Dissertation

Submitted to the

Combined Faculties of Natural Sciences - Mathematics

of the

Ruprecht-Karl University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Marc Bleumink

BSc. Medical Biochemistry MSc. Biomedical Sciences

Born in: Lichtenvoorde, the Netherlands

Oral-examination: 2nd October 2007

Resensitization of HTLV-1 infected T cells towards apoptosis by rocaglamide involves inhibition of protein translation

Referees: Prof Dr. Peter H. Krammer
(German Cancer Research Center)
Prof Dr. Michael Brunner
(Faculty for Biosciences, University of Heidelberg)

ACKNOWLEDGEMENTS

This was an exceptionally interesting and rewarding period for me and I would like to thank those who helped during this time.

I would like to express my gratitude to Prof. Dr. Peter H. Krammer for his support during this time at the Division of Immunogenetics at the German Cancer Research Center. I appreciate his vast knowledge in many fields and would like to thank him for his supervision and the opportunity to perform my PhD in his lab.

I also would like to thank Prof. M. Brunner from the Faculty of Biosciences, University of Heidelberg for taking time to serve as my second supervisor.

My special thanks goes to Dr. Min Li-Weber for having given me the opportunity to work in the interesting field of drug research. Under her supervision I developed a focus and became interested in traditional Chinese drugs. I appreciate her skills and her direct supervision during my PhD.

I also want to acknowledge Dr. Karsten Gülow, Dr. Rüdiger Arnold and Dr. Inna Lavrik for their suggestions during the project. I also would like to thank them, together with Dr. Christian Frey, for critically reading and correcting this thesis. Besides the people already mentioned, also other members of the lab provided continuous and invaluable help during my PhD, especially Dr. Heiko Weyd, Dr. Dirk Brenner and Marcin Kaminski. Appreciation also goes to Heidi Sauter for all her administrative assistance throughout my PhD. She was always there for questions and help with all kinds of forms.

Also thanks to the members of the subgroup of Dr. Min Li-Weber, especially Marco Giaisi, Monica Walker, Ana Tan and Dr. Konstatina Bourkoula, for the nice time and always being there and helping me with technical questions. A special thank you goes to Kinga Przibilla and Stefanie Koppenhöfes who provided me with technical support during my PhD.

I also would like to thank the whole lab for the good atmosphere. I had a great time in the lab with all kinds of discussions we had during work. Besides that, I want to mention the time outside the lab. I learned a lot about German culture and I am happy to tell the people back in the Netherlands a little bit more about our eastern neighbours. One of the things I would like to mention was the unforgettable time for me as a Dutch guy, being part of the celebrations during the World Cup Football 2006 here in Germany. Before I

came to Heidelberg, I would never have thought that I would cheer for the German national team. Although I have to mention that I was also happy with my ‘Dutch orange’ corner in the writing room.

I would also like to thank my friends in Heidelberg, most of them also PhD students coming from countries all over the world. I learned a lot about science and other topics during our multicultural discussions and exchanges of experiences. You all really enriched the experience here in Germany.

Back in the Netherlands, I wish to thank my parents and my brothers Leon and Roy and my sister Sabine for the support they provided through my PhD and their understanding of my decision to perform my PhD here in Heidelberg.

ZUSAMMENFASSUNG

Der Humane T-Zell Leukämie-Virus-Typ 1 (HTLV-1) ist ein Retrovirus, der neben anderen Krankheiten auch die Akute T-Zell Leukämie (ATL) hervorruft. Aufgrund der Apoptose-Resistenz HTLV-1 infizierter T-Zellen kann mit bisherigen Therapien ATL nur unzureichend behandelt werden.

Die erhöhte Expression anti-apoptotischer Proteine, die in der Modulation des intrinsischen Signalwegs der Apoptose beteiligt sind, wurde als ein Mechanismus der Apoptose-Resistenz postuliert.

In vorangegangenen Arbeiten konnten wir zeigen, dass HTLV-1-infizierte T-Zellen im Vergleich zu nicht-infizierten Zellen eine höhere Resistenz gegenüber sowohl CD95L- als auch TRAIL-induzierter Apoptose aufweisen. Dies deutet auf einen gemeinsamen Mechanismus der Resistenz gegenüber Todesrezeptor-vermittelter Apoptose hin. Vor kurzem konnte unsere Gruppe weiterhin zeigen, dass der extrinsische Signalweg der Apoptose in HTLV-1-infizierten T-Zellen durch erhöhte Expression von c-FLIP blockiert ist. Durch Kompetition mit Procaspase-8 um die Bindungsstellen von FADD bei der Bildung des DISC-Komplexes inhibiert c-FLIP die Todesrezeptor-vermittelte Apoptose. Zur Überwindung der Resistenz wurden HTLV-1-infizierte T-Zellen mit CD95L bzw. TRAIL allein oder in Kombination mit Rocaglamide behandelt. Rocaglamide ist ein pflanzlicher Inhaltsstoff, der Anwendung in der traditionellen chinesischen Medizin (TCM) findet. Wir konnten zeigen, dass ein aktives Rocaglamide-Derivat (Roc-AR) HTLV-1-infizierte T-Zellen durch Reduktion der c-FLIP-Expression auf posttranskriptionaler Ebene gegenüber CD95L- und TRAIL-vermittelter Apoptose sensitiviert. Weitere Untersuchungen ergaben, dass der von Roc-AR-vermittelte Mechanismus der Inhibition der Translation sich von den Mechanismen anderer Inhibitoren der Translation unterscheidet: Roc-AR hemmt den Ras-Signaltransduktionsweg, was wiederum eine Hemmung von Mnk-1, einer Proteinkinase, die den eukaryotischen Initiationsfaktors der Translation 4E (eIF4E) aktiviert, hervorruft. Die Roc-AR-vermittelte Blockade der Aktivierung von eIF-4E hemmt die cap-abhängige eukaryotische Translation in der Phase der Initiation der Translation. Im Gegensatz dazu hat Roc-AR keinen Einfluss auf die CD95L- und TRAIL-induzierte Apoptose peripherer nicht-infizierter T-Zellen. Durch die spezifische Sensitivierung infizierter

Zellen durch Roc-AR besteht die Möglichkeit, Roc-AR in Kombination mit CD95L- bzw. TRAIL zur Therapie von ATL und anderer Arten von T-Zell-Tumoren einzusetzen.

ABSTRACT

Human T cell Leukemia Virus Type 1 (HTLV-1) is a retrovirus, associated with several diseases including Adult T-cell Leukemia/Lymphoma (ATL). Because of apoptosis resistance treatment provides only limited benefits for ATL.

CD95/CD95L-mediated apoptosis is an important mechanism of T cell homeostasis. We have previously shown that HTLV-1 infected T cells are more resistant to CD95L-induced apoptosis as compared to non HTLV-1 infected T cells. In this study we showed that HTLV-1 infected T cells are also resistant towards TRAIL, which suggests a general mechanism of resistance towards death receptor-mediated apoptosis.

The basis of apoptotic resistance in HTLV-1 infected T cells was suggested to be due to the elevated expression of several anti-apoptotic proteins involved in modulation of the intrinsic cell death pathway. Recently our group further found that apoptosis is also blocked within the extrinsic cell death pathway by high c-FLIP expression. C-FLIP is an anti-apoptotic protein that blocks death receptor-mediated apoptosis at the DISC level.

To overcome resistance, we have treated HTLV-1 infected T cells with CD95L or TRAIL in combination with an herbal compound, Rocaglamide, derived from a Traditional Chinese Medicinal plant (TCM). We showed that one of the Rocaglamide derivatives tested, Roc-AR, sensitizes HTLV-1 infected T cells towards CD95L- and TRAIL-mediated apoptosis *via* down-regulation of c-FLIP expression at the translational level. Further investigation of the molecular mechanisms by which Roc-AR suppresses c-FLIP translation, revealed a mechanism different from other known translation inhibitors. Roc-AR strongly inhibits the Ras pathway leading to the inhibition of Mnk-1, a protein kinase essential for the activation of the translation initiation factor 4E (eIF4E). Thus, blocking activation of eIF4E by Roc-AR leads to inhibition of cap-dependent eukaryotic translation at the initiation stage. Most importantly, Roc-AR does not sensitize normal peripheral blood T cells to CD95L-and TRAIL-induced apoptosis. Our study raises the possibility to develop Roc-AR as CD95L or TRAIL adjuvant for treatment of ATL and other types of T-cell tumors.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Introduction to HTLV-1

Human T Cell Leukemia Virus (HTLV) is a type C retrovirus and is the first human oncogenic retrovirus identified. It was isolated from a cell line derived from a patient with cutaneous T-cell lymphoma (Poiesz *et al*., 1980). Independently, a related retrovirus was isolated from another patient with adult T-cell leukemia/lymphoma (ATL) and was described as Adult T-cell Leukemia Virus (ATLV) (Yoshida *et al*., 1982). HTLV and ATLV were later demonstrated to be the same retrovirus based on homology between the viral genome and viral antigens. This retrovirus was renamed HTLV-1 (Watanabe *et al*., 1984).

HTLV-1 has infected ~ 15-20 million people worldwide and is endemic in Southwest Japan, the Caribbean islands, southeastern parts of USA and parts of Central and South Africa (De The *et al*., 1993; Edlich *et al*., 2000). HTLV-1 infects various cell types including $CD8^+$ T cells, B cells, dendritic cells and fibroblasts. However, the HTLV-1 provirus is predominately found in $CD4^+$ T cells (Hanon *et al*., 2000; Yasunaga *et al*., 2001). Most viruses are spread by causing the infected cell to release thousands of virus particles. In contrast, HTLV-1 needs cell to cell contact for transmission, which takes place upon forming of a virological synapse between an infected and non-infected cell (Nejmeddine *et al*., 2005). It is thought that this is partly mediated by the interaction between the Glucose-transporter GLUT-1 on the non-infected cell and the envelope protein (Env) on the infected cell (Manel *et al*., 2003).

HTLV-1 infected cells enter the human body *via* three major routes, namely transmission from mother to child through breast feeding, sexual transmission and parenteral transmission through infected blood, blood components or by infected needles (Tajima, 1988; Chen *et al*., 1989). Besides these routes, transplacental transfer has also been reported (Komuro *et al*., 1983).

1.2 HTLV-1 genomic structure and Tax protein

The size of the HTLV-1 genome is ~ 9 kb. It is comprised out of two copies of a single-stranded RNA. The genome is reverse transcribed into a double-stranded DNA that integrates into the host cell genome referred to as provirus. The HTLV-1 provirus includes, like other retroviruses, Gag, Pol, and Env genes encoding the viral matrix, the capsid, and envelope proteins, as well as enzymes such as reverse transcriptase and integrase (Seiki *et al*., 1983). In addition, the HTLV-1 genome encodes for several regulatory and accessory proteins *via* alternative splicing and an internal initiation codon located between the Env gene and the 3'-end of the genome (Fig. 1.1). The region encoding these proteins is called the pX region (Seiki *et al*., 1983).

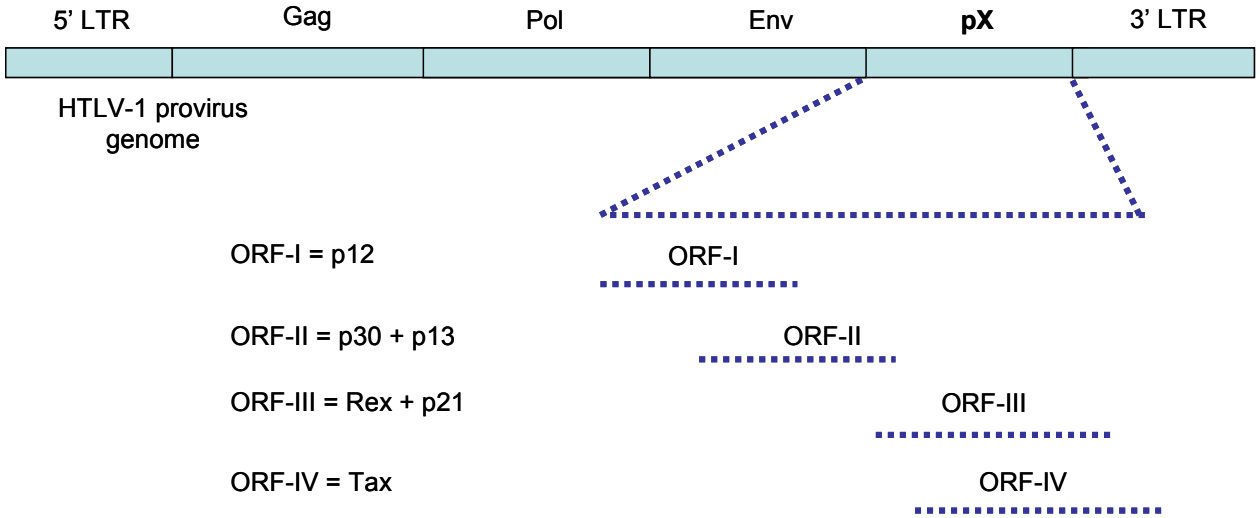


Figure 1.1: Genomic organization of HTLV-1

The HTLV-1 provirus genome encodes for Gag, Pol, and Env genes. In addition, the HTLV-1 genome contains a pX region, which contains four partially overlapping open reading frames (ORF), X-I – X-IV, encoding for several regulatory and accessory proteins. ORF-IV encodes for the Tax protein, which is considered as the major player involved in transformation events of HTLV-1 infection.

The pX region consists of four partially overlapping open reading frames (ORF), ORF-I – IV. ORF-I and II encode for the accessory proteins p12, p13 and p30 which have a role in transcriptional regulation, viral persistence and virus assembly (Albrecht and Lairmore, 2002). ORF-III codes for the Rex protein which is necessary for the export

of unspliced viral RNA from the nucleus (Heger *et al*., 1999) and codes as well for p21, a protein of unknown function. ORF-IV encodes the Tax protein which is thought to be the major player in transformation events of HTLV-1 infection (Felber *et al*., 1985).
Tax is a 40 kDa (353 amino acids) protein which is predominantly located in the nucleus of the host cell. Tax induces cell immortalization and transformation *in vitro* (Grassmann *et al*., 1989; Pozzatti *et al*., 1990; Grassmann *et al*., 1992) as well as tumor formation in transgenic mice (Nerenberg *et al*., 1987). Tax was initially identified as a trans-acting transcriptional activator of the HTLV-1 promoter in the LTR (Felber *et al*., 1985; Seiki *et al*., 1986; Sodroski *et al*., 1985), later it was shown that Tax can also trans-activate transcription of various cellular genes.

1.3 HTLV-1 associated diseases

HTLV-1 infection can lead to the development of Adult T Cell Leukaemia/Lymphoma (ATL), a malignancy of the clonal proliferation of infected mature $CD4^+$ T cells (Yoshida *et al*., 1982). The infected malignant $CD4^+$ T cells have characteristic large, irregular nuclei, called ‘leukemic flower cells’. The clinical course of ATL is progressive and can be divided into four clinical stages: pre-ATL, smoldering, chronic and acute ATL.
The chronic and smoldering stages of the disease have an indolent early course but progress to the acute form after a variable period of time (Shimoyama, 1991). During the acute stage of ATL, a tumor burden is observed, including massive lymphadenopathy, hepatosplenomegaly, infiltrative skin lesions, central nervous lesions and hypercalcemia (Watanabe *et al*., 1990). Only 2-4% of the carriers develop ATL which starts after a latent time of 20 to 40 years after infection (Yamaguchi, 1994). Besides ATL, HTLV-1 infection was also found to cause the neurological disease: tropical spastic paraparesis (TSP) (Gessain *et al*., 1985) and HTLV-1-associated myelopathy (HAM), later identified to be identical myelopathies (TSP/HAM) (Osame *et al*., 1986). TSP/HAM is a chronic, progressive neurological disease characterized by degeneration of the spinal cord and the presence of infiltrating T cells in both peripheral blood and cerebrospinal fluid. Patients have high levels of circulating HTLV-1-specific $CD8^+$ cytotoxic T cells which mainly recognize the same epitope of the HTLV-1 Tax protein (Tax 11-19) (Bangham, 2000; Mosley *et al*., 2005). Furthermore, HTLV-1 infection indirectly causes other

diseases *via* induction of immunodeficiency, such as chronic lung diseases and infection with *strongyloides stercoralis* (Porto *et al*., 2001; Carvalho and Porto, 2004). HTLV-1 is also associated with diseases, such as uveitis, which involves the inflammation of the eye and Sjogren syndrome, an autoimmune disorder in which immune cells attack and destroy the glands that produce tears and saliva (Terada *et al*., 1994; Mochizuki *et al*., 1992; Nishioka *et al*., 1989).

1.4 HTLV-1 persistence

HTLV-1 has been shown to activate and immortalize human T cells *in vitro*, although the exact mechanisms are still not fully understood (Gazzolo *et al*., 1987; Kimata *et al*., 1991). HTLV-1 transformation appeared to be a multi-step oncogenic process in which HTLV-1 infection represents the first event (Franchini, 1995). Shortly after infection the virus enters a latent state, rendering the infected individuals asymptomatic seropositive healthy carriers. During this latency period the viral gene expression in the infected peripheral $CD4^+$ cells is very low (Richardson *et al*., 1997). Also, very low levels of viral proteins are detectable in the infected cells (Kinoshita *et al*., 1989). It has been thought that antibody responses against viral antigens and HTLV-1-specific $CD8^+$ cytotoxic T cells might be important immune effectors that suppress the outgrowth of HTLV-1-transformed T cells and thus, reduce the risk of ATL development in healthy carriers (Mitsuyu *et al*., 1983; Jacobson *et al*., 1990; Kannagi *et al*., 2005). It was shown that infected $CD4^+$ T cells isolated from healthy carriers started to produce high viral gene expression when cultured *in vitro* (Asquith *et al*., 2000; Tochikura *et al*., 1985; Hanon *et al*., 2000). Addition of sera from the carrier into the culture medium inhibited viral expression whereas sera of uninfected control donors did not (Tochikura *et al*., 1985). Depletion of antibodies from the sera abolished this inhibition. It was also shown that the amount of infected $CD4^+$ T cells increased after HTLV-1-specific $CD8^+$ cytotoxic T cells had been depleted from the blood samples. Therefore, the viral load in HTLV-1 infected individuals could increase during the latency time primarily by proliferation of the proviral DNA-harboring cells rather than by repeated cycles of cell to cell infection of new uninfected cells (Etoh *et al*., 1997). Since ATL develops only in 2-4% of the people infected with HTLV-1 after a long latent period, it is possible that the virus infected T

cells are not killed by HTLV-1-specific $CD8^+$ cytotoxic T cells in a subpopulation of HTLV-1 carriers with a specific immunogenetic background (Uzuku *et al*., 1988; Sonoda *et al*., 1996). Recent studies have found that the frequency, diversity and function of HTLV-1-specific $CD8^+$ cytotoxic T cells are reduced in ATL patients as compared to healthy donors (Kozako *et al*., 2006). Furthermore, in TSP/HAM patients, a strong increase in the viral load is found as compared to healthy donors. The increase in the viral load was mainly dependent on the 'efficiency' of their HTLV-1-specific $CD8^+$ cytotoxic T cells (Bangham 2003; Vine *et al* 2004; Asquith *et al* 2005). $CD8^+$ cytotoxic T cells kill virus infected cell mainly by two mechanisms namely by the CD95 system mediated by CD95L on their surface or by the perforin/granzyme B system. Activation of CD95 or the entry of Granzyme B in the virus infected cell leads to the cleavage and activation of caspases and cell death. Accumulating evidence shows that HTLV-1 infected T cells are resistant towards CD95-induced apoptosis (Copeland *et al*., 1994). The survival mechanisms of HTLV-1 infected cells are poorly understood. It is widely believed that modulation of cell proliferation and resistance towards apoptosis play both a major role in persistence of HTLV-1 infection and disease development.

1.5.1 Introduction to the cell cycle

The cell cycle describes the process of cell duplication. It is the basis for the reproduction and sustained growth of all living organisms. Whether cells progress through the cell cycle or not, depends to a large extent on presense of growth factors. The eukaryotic cell cycle is divided into four phases namely the G1, S, G2 and M phase (Fig.1.1). In addition to these cell cycle phases, the term G0 phase is used to describe cells that have exited the cell cycle and become quiescent. The cell cycle regulation is tightly controlled by cyclins and the cyclin dependent kinases (CDKs) (Kato, 1999). Cyclins are activating subunits of CDKs that interact with their specific CDKs and regulate their activity. The first cyclin-CDK complex activated during the G_1-phase is composed of D-type cyclins in association with CDK4 or CDK6.

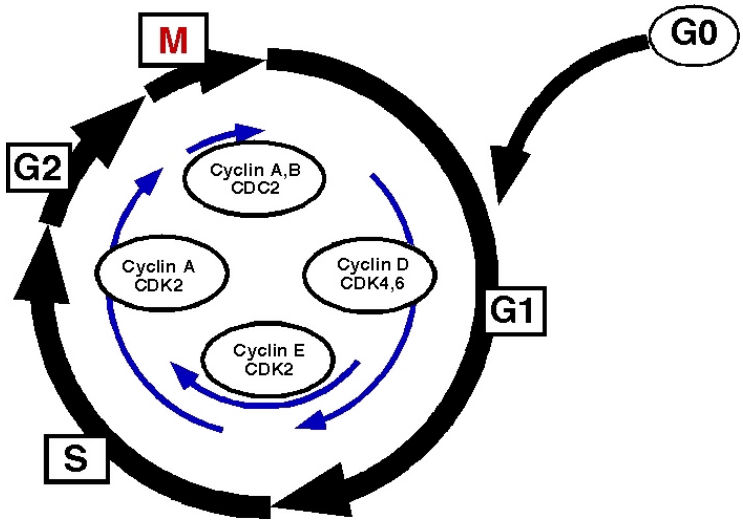


Figure 1.1: Cell cycle and cyclin dependent kinases (CDKs).

The G1 phase is the phase when the cell becomes committed to DNA replication and the decision on entering cell cycle is made (Hartwell et al., 1974; Nurse, 1975). During the S phase DNA synthesis occurs and during the G2 phase the cell prepares for the process of division. The phase in which the replicated chromosomes are segregated into separate nuclei is called mitosis (M-phase). In this phase, the cell divides into two daughter cells.

As cells progress through the G1 phase, cyclin E is expressed and associates with CDK2, which is essential for entry in the S phase. In the S phase, cyclin E is degraded and CDK2 associates with cyclin A. Finally, cyclin A and B associate with CDK1 and promote entry into mitosis. In G0 cells, the levels of D-type cyclins (D1, D2, and D3) are controlled at several levels like transcription, translation and protein stability. Stimulation of cells with growth factors leads to the transcription of D-type cyclins *via* the signal transduction pathways ERK and PI3K-Akt (Cheng *et al*., 1998; Klippel *et al*., 1998, Pouyssequr *et al*., 2007).

1.5.2 Regulation of the cell cycle

The cell cycle is not only regulated at the transcriptional level but can also be regulated directly on the translational level (Proud and Averous, 2006). Protein translation is considered as a three-stage process, consisting of an initiation, elongation and termination phase (Feliers *et al*., 2006). Protein translation is tightly regulated, predominantly at the level of initiation by the modification of the eukaryotic initiation factors (eIFs) (Sonenberg and Frederickson, 1992).

In eukaryotes, most mRNAs are translated in a cap-dependent manner. The cap structure, m^7GpppN (where N is any nucleotide), is present at the 5' terminus of all cellular eukaryotic mRNAs. The mRNA 5' cap structure is recognized and bound by the translation initiation factor eIF4E. After binding to the 5' cap structure, eIF4E interacts with eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex. The eIF-4F complex is directed to the 5' terminus of the mRNA and unwinds the mRNA 5' secondary structure to facilitate ribosome binding which promotes ribosome recruitment and translation (Gingras *et al*., 1999b).

An alternative mechanism for the recruitment of ribosomes to mRNA is the so called internal ribosome binding (Jackson, 1995). This process, which is mediated by the direct binding of the ribosome to an Internal Ribosome Entry Side (IRES) bypassing the 5' cap, was first reported for picornaviral mRNAs (Sonenberg, 1996). Viral IRES-dependent translation is efficient even when host cell cap-dependent translation is inhibited. This is mediated *via* the tertiary structure of the IRES, which is in turn stabilized by binding to cellular factors which interact directly with the translational machinery (Le and Maizel, 1997).

As mentioned, in eukaryotes, most mRNAs are translated in a cap-dependent manner by binding of the translation initiation factor eIF4E. The amount of the translation initiation factor eIF4E is limited as compared with other initiation factors and is therefore an important target for translational regulation (Duncan *et al*., 1987; Sonenberg, 1996). The activity of eIF4E is regulated by several mechanisms. At the transcriptional level, eIF4E mRNA levels are increased in response to growth factors. Second, the assembly of the eIF4F complex is inhibited by the translational repressor of eIF4E-binding proteins

(4E-BP1). In their hypophosphorylated state, they interact with eIF4E and prevent the recognition and binding to the 5' cap structure. Upon phosphorylation, the interaction between 4E-BP1 and eIF4E disrupts, allowing eIF4E to bind to the 5'cap structure and assemble the translation initiation complex. 4E-BP1 is phosphorylated by the kinase mTOR (molecular target of rapamycin), which, in turn is activated upon stimulation with growth factors by the PI3K/Akt pathway (Fig. 1.2). Rapamycin treatment inhibits mTOR activation and thereby inhibits 4E-BP1 phosphorylation. This increases the interaction between eIF4E and 4E-BP, and consequently inhibits cap-dependent translation.

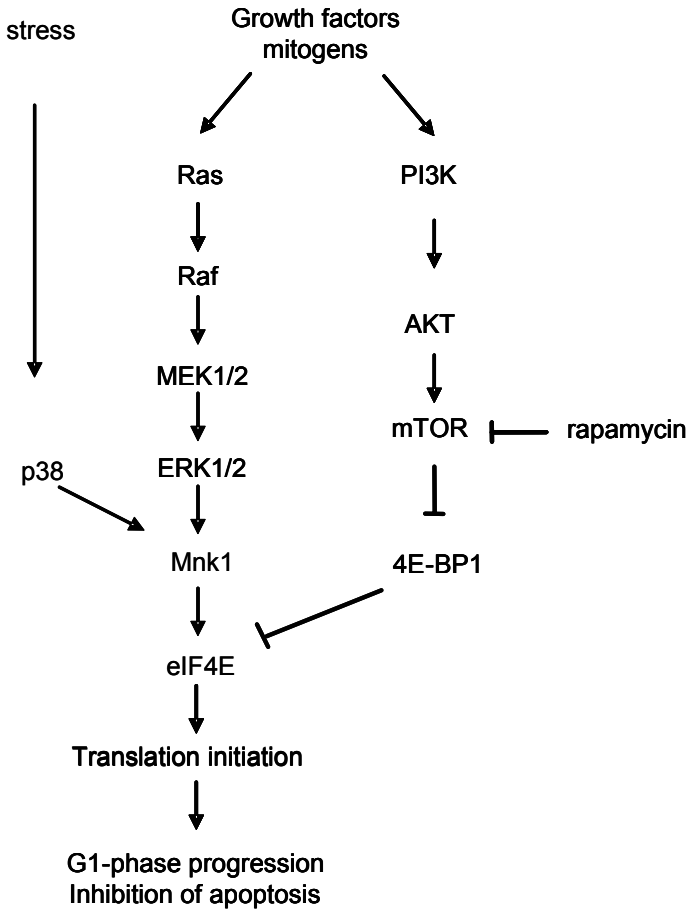


Figure 1.2: The intracellular signaling pathways regulating translation.
The activity of eIF4E is regulated by several pathways. The activation the PI3K/AKT pathway leads to the phosphorylation of 4E-BP1 mediated by the kinase mTOR. The growth factor induced ERK pathway and the stress-induced p38 MAPK pathway phosphorylate the kinase Mnk1 which in turn phosphorylates the initiation factor eIF4E. Phosphorylation of eIF4E increases its affinity for the 5' cap structure and thereby initiates translation.

