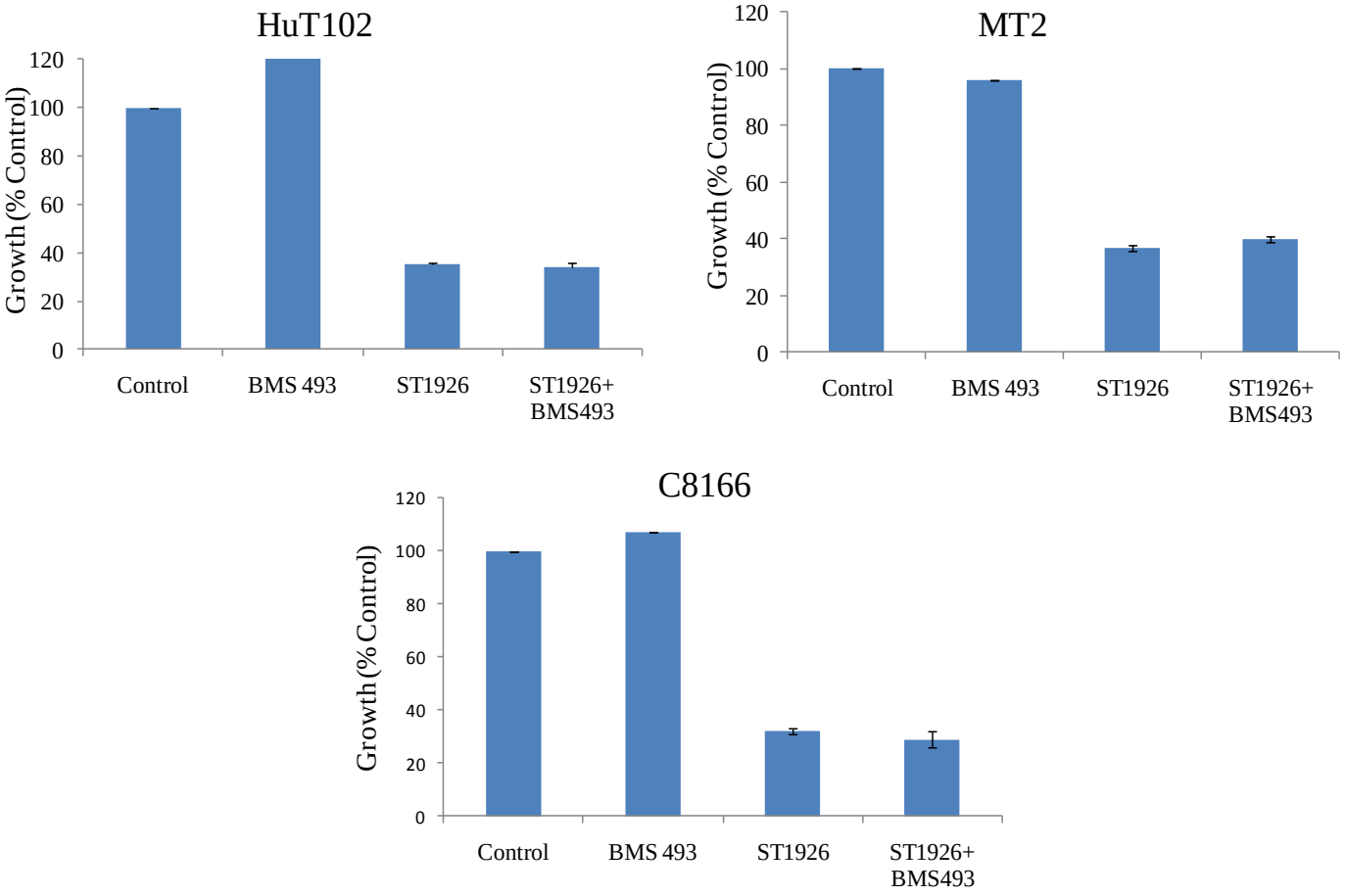


Figure 27. ST1926 treatment of HTLV-1 positive cells in the absence or presence of the pan RAR inverse agonist BMS493. HuT-102, C8166, and MT2 were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO, $1x10^{-6}$ M BMS493 or with both BMS493 and $1x10^{-6}$ M ST1926 for 24 h. Cell growth was assayed in quadruplicate wells with the CellTiter96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of two independent experiments ± SD.

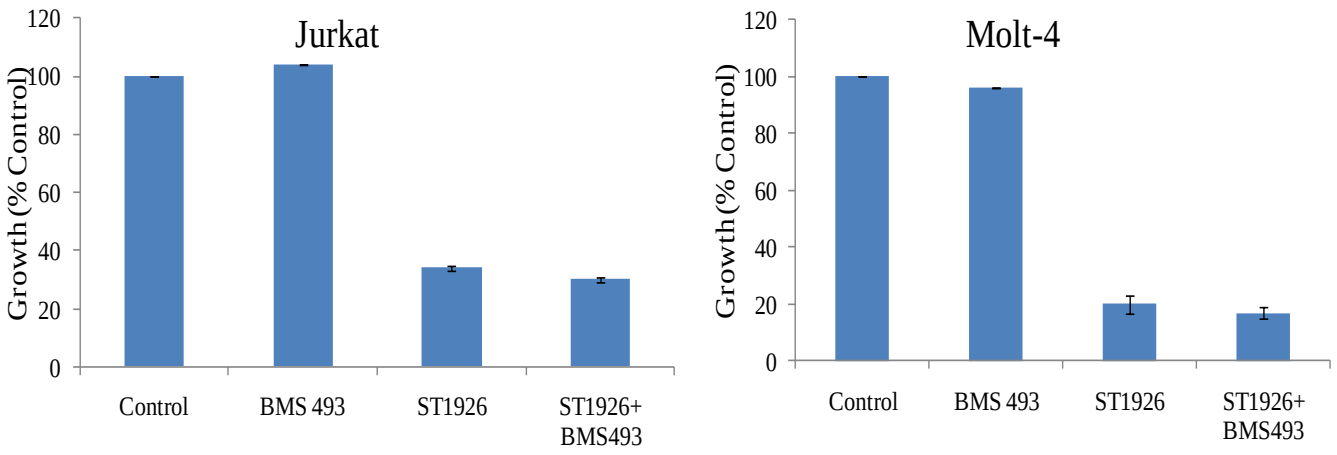


Figure 28. ST1926 treatment of HTLV-1 negative cells in the absence or presence of the pan RAR inverse agonist BMS493. Jurkat and Molt-4 were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO, $1x10^{-6}$ M BMS493 or with both BMS493 and $1x10^{-6}$ M ST1926 for 24 h. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of two independent experiments ± SD.

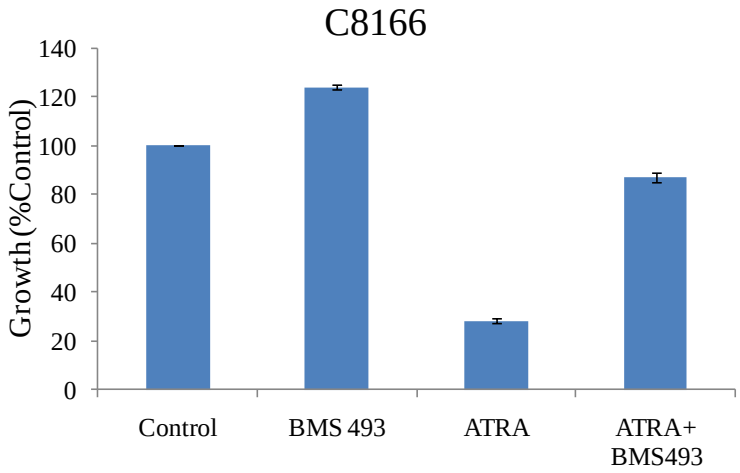


Figure 29. ATRA treatment of the HTLV-1 positive cell line C81 in the absence or presence of the pan RAR inverse agonist BMS493. C8166 were seeded at a density of $2x10^5$ cells/ml, and treated with 0.1% DMSO, $1x10^{-6}$ M BMS493 or with both BMS493 and $3x10^{-6}$ M ATRA for 72 h. Cell growth was assayed in quadruplicate wells with the CellTiter 96® non-radioactive cell proliferation kit. The results are expressed as percentage of control (0.1% DMSO). Results are an average of two independent experiments.