The phosphorylation state of S209 correlates with the translation rate and growth status of the cell (Kleijn *et al*., 1998). Phosphorylation of eIF4E has been thought to increase its affinity for the 5' cap structure and thereby stimulates translation. As increased phosphorylation of eIF4E was found in Ras-transformed cells (Rinker-Schaeffer *et al*., 1992), an important role has been suggested for the Ras/Raf/ERK pathway. The activation of the ERK signaling pathway and thereby the phosphorylation of eIF4E occurs by a variety of extracellular stimuli such as hormones, cytokines or growth factors (Flynn and Proud, 1997; Wang *et al*., 1998). Also the p38 MAPK pathway is known to phosphorylate eIF4E (Wang *et al*., 1998). This pathway is induced by cellular stress, including heat shock, UV irradiation or anisomycin treatment (Waskiewicz and Cooper 1995; Kyriakis and Avruch, 1996). Induction of phosphorylation of eIF4E by anisomycin is prevented by the p38 MAPK inhibitor SB203580 (Morley and Mckendrick, 1997; Wang *et al*., 1998). It has been demonstrated that the phosphorylation of eIF4E by the mitogen induced ERK pathway and the stress induced p38 MAPK pathway converge on the eIF4E kinase Mnk1 (MAP kinase interacting kinase 1). Stimulation of either pathway resulted in Mnk1 phosphorylation and subsequent activation (Fukunaga *et al*., 1997; Waskiewicz *et al*., 1997). Mnk1 was found to efficiently phosphorylate eIF4E *in vitro*, and appeared to be the candidate to phosphorylate eIF4E after stimulation of one of the two pathways. Translational control has important implications for cell growth, cell survival and apoptosis. eIF4E plays an important role in the regulation of cell cycle progression, particularly in the G1/S progression by increasing the levels of cyclin D1 and D2 (Tan *et al*., 2000). eIF4E can also rescue cells from apoptosis, by inhibiting mitochondrial cytochrome c release through an increase in Bcl-X_L mRNA translation (Li *et al*., 2003). Synthesis of proteins that regulate cell proliferation can alter the balance between cell survival and cell death. eIF4E plays a key role in the control of this balance by regulating protein translation. This balance is disturbed in a variety of cancers overexpressing eIF4E, including breast, lung, prostate, cervix, Hodgkin's lymphma and colon cancer (Tan *et al*., 2000; Clemens, 2001; Dua *et al*., 2001; Li *et al*., 2003). Inhibitors of the pathways controlling eIF4E and thus cap-dependent translation are therefore promising therapeutic targets for the treatment of cancer.

1.5.3 Modulation of cell cycle by Tax

Tax expressing cells display an accelerated progression at the G_1-phase (Lemoine *et al*., 2001). Several different mechanisms have been proposed to explain the accelerated progression of the G_1-phase in Tax-expressing cells. First, studies have shown that signaling pathways are activated in HTLV-1 infected T cells. In HTLV-transformed Rat-1 cells, the PI3K-Akt pathway was found to be constitutively activated and to be involved in cell transformation (Jeong *et al*., 2005; Liu *et al*., 2001). Currently, it remains unclear how Tax stimulates this pathway. The ERK-JNK cascade has also been shown to be constitutively activated in Tax-transformed murine fibroblasts, in human lymphocytes transformed *in vitro* by HTLV-1, and in leukocytes isolated from ATL patients. However, this activation is not induced by Tax alone (Xu *et al*., 1996).

Second, it has been shown that Tax induces cyclin D2 expression (Santiago *et al*., 1999). The increased cyclin D2 expression also coincides with expansion of cyclin D2 binding partners Cdk4/6 (Santiago *et al*., 1999; Huang *et al*., 2001). Third, Tax may directly bind to cyclin D2 and stabilize the cyclin D/Cdk4 complex (Haller *et al*., 2002). Additionally, Tax may also directly bind to CDK inhibitor (CDKI) $p16^{INK4A}$, thereby preventing it from binding to Cdk4 and Cdk6 (Low *et al*., 1997). Taken together, Tax is able to generate an abundance of activated cyclin D/Cdk complexes, and promote G_1- to S-phase transition and cell proliferation.

1.6.1 Introduction to Apoptosis

Development and maintenance of a multicellular organism does not only require cell proliferation but also a balance of proliferation and cell death. Already in 1842, Carl Vogt described that cells can die in a regulated process (Vogt *et al*., 1842), an observation later on also found to be involved in embryogenesis (Gluecksmann *et al*., 1951). This regulated process of death was called programmed cell death. Later on, the term 'apoptosis' was proposed by Kerr, Wyllie and Currie in 1972 to describe the morphological processes of controlled cellular self-destruction. (Kerr *et al*., 1972). The word ‘apoptosis’ is of Greek origin and describes the process of leaves falling from trees. This implies that death is an active and defined process of the life cycle of an organism.

The apoptotic form of cell death is essential for regulation of development, differentiation and maintenance of cell populations in tissues, especially in the immune system (Krammer, 2000; Los *et al*., 1999; Vaux and Korsmeyer., 1999). On the one side, failure in the apoptotic process may result in autoimmune diseases, cancer and spreading of viral infections (Thompson, 1995). On the other side, excessive apoptosis has been associated with other diseases, such as AIDS and several neurodegenerative disorders (Cacabelos *et al*., 1996). A cell undergoing apoptosis shows characteristic biochemical and morphological changes. In an early stage of apoptosis, the cell shrinks and looses contact with neighbouring cells followed by chromatin condensation and plasma membrane blebbing. Finally the cell is fragmented into closed membrane structures, which are called 'apoptotic bodies' containing the cytosol, the condensed chromatin and organelles. These events are the result of the activation of proteolytic enzymes that mediate the cleavage of a multitude of specific protein substrates which are normally important in the integrity and shape of the cell and its organelles. Further on, these enzymes lead to the cleavage of DNA into oligonucleosomal fragments in approximately 180 to 200 bp fragments, which can be visualized by the typical 'DNA ladder' formation, seen after separation in an agarose gel (Wyllie *et al*.,1980; Steller *et al*., 1995). The apoptotic form of cell death is different from necrosis (Fig. 1.3). Necrosis occurs in response to a major insult as injury by toxins, or ischemia. Necrosis shows cell swelling, eventually the destruction of the cellular organelles, rupture of the plasma membrane and finally leakage of the cellular content into the cellular environment. Often, necrotic cell death is associated with damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Okada *et al*., 2004).

Apoptosis lead to the activation of a family of cysteinyl proteases, called caspases (cysteinyl aspartate-specific protease). The caspases are synthesized as non-active zymogens, procaspases, and upon activation cleavage of substrates occurs on the carboxyl-side of an aspartate residue (Cohen, 1997; Stennicke, 1998; Thornberry, 1998). Upon death signals, the caspases are proteolytically processed, generating a heterotetramer consisting of each two small and two large subunits. There are 14 mammalian caspases identified which can be grouped into two subclasses involved in apoptosis. The first subclass contains the initiator caspase-8, -9 and -10. These caspases contain either a death effector domain (DED) or a caspase recruitment domain

(CARD) (Hoffmann, 1997). The DED is found in caspase-8 and -10 and is necessary for interaction of these caspases with the adaptor protein FADD (FAS associated protein with death domain). The CARD domain found in caspase-9 is involved in promoting interactions with other caspases and with other regulatory proteins such as APAF-1. The second subclass of caspases is the effector or executioner caspases, including caspase-3, -6 and -7. These caspases cleave several cellular substrates, ultimately leading to the typical apoptotic morphology of the cell (Muzio *et al*., 1998). The anti-apoptotic proteins of the IAP family (inhibitors of apoptotic proteins) can directly inhibit caspase activity. The IAP family proteins, including XIAP, cIAPs and survinin, bind specifically to the effector caspase-3 (Roy *et al*., 1997). In this way they can inhibit its proteolytic activation.

Caspases can be induced by external or internal stimuli by the induction of two major pathways, namely the death receptor (extrinsic) cell death pathway and the mitochondrial (intrinsic) cell death pathway.

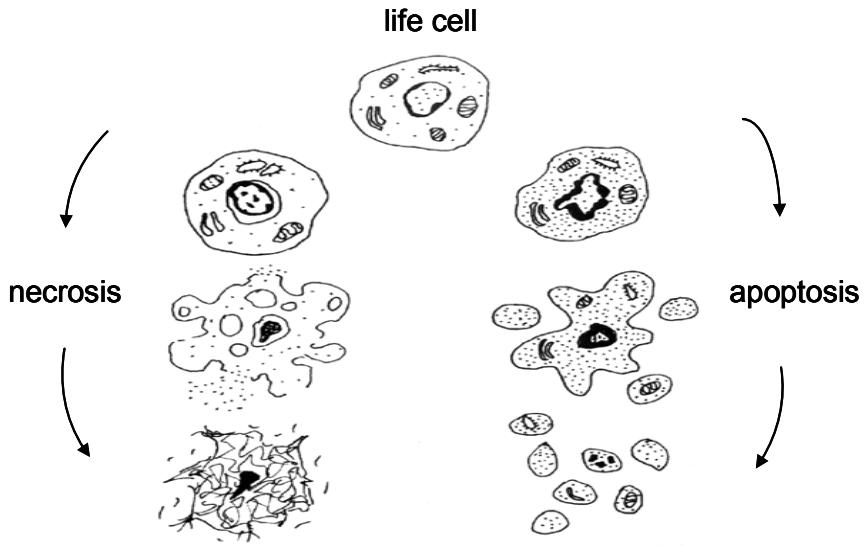


Figure 1.3: Hallmarks of the apoptotic and necrotic cell death process.

Apoptosis involves cellular shrinking, chromatin condensation, plasma membrane blebbing and formation of membrane-bound 'apoptotic bodies', which are phagocitized without triggering inflammatory processes. The necrotic cell death is characterized by swelling, plasma membrane rupture and release of its content into the surrounding tissue resulting in inflammation.

1.6.2 The death receptor (extrinsic) cell death pathway

The tumor necrosis factor receptor (TNF-R) superfamily regulates a large number of biological functions, such as growth, differentiation and apoptosis (Locksley *et al*., 2001). The extracellular ligand binding sites of these receptors are characterized by a number of cysteine rich domains which are necessary for specific ligand recognition (Naismith and Sprang, 1998). Death receptors (DR) belong to a subfamily of the TNF-R superfamily (Fig. 1.4). Until now, six members of the subfamily of death receptors have been characterized: tumor necrosis factor receptor 1 TNF-R1 (DR1, CD120a), CD95 (DR2, APO-1, Fas), DR3 (APO-3, LARD, TRAMP, WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, DR4, APO-2), TRAIL-R2 (DR5, KILLER, TRICK2) and DR6 (ectodysplasin) (Schulze-Osthoff *et al*., 1998; Ashkenazi and Dixit, 1999; Zola *et al*., 2005). Death receptors differ from the other TNF-R members by a common cytoplasmic conserved sequence of 80 amino acids, called death domain (DD), which is important for the transduction of the apoptotic signal (Itoh *et al*., 1993; Tartaglia *et al*., 1993). CD95 (APO-1/FAS) is one of the most studied death receptors and was discovered by the generation of monoclonal antibodies which induced apoptosis in various human cell lines (Trauth *et al*., 1989; Yonehara *et al*., 1989). CD95 is a type I transmembrane glycoprotein with a molecular mass of approximately 45 to 52 kDa (Itoh *et al*., 1991; Oehm *et al*., 1992). CD95 is expressed in many tissues and cells, mostly in thymus, heart, lung and liver. Under normal physiological conditions CD95-mediated apoptosis is triggered by its natural ligand, CD95L (APO-1L/CD178).

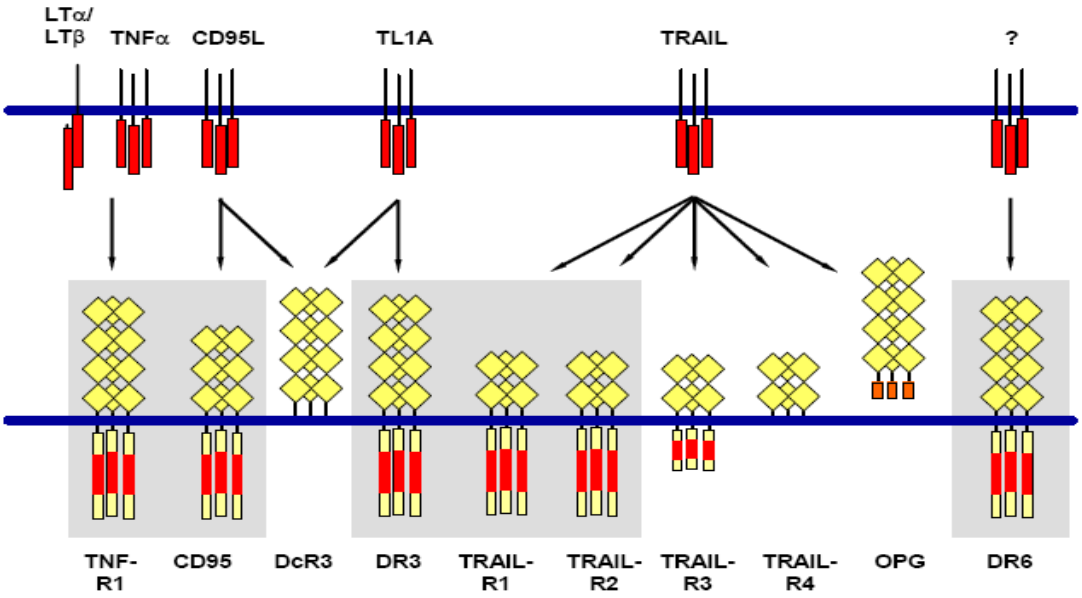


Figure 1.4: Death receptors, decoy receptors and their ligands.

Members of the subfamily of death receptors are characterized by cysteine rich domains (yellow squares) and a cytoplasmic conserved death domain (DD) (red). Death receptors are shown using a gray background.

CD95L is a type II membrane protein of the TNF family with a molecular mass of 40 kDa (Suda *et al*., 1993; Takahashi *et al*., 1994; Yu *et al*., 1999). In contrast to the widespread expression of CD95, CD95L expression is restricted to the immune system, mainly to $CD4^+$, $CD8^+$ and natural killer (NK) cells and a few tissues including the immune-privileged areas such as the iris (Griffith *et al*., 1995). CD95L is also expressed in several types of tumor cell lines (O'Connell *et al*., 1996; Strand *et al*., 1996). Expression of CD95L can be induced in T cells through activation of the T cell receptor. CD95L exists in a membrane-bound as well as in a soluble form. The soluble form of CD95L is generated from cleavage of the transmembrane form by metalloproteases (Kayagaki *et al*., 1995; Mariani *et al*., 1995; Tanaka *et al*., 1998). Besides it's ligand, CD95-mediated apoptosis can also be induced by agonistic antibodies (Trauth *et al*., 1989).

The CD95 system plays an important function in maintaining homeostasis in the immune system by inducing apoptosis in T cells, and eliminating auto-reactive lymphocytes in the periphery. Mice lacking functional CD95 (lpr/lpr) (lymphoproliferation) show the

phenotype of the autoimmune disease lupus. Similar to lpr$^{-/-}$ mice, mice lacking functional CD95L (gld/gld) (generalized lymphoproliferative diseases) fail to appropriately remove autoreactive lymphocytes from their immune systems (van Parijs and Abbas, 1996). In humans a similar disease with a dysfunction of the CD95 system is found called autoimmune lymphoproliferative syndrome (ALPS). These patients show massive, non-malignant lymphadenopathy, an altered and enlarged T-cell population and severe autoimmunity (Lenardo, 2003).

Besides CD95, also other death receptors play an important role in the immune system. TRAIL-R1 and R2 are activated by their ligand TRAIL (TNF-related apoptosis-inducing ligand). Besides TRAIL-R1 and R2, many cells also express the decoy receptors Dc-R1 (TRAIL-R3, CD263) and Dc-R2 (TRAIL-R4, CD264). Dc-R1 lacks the characteristic death domain and Dc-R2 contains a truncated non-functional death domain. These decoy-receptors are believed to negatively regulate TRAIL-induced apoptosis by competing for ligand binding (Sheridan *et al*., 1997). However, many cancer cells appear not to express these decoy receptors, making TRAIL-R1 and R2 to be potential targets for anti-cancer treatment. TRAIL knockout mice suffer form higher incidence of developing autoimmune diseases, suggesting that TRAIL also plays a role in the apoptotic depletion of autoreactive T lymphocytes. The CD95 and TRAIL-R1/TRAIL-R2 apoptotic signaling pathways are initiated upon binding to trimerized CD95L or TRAIL. This leads to trimerization of the receptor and clustering of the DD of the activated receptor in the cytoplasm. Several proteins associate with the DD of activated CD95 or TRAIL receptors and form a Death Inducing Signaling Complex (DISC) (Fig. 1.5). The adaptor molecule FADD (Fas-Associated Death Domain), which also contains a DD, is recruited and interacts with the DD of the activated receptor (Kischkel *et al*., 1995; Bolding *et al*., 1995; Chinnaiyan *et al*., 1995). FADD also contains a death effector domain (DED). Upon recruitment of FADD to the receptor, its DED interacts with the N-terminal tandem DED of the initiator caspase, procaspase-8 (Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997) or procaspase-10 (Kischkel *et al*., 2001; Wang *et al*., 2001; Sprick *et al*., 2002). However, whether caspase-10 can trigger cell death in the absence of caspase-8 in response to CD95 or TRAILR1/R2 stimulation is controversial (Walczak *et al*., 2002). Two types of cell death were established. Cells that have the ability to form high levels of CD95 DISC can effectively activate caspase-8 and directly

activate downstream effector caspases. These cells are called type I cells. Cells that have low levels of CD95 DISC formation must amplify the signal for cell death *via* the mitochondrial (Intrinsic) apoptotic pathway. These cells are called type II cells.

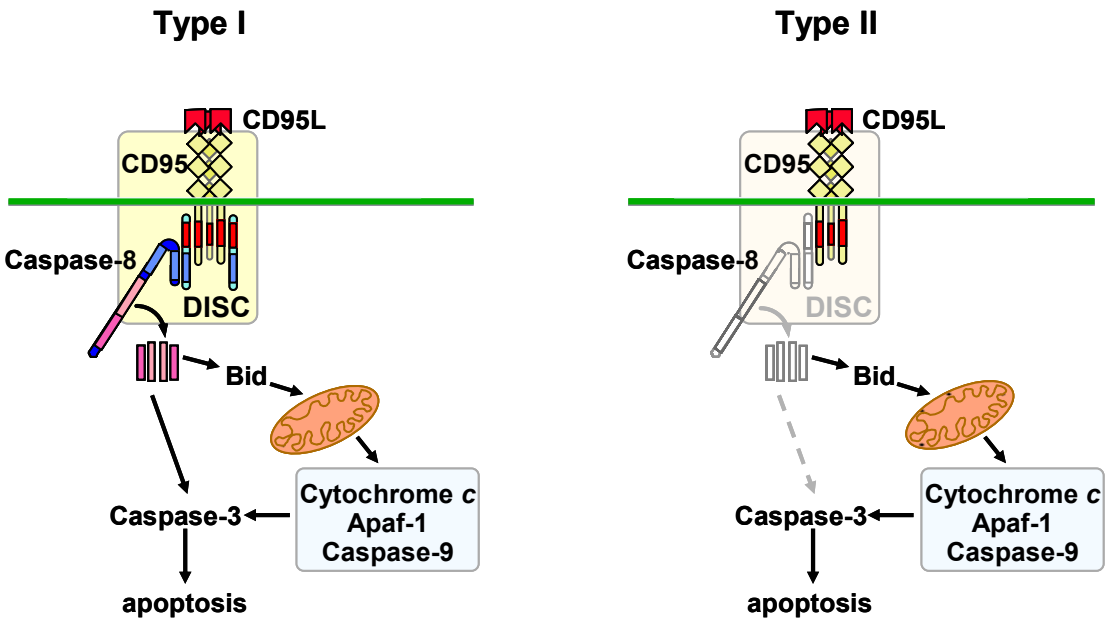


Figure 1.5: CD95 signaling pathways used in type I and type II cells.

CD95 signaling used in type I cells is shown on the left side whereas signaling used in type II cells is shown on the right side. Type I cells have the ability to form high levels of CD95 DISC formation and can directly activate downstream effector caspase-3. Type II cells have low levels of CD95 DISC formation. Therefore, the signal must be amplified via the mitochondrial apoptotic pathway mediated by the pro-apoptotic BH3 only family member Bid.

1.6.3 The mitochondrial (intrinsic) cell death pathway

The intrinsic cell death pathway can be induced by DNA damage, cytotoxic agents, hypoxia and deprivation of growth factors (Norbury and Zhivotovsky, 2004). An important trigger for the intrinsic cell death pathway is the mitochondrial outer membrane permeabilization (MOMP) which is largely regulated by members of the Bcl-2 (B-cell lymphoma-2) family (Chao and Korsmeyer, 1998). Bcl-2 family members are composed of pro- and anti-apoptotic proteins. Anti-apoptotic Bcl-2 family members such as Bcl-X_L, Mcl-1 and Bcl-2 contain four conserved domains which are called Bcl-2 homology (BH) domains (BH1-BH4). Pro-apoptotic Bcl-2 family members can be further divided into a group of proteins containing the BH1-BH3 domains, such as Bax and Bak and a BH3-only group such as Bim, Bad, Puma and Bid. The intrinsic cell death pathway is tightly controlled by these pro-and anti-apoptotic proteins of the Bcl-2 family. Following an apoptotic stimulus, Bax undergoes homodimerization and associates with the mitochondrial membrane. This results in changes of the mitochondrial permeabilization and the breakdown of the mitochondrial membrane potential (Petit *et al*., 1995; Zamzami *et al*., 1995). Bcl-2 or Bcl-X_L, are shown to associate with the mitochondrial membrane and maintain the mitochondrial membrane potential by antagonizing the pro-apoptotic Bcl-2 family members Bax and Bak (Zha *et al*., 1997). Depolarization of the mitochondrial potential results in the release of pro-apoptotic proteins from the mitochondria such as Smac/Diablo (second mitochondria-derived activator of caspase/direct IAP binding protein with low PI) and cytochrome c in the cytoplasm. Smac/Diablo then inhibits the anti-apoptotic function of the IAPs. Released cytochrome c associates with Apaf-1 (apoptosis protease-activating factor 1) in an ATP-dependent way. Apaf-1 recruits and interacts *via* its CARD domain with the CARD domain of procaspase-9 (Hoffmann 1997). This protein complex is called the apoptosome and leads to the activation of caspase-9 which in turn activates effector caspases such as caspase-3, -6 and -7 (Acehan *et al*., 2002; Zou *et al*., 1999). The activation of these effector caspases lead to the activation of other caspases and the cleavage of substrates and ultimately to apoptosis. The death receptor pathway and the

mitochondrial apoptotic pathway are linked by the pro-apoptotic BH3 only family member, Bid. Upon stimulation, Bid is cleaved by caspase-8 to a truncated form (tBID) which translocates to the mitochondria where it acts together with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the intrinsic cell death pathway (Scaffidi *et al*., 1998).

1.6.4 c-FLIP, a regulator of death receptor-induced apoptosis

To avoid uncontrolled cell death or tissue damage, apoptosis is tightly controlled on several levels. Receptor-mediated apoptosis can be regulated at the DISC by the presense of the cellular FLICE-like inhibitory protein (c-FLIP, FLAME-1, I-FLICE, Casper, CASH, MRIT, CLARP and usurpin), originally found in the family of herpesviruses. Several splice variants of c-FLIP have been reported to excist as RNA, but so far only three c-FLIP proteins have been found to be expressed. These include c-FLIP$_S$, and c-FLIP$_R$ and c-FLIP$_L$ (Irmler *et al*., 1997; Scaffidi *et al*., 1999; Golks *et al*., 2005) (Fig. 1.6). All FLIPs are characterized by two DEDs and can function as a dominant-negative inhibitor to block the recruitment of procaspase-8 to the DISC and thereby block cleavage and activation of caspase-8 (Krueger *et al*., 2001). c-FLIP$_S$ is similar in structure to viral FLIP (v-FLIP) of the herpesviruses, except that the two DEDs of c-FLIP$_S$ are followed by a short carboxy-terminal extension of around 20 amino acids that seems to be crucial for its ubiquitylation and therefore its proteasomal degradation. c-FLIP$_R$ also contains two DEDs but lacks the additional carboxy-terminal amino acids. c-FLIP$_L$ contains a longer C-terminal extension with a caspase-like region that is similar to procaspase-8 and procaspase-10. However, the C-terminal part of c-FLIP$_L$ lacks caspase enzymatic activity, due to the substitution of several amino acids, such as the cysteine residue, which is required for catalytic activity (Han *et al*., 1997; Hu *et al*., 1997; Inohara *et al*., 1997 Goltsev *et al*., 1997).

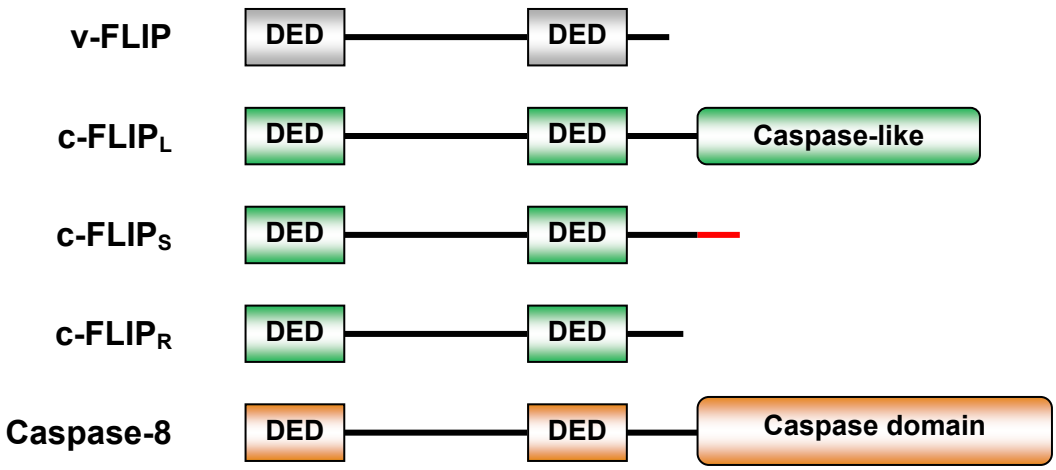


Figure 1.6: Molecular structure of viral and cellular c-FLIPs.
All FLIP molecules contain two death-effector domains (DEDs), similar to caspase-8. The carboxy (C)-terminal regions vary in length and sequence. Viral FLIP (v-FLIP) was initially detected in γ-herpesviruses. C-FLIP$_S$ and c-FLIP$_R$ are similar in structure to the v-FLIP except that c-FLIP$_S$ contains an extension of around 20 amino acids that is thought to be crucial for its ubiquitylation (red color). C-FLIP$_L$ contains a C-terminal inactive caspase-like domain, which is similar to procaspase-8 and procaspase-10. The short splice variants c-FLIP$_S$ and c-FLIP$_R$ lack such a caspase-like domain.

Besides competing with procaspase-8 for recruitment to the DISC, c-FLIP$_L$ can form heterodimers with procaspase-8, which leads to the generation of a 43 kDa FLIP fragment (p43). FLIP-p43 can recruit other signaling molecules, such as Receptor-interacting protein (RIP), TNF Receptor-Associated Factor 1 and 2 (TRAF1 and TRAF2) and promote thereby the activation of NF-κB and extracellular signal-regulated kinase (ERK)-mediated gene expression (Chaudhary *et al*., 2000; Kataoka *et al*., 2000; Kataoka and Tschopp, 2004). Therefore, CD95 also signals proliferation and differentiation (Zuliani *et al*., 2006). As c-FLIPs are key controllers of CD95L and TRAIL-R1/2 -induced apoptosis, their expression is strongly controlled. At the transcriptional level c-FLIP has been reported to be regulated by the nuclear factor of activated T cells (NFAT). Furthermore, it has been reported that c-FLIP is regulated by the transcription factor NF-κB (Micheau *et al*., 2001; Kreuz *et al*., 2001). It has also been reported that c-FLIP expression in normal cells and tumor cells is induced at the translational level by the PI3K/Akt pathway and can thereby induce resistance towards TRAIL and CD95 induced apoptosis (Panka *et al*., 2001; Suhara *et al*., 2001; Poulaki *et al*., 2002; Uriarte *et al*.,

2005). In activated T cells, c-FLIP expression has been shown to be dependent on the ERK/MAPK pathway. The addition of a dominant active MKK1 (MEK-1) induced c-FLIP expression in this case (Yeh *et al*., 1998).

1.6.5 Modulation of apoptosis by Tax

The viral protein Tax does not only affect cell proliferation but also apoptosis. HTLV-1 infected T cell lines were shown to be resistant towards CD95-induced apoptosis (Copeland *et al*., 1994). A similar resistance towards CD95-mediated apoptosis was observed in T cells derived from transgenic mice carrying the tax gene (Kishi *et al*., 1997). Analyses of these mice suggested that protection of peripheral T cells from CD95-mediated apoptosis by Tax is one of the important underlying mechanisms required for the immortalization of T cells and the development of ATL.

Besides resistance towards CD95 mediated apoptosis, HTLV-1 infected T cell lines have also been shown to be resistant towards TRAIL mediated apoptosis (Matsuda *et al*., 2005). The basis of apoptotic resistance in HTLV-1 infected T cells is the altered expression of proteins involved in the mitochondrial apoptotic pathway such as Bax and Bcl-X_L. It has been shown that Tax can downregulate the expression of the pro-apoptotic protein Bax (Brauweiler *et al*., 1997) and up-regulate the expression of the anti-apoptotic protein Bcl-X_L by induction of NF-κB (Tsukahara *et al*., 1999; Mori *et al*., 2001).

1.7.1 Regulation of transcription by NF-κB

NF-κB represents a family of structurally related inducible transcription factors that regulate diverse biological processes, including cell growth and survival of both T cells and nonlymphoid cells (Karin and Lin, 2002; Li and Verma, 2002). NF-κB proteins form homo- or heterodimers. At least five NF-κB proteins have been identified in mammalian cells, including p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and Rel A, which can transactivate target genes containing a NF-κB binding site (Siebenlist *et al*., 1994; Verma *et al*., 1995; Baldwin, 1996). The NF-κB proteins are normally sequestered in the cytoplasm by physical interaction with inhibitors of NF-κB (IκBs) (Verma *et al*., 1995; Baldwin, 1996). The latent NF-κB complexes can be activated by diverse immune stimuli, such as antigens, cytokines and microbial components, which target two

alternative NF-κB signaling pathways, namely the canonical or noncanonical pathway (Pomerantz and Baltimore, 2002). The canonical pathway is induced by several stimuli which lead to the phosphorylation of IκBα. Phosphorylated IκBα is then subjected to ubiquitination and degradation by the 26S proteasome. The degradation of IκBα releases the p50/RelA and p50/cRel NF-κB dimers which translocate to the nucleus and activate NF-κB-responsive gene expression (Didonato *et al*., 1996; Chen *et al*., 1996; Karin and Ben-Neriah, 2000). Phosphorylation of IκBα is carried out by an IκB kinase (IKK) complex comprised of two catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ (Zandi *et al*., 1997; Yamaoka *et al*., 1998). IKKγ, which is also known as NF-κB essential modulator (NEMO), has no kinase activity but likely functions as a scaffold protein, which assembles the IKK complex (Li *et al*., 2001). The IKK complex is activated by upstream kinases such as NF-κB inducing kinase (NIK) and the mitogen-activated protein kinase /ERK kinase kinase-1 (MEKK1) (Nakano *et al*., 1998; Mercurio *et al*., 1997) which activate the catalytic subunits, IKKα and IKKβ. The kinase HPK1 (Hematopoietic progenitor kinase 1) can also activate the IKK-complex and phosphorylates IKKβ *in vitro* (Brenner *et al*., 2005). After T-cell stimulation, caspase-3 can cleave HPK-1 and the C-terminal part of HPK-1 which can in turn inhibit NF-κB activity (Arnold *et al*., 2001; Brenner *et al*., 2005).

The noncanonical NF-κB pathway does not require the IKK complex but activation is mediated by upstream kinases and the IKKα subunit (Senftleben *et al*., 2001). This pathway is required for the activaton of specific NF-κB dimers as p52/RelB (Derudder *et al*., 2003) and is only used in specific cell types, such as B cells. In T cells and in most other cell types the signals are mediated by the canonical NF-κB pathway.

To achieve temporal activation, the NF-κB pathway has a negative feedback mechanism. NF-κB induces the expression and synthesis of the inhibitory protein IκBα (Scott *et al*., 1993). IκBα is able to enter the nucleus and can stop the induction of NF-κB dependent gene expression.

1.7.2 Activation of NF-κB by Tax

In contrast to the control of the NF-κB pathway in normal T cells, NF-κB is constitutively activated in freshly isolated ATL cells and HTLV-1-transformed T cell lines (Ballard *et al*., 1988; Ruben *et al*., 1988; Leung *et al*., 1988). This constitutive activation is mediated by the Tax protein which serves as an intracellular NF-κB inducer and bypasses negative feedback mechanisms. Tax has been reported to activate NF-κB through various mechanisms and it has been thought to physically interact with several NF-κB family members such as p50 and RelA (Beraud *et al*., 1994). Later, it was also reported that Tax-mediated NF-κB activation occurs at the level of NF-κB nuclear translocation (Kanno *et al*., 1994; Mckinsey *et al*., 1996). This was also shown with a Tax mutant, defective in nuclear localization, which could still activate NF-κB-dependent gene transcription (Nicot *et al*., 1998). Furthermore it was shown that Tax can induce the phosphorylation and degradation of I-κBα which further suggested that the NF-κB signaling pathway is involved in Tax-mediated NF-κB activation (Brockman *et al*., 1995; Kanno *et al*., 1994; Sun *et al*., 1994). Tax physically associated with the canonical IKK complex, in which IKK was constitutively phosphorylated and activated (Chu *et al*., 1998; Carter *et al*., 2001) (Fig. 1.7). The activation of the IKK complex was dependent on the physical interaction between Tax and the IKK regulatory subunit IKKγ. IKKγ serves as an adaptor for recruiting Tax to the IKK catalytic subunits IKKα and IKKβ (Geleziunas *et al*., 1998; Uhlik *et al*., 1998). Constitutive activity of the IKK complex was detected in both Tax-transfected cells as in HTLV-1-infected cells. The physical interaction of Tax with the canonical IKK complex is probably the major mechanism by which HTLV-1 induces persistent activation of NF-κB. However, other mechanisms may be involved in the maintenance of constitutive NF-κB activation.

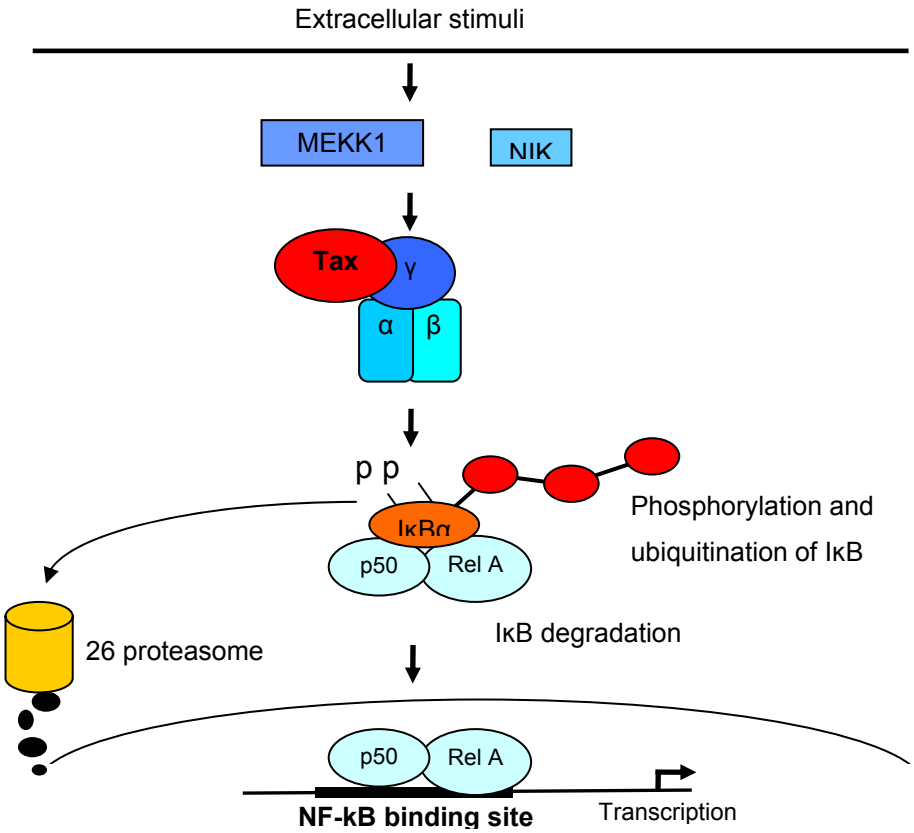


Figure 1.7: Activation of NF-κB signaling by Tax.
NF-κB is constitutively activated in HTLV-1 infected T cell lines by the interaction of Tax with the IKK regulatory subunit IKKγ. IKKγ serves as an adaptor for recruiting Tax to the IKK catalytic subunits IKKα and IKKβ, which are phosphorylated and activated. This leads to the phosphorylation and ubiquitination of IκBα, which is degradated by the 26S proteasome. The degradation of the I-κBs releases p50/RelA NF-κB dimers, which translocate to the nucleus and lead to the activation of NF-κB-responsive gene expression.

The constitutive activation of the NF-κB pathway in both HTLV-1 infected T cell lines and freshly isolated ATL cells suggests a critical role for NF-κB in the development of ATL. NF-κB may contribute to the abnormal growth and survival of infected T cells during the early stages of ATL disease progression by the induction of genes involved in T-cell proliferation and apoptosis inhibition. In addition, the constitutive NF-κB activity also likely promotes the genetic changes that drive the progression of T cell transformation.

1.7.3 Activation of CREB by Tax

Tax does not only have the capacity to activate NF-κB but can also activate other transcription factor such as the cAMP responsive element (CRE) binding factor (CREB) (Yoshida, 2001). In the normal regulation of CRE dependent transcription, is phosphorylated by protein kinase A upon stimulation of the cell, and phosphorylation allows its binding to a transcriptional co-activator, CBP (CREB binding protein). CBP associates with a specific 21-pb enhancer sequence and transcriptional initiation can occur. Tax interacts with CBP and forms a bridge between CREB and CBP without any specific phosphorylation of CREB (Brady *et al*., 1987; Jeang *et al*., 1988; Suzuki *et al*., 1993; Franklin and Nyborg, 1995). In this way, Tax trans-activates the HTLV-1 genome by directing the Tax-CBP/p300 complex to a 21-bp enhancer sequence located in the HTLV-1 LTR but it can also activate several cellular genes using similar mechanisms. Besides trans-activation, Tax also has the ability to trans-repress the activity of CREB (Franklin and Nyborg, 1995; Azran *et al*., 2004). High affinity of the Tax-CBP/p300 complex to transcription factors results in trans-activation, but weak affinity results in trans-repression.

The activation of transcription factors by Tax leads to the modulation of expression of a wide range of cellular genes (Yoshida, 2001). Some of them are directly involved in the activation of T-cell proliferation, such as interleukin 2 (IL-2) (Good *et al*., 1996), the α-subunit of its receptor (IL-2Rα) (Crenon *et al*., 1993), IL-15 (Azimi *et al*., 1998) and its receptor (IL-15R) (Mariner *et al*., 2001), granulocyte-macrophage colony stimulating factor (GM-CSF) (Himes *et al*., 1993) and others. The induction of IL-2Rα and IL-15R and their ligands by Tax lead to an autocrine activation loop (Maruyama *et al*., 1987).

All together, the activation of a wide range of cellular genes leads to the deregulation of many important processes, including cell cycle regulation and apoptosis, resulting in transformation of HTLV-1 infected T cells.

1.8 Therapeutic approaches

Despite advances in the knowledge of the molecular biology of HTLV-1 and ATL no effective treatment is available. The prognosis for ATL remains poor with a median survival of less than 1 year. Treatment of ATL has been of limited success due to the resistance of ATL cells towards apoptosis, the associated immunosuppression and the poor performance status of the patients (Siegel *et al*., 2001). ATL is generally treated with an aggressive multidrug approach of chemotherapeutic agents. So far, the combination of cyclophosphamide, adriamycin, vincristine and prednisone (CHOP) has been the first line therapy. In some clinical studies CHOP has been combined with other agents as etoposide, vindesine, ranimustine and mitoxantrone (Taguchi *et al*., 1996). Also, clinical studies are performed with an aggressive multidrug approach with a combination of vincristine, cyclophosphamide, doxorubicin and prednisone (VCAP), doxorubicin, ranimustine, and prednisone (AMP), and vindesine etoposide, carboplatin, and prednisone (VECP). Although these combinations are generally associated with an increase in response rate and response duration, the overall survival remains poor with survival rates between 5.5 and 13 months (Yamada *et al*., 2001). Besides chemotherapy, several new approaches for the treatment of patients with ATL have been proposed. Combination of the antiretroviral drug zidovudine and interferon-α (IFN-α) was reported to have significant activity in patients with ATL including those where chemotherapy had failed before. The constitutive activation of NF-κB in HTLV-1 infected T cells makes it an attractive target for therapy. A number of different approaches point to the therapeutic potential of NF-κB inhibition. The NF-κB inhibitor, Bay 11-7082, down-regulates the expression of the anti-apoptotic protein Bcl-X_L which is highly upregulated in HTLV-1 infected T cells. Inhibition of the proteasome by PS-341 (bortezomib) blocked the degradation of IκBα and thereby the inhibition of NF-κB. Both approaches lead to the induction of apoptosis in HTLV-1 infected T cells *in vitro* (Tan *et al*., 2002; Satou *et al*., 2004).

An alternative approach is to target cell differentiation markers on the HTLV-1 infected T cells with monoclonal antibodies. The high expression of the IL-2α receptor (CD25) has made it an attractive target. Patients are treated with an anti-CD25 monoclonal antibody

(daclizumab) with or without labeled Yttrium^{90} (Waldmann *et al*., 1988; Waldmann *et al*., 1995). A study of humanized anti-CD25 antibody therapy in combination with CHOP chemotherapy is currently ongoing.

Currently there is no clear evidence to support the value of any particular treatment approach with respect to treatment response. Although some therapies were reported to be partly effective, no convincing therapeutic benefits have been established. Therefore new molecular targeting strategies and the development of new drugs are important.

1.9.1 Plants, sources of new drugs

Plant-derived substances have traditionally played an important role in the treatment of human diseases. About 80% of the world population living in third world countries, almost entirely rely on plant products for their health care. Also in the western world pharmaceuticals are frequently derived directly from plant products. Examples include aspirin (originally derived from the *Rosacea Filipendula ulmaria*) and the prescription drug morphine (derived from *Papaveracea Papaver somniferum*). Although plant extracts have been used for over 3,500 years in the treatment of several diseases, it was only since the late 1950s that it became clear that plant-derived compounds could also play an important role in the treatment of cancer therapy. These compounds exert their antiproliferative effect on various intracellular targets. Many if not all applications of these drugs finally result in an inhibition of cell proliferation depending on the concentration used in the individual experiments.

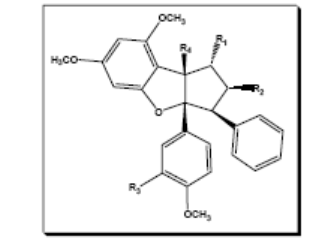
1.9.2 The plant genus Aglaia and its rocaglamide derivatives

Many research institutions and industrial pharmaceutical laboratories apply random screening approaches of plants extracts for interesting compounds. Such strategies have been estimated to require the evaluation of approximately 20.000 candidate plant extracts to obtain one clinical useful drug. In one of these screenings it was found that the plant genus *Aglaia* of the family *Meliaceae* could be a potential source of interesting compounds (Fig.1.9).

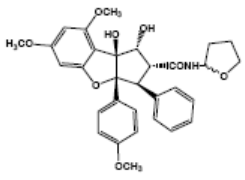
Figure 1.9: Aglaia odorata of the family Meliaceae, source of cyclopenta[b]benzofuran compounds (Rocaglamide).

The genus *Aglaia* consists of approximately 130 species mostly found in countries in South-East Asia. Plant extracts from several *Aglaia* species are already used in traditional medicine for the treatment of inflammatory skin diseases and asthma. The active compounds isolated from these plant extracts are derivatives of cyclopenta[b]benzofuran, also called rocaglamide (King *et al*., 1982). Since rocaglamide was found to be the active compound, investigation has led to the isolation of many related compounds. To date, 60 naturally occurring rocaglamide derivatives have been isolated from over 30 *Aglaia* species.

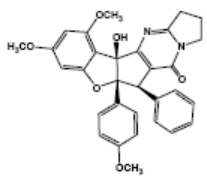
The majority of these naturally occurring derivatives have strong insecticidal activity as analyzed in the larvae *Spodoptera littoralis*. Most of the rocaglamide derivatives tested had a LC_{50} value ranging between 1-2 ppm which is comparable with the LC_{50} of the insecticide azadirachtin (Brader *et al*., 1998). Besides their insecticidal activity, rocaglamide derivatives have also been tested for their possible antiproliferative activity in cancer cell lines. Up to now, over 40 rocaglamide derivatives have been tested in *in vitro* assays against several cancer cell lines, and most of these compounds showed significant cytostatic activity with IC values comparable to that of the well known anti-cancer agent vinblastine (Lee *et al*., 1998; Kim *et al*., 2006). Rocaglamide derivatives differ usually from each other by the nature of their substituents at the positions, R1 (-OH) vs. –OAC), R2 (different amino acyl substituents vs. – $COOCH_3$ group) and/or R3 (-H vs. –OH or $–OCH_3$) whereas the substitution pattern at the remaining carbons of the rocaglamide skeleton as well as the absolute configuration of the various congeners are remarkably stable (Nugroho *et al*., 1997) (Fig. 1.10). The activity of the several rocaglamide derivatives is dependent on the different substituents, as analyzed in previous studies (Bohnenstengel *et al*., 1999).



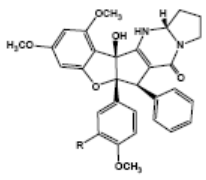
	R_1	R_2	R_3	R_4
1.	OH	$CON(CH_3)_2$	H	OH
2.	OH	$CON(CH_3)_2$	OH	OH
3.	OH	$CON(CH_3)_2$	OCH_3	OH
4.	$OCOCH_3$	$CON(CH_3)_2$	H	OH
5.	$OCOCH_3$	$CON(CH_3)_2$	OH	OH
6.	OH	$CONHCH_3$	H	OH
7.	OH	$CONHCH_3$	OH	OH
8.	$OCOCH_3$	$CONHCH_3$	H	OH
9.	$OCOCH_3$	$CONHCH_3$	OH	OH
10.	OH	$CONH_2$	H	OH
11.	OH	$CONH_2$	OH	OH
12.	OH	$CONH(CH_2)_4OH$	H	OH
13.	$OCOCH_3$	$CONH(CH_2)_4OH$	H	OH
14.	OH	$COOCH_3$	H	OH
15.	OH	$COOCH_3$	OH	OH
16.	OH	$COOCH_3$	OCH_3	OH
17.	$OCOCH_3$	$COOCH_3$	OH	OH
18.	OCHO	$COOCH_3$	H	OH
19.	OCHO	$COOCH_3$	OH	OH
20.	NOH	$COOCH_3$	OCH_3	OH
21.	OH	H	H	OH
22.	OH	H	OCH_3	OH
23.	OH	H	O-Rham	OH
24.	OH	H	H	OC_2H_5



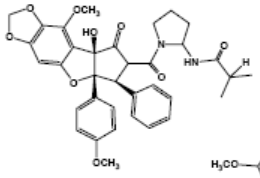
25. *R*-isomer
26. *S*-isomer



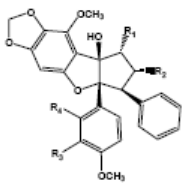
27



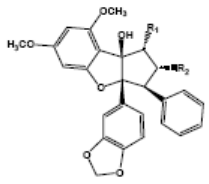
28



29



	R_1	R_2	R_3	R_4
30.	OH	$CON(CH_3)_2$	H	H
31.	OH	$CON(CH_3)_2$	OCH_3	H
32.	OH	$CON(CH_3)_2$	OCH_3	OH
33.	OH	$CONHCH_3$	H	H
34.	$OCOCH_3$	$CONHCH_3$	O	H
35.	OH	$CONHCH_3$	OCH_3	H



	R_1	R_2
36.	OH	$COOCH_3$
37.	OH	H
38.	OCHO	$COOCH_3$
39.	$OCOCH_3$	$COOCH_3$
40.	=O	H

Figure 1.10: Chemical structures of several Rocaglamide derivatives.
The structure of the rocaglamide derivative used in this study, 1-oxo-11, 12-methylendioxy-rocglaol, abbreviated as Roc-AR, is number 38.

Although the rocaglamide derivatives are known to exhibit cytostatic activity, their molecular target(s) have not been identified. Recent investigations have focused on the cellular mechanism of action of these compounds. It has been reported that some members of this class of compounds inhibit cell proliferation in human monocytic leukemia cell lines (Bohnenstengel *et al*., 1999). Later on, it was reported that rocaglamide derivatives can inhibit TNF- and PMA (phorbol 12-myristate 13-acetate)-induced NF-κB activity (Baumann *et al*., 2002). More recently, our group has shown that rocaglamide derivatives inhibit NFAT activity in activated T cells (Proksch *et al.,* 2005). It was demonstrated that rocaglamide derivatives selectively inhibit the NFAT-dependent gene expression of several cytokines after stimulation with anti-CD3/anti-CD28 or PMA/ionomycin in peripheral blood T cells. The tested rocaglamide derivatives inhibited NFAT at a concentration between 25-100 nM whereas NF-κB activity was not affected (Proksch *et al*., 2005). Recent new data in our group suggests that these compounds can induce apoptosis in leukemia cells but not in normal peripheral blood lymphocytes. Taken together, rocaglamide derivatives show antiproliferative activity observed *in vitro* in several human cancer cell lines and *in vivo* in an experimental mouse model. Therefore, they are promising chemotherapeutic agents. The mechanism of the rocaglamide-mediated antiproliferative effect is not completely understood. Natural compounds exert their antiproliferative effect on various intracellular targets. For example, vinblastine inhibits tubulin polymerization (Bayssass *et al*., 1980), actinomycin D inhibits DNA replication (Guy and Taylor, 1978) and cycloheximide inhibits protein biosynthesis *via* the ribosomal machinery (Korner, 1966). Other natural products interfere with signal transduction pathways by inhibiting protein kinases such as the inhibition of the kinase mTOR by Rapamycin (Tsang *et al*., 2007). Applications of these drugs results in inhibition of cell proliferation which depends on the concentration used. The specificity of the drug for a certain molecular target and the amount needed for treatment decide largely about its use as a therapeutic agent.

1.10 AIM OF THE STUDY

Human T cell Leukemia Virus Type 1 (HTLV-1) is a retrovirus, associated with several diseases including Adult T-cell Leukemia/Lymphoma (ATL). Because of apoptosis resistance therapeutic treatment provides only very limited benefits for ATL.

CD95/CD95L-mediated apoptosis is an important mechanism of T cell homeostasis. We have previously shown that HTLV-1 infected T cells are more resistant towards apoptosis induced by CD95L as compared to non infected T cells. In this study, we address the question, whether these cells are more resistant towards death receptor-mediated apoptosis in general and compare to other death receptors, like TRAIL receptors.

The basis of apoptotic resistance in HTLV-1 infected T cells was suggested to be due to the elevated expression of several anti-apoptotic proteins involved in modulation of the intrinsic cell death pathway. Recently our group found that apoptosis is also blocked within the extrinsic cell death pathway by high c-FLIP expression. C-FLIP is an anti-apoptotic protein that blocks death receptor-mediated apoptosis at the DISC level.

The aim of this study is to develop a new approach to overcome resistance of HTLV-1 infected T cells towards death receptor-mediated apoptosis. The herbal compound, Rocaglamide, derived from a Traditional Chinese Medicinal plant (TCM) should be tested to sensitize HTLV-1 infected T cells towards cell death.

Although our lab recently showed that Rocaglamide derivatives exhibit antiproliferative activity on different leukemia cell lines and in leukemia cells freshly isolated form patients, their molecular target(s) have not been identified. Therefore, it is intended to find the molecular mechanism of resensitization by Rocaglamide. The ultimate goal of this study is to develop a strategy for the treatment of ATL and other types of T-cell tumors.

2 MATERIALS & METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals, unless stated otherwise, are purchased from Merck (Darmstadt), Roth (Karlsruhe), Fluka (Neu-Ulm), Serva (Heidelberg) and Sigma (München).