O. RAR and RXR Protein Profiles in HTLV-1 Positive and Negative Cell Lines Upon ST1926 Treatment

Normal lymphocytes express RARα, RARγ and RXRα genes. HuT102, Jurkat and Molt-4, unlike MT2 express RARα (Darwiche, unpublished results). RARγ protein levels are expressed in HTLV-1 positive cells while they are not detectable in HTLV-1 negative cells (Darwiche 2001; unpublished results). In contrast, RXRα proteins are highly expressed in all tested cell lines (Darwiche 2001, unpublished results).

In order to determine the effects of ST1926 on the retinoid receptor proteins in malignant T-cells, we treated HuT102, MT2, Jurkat and Molt-4 with 1μM ST1926 for 2, 12 and 24 h and assessed the profile of RAR and RXR by western blot analysis. In HTLV-1 positive cells (HuT102 and MT2) and upon treatment with ST1926, the protein levels of RARγ and RARα started to decrease following 12 h of treatment. On the other hand, we did not detect a major change in the expression of RXRα in HuT102 and MT2 after 24 h of treatment with 1 μM ST1926 (Figure 30). As for the expression of RARα and RXRα in HTLV-1 negative cells, we were only able to detect a decrease in the expression of RARα in Jurkat and Molt-4 after 12 h of treatment with ST1926 (Figure 31). RXRα levels minimally decreased after 24 h of treatment and we were not able to detect RARγ. ST1926 induces the degradation of RARα and RXRα in the tested HTLV-1 positive and RARα in the tested negative cell lines, which may be due to proteasomal-mediated degradation (Zhu 1999).

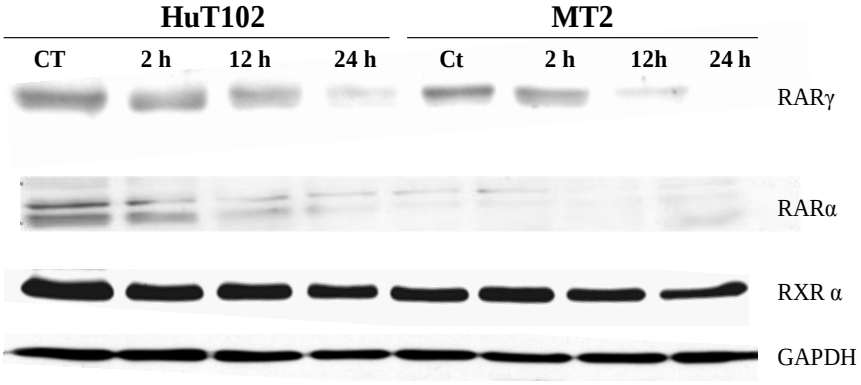


Figure 30. ST1926 induces the degradation of RARα, RARγ proteins in HTLV-1 positive cells. HuT102 and MT2 cells were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12, 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against RARα, RARγ and RXRα antibodies. All blots were re-probed with GAPDH to ensure equal protein loading. Similar trends in protein levels were observed in two independent experiments.

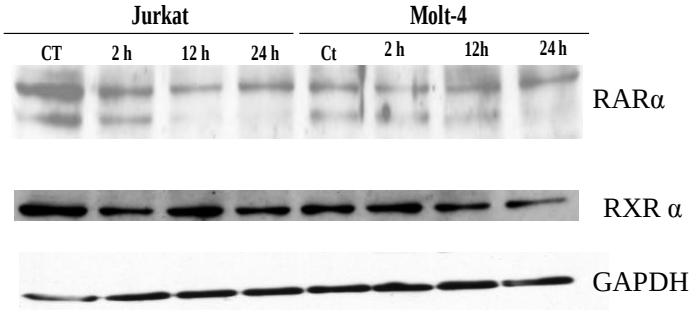


Figure 31. ST1926 induces the degradation of RARα protein in HTLV-1 negative cells. Jurkat and Molt-4 were seeded at a density of $2x10^5$ cells/ml and then treated with $1x10^{-6}$ M ST1926 for 2, 12, 24 h. Whole SDS protein lysates (50 μg/lane) were prepared and immunoblotted against RARα and RXRα antibodies. All blots were re-probed with GAPDH antibody to ensure equal protein loading. Similar trends in protein levels were observed in at least two independent trials.