2.1.2 Instruments

Instrument	**Manufacturer**
Agarose gel electrophoresis apparatus	Gibco BRL
Autoradiography-Film (Kodak X-Omat^{TM}LS)	Kodak
Bacterial shaker Certomat HK	Braun
Biofuge A	Heraeus
Biofuge Fresco	Heraeus
Cell culture Hood SG600	Baker Company
Cell culture incubator	Forma Scientific
Centrifuge 5402	Eppendorf
Developing system for X-Ray films Curix 160	Agfa-Gevaert
Electrophoresis power supply Consort 865	Renner
FACS scan flow cytometer	Becton Dickinson
FACS calibur flow cytometer	Becton Dickinson
Fine scale PE 3600	Mettler
Freezer -20°C	Liebherr
Freezer -80°C	Forma Scientific
GeneAmp 5700	PE Applied Biosciences
Heat block Thermostat 5320	Eppendorf
Hemacytometer Neubauer	HBG

Instrument	Manufacturer
Light microscope ID 02	Zeiss
Megafuge 1.OR	Heraeus
Microwave HMG730B	Bosch
Mini gel electrophoresis apparatus	Bio-Rad
Nucleofector Electroporator	Amaxa
pH meter WTW	WTW
Phase contrast microscope	Leitz
quartz cuvettes Suprasil	Hellma
Scale AE 240	Mettler
Semi Dry Blot Apparatus 20 x 25 cm	CTI/Biorad
Sorvall RC 3B PLUS, 5C PLUS	Beckmann
Spectrophotometer 6505 UV/Vis	Jenway
Spectrophotometer U-1100	Hitachi
Thermocycler	Perkin Elmer
UV transilluminator	Konrad Benda
Vertical Gel Electrophoresis	BRL
Video Graphic Printer UP-860 CE	Sony
Water bath	Köttermann
x-ray developer Curix 160	Agfa-Gevaert

2.1.3 Solutions and buffers

Buffer	concentration	Reagents
PBS:	137 mM	NaCl
	8.1 mM	Na_2HPO_4
	2.7 mM	KCl
	1.5 mM	KH_2PO_4 (pH = 7,4)
FACS buffer (in PBS)	5% (w/v)	FCS
	0.1% (w/v)	NaN_3
Luciferase buffer	470 µM	Luciferin (Photinus pyralis)
	1.07 mM	$(MgCO_3)_4Mg(OH)_2.5H_2O$
	20 mM	Tricin
	2.67 mM	$MgSO_4$
	100 µM	EDTA
	33.3 mM	DTT
	270 µM	EDTA
	33.3 mM	DTT
	270 µM	Acetyl-CoA
	530 µM	ATP
Nicoletti lysis buffer	0.1% (w/v)	Sodium citrate (pH 7.4)
	0.1% (w/v)	Triton X-100
	50 µg/ml	Propidium iodide
TBE (10x)	0.45 M	Tris base
	0.45 M	Boric Acid
	10 mM	EDTA (pH = 8.3)
TE	10 mM	Tris base
	1 mM	EDTA (pH = 7.5)

Buffer (SDS-PAGE)	**concentration**	**Reagents**
RIPA Lysis buffer	150 mM	NaCl
	1 mM	DTT
	1% (w/v)	Triton X-100
	1 tablet / 50 ml	Protease inhibitor Cocktail (Roche)
	50 mM	Tris-HCL, pH= 8.0
	0.5%	Na-deoxycholate
	2 mM	EDTA (ethylene-diamine-tetra-acetate)
	0.1%	SDS
	200 µM	Na_3VO_4
	25 mM	NaF
Laemmli Stacking gel buffer (5%)	24 mM	Tris base (pH = 6.8)
	5% (w/v)	Acrylamide/Bisacrylamide 37.5:1
	0.1% (w/v)	SDS
	0.1% (w/v)	Ammoniumpersulfat (APS)
	0.1% (w/v)	Tetramethylethylendiamine (TEMED)
Laemmli Resolving gel	37.5 mM	Tris base (pH = 8.8)
	7.5-15% (w/v)	Acrylamid/Bisacrylamid 37.5:1
	0.1% (w/v)	SDS
	0.03% (w/v)	APS
	0.1% (w/v)	TEMED
Transfer buffer (Western Blot)	25 mM	Tris base
	0.19 M	Glycine
	20% (v/v)	Methanol
	0,037% (w/v)	SDS
Blocking buffer	5% (w/v)	non-fat dry milk in TBST
SDS sample buffer (5 x)	50% (v/v)	Glycerol
	10% (w/v)	SDS
	50 mM	Tris base (pH = 6.8)
	25% (v/v)	β-Mercaptoethanol
	0.25 mg/ml	Bromphenol blue
Running buffer (SDS-PAGE)	0.19 M	Glycin
	0.1% (w/v)	SDS
	25 mM	Tris base (pH = 6.8)

Buffer (nuclear extract)	concentration	Reagents
Buffer A	10 mM	HEPES (pH 8.0)
	0.5 M	Sucrose
	50 mM	NaCl
	0.25 mM	EGTA
	1 mM	EDTA
	0.5 mM	Spermidine
	0.5 %	Triton x-100
	1 µg/ml	Trypsin
	0.5 mM	PMSF
	0.5 µg/ml	Leupeptin
	0.7 µg/ml	Pepstatin
	1 µg/ml	Aprotinin
	40 µg/ml	Bestatin
Buffer B	10 mM	HEPES (pH 8.0)
	25%	Glycerol
	500 mM	NaCl
	0.1 mM	EGTA
	1 mM	EDTA
	0.5 mM	Spermidine
	0.25 mM	DTT
	0.5 mM	PMSF
	0.5 µg/ml	Leupeptin
	0.7 µg/ml	Pepstatin
	1 µg/ml	Aprotinin
	40 µg/ml	Bestatin

2.1.4 Eukaryotic Cell lines and bacterial strains

Cell lines	Description
Jurkat	Human acute lymphoblastoid T cell line
CEM	Human acute lymphoblastoid T cell line
SP	HTLV-1 infected leukemia T cell line
MT-2	HTLV-1 infected lymphoblastoid T cell line
ATL-3	HTLV-1 infected leukemia T cell line
CHAMP	HTLV-1 infected leukemia T cell line

Bacterial strain	Source
DH5α	Bethesda Research Laboratories

2.1.5 Culture media bacteria

Bacterial culture Medium	Composition	
LB (Luria-Bertani) medium	90 g	NaCl
(10x)	90 g	Casein hydrolysate (Roth)
	45 g	Yeast extract (Gerbu)
	(Adjust pH to 7.5 with NaOH)	

For the preparation of 10 LB-plates, 4.5 g of bacto-agar were added to 300 ml of LB medium, autoclaved and cooled down to 50°C in a water bath. At this temperature ampicillin (50 µg/ml) was added. After cooling down, petri dishes were filled with the solution. The plates were stored at 4°C until usage.

2.1.6 Culture media eukaryotic cell lines

Cell culture medium 1640 is obtained from GibcoBRL in powder form and solved in H_2O as described. The medium was sterilized and stored at 4 °C. Before usage FCS, Gentamycin, L-glutamine were added. The final composition of the medium is shown below. Prior addition to the medium Fetal Calf Serum (FCS) was heat inactivated for 30 min at 56 °C. For primary human T cells IL-2 (25 U/ml) was added to the medium.

Human cell culture medium	**Composition**	
	900 ml	RPMI 1640 (GibcoBRL)
	10%	FCS (GibcoBRL)
RPMI medium (complete)	10 mg/ml	Gentamycin (GibcoBRL)
	2 mM	L-glutamine (Invitrogen Life Technologie)

2.1.7 Antibodies for western blot analysis

Name	antigen	Origin
C15 (IgG2b)	caspase-8	Scaffidi *et al*, 1997
NF6 (IgG1)	c-FLIP	Scaffidi *et al*, 1999
anti-FADD (IgG1)	FADD	Transduction Laboratories
anti-Erk1 (MK12)	Erk	BD Biosciences
anti-Mek1/2 (47G9)	Mek1/2	Cell Signaling Technology
anti-phospho-Mek1/2 (41G9)	phospho-Mek1/2	Cell Signaling Technology
anti-Caspase-3 (9662)	Caspase-3	Cell Signaling Technology
anti-phospho-ERK (E-4)	Phospho-Erk	Cell Signaling Technology
anti-phospho-mTOR (2971)	Phospho-mTOR	Cell Signaling Technology
anti-mTOR (2972)	mTOR	Cell Signaling Technology
anti-phospho-Mnk1 (2111)	Phosphor-Mnk1	Cell Signaling Technology
anti-phospho-eIF4E (9741)	Phospho-eIF4E	Cell Signaling Technology
anti-eIF4E (9742)	eIF4E	Cell Signaling Technology
anti-eIF4G (2441)	eIF4G	Cell Signaling Technology
anti-p38 (5F11)	p38	Cell Signaling Technology
anti-phospho p38 (V121A)	Phospho-p38	Promega
anti-Tubulin (clone B-5-1-2)	tubulin	Sigma
anti-Tax hybridomas (clone 168B17-46-34 and 168B17-46-50)	Tax	NIH AIDS Research & Reference Reagent Program
anti-Mnk1 (C-20) (sc-6965)	Mnk1	Santa Cruz Biotechnology
anti-Cyclin D1 (M-20)	Cyclin D1	Santa Cruz Biotechnology
anti-Cyclin D2 (C-17)	Cyclin D2	Santa Cruz Biotechnology
anti-Cyclin B (554178)	Cyclin A	BD Biosciences
anti-Cyclin E (E-4)	Cyclin E	Santa Cruz Biotechnology
anti-IgG, HRP (Goat, polyclonal)	Mouse IgG	Jackson
anti-IgG1, HRP	Mouse IgG1	Southern Biotechnology

(Goat, polyclonal)		
anti-IgG2a, HRP (Goat, polyclonal)	Mouse IgG2a	Southern Biotechnology
anti-IgG2b, HRP (Goat, polyclonal)	Mouse IgG2b	Southern Biotechnology
anti-Rabbit, HRP (Goat, polyclonal)	Rabbit IgG	Santa Cruz Biotechnology

2.1.8 Antibodies for FACS analysis

Name (serum/isotyp)	**Antigen**	**Origin**
anti-CD69-FITC	CD69	BD Pharmingen
anti-APO-1 (IgG3)	CD95	Trauth *et al*., 1989
NOK-1	CD95L	BD Pharmingen
HS101 (IgG1)	TRAIL-R1	Alexis
HS201 (IgG1)	TRAIL-R2	Alexis
HS301 (IgG1)	TRAIL-R3	Alexis
HS402 (IgG1)	TRAIL-R4	Alexis
anti-IgG1, PE (goat, polyclonal)	mouse IgG1	BD Pharmingen
anti-IgG1, FITC (goat, polyclonal)	mouse IgG1	Jackson

anti-IgG1 antibody was conjugated with FITC (Fluorescein-Isothiocyanat $\lambda_{Ex/Em}$/max 488/514 nm), or with PE (R-Phycoerythrin $\lambda_{Ex/Em}$/max 488/575 nm).

2.1.9 Reagents

Reagents	Reference
anti-APO-1	Trauth *et al*., 1989
Leucine zipper (LZ)–CD95L	Walczak *et al*., 1999
Super-TRAIL	Alexis
Cycloheximide	Sigma
Rocaglamide derivative Roc-AR	Proksch *et al*., 2005
Rapamycin	Sigma
Actinomycin D	Sigma
Phorbol-12-myristate-13-acetate (PMA)	Merck
Ionomycin	Merck

2.1.10 Plasmids

Name	Plasmid
pcDNA3	Eukaryotic expression vector (Invitrogen)
4 x NF-κB	Contains four copies of the NF-κB consensus sequence (GGA AAT TCC CC) (Li-Weber *et al*., 1998)

2.1.11 Primers

Target gene	Sequence
c-Flip$_L$ forward	5′-AAT TCA AGG CTC AGA AGC GA-3′
c-Flip$_L$ reverse	5′-GGC AGA AAC TCT GCT GTT CC-3′
c-Flip$_S$ forward	5′ GGC CGA GGC AAG ATA AGC AAG G-3′
c-Flip$_S$ reverse	5′-GCG CGG TAC CTC ACA TGG AAC AAT TTC CAA G-3′
β-actin forward	5′-GCC CCC CTG AAC CCC AAG GCC AAC-3′
β-actin reverse	5′-CCG CTC GGC CGT GGT GGT GAA GCT-3′
CD95L forward	5′-ATA GGA TCC ATG TTT CTG CTC TTC CAC CTA CAG AAG GA -3′
CD95L reverse	5′-ATA GAA TTC TGA CCA AGA GAG GCT CAG ATA CGT TGA C-3′
Tax forward	5′-ATG GCC CAT TTC CCA GG-3′
Tax reverse	5′-TCA GAC TTC TGT TTC TCG G-3′

The used DNA oligonucleotides are synthesized and obtained from MWG Biotech.

2.1.12 Kits

Enzyme/kit	Origin
Reverse Transcriptase	Appplied Biosystems
T4 DNA Ligase	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas
High Fidelity PCR Kit	Roche
RNA Microprep Kit	Stratagene
NucleoSpin RNA II kit	Macherey-Nagel
Gel Extraction Kit	Qiagen
Plasmid Maxi Kit	Qiagen

2.2 Molecular biological methods

2.2.1 Preparation of competent bacteria

For preparation of competent bacteria, 10 ml of an O/N culture of DH-5α *E.coli* were inoculated into 300 ml LB-media and further cultured at 37°C in a shaker until the OD_{600} = 0.6. The bacterial culture was cooled down for 10 min and then pelleted by centrifugation at 4000 rpm at 4°C for 5 min. The supernatant was discarded and the pellet was resuspended in 40 ml ice-cold 100 mM $CaCl_2$ buffer and incubated on ice for 10 min. This step was repeated two times. The pellet was resuspended in 8 ml ice-cold 100 mM $CaCl_2$ buffer, incubated on ice for 10 min and aliquoted. Aliquots of 100 µl cell suspension were stored at -80°C until usage.

2.2.2 Transformation of plasmid DNA in competent bacteria

Competent bacteria (100 µl) were thawn on ice and plasmid solution was gently mixed with the bacterial solution and incubated on ice for 30 min. After 30 min a heat shock was performed at 42 °C for 1 min. The reaction mix was transferred to a LB-agarose plate containing ampicillin (50 µg/ml). The plate was incubated O/N at 37 °C.

2.2.3 Plasmid DNA purification

The purification of plasmid DNA was performed with the Plasmid Maxi Kit (Qiagen). Bacteria containing the transformed plasmid were cultured in 300 ml LB medium containing 50 µg/ml Ampicillin, O/N at 37 ºC in an incubator (300 rpm). The next day, the bacteria were harvested by centrifugation at 6000 x g for 15 min at 4 ºC. After discarding the supernatant, the bacterial pellet was resuspended in 10 ml of the supplied buffer P1. To lyse the cells, 10 ml of buffer P2 were added to the samples, mixed by inverting the tube 4-6 times, and incubated at RT for 5 min. To stop the lysis, 10 ml of chilled buffer P3 was added, mixed well, incubated on ice for 20 min, and then centrifuged at 13.000 x g. The QIAGEN-tip 500 columns were equilibrated by applying 10 ml of buffer YBT, and the column was allowed to drain by gravity. The supernatant containing the plasmid was filtered through a filter paper into the column and allowed to enter the column by gravity.

The column was washed 2 times with 30 ml of buffer QC. To elute the plasmid DNA, 15 ml of buffer QF was applied to the column and the eluate was collected into a fresh tube. The DNA was precipitated by adding 0.7 volumes (10.5 ml) of isopropanol, followed by centrifugation at 4 ºC at 13000 x g for 30 min. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged again at 4 ºC for 15 min. After pouring off the ethanol, the pellet was dried at RT and then resuspended in the required amount of sterile water.

2.2.4 Restriction enzyme digestion and ligation of DNA fragments

The plasmid DNA and DNA fragments were prepared by cutting it with suitable restriction enzymes for 1hr at 37 ºC in a following typical reaction:

Cutting of DNA fragments obtained by PCR

5 µl	DNA fragment
1 µl	restriction enzyme
5 µl	buffer 10x
Add to 50 µl with H_2O	

Cutting of plasmid DNA

1 µg	plasmid
1 µl	restriction enzyme
5 µl	buffer 10x
Add to 50 µl with H_2O	

The fragments were separated on an agarose gel and purified out of the gel. For ligation, 1:3 molar ratio of vector:insert DNA fragments together with 1 µl of T4 DNA ligase were incubated in 1x ligation buffer in a total volume of 20 µl at 16 ºC O/N.

2.2.5 Measurement of DNA concentration

The concentration of DNA was determined by measuring the absorbance at 260 nm using a spectrophotometer. For measuring the absorbance, the spectrophotometer was first calibrated with 100 µl of H_2O. Then 5 µl of DNA sample was diluted with 95 µl H_2O, transferred to a quartz cuvette, and the absorbance was recorded at 260 and 280 nm. The concentration of DNA was calculated by using the following formula:
Absorbance at 260 nm (A_{260}) x dilution factor x 50 µg/ml = x µg/ml

2.2.6 Total RNA extraction

Total RNA was extracted from cells (5 x 10^6) using the NucleoSpin RNA II kit (Macherey-Nagel). Briefly, cells are centrifuged at 1500 rpm for 5 min and a mixture of 350 µl Buffer RA1 and 3.5µl β-ME was added to the pellet. The sample are passed through a syringe fitted with a 20-gauge needle four to six times and then loaded on a column and centrifuged at 13000 rpm for 1 min. The flow through was mixed with 350 µl 70% ethanol and the sample is loaded in a NucleoSpin column and centrifuged for 13000 rpm for 30 sec. The DNase I reaction buffer is prepared by adding 10 µl DNase I to 90 µl DNase I reaction buffer, loaded on a column and incubated for 15 min at RT. After adding 200 µl buffer RA2, the sample is centrifuged for 13000 rpm for 30 sec and the NucleoSpin column is placed into a new 2 ml collection tube. Then the samples are washed with 600 µl buffer RA3 and centrifuged at 13000 rpm for 30 sec. This step is repeated and the NucleoSpin column is placed into a 1.5 ml microcentrifuge tube. The RNA is eluted by adding 60 µl Nuclease-free water and centrifuged at 13000 for 1 min. The mRNA samples were stored at -80°C.

2.2.7 Reverse-Transcription reaction for cDNA generation (RT)

Total RNA was isolated from cell lines with the NucleoSpin RNA II kit. Buffers and reagents were obtained from the RNA PCR Core Kit (Applied Biosystems). For the Reverse Transcriptase reaction the following components were prepared:

volume	**Reagents**
2 µg	RNA
8 µl	25 mM $MgCl_2$
4 µl	10 x PCR buffer II
4 µl	dNTP-Mix (each 10 mM dATP, dCTP, dGTP, dTTP)
2 µl	100 mM Oligo-$(dT)_{16}$
2 µl	RNase inhibitor
2 µl	MuLV Reverse Transcriptase
Filled to 40 µl	RNase-free H_2O

The mixture was incubated in a Thermocycler (Biorad) at the following temperatures:

15 min	25ºC
45 min	42ºC
5 min	95ºC

The cDNA is stored at -20ºC. For PCR reactions, 5 µl is used in a total volume of 50 µl.

2.2.8 Polymerase Chain Reaction (PCR)

First, the template DNA must be denatured, which means that the strands of its helix must be unwound and separated-by heating to 95°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase, called the elongation step. The PCR products were electrophoretically separated on a 1% agarose gel containing ethidium bromide.

The following components are used in the PCR reaction mixture:

volume	**Reagents**
5 µl	10 x PCR buffer
1 µl	dNTP-Mix (each 10 mM dATP, dCTP, dGTP, dTTP)
1 µl	Primer forward (100 pmol)
1 µl	Primer reverse (100 pmol)
20 ng	DNA template
1µl	*Taq* DNA-polymerase
	Add to 50 µl with H_2O

The PCR reaction contained the following steps.

		time	Temperature
	start	5 min	95 ºC
25-30 cyclus	Denaturation	1 min	95 ºC
	Annealing	1 min	58 ºC
	Elongation	2 min	72 ºC
	Termination	10 min	72 ºC

2.2.9 Purification of PCR products

Purification of PCR products prior to restriction enzyme digestion was done with the PCR purification kit (Qiagen). All centrifugation steps were performed at 10.000 g in a conventional table top micro centrifuge. 5 volumes of buffer PB, which is provided by the kit, was mixed with 1 volume of the PCR sample. A QIAquick spin column was placed in a 2 ml collection tube. The mixture was applied to the QIAquick column and centrifuged for 1 min. The flow through was discarded. 0.75 ml wash buffer PE from the kit was added to the QIAquick column and centrifuged for an additional 1 min to completely remove the residual buffer. The QIAquick column was transferred in a clean 1.5 ml micro centrifuge tube and the DNA was eluted. 50 µl H_2O was placed directly on the filter and left on the column for 2 min. Then the column was centrifuged for 1 min. DNA concentration was analyzed, using spectrophotometry.

2.2.10 Quantitative real-time PCR

Cells were treated as described and total RNA was extracted, quantified and reverse transcribed. The cDNA obtained from reverse transcription of 10 ng of total RNA was used for each reaction. First the PCR master mix was prepared as follows:

Volume in µl	**Reagents**
1.25	PCR buffer (2x)
2.50	$MgCl_2$ (10 mM)
1.00	dNTP-Mix (200 µM)
0.0625	Hot Gold Star enzyme (0.05 U)
0.125	Uracil-N-glycosylase(0.02 U)
1.30	H_2O

The cDNA was pipetted into a 96 well plate and the following components were added to each reaction. Each reaction was done in triplicates in a total volume of 25 µl.

Reagents	**c-FLIP$_s$**	**β-actin**
Forward primer	5.3	5.3
Reverse primer	5.3	15.8
Probe Taq	3.5	3.5
cDNA template	4.4	4.4
PCR mix	43.8	43.8
H_2O	25.4	14.9

Quantitative RT PCR was performed using a GeneAmp 5700 RT PCR detection system for 40-45 cycles. The median Ct values were taken, and the standard deviations were calculated from the triplicates. Gene expression levels were determined by relative quantification using β-actin as internal control gene for normalization.

2.2.11 Agarose gel electrophoresis

Double stranded DNA fragments can be separated according to their size on agarose gels. Agarose was added to 0.5 x TAE-buffer to obtain a final concentration between 1.0% (w/v)- 2.0% (w/v). The agarose supension was boiled in a microwave until it is completely solubilized. The agarose is cooled down to ~ 50ºC and then ethidium bromide was added to the final concentration of 0.5 µg/ml. DNA samples were mixed with 1/6 volume of 6x loading buffer and loaded on the agarose gel. Besides the samples, also a DNA-size marker of 10 kb was loaded. The gel was run for 0.5-1 h with 100 V. DNA fragments were visualized under UV illuminiation (366 nm) in a BioRad Gel Documentation system (BioRad life sciences).

2.2.12 Purification of DNA fragments from agarose gels

To obtain the desired DNA fragments, the gel was cut by using a scalpel and transferred to an Eppendorf tube. DNA was purified according to the QIAquick Gel Extraction kit (Qiagen). Briefly, the DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was transferred in a tube and 3 volumes of buffer QG were added to 1 volume of gel. The mixture was incubated at 50 ºC with shaking for 10 min to dissolve the gel. When the gel was dissolved completely, one gel volume of isopropanol was added to the mixture. The mixture was applied to a QIAquick column and centrifuged for 1 min. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. The DNA was washed with 0.75 ml of buffer PE and centrifuged again for 1 min. The flow-through was discarded and the QIAquick column was centrifuged for an additional min and the QIAquick column was placed into a 1.5 ml Eppendorf tube. DNA was eluted by adding 50 µl buffer EB to the center of the membrane and centrifuged for 1 min. DNA was stored at -20 ºC before further use.

2.2.13 Preparation of protein lysates for SDS-PAGE

1 x 10^7 cells were washed twice with ice-cold PBS and then resuspended in 200 µl of RIPA buffer and incubated for 20 min on ice. The cells were centrifuged for 20 min at 13000 rpm at 4ºC and the supernatant was transferred to a new tube. 5 x sample loading buffer was added to the samples and incubated at 95ºC for 10 min and stored at -20ºC until used for SDS-PAGE.

2.2.14 Preparation of nuclear extracts

The entire preparation procedure was carried out at 4 ºC and all reagents must be kept on ice. 1 x 10^8 cells were resuspended in buffer A (10x pellet volume) and after vortexing the nuclei were collected by centrifugation at 3500 rpm for 5 min. The nuclei were resuspended in 500 µl buffer A and centrifuged at 4000 rpm for 5 min. Then the nuclei were lyzed in 500 µl buffer B by vortexing several times during incubation for 1 hr at 4 ºC. The extracts containing the nuclear proteins were collected by centrifugation at 13000 rpm for 10 min. The nuclear extracts were stored at -80 ºC.

2.2.15 SDS PAGE

The glassplates, comb, and separators were washed and dried. The plates, separators, and gel apparatus were carefully assembled to prevent leaks. The buffer for the resolving gel was made in a 50 ml disposable tube by mixing the above ingredients. The TEMED and 10% APS were added at last. The solution was mixed well by swirling the solution. The solution was pored using a P1000 from the side of the glassplate and covered with a 5 mm 80% ethanol layer on top of the gel. After polymerization of the gel for about 20 min the gel apparatus was hold upside-down to drain the 80% ethanol. The stacking gel was made in a 50 ml disposable tube by mixing above ingredients and again pored with a P1000 on top of the separating gel. Then the comb was inserted carefully and the stacking gel was polymerized for about 20 min. The comb was removed, the gel was washed with distilled H_2O and the gel is transferred into a running container. Both chambers were filled with running buffer, making sure that all the wells were full of running buffer and that air bubbles were displaced from the wells. The samples were prepared as described before and loaded into the wells. The use of special narrow pipette tips can maximize the volume that can be loaded by starting at bottom of the well and slowly raising tip during loading process. Unused wells were loaded with the same SDS-containing buffer used to prepare the samples.The gel was run at 40 mA until the dye front reached the bottom of the gel.

2.2.16 Western blot

After running the gel with SDS-PAGE the proteins were transferred to a nitrocellulose membrane (Amersham) using a Trans-Blot Semi-dry Transfer chamber (BioRad).
Therefore the gel was rinsed by submerging it briefly in the chilled transfer buffer and transferred to the transfer chamber on top of three pre-wet filter papers and a nitrocellulose membrane presoaked in transfer buffer. A 15 ml disposable tube was used to gently remove any bubbles that may be trapped underneath the gel. Three pre-wet filter papers were placed on top of the gel. Transfer took place at 60 Volts for 120 min. After transfer, the gel was removed from the transfer chamber and was transferred to a small container containing blocking milk solution and was incubated for 1 hr at RT on a shaker to decrease unspecific binding of the primary antibody. Immediately after

blocking, the diluted primary antibody solution was added and incubated in a closed container at 4ºC O/N. Then the membrane was washed three times for 5 min with wash buffer. After washing the membrane, the secondary antibody was incubated for 1 hr at RT and washed again three times with wash buffer for 5 min and placed in a film cassette. For developing the Western-Blot, the detection kit ECL reagent (PerkinElmer Life Sciences) was used according to the protocol. Briefly, the detection solution A and B were mixed in a ratio of 1:1, and the membrane was incubated with detection solution for 1 min. After incubation, detection solution was drained off and the membrane was covered with a plastic layer and placed in a cassette. The development of the film was carried out in a dark room. Therefore, a sheet of autoradiography film (FujiRX film) was put on top of the membrane and exposed for 10 sec. The film was developed and fixed immediately with an x-ray developer Curix 160 (Agfa-Gevaert). Depending on the intensity of the band, the second film was exposed either for a longer or shorter period of time.

2.2.17 *In vitro* translation assay

The *in vitro* translation assay was performed by using the Rabbit Reticulocyte Lysate system (Promega). The amino acid mixtures, lacking Leucine and methionine were used in this reaction. By using both incomplete mixes, a sufficient concentration of all amino acids was obtained. Briefly, the following components were incubated:

volume	**Reagents**
35 µl	Rabbit Reticulocyte Lysate
0.5 µl	Amino acid mixture minus Leucine (1 mM)
0.5 µl	Amino acid mixture minus Methionine (1 mM)
1 µl	RNasin Ribonuclease Inhibitor (40 U/µl)
1 µl	Luciferase RNA (1 µg/µl)
12 µl	Nuclease-free water

The reactions were incubated at 30ºC for the indicated time points and the synthesis of luciferase was assayed with a luminometer (Duolumat LB9507 Berthold).

2.3 Cellular biological methods

2.3.1 Cell culture

All cell culture work was done under sterile conditions. All cell lines were cultured at 37°C in 5% CO_2. Cells were grown as suspension cultures and maintained by the replacement of RPMI medium every third day of culture. Cell density was kept between 1 x 10^5 and 4 x 10^5 cells/ml and counted with a Neubauer cell counting chamber.

2.3.2 Storage of eukaryotic cell lines

Cell freezing

Cell lines were stored for a long-term use in liquid nitrogen or for a short-term use in a freezer at -80ºC. For storages of cell lines, cells were centrifuged at 1500 rpm for 5 min. at 4ºC, resuspended in an adequate medium (1-2 x 10^6 cells/ml), supplemented with 10% FCS and 10% DMSO. Additon of DMSO provides protection against formation of ice crystals inside the cells during the freezing procedure. After dispensing of cells into 2 ml cryotubes, cell suspensions were transferred immediately into an -80ºC freezer for short-term storage and stored in liquid nitrogen (-196 ºC) for long-term storage.

Cell thawing

Frozen cells were thawed directly at 37ºC in a waterbath. Immediately after thawing, the cell solution was transferred into a 10 ml Falcon tube and resuspended in RPMI with 10% FCS, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cells were resuspended in 10 ml fresh pre-warmed RPMI with 10% FCS.

2.3.3 Apoptosis analysis

Apoptotic cell death was examined by DNA fragmentation according to the method of Nicolletti (Nicoletti *et al*., 1991). Briefly, LZ-TRAIL, LZ-CD95L or drug-treated cells (1 x 10^6) were collected by centrifugation at 1500 rpm for 5 min. The cells were washed with 200 µl PBS and centrifuged again at 1500 rpm for 5 min. The cells were lysed in 200 µl of a hypotonic fluorochrome solution containing propidium iodide and stored O/N at 4 ºC in dark. The propidium iodide stained DNA fragments were quantified by flow cytometry (Becton Dickinson). Specific DNA fragmentation was calculated (percentage of experimental DNA fragmentation – percentage of spontaneous DNA fragmentation) / (100 – percentage of spontaneous DNA fragmentation) x 100%.

2.3.4 Cell cycle analysis

Drug-treated cells were collected and stained with Nicoletti lysis buffer as described in 2.3.3. Quiescent and G1 cells have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have two copies of DNA and therefore, will have 2X intensity. Since the cells in S phase are synthesizing DNA, they will have fluorescence values between the 1X and 2X populations. The resulting histrogram consists of three populations: two Gaussian curves (1X and 2X peaks) and the S-phase population. Adjacent populations overlap each other. Therefore, a population of cells at different cell cycle state can be examined by measuring the amount of fluorescence-labelled DNA by flow cytometry.

2.3.5 Cell surface staining

For analysis of the surface expression levels of TRAIL and CD95 receptors, cells (5 x 10^5) were washed with PBS and incubated with 1 µg/mL of corresponding antibodies for 30 min at 4°C, washed with PBS and incubated for 30 min with phycoerythrin-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany), and analyzed by flow cytometry with a FACScan Cytometer (Becton Dickinson). A minimum of 10 000 cells per sample was analyzed. For analysis of the TRAIL receptors the following antibodies were used: HS101 (TRAIL-R1), HS201 (TRAIL-R2), HS301 (Dc-R1), and HS402 (Dc-R2) (mIgG1, Alexis, San Diego).

2.3.6 Isolation of peripheral blood lymphocytes (PBL)

Human peripheral blood lymphocytes (PBL) were isolated from heparinized blood of healty donors using standard Ficoll-Hypaque gradient centrifugation. Briefly, 30 ml blood was loaded gently on the surface of 15 ml Ficoll in a 50 ml Falcon tube, without disturbing the interphase and centrifuged at 20 ºC, 2420 g for 20 min. According to different sizes of blood cells, lymphocytes and myeloid cells formed a gradient band at the interphase. Cells at the interphase were collected and diluted with 1 volume of RPMI medium (without FCS) and then centrifuged at RT, 1500 rpm for 5 min. The cell pellets were resuspended in 15 ml of RPMI medium and incubated in a culture flask. The myeloid cells were removed from lymphocytes by incubation of the culture flask at 37 ºC for 1 hr (myeloid cells adhere on the surface of the flask). T lymphocytes were further purified from lymphocytes by a rosetting technique using sheep erythrocytes. Briefly, 8 x 10^6 cells/ml were mixed with 1:1 volume of 2-amino-ethylisothyo-uronium-bromide-treated (AET-) sheep erythrocytes. The T cells can be separated from the B cells by centrifugation at RT, 1000 rpm for 10 min. The supernatant containing the B cells was discarded and the pellet containg the T cells and sheep erythrocytes was suspended in 4 volumes of ACK buffer to lyse the erythrocytes. Purified T cells were suspended at a concentration of 2 x 10^6 cells/ml.

2.3.7 Preparation of AET-sheep erythrocytes

For preparation of a 2% erythrocyte solution, 25 ml of sheep erythrocytes were washed with sterile PBS for several times until the supernatant becomes clear. The erythrocytes were suspended in AET solution (0.5 g AET in 12.5 ml H_2O (pH = 9.0)) and incubated at 37 ºC on a roller for 15 min. The erythrocytes were then washed again with PBS until the supernatant becomes clear. The pellet containing the erythrocytes were then resuspended in RPMI at a concentration of 2% erythrocytes. The erythrocytes could be kept for three days at 4ºC.

2.3.8 Transfection

This transfection method is based on the fact that cell membranes can be made transiently permeable by the application of short electric pulses. A NF-κB luciferase reporter construct containing four copies of the NF-κB consensus binding site and a pcDNA3 expression plasmid containing the Tax cDNA were transiently transfected into Jurkat T cells by electroporation. Briefly, cells (5×10^6) suspended in 400 µl of FCS-free RPMI medium, mixed with plasmid DNA (5 µg/ml) and cooled on ice. Subsequently, cells were electroporated using a gene pulser (Bio-Rad) at 240 V / 950 µF with constant duration time of a pulse. The electroporated cells were plated out immediately mixed with an equal volume (400 µl) of fresh medium. Life cells were transferred into a 6 cm Petri dish containing 3.5 ml fresh medium.

2.3.9 Luciferase assay

Jurkat T cells were transfected with reporter constructs and cultured O/N as described. Next day, the cells were centrifuged, washed with PBS and lysed in 50 µl of Passive Lysis Buffer (Promega) for 20 min on ice. The samples were centrifuged for 6000 rpm for 3 min and analyzed with a Luminometer (DuoLumat, Berthold). Therefore 2 x 10 µl aliquots were analyzed after adding 50 µl Luciferase-reagents in the reaction chamber and light emission was detected for 10 sec. The mean and the standard deviation was calculated out of two measurements and further analyzed.

2.3.10 Metabolic labeling

Protein synthesis was estimated by measuring the amount of incorporated ^{35}S methionine. Briefly, cells (1 x 10^5) were incubated for 2 hr with 1 ml of methionine-free medium (Sigma) in a 24-wells plate. Then 10 µl pro well of ^{35}S protein-labeling mix (7 mCi (2.59MBq)) (Perkin Elmer) was added and cells were treated with different drugs as indicated. After incubation, cells were washed twice with PBS and lysed in ice-cold RIPA-lysis buffer for 15 min on ice and centrifuged (20 min, 13000 rpm). 4 µl of each lysate, was loaded on a filter paper, followed by precipitation with 10% Trichloroacetic Acid (TCA) by incubating the filter paper three times in TCA precipitation buffer for 5 min. After drying, the filters papers were incubated in 1 ml of Liquid Scintillation Cocktail solution (Beckman coulter, CA) and radioactivity was determined with Liquid Scintillation counting.

3. RESULTS

3.1 Molecular basis of apoptosis resistance of HTLV-1 infected T cells

3.1.1 HTLV-1 infected T cells are relatively resistant to CD95-induced apoptosis

Generally, deletion of virus infected cells by the immune system takes place by $CD8^+$ cytotoxic T cells. One important mechanism, whereby $CD8^+$ T cells kill virus infected cells is mediated by the CD95/CD95L system. However, HTLV-1 infected T cells were found to be resistant towards CD95-induced apoptosis (Copeland *et al*., 1994). A similar resistance was also observed in T cells derived from transgenic mice carrying the *tax* gene (Kishi *et al*., 1997). Analysis of these mice suggested that protection of peripheral T cells from CD95-induced apoptosis is one of the important underlying mechanisms leading to the immortalization of T cells. To further investigate the mechanism by which HTLV-1 infected T cells escape apoptosis, four HTLV-1 infected T cell lines, ATL-3, CHAMP, MT-2 and SP derived from HTLV-1 infected patients were examined for their sensitivity towards CD95-induced apoptosis. Cells were treated with different concentrations of recombinant LZ-CD95L or the agonistic antibody anti-APO-1 for 16 hr. Apoptotic cell death was examined by DNA fragmentation according to the method of Nicoletti (Nicoletti *et al*., 1991). Apoptotic cell death of the HTLV-1 infected T cell lines was compared with the control T cell lines CEM and Jurkat. After treatment with LZ-CD95L (200 ng/ml), 65-75% of CEM and Jurkat underwent apoptosis, whereas only 25-35% of the HTLV-1 infected T cells showed apoptotic cell death (Fig. 3.1A). Similar results were obtained upon treatment with anti-APO-1 (Fig. 3.1B). At a concentration of 50 ng/ml anti-APO-1, the control T cell lines Jurkat and CEM underwent 70-85% apoptotic cell death but only 25-34% cell death was observed in the HTLV-1 infected T cell lines (Fig. 3.1B). This indicates that HTLV-1 infected T cell lines were more resistant towards CD95-induced apoptosis.

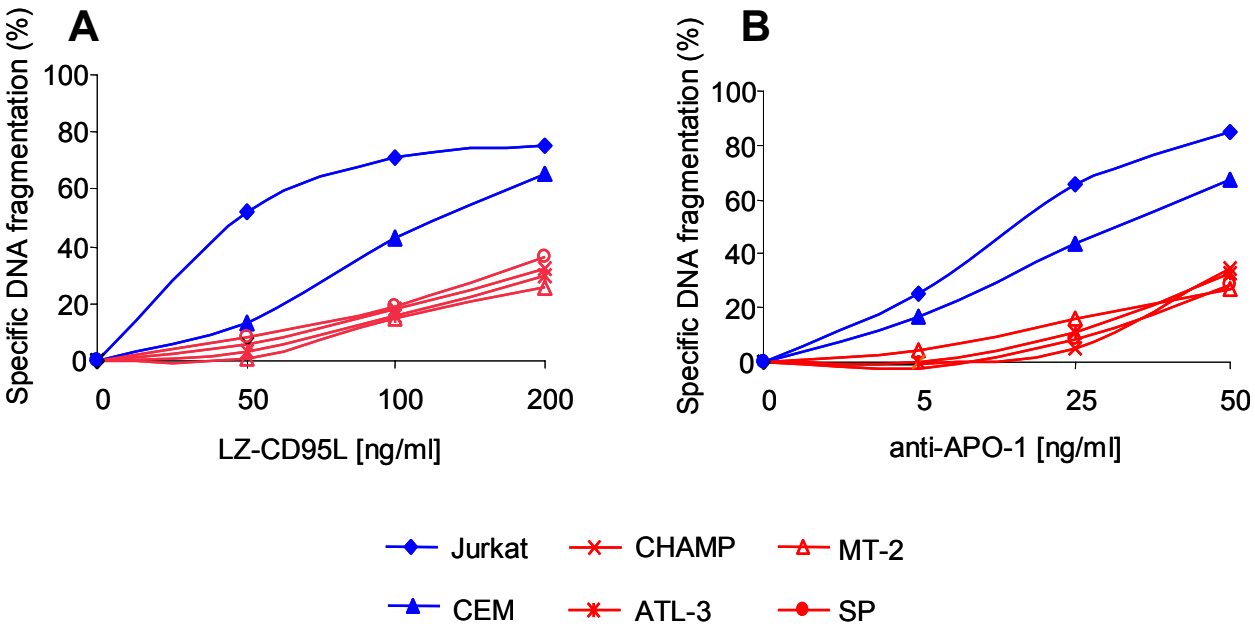


Figure 3.1: HTLV-1 infected T cells are relatively resistant towards CD95-induced apoptosis.

1 x 10^6 cells of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP and control T cell lines CEM and Jurkat were incubated with various concentrations of LZ-CD95L (50, 100, 200 ng/ml) (A) or anti-APO-1 (5, 25, 50 ng/ml) (B) for 16 hr. Apoptotic cell death was determined by Nicoletti staining. The data are representative of three independent experiments.

3.1.2 HTLV-1 infected T cell are resistant towards TRAIL-induced apoptosis.

Since HTLV-1 infected T cells were shown to be relatively resistant towards CD95-induced apoptosis, we analysed whether these cells are more resistant towards death receptor-mediated apoptosis. To investigate this, TRAIL, was applied to the HTLV-1 infected T cells. ATL-3, CHAMP, MT-2 and SP were treated with different concentrations of recombinant LZ-TRAIL for 16 hr and apoptotic cell death was examined by DNA

fragmentation. The non-infected T cell lines CEM and Jurkat were used as control. The experiment showed that 35-55% of the control T cell lines CEM and Jurkat underwent apoptotic cell death after treatment with LZ-TRAIL (100 ng/ml). In contrast, only 4-8% apoptotic cell death was observed in HTLV-1 infected T cells (Fig. 3.2). Thus, HTLV-1 infected T cells are not only resistant towards CD95L but also to TRAIL-induced apoptosis.

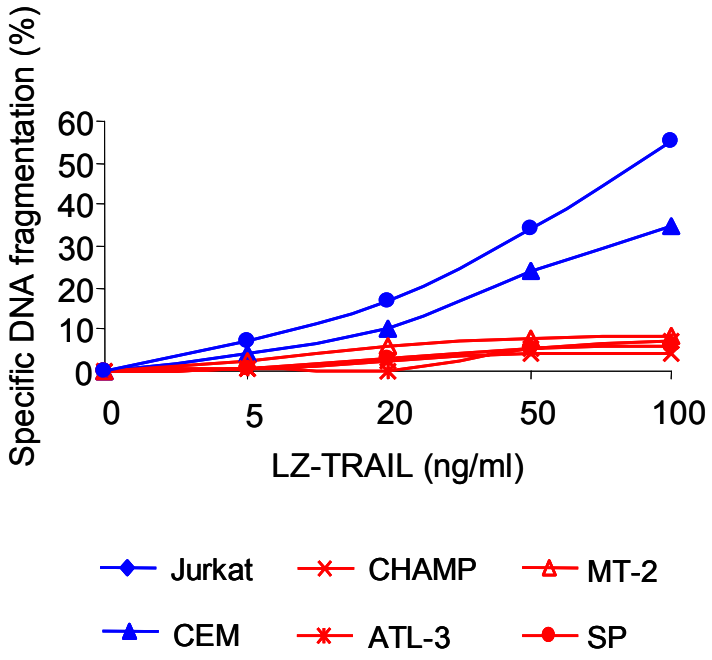


Figure 3.2: HTLV-1 infected T cells are resistant towards TRAIL-induced apoptosis.

$1x10^6$ cells of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP and the control T cell lines CEM and Jurkat were incubated with various concentrations of recombinant LZ-TRAIL (0-100 ng/ml) for 16 hr. Apoptotic cell death was determined by DNA fragmentation. The data are representative of three independent experiments.

3.1.3 Non-infected and HTLV-1 infected T cells express comparable amounts of CD95 and TRAIL receptors

Resistance of HTLV-1 infected T cells towards CD95L- and TRAIL-induced apoptosis might be due to a lower level of receptor expression. To examine this assumption, surface expression levels of CD95 and different TRAIL receptors in the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were compared with the control T cell lines CEM and Jurkat. The experiments showed that all four HTLV-1 infected T cell lines display a similar CD95 surface expression as compared to control T cell lines (Fig. 3.3). The difference in sensitivity towards CD95-induced apoptosis is therefore not due to a difference in surface expression of CD95.

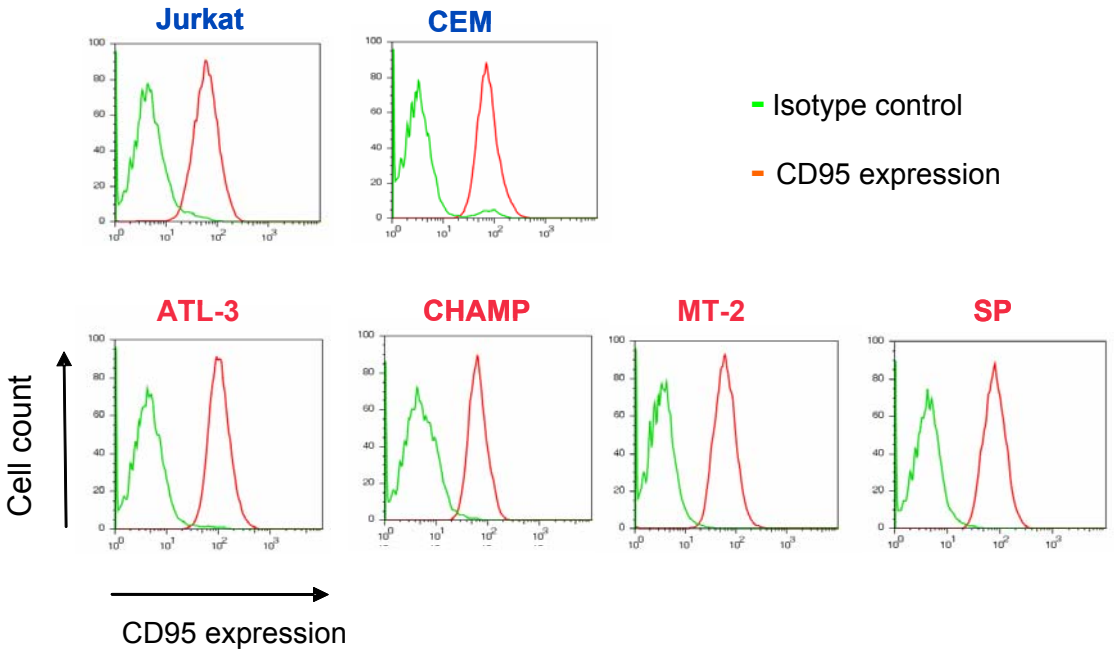


Figure 3.3: Non-infected T cells and HTLV-1 infected T cells express comparable amount of CD95

Cells ($1x10^6$) of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP and the non-infected T cell lines Jurkat and CEM were incubated with anti-APO-1 (1 µg/mL) or an IgG3 isotype control antibody for 30 min on ice. CD95 surface expression was analyzed by flow cytometry. The data are representative of two independent experiments.

Four TRAIL receptors are expressed. TRAIL-R1 and TRAIL-R2 are death receptors that mediate TRAIL-induced apoptosis, whereas, Dc-R1 and Dc-R2 are believed to negatively regulate TRAIL-induced apoptosis by competing for ligand binding (Sheridan *et al*., 1997). Therefore, the surface expression levels of all four receptors were analyzed. Both, HTLV-1 infected and non-infected T cell lines express TRAIL death receptors, however there is no clear correlation between the expression levels of different TRAIL receptors and the sensitivity of these cell lines to TRAIL-induced apoptosis. For example, MT-2 cells express similar amounts of TRAIL-R1 and TRAIL-R2 as Jurkat T cells. However, Jurkat T cells were sensitive whereas MT-2 cells were resistant to TRAIL-induced apoptosis (Fig 3.4). Furthermore, CHAMP expresses the decoy receptor Dc-R2 at a similar level as the decoy receptor Dc-R1 in Jurkat T cells. In contrast to Jurkat T cells, CHAMP cells were resistant to TRAIL-induced apoptosis. The data indicate that resistance of HTLV-1 infected T cells to death receptor-mediated apoptosis is not due to the lack of surface expression of death receptors or upregulation of decoy receptors.

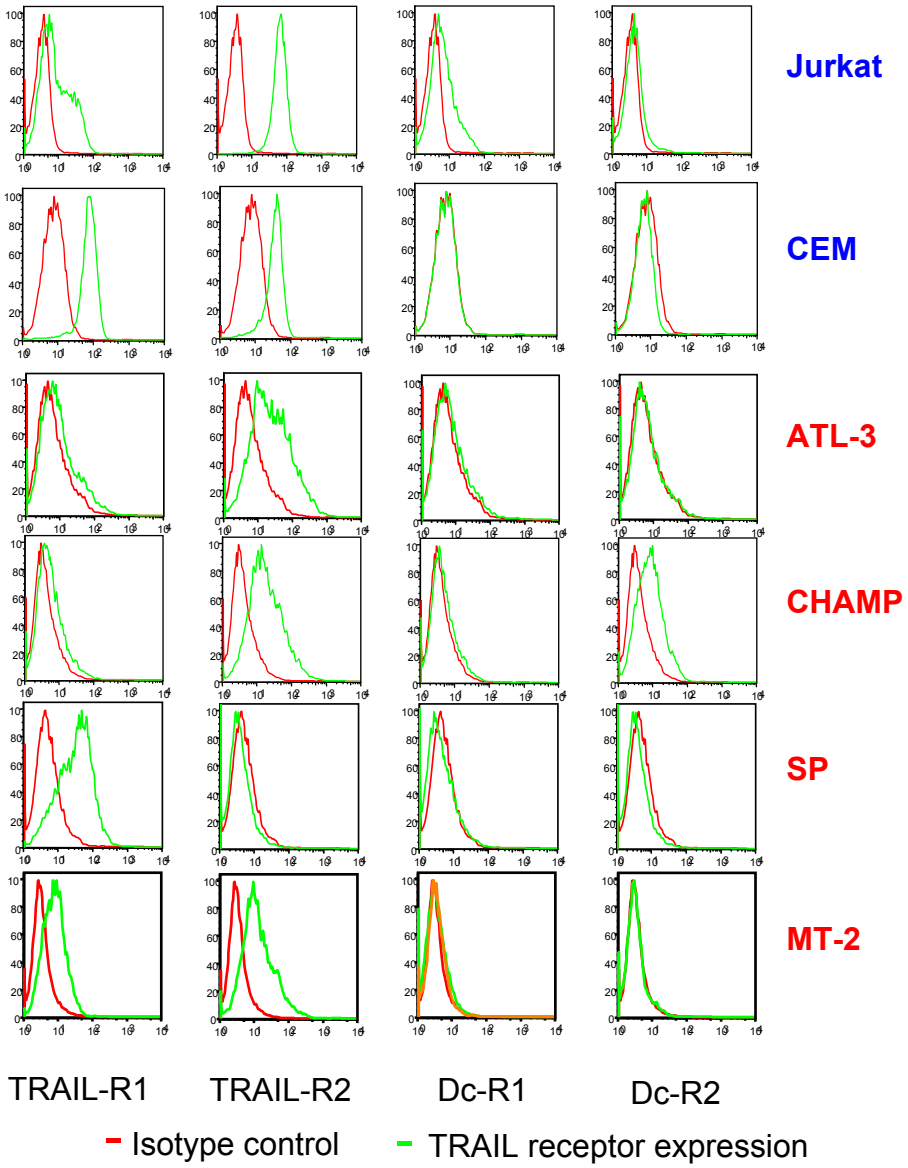


Figure 3.4: Surface expression of different TRAIL receptors in HTLV-1 infected T cells and control T cell lines.

Surface expression of TRAIL-R1, TRAIL-R2, the decoy receptors Dc-R1 and Dc-R2 in HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were compared with that in control T cell lines CEM and Jurkat. Cells were analyzed with HS101 (TRAIL-R1), HS201 (TRAIL-R2), HS301 (Dc-R1), HS402 (Dc-R2) for surface expression of different TRAIL receptors or an IgG1 isotype control antibody. The data are representative of two independent experiments.

3.1.4 HTLV-1 infected T cells express elevated amounts of c-FLIP

The basis of apoptotic resistance in HTLV-1 infected T cells has been partly explained by dysregulated expressions of proteins involved in the cell death pathway. The anti-apoptotic Bcl-2 family protein Bcl-X_L and Bcl-2 have been shown to be up-regulated in HTLV-1 infected T cells (Mori *et al*., 2001). However, Bcl-2 and Bcl-X_L are considered to mainly regulate apoptosis at the mitochondrial level. In our previous work CD95-induced apoptosis was found to be blocked at the DISC level upstream of caspase-8 in HTLV-1 infected T cells (Krueger *et al*., 2006). The only molecule known to inhibit CD95 and TRAIL signaling at the DISC level is c-FLIP. Therefore, we compared c-FLIP expression levels of HTLV-1 infected T cells with that of control T cells. As controls, expression levels of pro-caspase-8 and tubulin were analyzed. Western blot analysis revealed that all four Tax-expressing HTLV-1 infected T cell lines express increased levels of both c-FLIP isoforms, c-$FLIP_S$ and c-$FLIP_L$ (Fig. 3.5). Consistent with the previous reports, Bcl-2 and Bcl-X_L expression were shown to be also increased in HTLV-1 infected T cells. In previous experiments, we have shown that the elevated expression of c-FLIP is regulated by the Tax protein. Activation of Tax in an inducible system led to both resistance towards CD95-induced apoptosis and an increased expression of c-FLIP. Downregulation of c-FLIP expression by using siRNA abolished apoptosis resistance of the HTLV-1 infected T cells (Krueger *et al*., 2006).

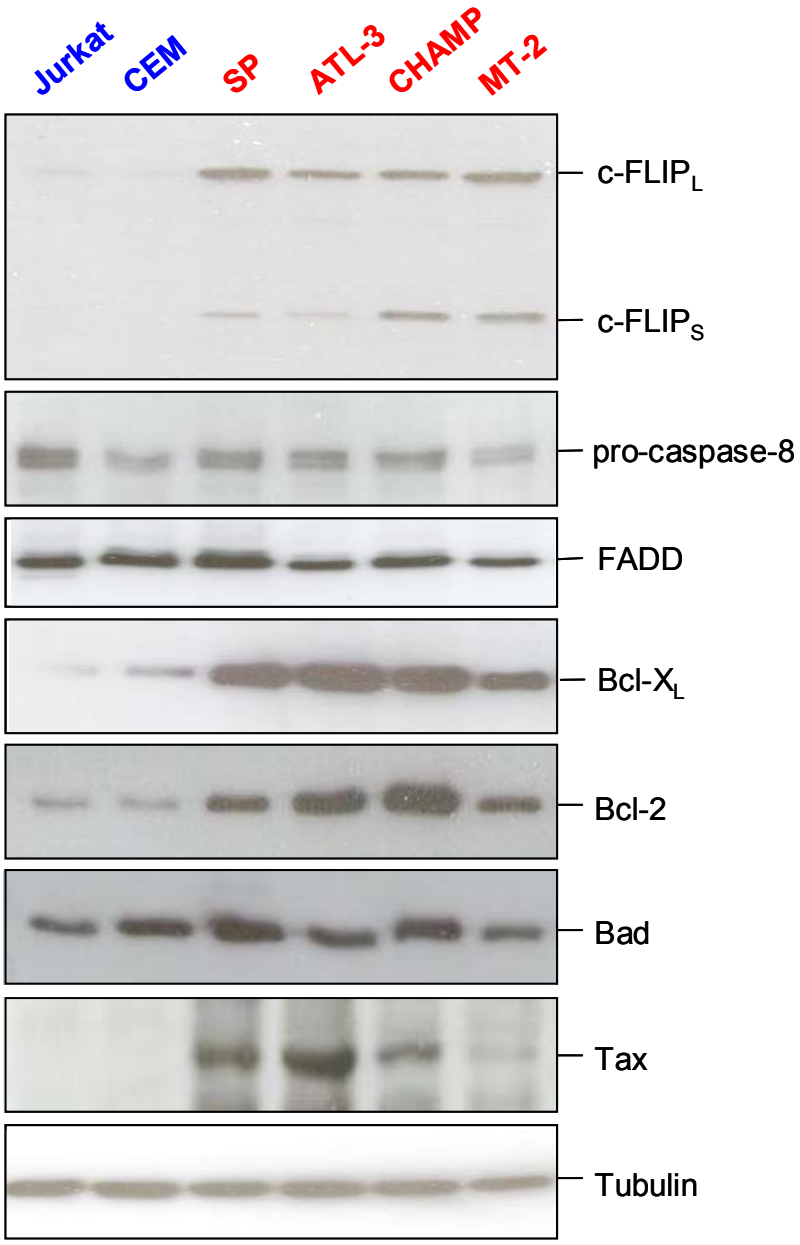


Figure 3.5: HTLV-1 infected T cells express increased amounts of c-FLIP.
c-FLIP levels in HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP and non-infected T cell lines Jurkat and CEM were analyzed by Western blot with antibodies against c-FLIP, Bcl-X_L, Bcl-2, Bad, pro-caspase-8 and the viral protein Tax. Tubulin levels served as a control for equal loading of proteins. The data are representative of two independent experiments.

3.2 Sensitization of HTLV-1 infected T cells towards death receptor-mediated apoptosis by Rocaglamide

3.2.1 Chemical structure of the Rocaglamide derivative Roc-AR

Recently, our group showed that Roc derivatives can induce apoptosis in different leukemia cell lines and in leukemia cells freshly isolated from patients, but not in normal peripheral blood lymphocytes (Zhu *et al*., 2007). The low toxicity of Rocaglamide in normal lymphocytes prompts us to investigate whether these compounds can induce apoptosis in HTLV-1 infected T cells and whether they can sensitize HTLV-1 infected T cells. Therefore we tested one of these derivatives, 1-oxo-11,12-methylendioxy-rocglaol, abbreviated as Roc-AR (Fig. 3.6) on the CD95L- and TRAIL- resistant HTLV-1 infected T cell lines. For all experiments, Roc-AR was used at a purity of at least 98% (Baumann *et al*., 2002).