CHAPTER IV

DISCUSSION

Multiple pathways have been reported in cancer cells concerning the molecular events mediating the antitumor activities and apoptosis induction by synthetic retinoids. ST1926, a novel adamantyl retinoid, orally bioavailable and currently tested in phase I clinical trials (Valli 2008). This synthetic retinoid is more bioavailable, less toxic and well-tolerated *in vivo* than the previously tested synthetic retinoid CD437. ST1926 was tested on different solid tumors such as ovarian carcinoma, lung carcinoma, neuroblastoma, teratocarcinoma and on hematological malignancies such as acute myeloid leukemia (Cincinelli 2003; Garattini 2004). The mechanism of action of ST1926 differed in the various cell systems and studies have shown that the exposure of tumor cells to ST1926 rapidly induces G_0/G_1 or G_2/M cell cycle arrest. Consequently, apoptosis induction is often associated with p21 upregulation and DNA repair gene expression. Moreover, ST1926 has the ability to induce both p53-dependent and -independent apoptosis (Cincinelli 2003). Yet, all these mechanisms appear to be cell type specific.

Based on all these promising results for the use of ST1926 in the cancer setting, we aimed at investigating the mode of action of ST1926 in ATL and T Lymphoma cells. ATL, an aggressive lymphoproliferative disorder of mature activated T-helper lymphocytes, has a poor prognosis due to immunosuppression and resistance to chemotherapy. Depending on the clinical picture and the subtype of ATL in each patient, several treatment modalities were tested ranging from antiretroviral therapy, induction chemotherapy, antibodies against VEGF, tyrosine kinase inhibitors, inhibitors of the NF-κB pathway and allogeneic bone marrow transplantation (Bazarbachi 2004). Synthetic retinoids such as HPR and CD437 were tested on HTLV-1

positive and negative malignant T-cells, at concentrations ranging from 0.5 μM and 10 μM, and induced growth inhibition, G_0/G_1 arrest, and apoptosis in all tested malignant T-cells lines (Darwiche 2004; unpublished data).

Here we report that ST1926 induces growth suppression and apoptosis in HTLV-1 positive and negative malignant T-cells at five to ten fold lower concentrations than HPR and CD437. We used a set of concentrations ranging between 0.05 μM and 5 μM to screen for the concentration that induces around 50% growth inhibition in the above cell lines. Most of HTLV-1 positive and negative cell lines showed more than 50% growth inhibition at 1μM ST1926 at 24 h of treatment. This concentration was shown to be the peak plasma concentration achieved in patients after oral administration of 200 mg ST1926/day (Valli 2008).

Furthermore, to assess the cytotoxicity potential of ST1926, the PBMCs of three healthy donors were treated up to 10 μM ST1926 for 48 h and these cells showed resistance to the drug. Since 1 μM is a physiologically achievable and a non-cytotoxic concentration and causes more that 50% growth inhibition at 24 h in all tested malignant T-cells with no effect on normal lymphocytes, we decided to test the effect of this concentration on cell death mechanisms and on retinoid signaling pathways. Cell cycle analysis revealed a higher percentage of cells in the pre G_1 phase in HTLV-1 negative cell lines as compared to their positive counterparts (around 20% in HTLV-1 positive cells, 30% in Molt-4 and 50% in Jurkat) at 1 μM ST1926 after 24 h of treatment. These results suggest that ST1926 presumably induces apoptosis in HTLV-1 positive and negative cell lines at 1 μM concentration as early as 24 h of treatment. To further confirm apoptosis induction by ST1926, we checked for DNA fragmentation, which occurs when nuclear enzymes become active and destroy the normal chromatin structure of DNA. For this reason, we used the TUNEL assay that detects DNA fragmentation as a late apoptotic

marker. HTLV-1 positive cells (HuT102 and MT2) showed no TUNEL positivity at 1 μM ST1926 after 24 h of treatment. However, the percentage of TUNEL positive cells increased approximately to 20% after 48 h of ST1926 treatment in HuT102 and MT2 cells. On the other hand, the HTLV-1 negative cells showed approximately 70% TUNEL positivity after 24 h of treatment with 1 μM ST1926. Apoptosis induction as measured by TUNEL positivity suggests that although HTLV-1 positive and negative cell lines had similar sensitivity to ST1926 as detected by the MTT assay, yet apoptosis induction is delayed in HuT102 and MT2 cells. By altering several cellular pathways, the oncoprotein Tax might play a role in affecting ST1926-induced apoptosis in HTLV-1 positive cell lines.

Furthermore, we measured the changes in the mitochondria as being one of the earliest measurable changes in intrinsic apoptosis that occurs when the outer membrane becomes permeable and apoptotic regulators in the mitochondria are released into the cytosol. The regulation and dissipation of mitochondrial membrane potential (MMP) involves a number of proteins, particularly those from the Bcl-2 family. The pro-apoptotic Bcl-2 family members, Bax and Bak, assist in the dissipation of MMP, resulting in the eventual activation of procaspase-9. In contrast, the anti-apoptotic proteins Bcl-2/Bcl-X_L suppress apoptosis by interacting with or preventing the pro-apoptotic proteins from carrying out their anti-apoptotic functions. It was also obvious that ST1926 effect was more pronounced at the mitochondrial level in HTLV-1 negative cell lines as compared to HTLV-1 positive cells. MMP as being one of the early markers of intrinsic apoptosis indicates that ST1926 induces cell death earlier in Jurkat and Molt-4 cells as compared to the HTLV-1 positive cells. Dwelling more in the apoptosis mechanism induced by ST1926 in both HTLV-1 positive and negative cell lines, we determined changes in the expression of several apoptotic regulators on the protein level. The protein levels of Bax and

Bcl2 were unchanged in all tested cell lines, suggesting that ST1926-induced cell death is Bcl2-independent. Furthermore, we detected the effect of ST1926 on both caspase 3 activation and PARP cleavage where both HTLV-1 positive and negative cell lines showed caspase 3 and PARP cleavage. PARP is not a sole substrate for caspase 3, and other caspases may be involved in ST1926-induction of apoptosis. Hence, other caspases status such as caspase 7 should be investigated. In addition, the pretreatment with the pan caspase inhibitor, z-VAD, partially reversed ST1926- induced growth inhibition in both HuT102 and Jurkat, hence emphasizing the fact that the caspase cascade is partially implicated in ST1926-induced cell death.

Tax, is a powerful oncoprotein that contributes to ATL malignant transformation. We, therefore, studied the effect of ST1926 on Tax status in HTLV-1 positive cells. Indeed, ST1926 caused degradation of Tax after 12 h of treatment with 1 μM ST1926 as detected in HuT102 cells and completely disappeared 24 h post-treatment. After 48 h, and in the absence of Tax, mitochondrial membrane dissipation increased and apoptosis induction was pronounced in the HTLV-1 positive cells. These results emphasize that Tax has the ability to delay ST1926-induced apoptosis in ATL cells.

P21, a direct regulator of cyclin-dependent kinase (CDK) activity, is a mediator of p53-induced growth arrest, whose expression is induced with apoptosis and cellular senescence. P21 is a member of the Cip/Kip family of CDK inhibitory proteins (CKIs), along with $p27^{Kip1}$ and $p57^{Kip2}$, which share significant sequence homology in the amino-terminal region. The unique carboxy-terminal domain of p21 associates with proliferating cell nuclear antigen (PCNA) to inhibit DNA replication. Interestingly, p21 expression is elevated at both the mRNA and protein levels in HTLV-1-infected T-cells, as Tax transactivates the p21 promoter (Grassmann 2005). However, in ATL cells, elevated levels of p21 induce cell proliferation and inhibit cell death.

We showed that p21 was degraded as early as 12 h post treatment with 1 μM ST1926 in both HuT102 and MT2 cells. p21 degradation occurred at the same time point as Tax degradation. This decrease in p21 levels upon ST1926 treatment may explain the enhanced sensitivity of HTLV-1 positive cells to ST1926-induced apoptosis revealed after 48 h of treatment, particularly because p21 prevents apoptosis in HTLV-1-immortalized and Tax-transformed cells.

Tax acts on different levels to initiate and maintain NF-κB signaling which is in turn responsible for activating most of the cellular genes in HTLV-1 infected cells (Kfoury 2005). For this reason, we investigated the effect of ST1926 on one of the inhibitory proteins of the NF-κB pathway, IκB-α. IkB-α, the best studied family member, is a 37 kDa protein, which becomes phosphorylated at serines 32 and 36 upon cellular activation and regulates the activity of NF-kB through both the cytoplasmic retention of NF-κB dimers and the inhibition of the NF-κB binding to the DNA (Kfoury 2005). Free IκB-α may also be imported to the nucleus where it can interact with NF-kB dimers, destabilizing their interaction with DNA (Kfoury 2005). Although we did not detect any changes in the expression of this inhibitory protein in both HTLV-1 positive cell lines, HuT102 and MT, yet this does not eliminate the ability of ST1926 to interfere with the NF-κB pathway *via* other players. Therefore, the effect of ST1926 on NF-κB and activity of other regulators of the NF-κB signaling pathway such as IKKα and IκB-β needs to be further investigated in Tax expressing cells.