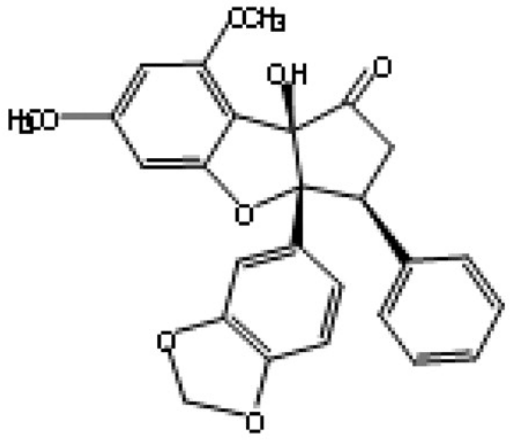


Figure 3.6: Chemical structure of the Rocaglamide derivative, 1-oxo-11, 12-methylendioxy-rocglaol (Roc-AR).

3.2.2 Roc-AR sensitizes HTLV-1 infected T cell lines towards CD95L and TRAIL-induced apoptosis

To study the effect of Roc-AR on HTLV-1 infected T cells, ATL-3, CHAMP, MT-2 and SP were treated with Roc-AR alone or in combination with LZ-CD95L. Apoptotic cell death was examined by DNA fragmentation. The experiment showed that 5-15% of the cells underwent apoptotic cell death after treatment for 16 hr with 100 nM Roc-AR alone and 2-10% apoptotic cell death after treatment with LZ-CD95L. However, treatment in combination of Roc-AR and LZ-CD95L resulted in a significant increase in apoptosis (30-50%) (Fig. 3.7).

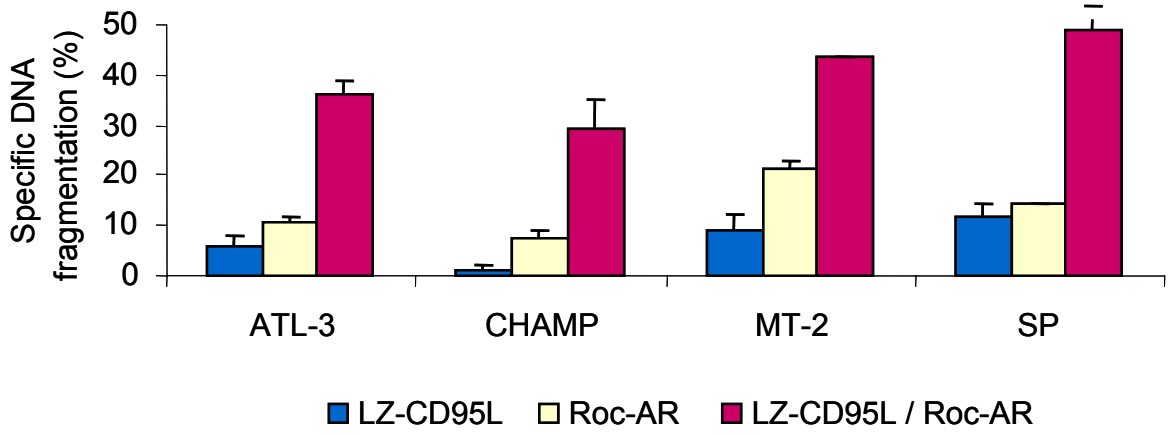


Figure 3.7: Roc-AR sensitizes HTLV-1 infected T cell lines towards CD95-induced apoptosis.

1 x 10^6 cells of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were left untreated or were treated either with LZ-CD95L (100 ng/ml) or Roc-AR (100 nM) alone or in combination for 16 hr. The results shown are representative of three independent experiments.

To investigate whether Roc-AR can also sensitize TRAIL-mediated apoptosis, different HTLV-1 infected cell lines were treated with LZ-TRAIL (100 ng/ml), Roc-AR (100 nM) alone or in combination. The experiment showed that 1-5% of the cells underwent apoptotic cell death after treatment for 16 hr with LZ-TRAIL alone and 8-12% apoptotic cell death with Roc-AR alone. However, the combination of Roc-AR and LZ-TRAIL resulted in 26-50% apoptotic cell death (Fig. 3.8). These data indicate that Roc-AR can sensitize HTLV-1 infected T cell lines towards CD95- and TRAIL-induced apoptosis.

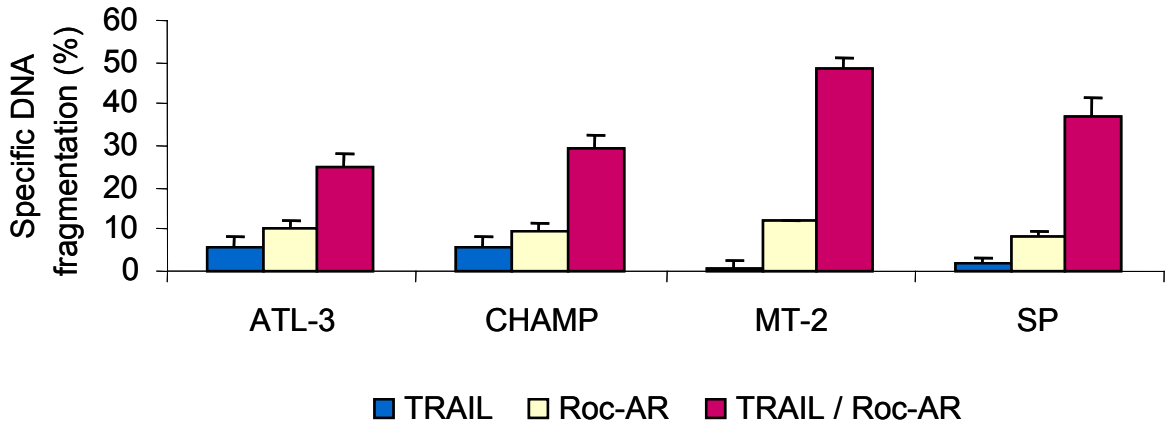


Figure 3.8: Roc-AR sensitizes HTLV-1 infected T cell lines towards TRAIL-induced apoptosis.

1 x 10^6 cells of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were treated with either LZ-TRAIL (100 ng/ml) or Roc-AR (100 nM) alone or in combination for 16 hr. The results shown are representative of three independent experiments.

3.2.3 Roc-AR enhances CD95L and TRAIL induced caspase processing

Processing and activation of pro-caspase-8 is one of the earliest events after death receptor triggering. Activation of pro-caspase-8 is followed by processing and activation of downstream effector pro-caspase-3. To confirm the sensitization towards CD95L and TRAIL mediated cell death by Roc-AR *via* the extrinsic cell death pathway, we analyzed processing of pro-caspase-8 and pro-caspase-3. The HTLV-1 infected T cell lines SP and MT-2 were treated either with LZ-CD95L (100 ng/ml), LZ-TRAIL (100 ng/ml) or Roc-AR (100 nM) alone, or in combination for 4 hr processing of pro-caspase-8 and pro-caspase-3 was analyzed by Western blot. The experiment showed that treatment with Roc-AR enhanced LZ-CD95L- and LZ-TRAIL-induced cleavage of caspase-8 and consequently, enhanced cleavage of caspase-3 (Fig. 3.9). These findings further demonstrate that Roc-AR can sensitize HTLV-1 infected T cell lines towards CD95L and TRAIL-induced apoptosis *via* the extrinsic cell death pathway.

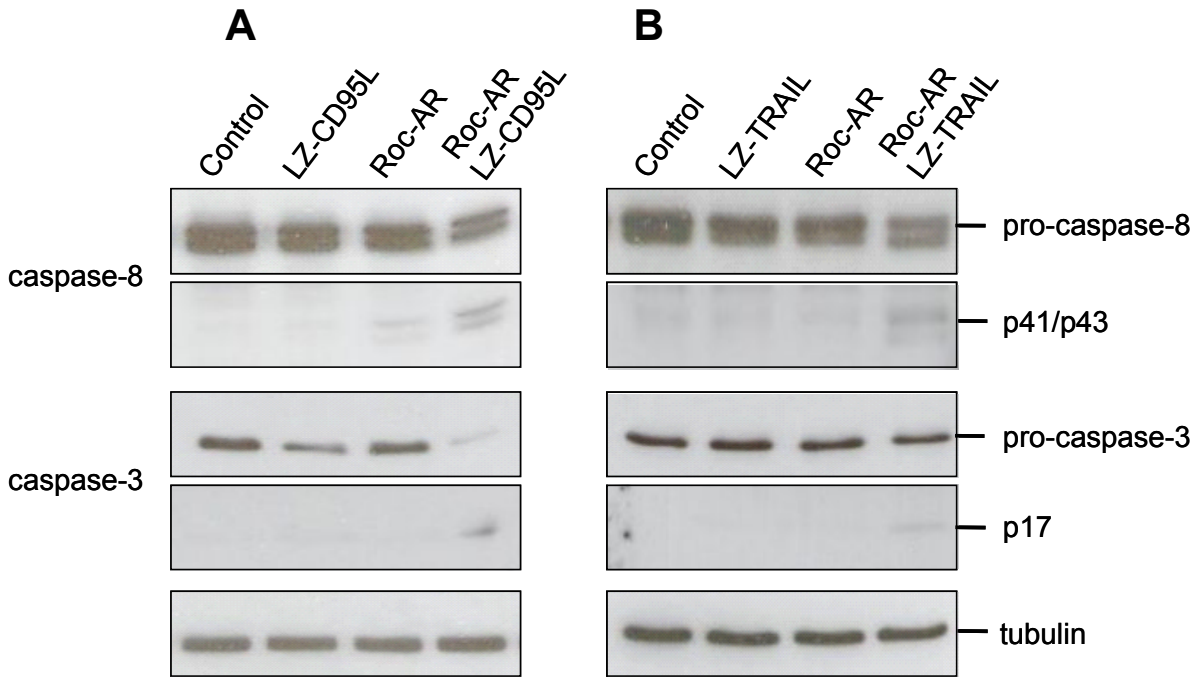


Figure 3.9: Roc-AR enhances LZ-CD95L and LZ-TRAIL induced caspase processing.

Cells (1 x 10^7) of the HTLV-1 infected T cell lines SP (A) and MT-2 (B) were left untreated or were treated with either LZ-CD95L (100 ng/ml), LZ-TRAIL (100 ng/ml) or Roc-AR (100 nM) alone or in combination as indicated for 4 hr. Analysis of tubulin expression served as control for equal protein loading. The results shown are representative of three independent experiments.

3.2.4 Roc-AR does not sensitize CD95L- and TRAIL-induced apoptosis in normal T cells

For therapeutic means, Roc-AR should have low toxicity on normal T cells. Therefore, the effect of Roc-AR on peripheral blood T cells was analyzed. Freshly isolated peripheral blood T cells from three healthy donors were incubated with LZ-CD95L (100 ng/ml), LZ-TRAIL (100 ng/ml) or Roc-AR (100 nM) alone, or in combinations for 16 hr. Cells were harvested and apoptotic cell death was examined by DNA fragmentation. In contrast to HTLV-1 infected SP cells, only 2-10% of healthy blood T cells underwent apoptotic cell death after treatment in combination with Roc-AR (Fig. 3.10). These data suggest that Roc-AR does not enhance CD95L- and TRAIL-induced apoptosis in freshly isolated T cells.

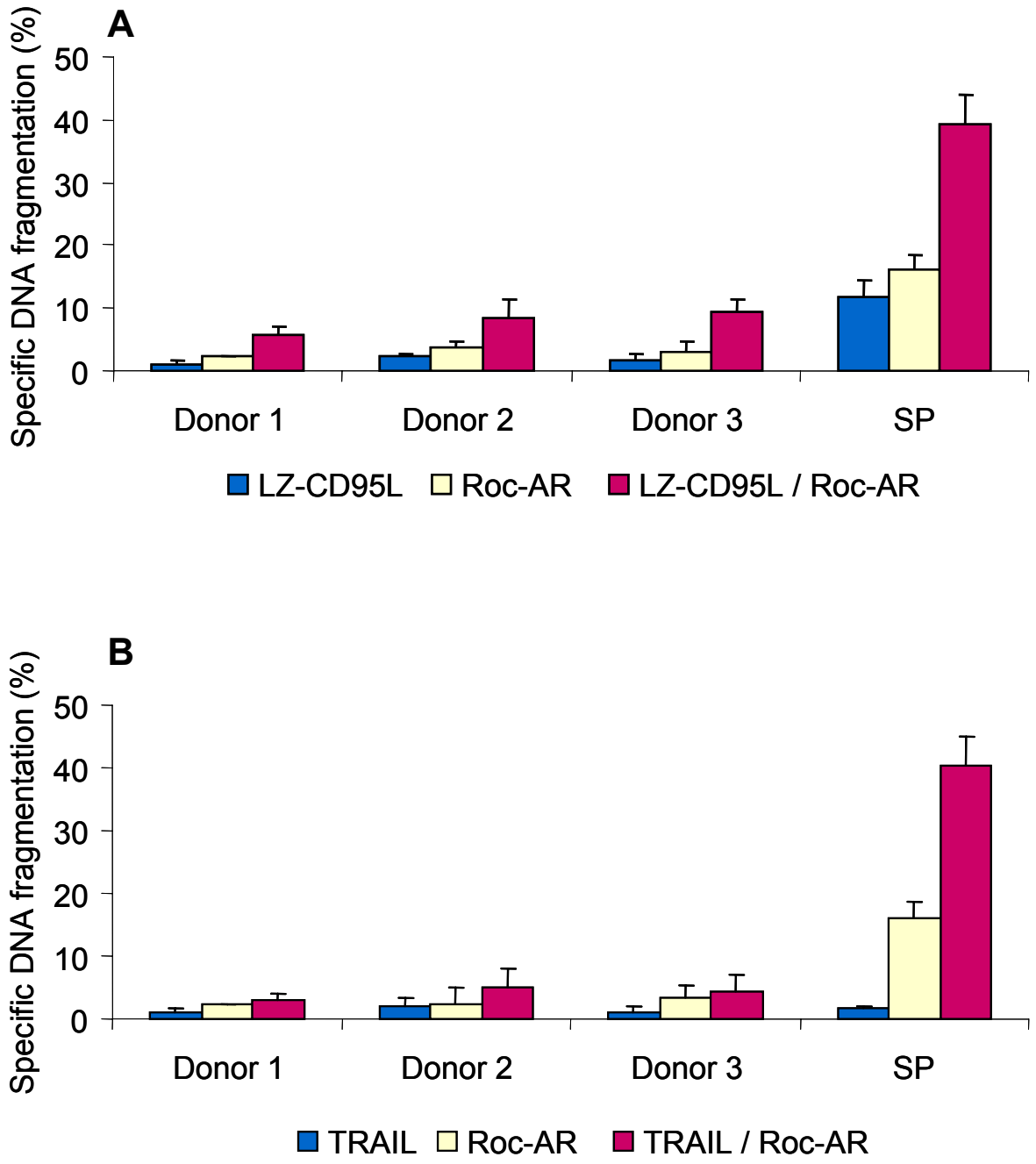


Figure 3.10: Roc-AR does not sensitize CD95- and TRAIL-induced apoptosis in normal T cells.

Freshly isolated peripheral blood T cells (1 x 10^6 cells) from three healthy donors were left untreated or were treated with Roc-AR (100 nM) alone or in combination with A) LZ-CD95L (100 ng/ml) or B) LZ-TRAIL (100 ng/ml) for 16 hr. HTLV-1 infected T cell line SP was used as control. The data are representative of three independent experiments.

3.3. Molecular mechanisms of Roc-AR-induced sensitization of death receptor-mediated apoptosis

3.3.1 Roc-AR does not affect CD95 or TRAIL receptor expression levels

Previous findings have shown that certain drugs induce upregulation of CD95 or TRAIL receptor expression and thereby sensitize cancer cells towards receptor-mediated apoptosis (Ganten *et al*., 2005). To examine this possibility, the effect of Roc-AR on expression of CD95 and the different TRAIL receptors was investigated. HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were treated with Roc-AR for 12 hr and surface expression of different receptors was analyzed by flow cytometry. No upregulation of surface expression of CD95 or TRAIL receptors could be observed in any of the cell lines tested (Fig. 3.11 and 3.12). Kinetic analysis of CD95 and TRAIL receptor surface expression levels after 2 hr to 24 hr Roc-AR treatment also showed no upregulation (data not shown). These experiments demonstrate that Roc-induced sensitization is not mediated by an upregulation of death receptors on the cell surface.

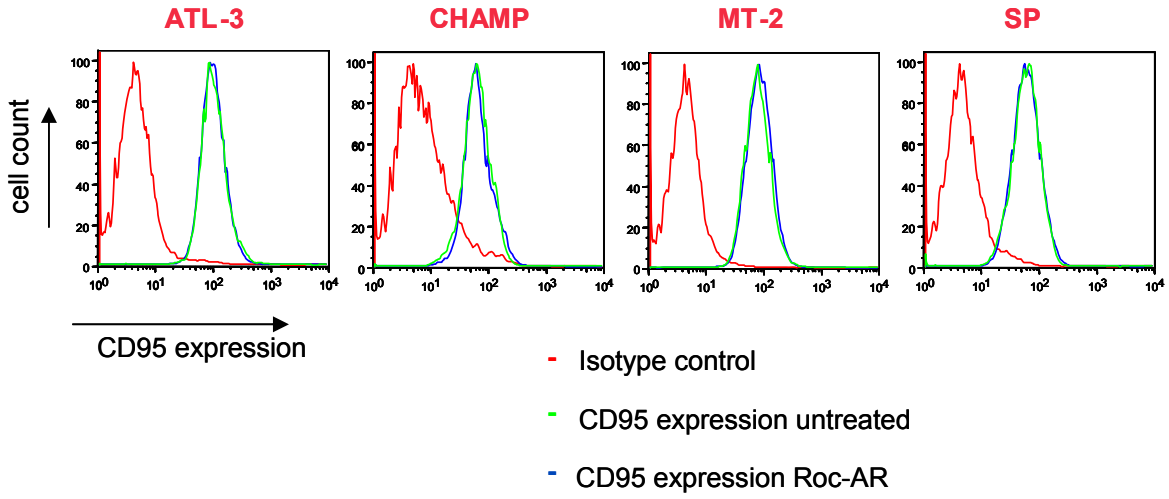


Figure 3.11: Roc-AR does not alter CD95 surface expression in HTLV-1 infected T cell lines.

Untreated or Roc-AR (100 nM for 12 hr) treated cells (1 x 10^6) of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were stained with anti-APO-1 (1 µg/mL) or an IgG3 isotype control antibody for detection of CD95 surface expression. The data are representative of two independent experiments.

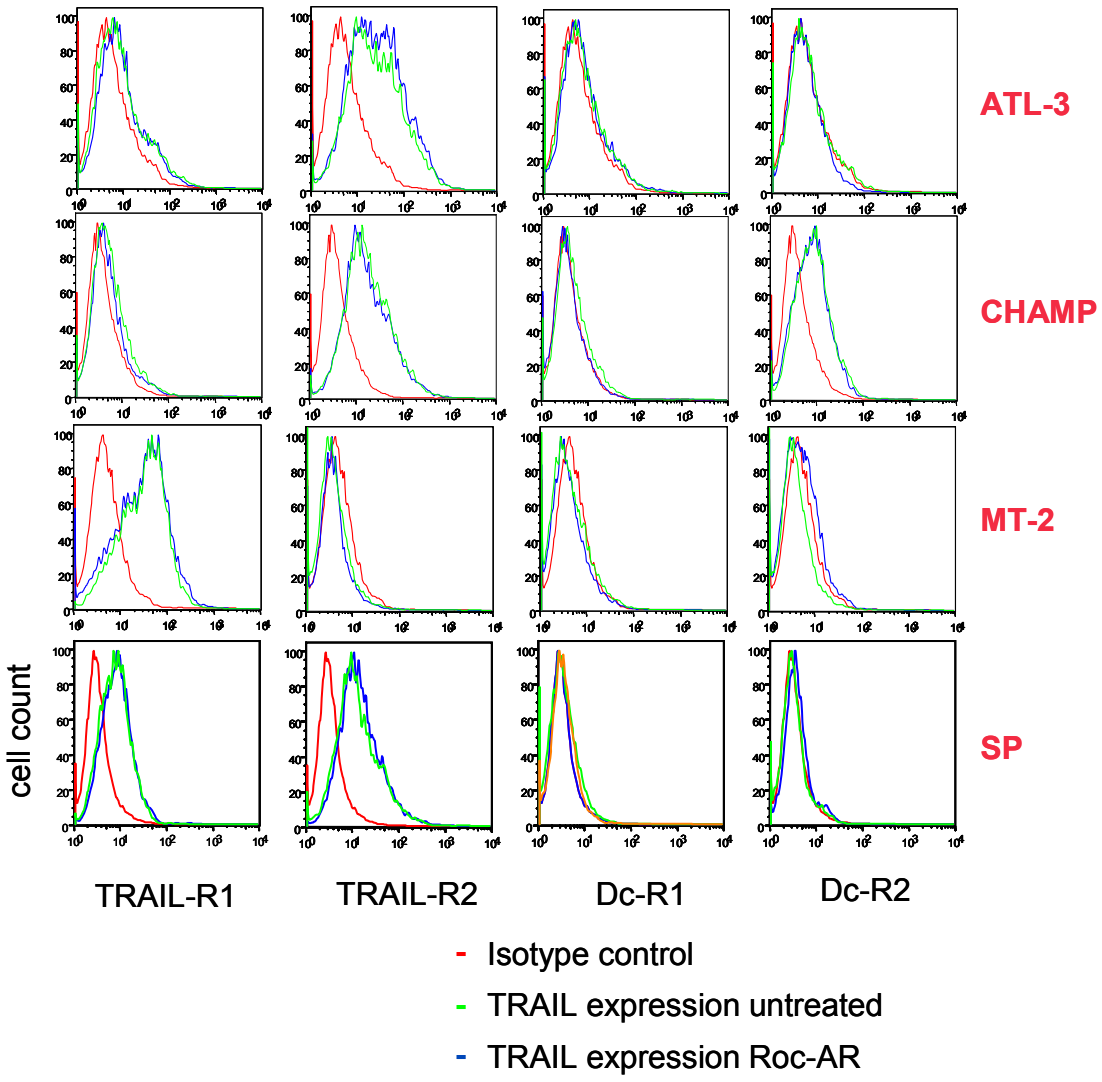
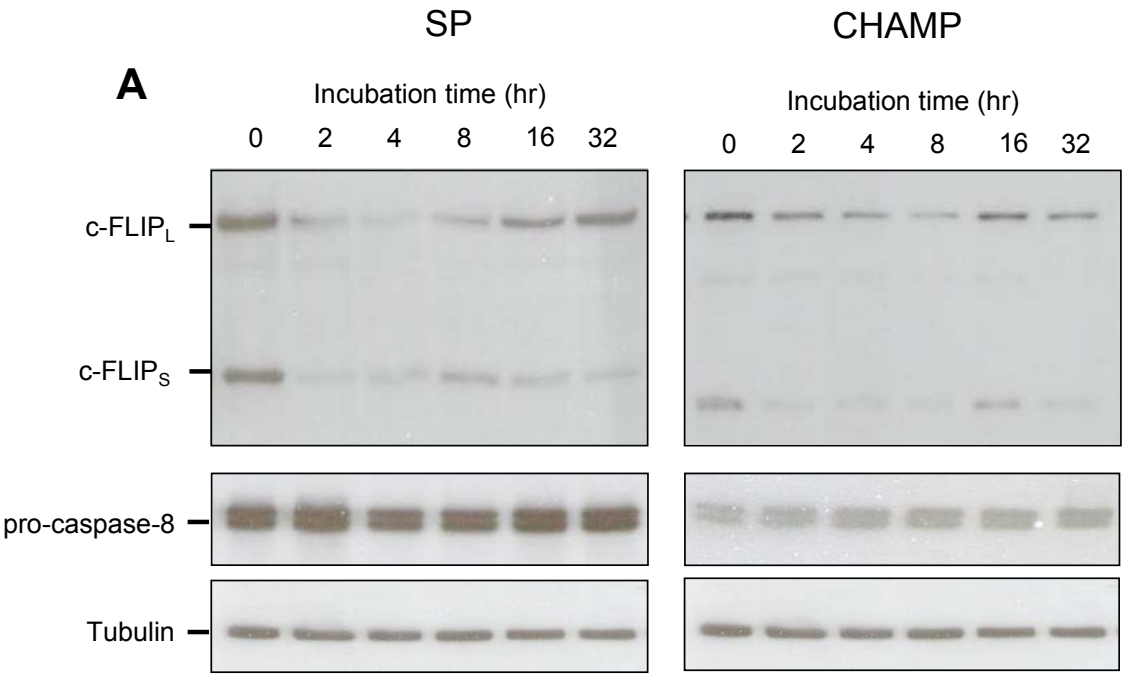


Figure 3.12: Roc-AR does not alter TRAIL receptor surface expression in HTLV-1 infected T cell lines.

Untreated or Roc-AR (100 nM for 12 hr) treated cells (1 x 10^6) of the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were analyzed with HS101 (TRAIL-R1), HS201 (TRAIL-R2), HS301 (Dc-R1), and HS402 (Dc-R2) or an IgG1 isotype control antibody for detection of TRAIL surface expression. The data are representative of two independent experiments.

3.3.2 Roc-AR sensitizes HTLV-1 infected T cells *via* suppression of the anti-apoptotic protein c-FLIP

Next we analyzed alterations of the apoptotic signaling pathways upon Roc-AR treatment. As shown before, HTLV-1 infected T cells contain a strongly elevated expression of c-FLIP, which is critical for the sensitivity towards CD95L and TRAIL-induced apoptosis (Fig. 3.5 and Krueger *et al*., 2006). Therefore, the expression level of c-FLIP$_L$ and c-FLIP$_S$ were analyzed after Roc-AR treatment. SP and CHAMP T cell line were treated with Roc-AR (100 nM) for various time periods (Fig. 3.13A) and concentrations of Roc-AR for 4 hr (Fig. 3.13B). c-FLIP expression was analyzed by Western blot. As shown, treatment with Roc-AR resulted in a strong decrease in expression of both isoforms of c-FLIP which could be detected as early as 2 hr after stimulation. The decrease in c-FLIP expression by Roc-AR was mediated in a dose-dependent way. In contrast, expression of pro-caspase-8 was not altered by Roc-AR treatment. Taken together, these results imply that loss of expression of c-FLIP$_L$ and c-FLIP$_S$ was responsible for the sensitization of HTLV-1 infected T cells towards CD95L and TRAIL-induced apoptosis.



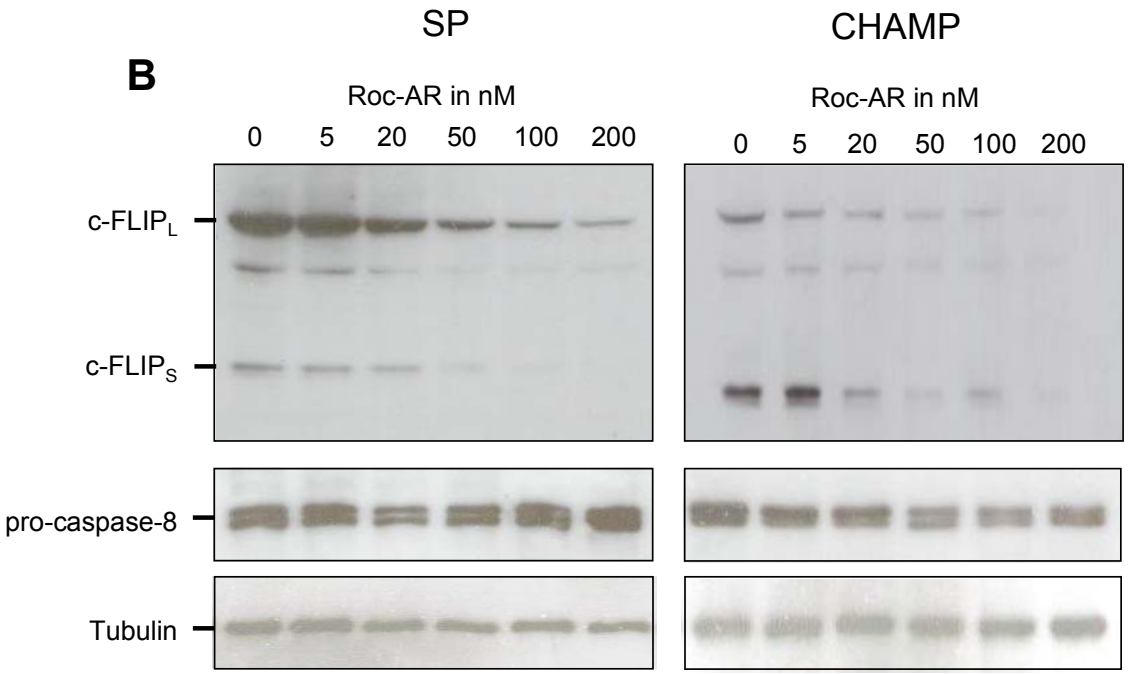


Figure 3.13: Roc-AR inhibits the expression of c-FLIP.
1 x 10^7 cells of the HTLV-1 infected T cell line SP and CHAMP were left untreated or were treated with Roc-AR (100 nM) for up to 32 hr (A) or were treated with various concentrations of Roc-AR (5, 20, 50, 100, 200 nM) for 4 hr (B). Cells were lysed and 20 µg of soluble protein was separated by SDS-PAGE. Protein levels were analyzed by Western blot with antibodies against c-FLIP and caspase-8. Tubulin expression served as a control for equal protein loading.

3.3.3 Roc-AR does not suppress c-FLIP expression by affecting the viral Tax protein

We have previously shown that in HTLV-1 infected T cells c-FLIP expression levels are increased by the HTLV-1 transactivator protein Tax (Krueger *et al*., 2006). Therefore, decrease in c-FLIP protein expression could be due to a Roc-AR mediated decrease in Tax protein levels. To examine this possibility, the HTLV-1 infected T cell line SP was incubated with Roc-AR (100 nM) for different time periods and the Tax expression levels were analyzed by Western blot. As shown in Fig. 3.14, Roc-AR did not alter the Tax protein expression, whereas c-FLIP expression was decreased. Therefore, the decrease in c-FLIP protein levels after treatment with Roc-AR is Tax-independent.

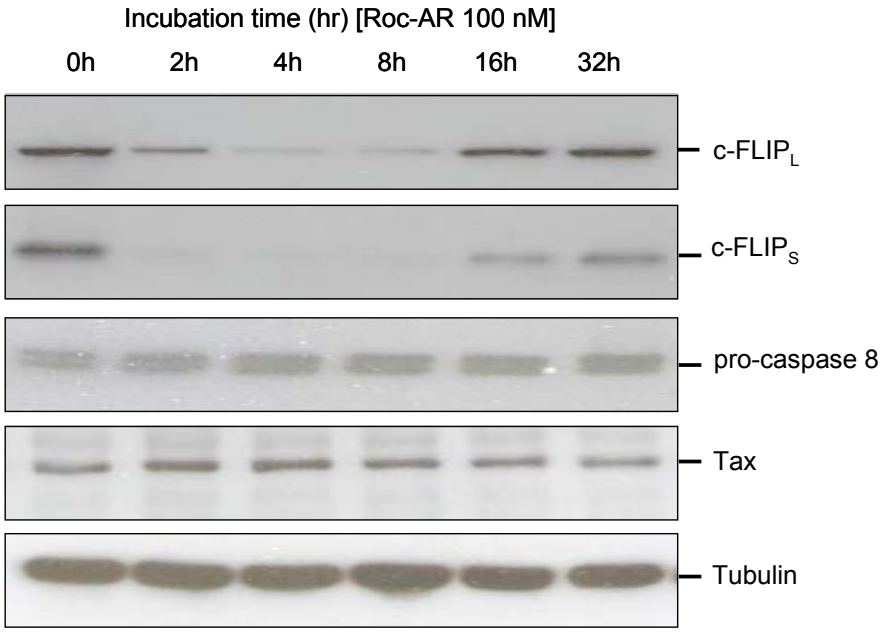


Figure 3.14: Roc-AR does not suppress expression of the viral Tax protein.
HTLV-1 infected T cell line SP (1 x 10^7) was left untreated or was incubated with Roc-AR (100 nM) for up to 32 hr. Cells were lysed and 20 µg of soluble protein were separated by SDS-PAGE. Protein level was analyzed by Western blot with antibodies against c-FLIP, pro-caspase-8 and Tax. Tubulin expression served as control for equal protein loading.

3.3.4 HTLV-1 infected T cells do not show increased nuclear localization of NFAT

In previous studies, our group showed that rocaglamide derivatives can suppress NFAT activation in T cells at concentrations of 25-100 nM without affecting other transcription factors such as NF-κB (Proksch *et al*., 2005). It has been reported that NFAT is constitutively activated in HTLV-1 infected T cells (Good *et al*., 1996). Furthermore, it has been reported that c-FLIP expression is regulated by the transcriptional activity of NFAT (Good *et al*., 1996; Rivera *et al*., 1998). This prompted us to investigate whether the decrease in c-FLIP expression is the consequence of downregulation of NFAT activity. Therefore, the activation status of NFAT in HTLV-1 infected T cells was examined by Western blot. Nuclear extracts were prepared from the HTLV-1 infected T cell lines CHAMP, MT-2 and SP and compared with non-infected T cell lines Jurkat (non-stimulated or PMA/Ionomycin (PMA/I) stimulated). Jurkat T cells show an increase in nuclear localization of NFAT upon treatment with PMA/I. However, no substantial increase in nuclear localization of NFAT in HTLV-1 infected T cells as compared with Jurkat T cells was seen (Fig. 3.14). This would indicate that NFAT is not constitutively activated in the studied HTLV-1 infected T cell lines.

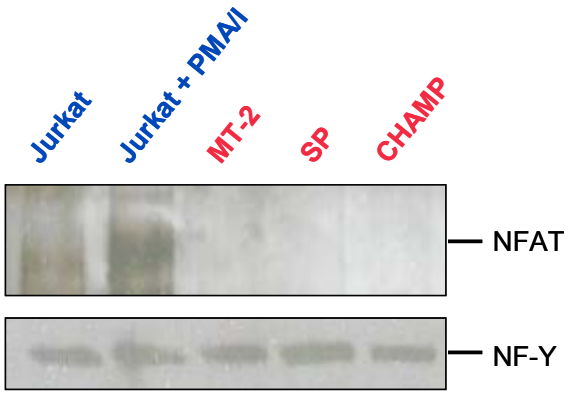


Figure 3.14: No detectable increase in nuclear localization of NFAT in HTLV-1 infected T cell lines as compared with non-infected Jurkat T cells.

Nuclear extracts were prepared from 1x10^8 cells from HTLV-1 infected T cell lines MT-2, SP and CHAMP or Jurkat cells untreated or treated for 2 hr with PMA (100 ng/ml) / Ionomycin (1 µM). From each sample, 20 µg of nuclear protein was separated by SDS-PAGE and NFAT level was analyzed by Western blot. NF-Y expression served as control for equal protein loading.

3.3.5 NFAT inhibitor CsA does not affect c-FLIP mRNA transcription

NFAT activity can be inhibited by the calcineurin inhibitor cyclosporin A (CsA). To further analyze whether inhibition of c-FLIP protein expression by Roc-AR was mediated by blocking the transcriptional activity of NFAT, the HTLV-1 infected cell line SP was treated with the NFAT inhibior CsA. mRNA analysis showed that the expression levels of c-FLIP were not affected by CsA (Fig. 3.15). These results imply that NFAT is at least not critically involved in the decrease of c-FLIP expression in HTLV-1 infected T cells after Roc-AR treatment.

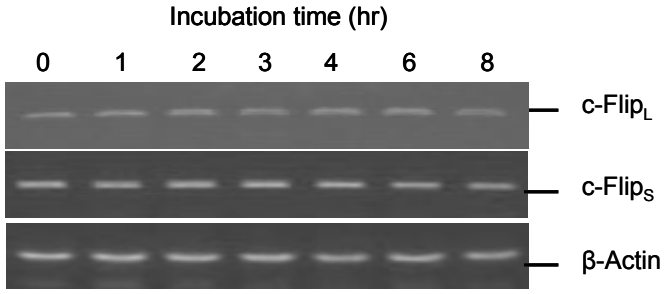


Figure 3.15: No influence of CsA on c-FLIP transcription.
HTLV-1 infected T cell line SP was treated with CsA (100 ng/ml) for the indicated time and mRNA expression levels of c-FLIP$_S$ and c-FLIP$_L$ were analyzed by RT-PCR. One representative experiment of two is shown.

3.3.6 Roc-AR does not sensitize HTLV-1 infected T cells towards apoptosis by inhibiting the transcription factor NF-κB

In freshly isolated ATL cells and HTLV-1-transformed T cells, NF-κB has been found to be constitutively activated (Ballard *et al*., 1988; Ruben *et al*., 1988; Leung *et al*., 1988). It has been reported that Tax-mediated increase in c-FLIP expression partially involves NF-κB activation (Yonehara *et al*., 2006). Rocaglamide derivatives were reported to have a suppressive effect on TNFα- and PMA-induced NF-κB activity (Baumann *et al*., 2002). In contrast, our group has shown that Rocaglamide derivatives have no effect on NF-κB activity when applied at concentrations of 50-100 nM (diploma thesis, Zhu). To investigate whether the observed decrease in c-FLIP expression by Roc-AR is mediated through downregulation of NF-κB activity, the status of NF-κB activity in HTLV-1 infected T-cell lines was analyzed. Nuclear extracts were prepared from the HTLV-1 infected T

cells lines MT-2, SP and CHAMP and the nuclear localization of the NF-κB subunits p50 and Rel A was compared with PMA/Ionomycin stimulated Jurkat T cells by Western blot. Consistent with the previous publications, HTLV-1 infected T cells showed a constitutive nuclear expression of p50 and Rel A, whereas Jurkat T cells showed nuclear localization of these subunits only after stimulation with PMA/Ionomycin (Fig 3.16A). Next, to investigate whether Roc-AR inhibits the NF-κB pathway and thereby decreases c-FLIP protein expression, the HTLV-1 infected T cell line SP was treated with different concentrations of Roc-AR for 4 hr and nuclear extracts were prepared and analyzed by Western blot. Treatment with Roc-AR up to 200 nM showed no decrease in nuclear localization of p50 and RelA in cell line SP, which indicates that NF-κB activity is not altered by Roc-AR treatment (Fig. 3.16B).

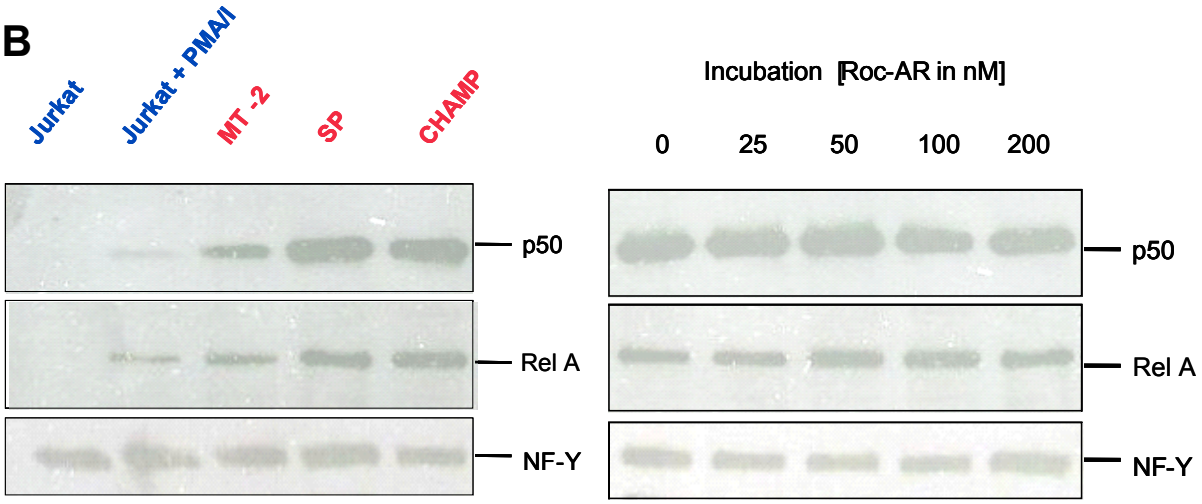


Figure 3.16: Roc-AR does not sensitize HTLV-1 infected T cell lines by inhibition of NF-κB activity

A) Nuclear localization of p50 and Rel A in HTLV-1 infected T cells. Nuclear extracts from 1 x 10^8 HTLV-1 infected T cell lines MT-2, SP, CHAMP, untreated or treated Jurkat cells (PMA (100 ng/ml) /I (1 μM) for 2 hr) were subjected to Western blot analysis. From each nuclear lysate, 20 μg of nuclear protein was separated by SDS-PAGE and analyzed with antibodies against p50 and Rel A. NF-Y expression served as control for equal protein loading. B) No Inhibition of nuclear localization of NF-κB subunits p50 and Rel A by treatment with Roc-AR. HTLV-1 infected T cell line SP (1 x 10^8 cells) was left untreated or was incubated with different concentrations of Roc-AR for 4 hr (25, 50, 100 or 200 nM) and analyzed as in A.

3.3.7 Roc-AR does not inhibit c-FLIP at the transcriptional level

NF-κB and NFAT are the two major transcription factors that have been reported to activate c-FLIP transcription. However, the above shown experiments do not support that Roc-AR suppresses c-FLIP expression by inhibition of NF-κB or NFAT. To investigate whether Roc suppresses c-FLIP expression at the transcriptional level, c-FLIP mRNA was analyzed after Roc-AR treatment. The HTLV-1 infected T cell line SP was treated with Roc-AR (100 nM) and total RNA was isolated at the indicated time. The mRNA transcription levels of c-$FLIP_S$ and c-$FLIP_L$ were analyzed by RT-PCR.

Analysis showed that c-FLIP mRNA expression levels were not altered after Roc-AR treatment (3.17A). To further confirm this result, c-$FLIP_S$ mRNA was analyzed after treatment with Roc-AR (100 nM) by quantitative real time PCR (3.17B). Again, no inhibition of c-$FLIP_S$ transcription could be detected. Taken together, these data show that the decrease in c-FLIP protein expression after Roc-AR treatment is not detectable at the transcriptional level.

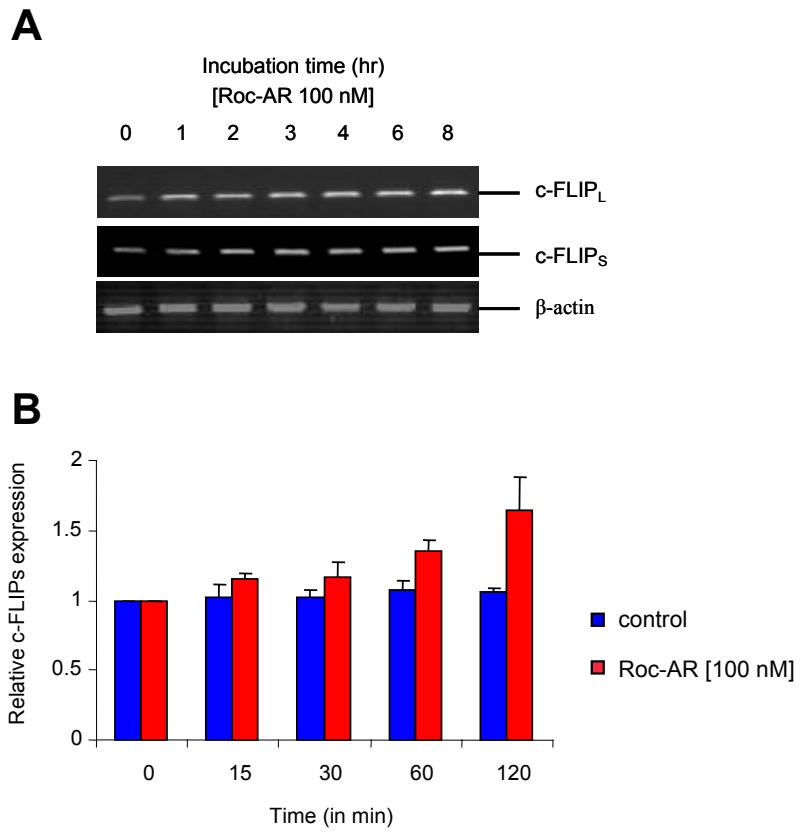


Figure 3.17: Roc-AR does not inhibit c-FLIP expression at the transcriptional level

A) HTLV-1 infected T cell line SP (1 x 10^8 cells) was treated with Roc-AR (100 nM) for the indicated time and mRNA expression levels of c-FLIP$_S$ and c-FLIP$_L$ were analyzed by RT-PCR. β-actin mRNA levels were analyzed as control.

B) HTLV-1 infected T cell line SP (1 x 10^8 cells) was left untreated or treated with Roc-AR (100 nM) for the indicated time and mRNA expression level of c-FLIP$_S$ was analyzed by quantitative real-time PCR. For both experiments, one representative experiment of two is shown.

3.3.8 Roc-AR inhibits protein expression at the translational level

To further investigate the mechanism by which Roc-AR down-regulates c-FLIP expression, the effect of Roc-AR on protein synthesis was examined. As a positive control, the well known protein synthesis inhibitor cycloheximide (CHX) was used in parallel. The HTLV-1 infected T cell line SP was incubated for 2 hr with methionine-free medium. Then ^{35}S-labelled methionine was added and cells were left untreated or were treated for 1 hr with different concentrations of Roc-AR (25-400 nM) or CHX (25-400 ng/ml) or incubated for different periods of time up to 4 hr with Roc-AR (100 nM) or CHX (100 ng/ml) as indicated.

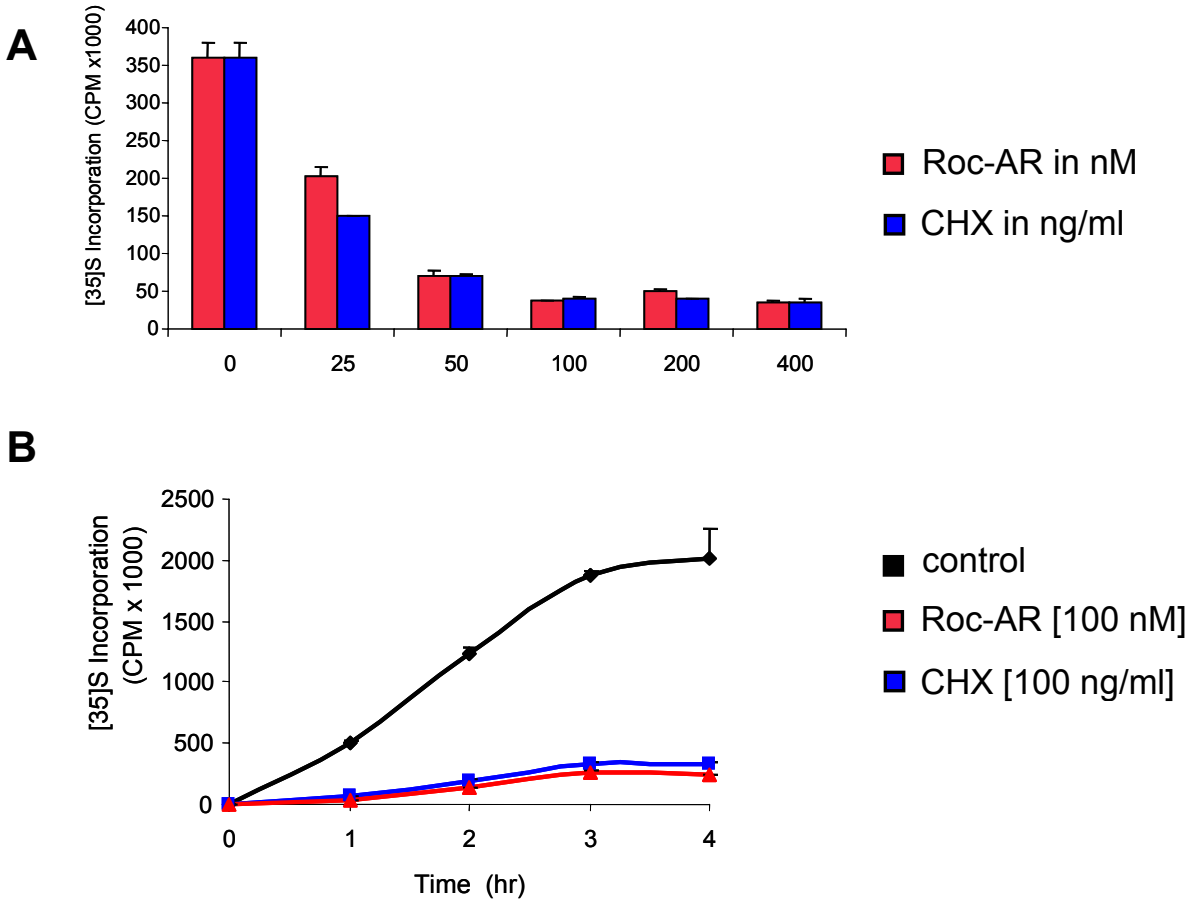


Figure 3.18: Roc-AR inhibits protein synthesis.

SP cells (1 x 10^5) were incubated with ^{35}S-labelled methionine for 1 hr as described in materials and methods and were then left untreated or treated with Roc-AR (100 nM) or CHX (100 ng/ml) for indicated times (A) or with different concentration for 1 hr (B). Data are representative of two experiments.

As shown in Fig. 3.18A, CHX inhibited protein synthesis with an IC_{50} of 25 ng/ml. An almost complete block of protein synthesis could be observed at a concentration of 100 ng/ml. Roc-AR inhibited protein synthesis with an IC_{50} of approximately 30 nM and almost completely inhibited protein synthesis at 100 nM (Fig. 3.18B). These data indicate that Roc-AR affects protein synthesis and thereby decreases c-FLIP protein expression.

3.3.9 Roc-AR inhibits protein expression upstream of the translation machinery

In order to investigate, whether Roc-AR inhibits protein synthesis on the translation level, the effect of Roc-AR on protein synthesis was studied by an *in vitro* translation system. The cell free rabbit reticulocyte lysate was incubated with luciferase mRNA in the absence or presence of different concentrations of Roc-AR (0-200 nM) or CHX (0-200 ng/ml). Synthesis of luciferase protein was quantified by luminescense. Similar to the [^{35}S]-methionine incorporation assay, there was a complete block of protein translation with CHX treatment at a concentration of 100 ng/ml (Fig. 3.19A). Surprisingly, 100 nM of Roc-AR, which was shown to completely block protein translation in the incorporation assay, showed no effect on protein translation in this *in vitro* system. Kinetics also showed also no inhibition of protein translation in the *in vitro* system (3.19B). The experiment indicates that Roc-AR is affecting protein translation only in living cells and probably acts upstream of the translation machinery.

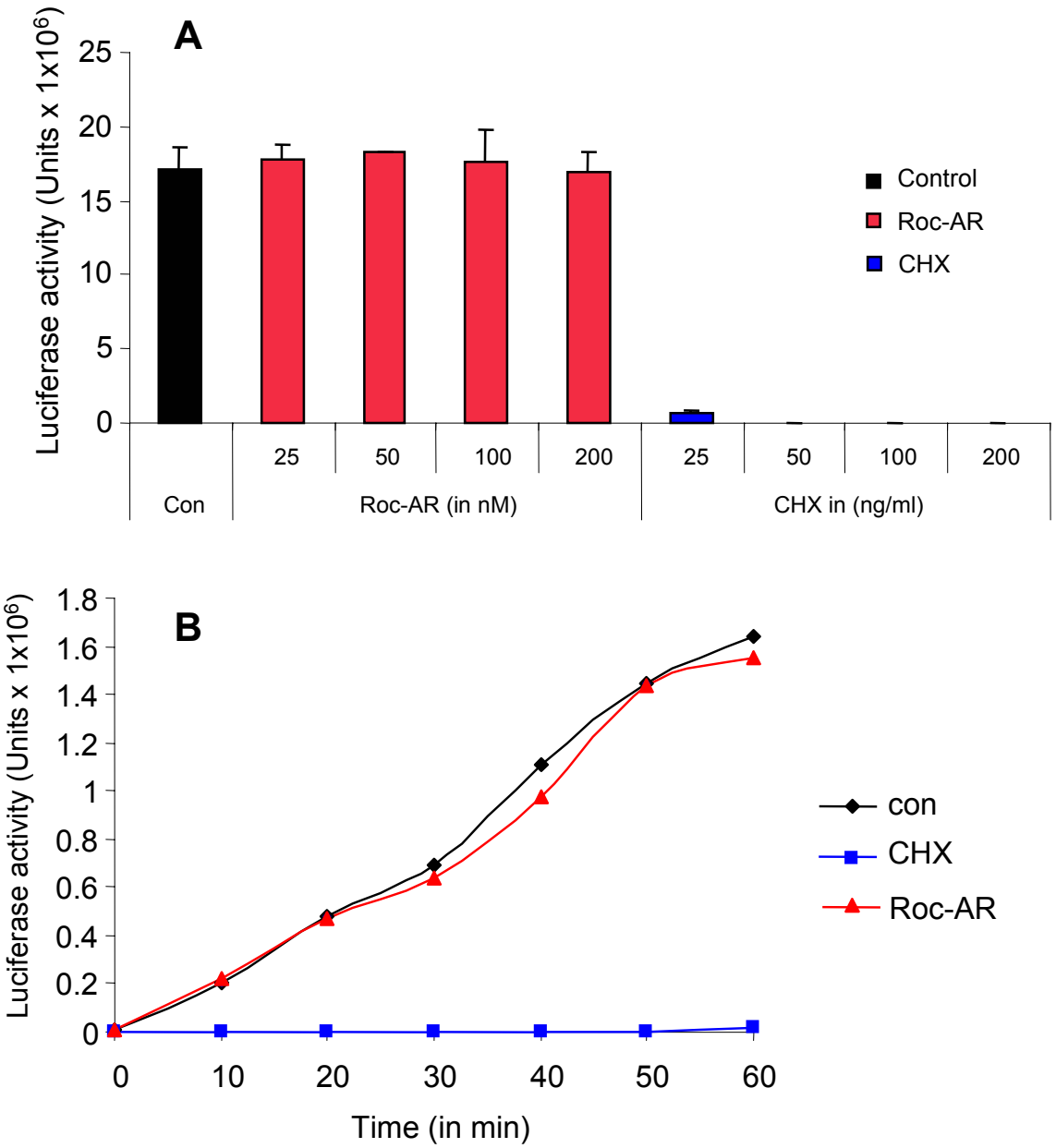


Figure 3.19: Roc-AR does not inhibit protein translation in vitro

The cell-free rabbit reticulocyte in vitro translation assay was performed in the absence or presence of the indicated concentration of Roc-AR or CHX for 60 min (A) or with Roc-AR (100 nM) and CHX (100 ng/ml) for the indicated time (B). Firefly luciferase activity (RLU) was measured as described in "Materials and methods". Error bars are ± SD for duplicate samples. Data are representative of triplicate experiments.