p53 is a DNA-binding transcription factor that guards against cellular DNA damage and transformation. The gene for *p*53 is mutated in 50% of human cancers. Interestingly, unlike most other cancers, only a small fraction of ATL cells have p53 mutations. This paradox was resolved by independent findings that p53 function is inactivated by Tax (Kfoury 2005). Tax-

dependent activation of the NF-κB pathway was reported to play an important role in the inhibition of p53 transactivation functions. Our results indicate that p53 was upregulated upon treatment with 1μM ST1926 in HTLV-1 positive cell lines. Furthermore, the active p53, phosphorylated at ser 15 was also shown to increase at the protein level upon treatment with ST1926. However, Jurkat cells that have a mutant p53 are particularly sensitive to ST1926-induced apoptosis. Altogether these results indicate that ST1926-induced apoptosis in malignant T-cells is p53 -dependent and -independent.

. It is noteworthy that we were not able to detect ROS production in both HTLV-1 positive and negative cell lines upon treatment with 1μM ST1926. This is in sharp contrast to the cell death effect of the synthetic retinoid HPR in HTLV-1 negative cells which was ROS-mediated (Darwiche 2007).

Synthetic retinoids have a favorable feature to induce growth inhibition and apoptosis independently of the retinoid receptors. Studies have shown that ATRA was a nonselective (pan-RAR) agonist, whereas CD437 was a selective RAR γ agonist (Fontana 2002). Although ST1926 was shown to be a stronger RARγ transactivator than ATRA, yet it can bind and trans-activate the three RAR receptors with similar affinity and, unlike CD437, lacked RAR γ selectivity (Parrella 2006). Both ST1926 and CD437 are poor RXR activators (Parrella 2006). In our tested malignant T-cells, the pan RAR inverse agonist BMS493 was not able to reverse the effects of ST1926-induced growth inhibition at 1 μM drug concentration after 24 h of treatment. Hence, we were able to show that ST1926 works independent of the retinoid receptors in both HTLV-1 positive and negative cell lines. Our conclusion is based on the fact that RAR agonists can autonomously activate transcription through heterodimerizing with RXRs and RXRs are unable solely to respond to RXR-selective agonists (rexinoids) in the absence of

RAR ligands (Germain 2006). Therefore, by blocking the action of RARs, using BMS493, we are at the same time blocking the RAR/RXR signaling pathway.

We studied the change in the protein levels of RARα, RARγ and RXRα upon ST1926 treatment of malignant T-cells. We were able to show that upon ST1926 treatment, the protein levels of RARα and RARγ started to decrease after 12 h in HTLV-1 positive cells. As for the expression of RARs and RXRs in HTLV-1 negative cells, we were only able to detect a decrease in the expression of RARα in Jurkat and Molt-4 cells. On the other hand, minimal change was detected in the expression of RXRα in all tested malignant T-cells upon ST1926 treatment. It would be interesting to test whether ST1926-induced RAR degradation is proteasomal-mediated.

Finally, in comparison to previously tested synthetic retinoids such as HPR and CD437, ST1926 effect on malignant T-cells was shown to be five to ten folds stronger (Darwiche 2004; unpublished results). Hence, ST1926 seems to be a promising agent and a potent synthetic retinoid in ATL/lymphoma and peripheral T-lymphomas treatment. Since ST1926 was shown to be a genotoxic agent at early time points, we can investigate its ability to induce DNA double strand breaks in both HTLV-1 positive and negative cell lines and compare its mechanism of action in both systems as they differ in the expression of the oncoprotein Tax. In addition, the extrinsic pathway of apoptosis needs to be studied as ST1926 might be using such pathway in addition to the intrinsic one to induce cell death. Future experiments using *in vivo* mouse models of ATL (Hasegawa 2006) are needed in order to determine whether to recommend ST1926 for ATL and peripheral T cell lymphoma clinical trials.

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