3.3.10 Roc-AR does not inhibit the Akt-mTOR pathway

Initiation of translation *in vivo* involves a highly regulated process which requires several initiation factors, initiation complexes, and their inhibitors (Gingras *et al*., 1999b). The rate-limiting step is the binding of the initiation factor eIF4E to the mRNA 5' cap structure (Duncan *et al*., 1987; Sonenberg 1996). The activity of eIF4E is regulated by signaling pathways upstream of the protein translation machinery (Kleijn *et al*., 1998). One of the signaling pathways known to regulate eIF4E is the phosphatidylinositol 3-kinase (PI3K/Akt) pathway and its downstream target, the kinase mammalian target of rapamycin (mTOR) (Sonenberg, 1996). mTOR activation disrupts the interaction between 4E-BP1 and eIF4E, allowing eIF4E to bind to the 5'cap structure. Rapamycin treatment inhibits mTOR activation and thereby increases the interaction between eIF4E and 4E-BP, and consequently inhibits cap-dependent translation. To investigate whether Roc-AR might also interfere with this pathway and thereby block the initation of translation, the HTLV-1 infected T cell line SP was treated with Roc-AR (100 nM) and the phosphorylation status of mTOR and its downstream target kinase, S6 was analyzed by Western blot. As control, cells were treated with 100 ng/ml rapamycin. As shown, phosphorylation of the kinase mTOR and the downstream kinase S6 was blocked after treatment with Rapamycin in a time-dependent way (Fig. 3.20A). However, phosphorylation was not inhibited or even seemed to be increased after Roc-AR treatment (Fig. 3.20B). These results suggest that Roc-AR does not inhibit the activation of the mTOR pathway.

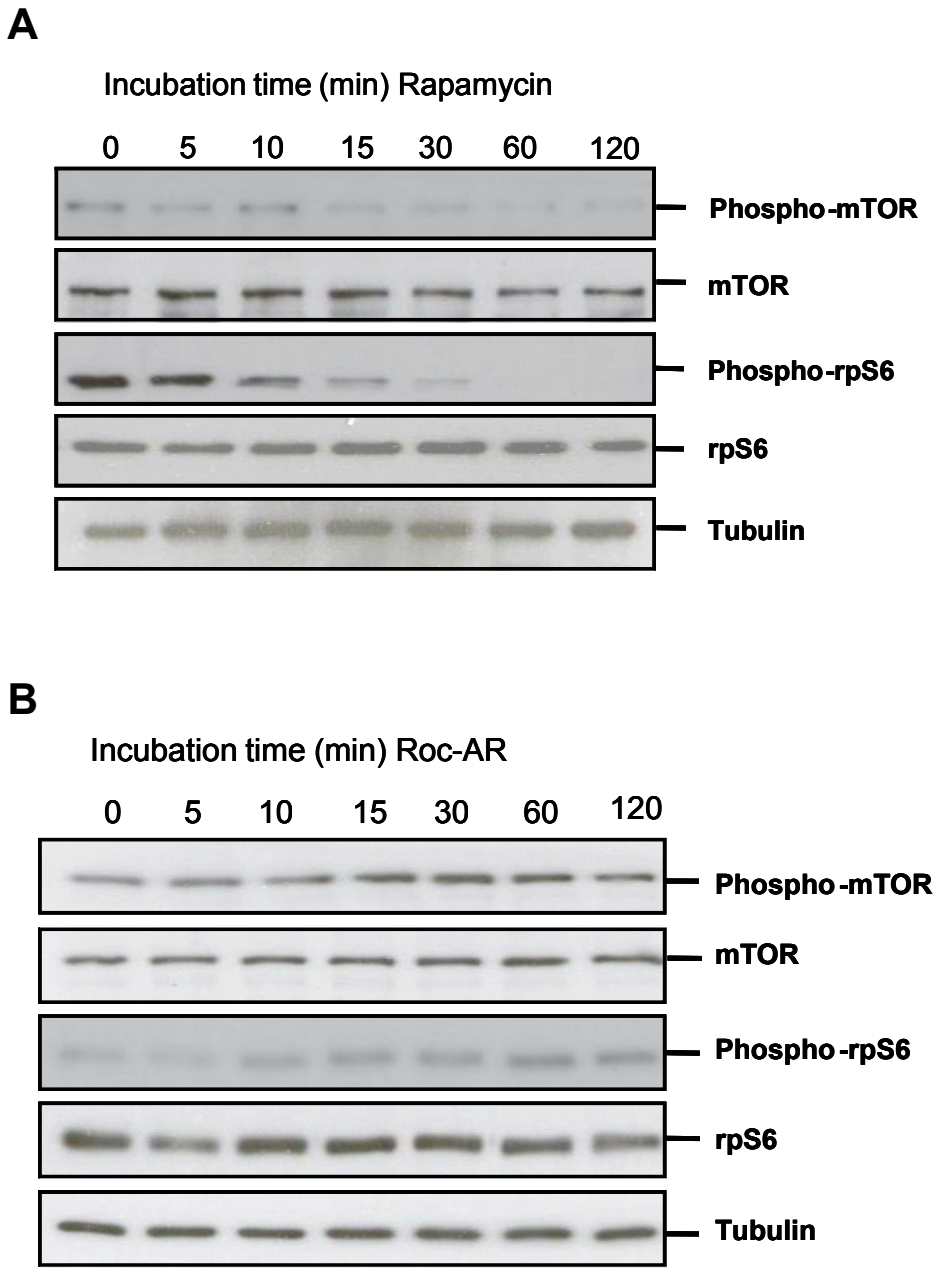


Figure 3.20: Roc-AR does not affect the mTOR pathway.

The HTLV-1 infected T cell line SP ($1x10^7$) was left untreated or was incubated with A) Roc-AR (100 nM) or B) rapamycin (100 ng/ml) for up to 120 min. Phosphorylation status of the mTOR pathway was analyzed by Western blot with antibodies against mTOR and S6. Tubulin expression served as control for equal protein loading. Data are representative of two reproducible experiments.

3.3.11 Roc-AR blocks the ERK-Mnk1-eIF4E pathway

Besides the mTOR pathway, the activity of eIF4E is also known to be regulated by the ERK pathway. ERK phosphorylates the kinase Mnk, which in turn phosphorylates eIF4E. Phosphorylation of eIF4E increases its affinity for the 5' cap structure and thereby stimulates translation. To investigate whether Roc-AR could block the ERK pathway, the phosphorylation status of Mek1/2, ERK, Mnk1, and eIF4E after Roc-AR treatment was analyzed by Western blot. As shown, treatment with 100 nM Roc-AR resulted in inhibition of Mek1/2, ERK, Mnk and eIF4E activation, which could be detected as early as 30 min (Fig 3.21). The results suggest that Roc-AR interferes with the ERK signal pathway, and, in this way, might influence the activitiy of eIF4E.

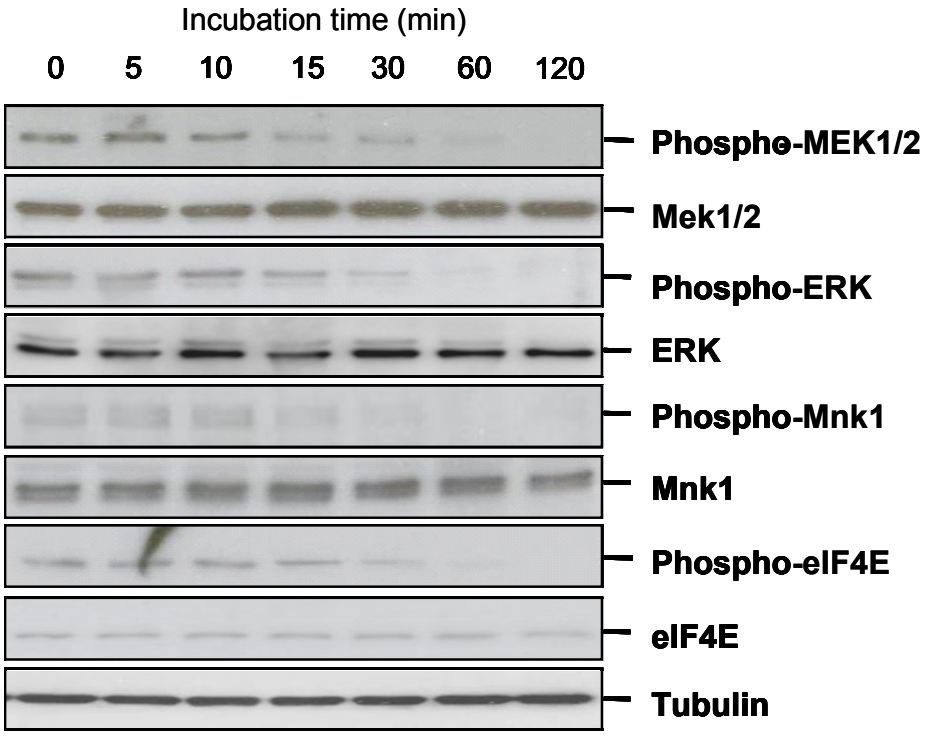


Figure 3.21: Roc-AR blocks the ERK-Mnk1-eIF4E pathway.
HTLV-1 infected T cell line SP ($1x10^7$) was left untreated or was incubated with Roc-AR (100 nM) for 0-120 min. The phosphorylation status of ERK pathway kinases was analyzed by Western blot with antibodies against Mek, ERK, Mnk and eIF4E. Tubulin expression served as control for equal protein loading. Data are representative of two experiments.

3.3.12 Roc-AR arrests the cell cycle at the G1 phase

De *novo* protein synthesis plays a critical role in the regulation of cell cycle progression, especially the transition from the G1 to the S phase (Mathews *et al*., 2000). Therefore the effect of Roc-AR on the different phases of the cell cycle was analyzed. HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were treated with Roc-AR (100 nM) for 24 hr and cell cycle was analyzed by flow cytometry. As shown, treatment with 100 nM Roc-AR, resulted in a decrease of S phase cells from 30% to 7%. G1 phase cells increased from 49 % to 77% (Fig. 3.22A). Similar results were observed with ATL-3, CHAMP and MT-2 cells after Roc-AR treatment (Fig. 3.22B).

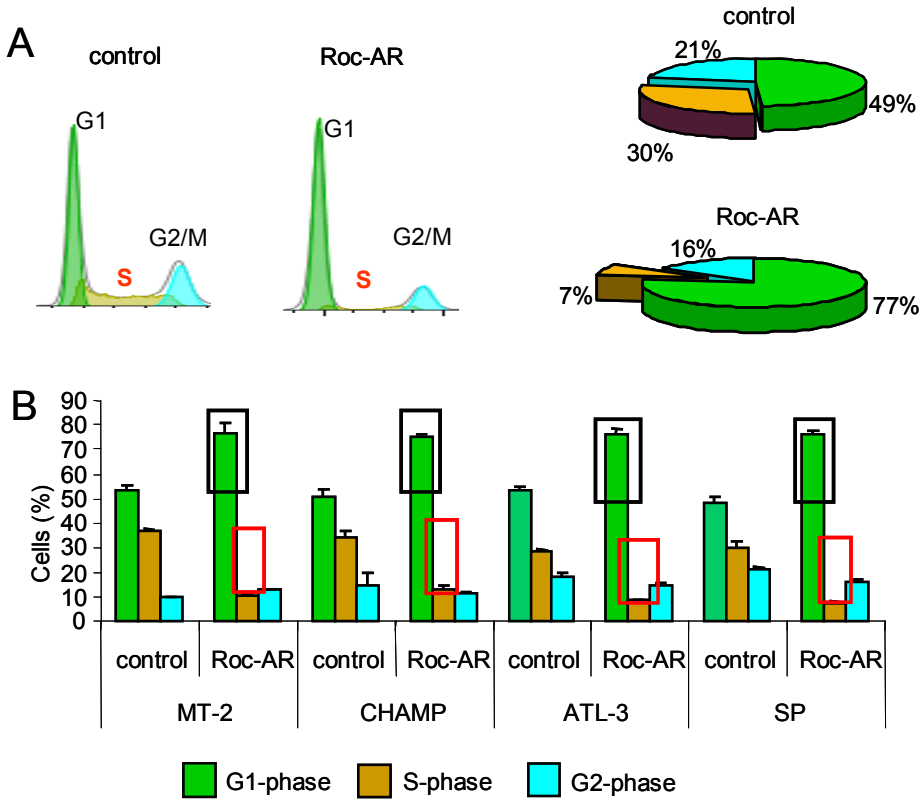


Figure 3.22: Roc-AR arrests cell cycle at G1 phase

Cell cycle analysis of HTLV-1 infected T cell lines MT-2, ATL-3, CHAMP and SP after treatment with 100 nM Roc-AR for 24 hr. 1 x 10^6 cells of each cell line were stained with Nicoletti buffer and the cellular DNA content was analyzed by flow cytometry. Data are analyzed with the program ModFit and are representative of three individual experiments. Increased G1-phase is marked by black boxes and decreased S phase is marked by red boxes.

3.3.13 HTLV-1 infected T cells express elevated levels of cyclin D1 and D2

Cell cycle progression is regulated by CDKs and their regulatory subunits, cyclins (Cheng *et al*., 1998; Klippel *et al*., 1998). HTLV-1 infected T cell lines were reported to have an accelerated progression into S-phase (Lemoine *et al*., 2001). The transition of G1 to S phase is regulated by the D-type cyclins. Cyclin D2 was reported to be upregulated in HTLV-1 infected T cells by the protein Tax (Santiago *et al*., 1999). Therefore, the protein expression levels of the major cyclins in the HTLV-1 infected T cell lines ATL-3, CHAMP, MT-2 and SP were analyzed by Western blot. Compared with the control T cell lines CEM and Jurkat, HTLV-1 infected T cell lines show high expression of cyclin D1 and D2 but similar comparable levels of cyclin B and E (Fig 3.23).

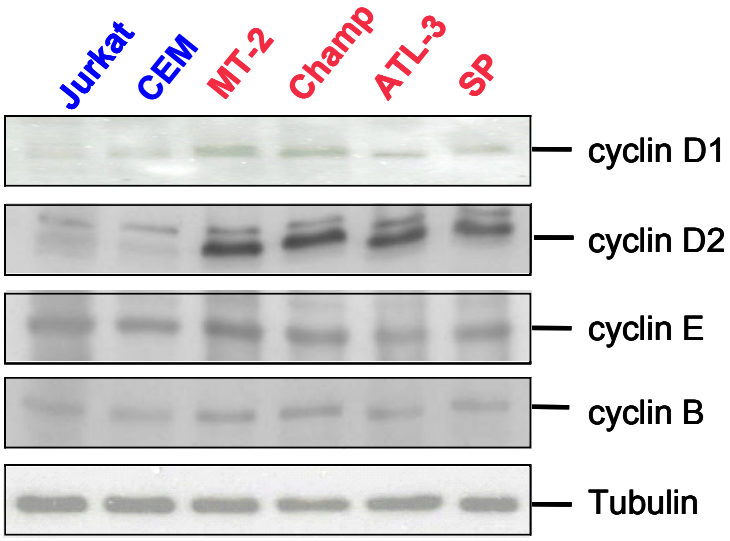


Fig 3.23 HTLV-1 infected T cells express elevated levels of cyclin D1 and D2
Expression of cyclins in HTLV-1 infected T cells (ATL-3, CHAMP, MT-2 and SP) as compared with the non-infected T cell lines (Jurkat, CEM). Cells (1 x 10^7) were lysed and 20 µg of soluble protein was separated by SDS-PAGE. Protein expression was analyzed by Western blot with antibodies against cyclin D1, D2, cyclin E and cyclin B. Tubulin expression served as control for equal protein loading. All data are representative of two reproducible experiments.

3.3.14 Roc-AR inhibits cyclin D1 and D2 protein expression

To investigate the antiproliferation activity of Roc-AR, the effect of Roc-AR on cyclins was analyzed. The HTLV-1 infected T cell line SP was treated with 100 nM Roc-AR, and protein expression of the cyclins D1, D2, B and E was analyzed by Western blot. As shown in Fig 3.23, protein levels of cyclin D1 and D2 was decreased after 2 hr of Roc-AR treatment whereas the other cyclins were not affected (Fig. 3.23). These data demonstrate that Roc inhibits D1 and D2 expression which led to cell cycle arrest at the G1 phase.

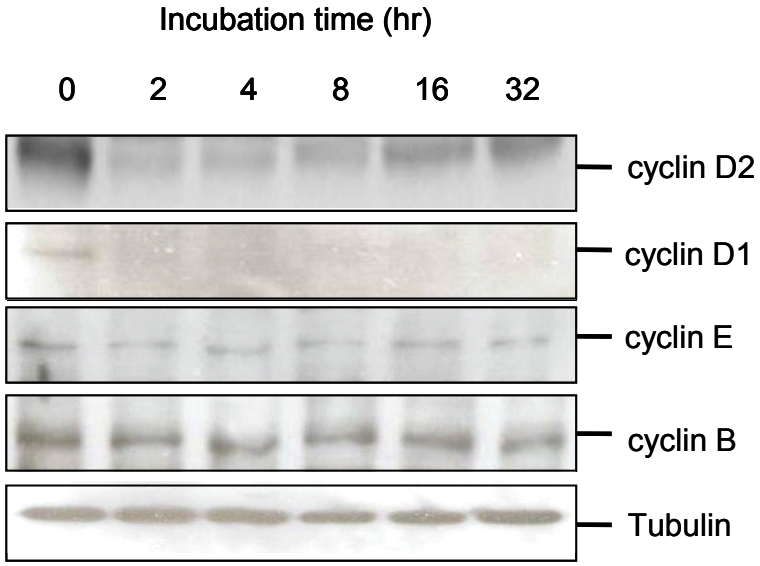


Figure 3.23: Roc-AR inhibits cyclin D1 and D2 expression.
$1x10^7$ cells of the HTLV-1 infected T cell line SP were treated with 100 nM Roc-AR for the indicated time. Cells were lysed and 20 µg of soluble protein was separated by SDS-PAGE. Protein level was analyzed by Western blot with antibodies against cyclin D1, D2, cyclin E and cyclin B. Tubulin expression served as control for equal protein loading. All data are representative of two reproducible experiments.

4 DISCUSSION

4.1 Molecular basis of Apoptosis Resistance of HTLV-1 infected T cells

HTLV-1 infection is causative for Adult T-Cell Leukaemia/Lymphoma (ATL) (Yoshida *et al*., 1982), a malignancy of clonal proliferation of infected mature $CD4^+$ T-cells.

HTLV-1 infection might lead to transformation of HTLV-1 infected cells and ultimately to development of ATL. The HTLV-1 Tax protein is required for the transformation events by interfering with processes that regulate DNA repair, cell cycle and apoptosis (Matsuoka and Jeang, 2005). For example, Tax abrogates DNA repair functions by inhibiting the expression of the DNA polymerase β, which is essential for base excision repair (BER) (Jeang et al., 1990; Philpott and Buehring, 1999), nucleotide excision repair (NER) (Kao *et al*., 2001) and mismatch repair (MMR) (Morimoto *et al* 2005). Furthermore, Tax stimulates proliferative factors that increase cell-cycle progression, especially at the G1–S-phase transition (Neuveut *et al*., 1998; Schmitt *et al.,* 1998). Tax increases cyclin D2 expression by direct activation of the promoter (Santiago *et al*., 1999). Tax also interacts and thereby activates the cyclin-dependent kinases (CDKs) CDK4 and CDK6 (Haller *et al*., 2002; Fraedrich *et al*., 2005), leading to hyper-phosphorylation of retinoblastoma (RB) protein. The phosphorylation followed by degradation of RB releases transcription factor E2F1 (Schmitt *et al*., 1998; Iwanaga *et al*., 2001), accelerating cell-cycle transition from G1 to S phase.

Tax is also known to interfere with the regulation of apoptosis. Elimination of virus infected T cells by the CD95/CD95L system is an important mechanism for killing of infected cells by $CD8^+$ cytotoxic T cells. Recent studies have found that the frequency, diversity and function of HTLV-1 specific $CD8^+$ cytotoxic T-cells are reduced in ATL patients as compared with healthy donors (Kozako *et al*., 2006). In addition, accumulating evidence showed that HTLV-1 infected T cells are resistant towards CD95-induced apoptosis (Copeland *et al*., 1994). Resistance towards CD95-induced apoptosis was also observed in T cells derived from transgenic mice carrying the viral

tax gene (Kishi *et al*., 1997). Analyses of these mice suggested that protection of peripheral T cells from CD95-induced apoptosis is one of the important underlying mechanisms required for the immortalization of T cells and the development of ATL. Our group previously investigated the molecular mechanisms of resistance mediated by HTLV-1 Tax. Our data showed that HTLV-1 infected T cell lines were resistant towards LZ-CD95L- and anti-APO-1-induced apoptosis (Fig. 3.1; Krueger *et al*., 2006). In this study we show that HTLV-1 infected T cells are also resistant towards TRAIL, which would suggest a general mechanism of resistance towards death receptor mediated apoptosis (Fig. 3.2). Multiple mechanisms might be involved in resistance to CD95L and TRAIL-mediated apoptosis (O'Connell *et al*., 1996; Whiteside and Rabinowich, 1998). One potential mechanism is deletion or downregulation of death receptors. Therefore the surface expression of the different receptors was analyzed. In our study, however, the CD95 surface expression of HTLV-1 infected T cell lines was not altered. Therefore resistance towards CD95-mediated apoptosis is not due to reduction of CD95 surface expression (Fig 3.3). Both, HTLV-1 infected and control T cell lines expressed various TRAIL receptors. As shown in Fig. 3.4, TRAIL-sensitive Jurkat and CEM T cell lines express both TRAIL receptors, TRAIL-R1 and -R2. However, the TRAIL-resistant HTLV-1 infected T cell line MT-2, also expressed both TRAIL receptors. Therefore, resistance towards TRAIL-induced apoptosis was not due to reduction of TRAIL-receptor surface expression. Since sensitivity can be also mediated by upregulation of TRAIL decoy receptors, surface expression of decoy receptors Dc-R1 and Dc-R2 was analyzed. Decoy receptors are believed to negatively regulate TRAIL-induced apoptosis by competing for ligand binding (Sheridan *et al*., 1997). The TRAIL-resistant HTLV-1 infected T cell line CHAMP expressed decoy receptor Dc-R2. However, the TRAIL-sensitive Jurkat T cell line also expressed one of the decoy receptors, Dc-R1. Therefore, no correlation between expression of decoy receptors and resistance towards TRAIL-induced apoptosis was found. Taken together, it is unlikely that apoptosis resistance is mediated at the receptor level. Although it could not be excluded that death receptors in HTLV-1 infected T cells were mutated at the ligand binding site or were truncated in their signaling transduction domain. Since the glycosylation status of cells has been shown to modulate their sensitivity towards death receptor-mediated apoptosis, the difference in sensitivity might also be mediated by differences in the

glycosylation status of the receptors (Peter *et al*., 1995).

Apoptosis resistance in HTLV-1 infected T cells has been partly explained by increased expression of anti-apoptotic proteins involved in the intrinsic cell death pathway. Bcl-X_L has been reported to be induced by Tax and Bcl-2 was highly expressed in HTLV-1 infected T cells independently of Tax (Mori *et al*., 2001). The increased expression of Bcl-X_L and Bcl-2 in HTLV-1 infected T cell lines has been confirmed in this study (Fig. 3.5). However, Bcl-X_L and Bcl-2 are mainly involved in the regulation of the intrinsic cell death pathway. So far, at the DISC, c-FLIP is the only protein known to inhibit CD95L- and TRAIL-induced apoptosis by preventing the activation of pro-caspase-8 (Fig. 4.1A). In our study we could show that HTLV-1 infected T cells expressed strongly elevated amounts of c-FLIP (Fig. 3.5; Krueger *et al*., 2006). Expression of Tax in an inducible system led to increased expression of c-FLIP and consequently, resistance towards CD95-induced apoptosis (Krueger *et al*., 2006). Downregulation of c-FLIP using siRNA abolished apoptosis resistance, thus, directly demonstrating the functional relevance of Tax-mediated upregulation of c-FLIP (Krueger *et al*., 2006). The expression level of c-FLIP has also been shown to be critically involved in regulation of TRAIL-induced apoptosis (Mitsiades *et al*., 2002). This would suggest that HTLV-1 infected T cells were generally more resistant towards Death Receptor-mediated apoptosis due to the high c-FLIP levels (Fig. 4.1B).

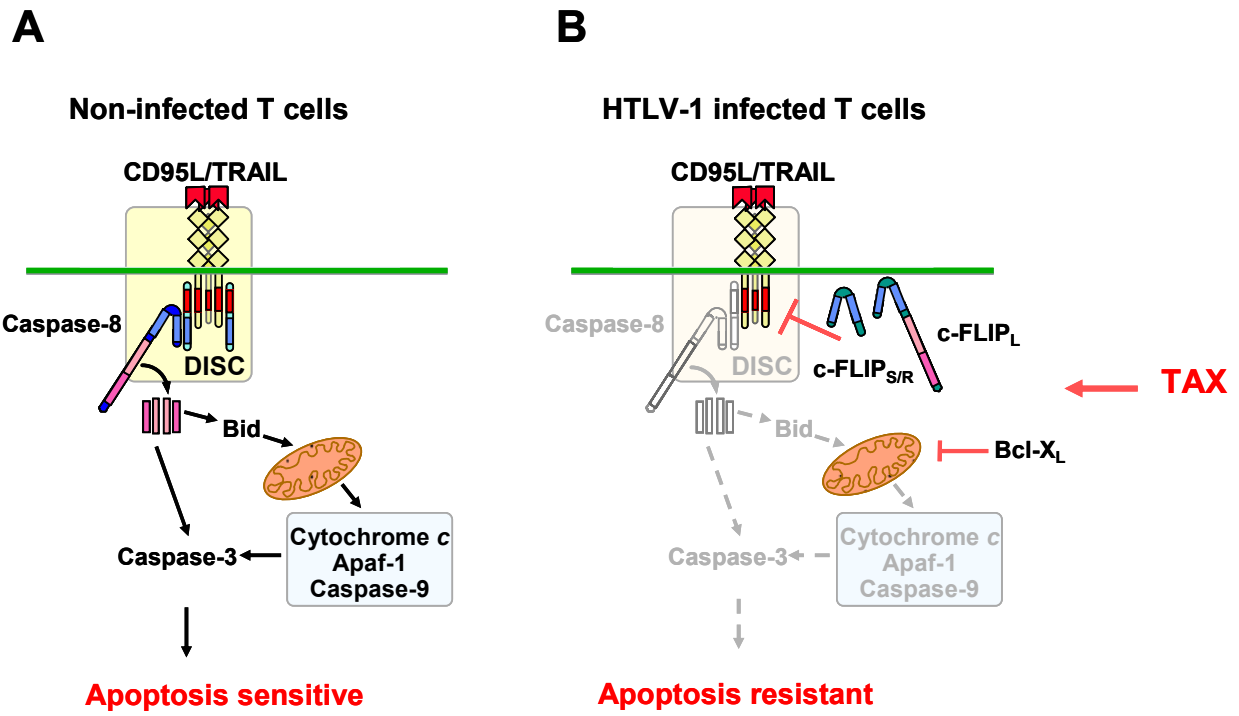


Figure 4.1: Mechanism of apoptosis resistance in HTLV-1 infected T cells towards CD95L and TRAIL.

A) Pathway of Death receptor-mediated apoptosis induced by CD95L and TRAIL. B) HTLV-1 infected T cells express Tax which upregulates the anti-apoptotic proteins Bcl-X_L and c-FLIP. Bcl-X_L inhibits apoptosis via the intrinsic cell death pathway, c-FLIP inhibits apoptosis via the extrinsic cell death pathway.

In conclusion, Tax-mediated induction of c-FLIP protein provides an important mechanism by which HTLV-1 infected T cells escape CD95L- and TRAIL-induced apoptosis by the immune system. The important role of c-FLIP in tumorgenesis has also been demonstrated in other cancers *e.g.* Hodgkin lymphoma (Dutton *et al.*, 2004). In addition, it has been demonstrated *in vivo* that tumor cells with increased c-FLIP expression promote tumor establishment and progression by preventing death receptor-induced apoptosis (Mathas *et al.*, 2004). Furthermore, it has been shown that c-FLIP overexpression plays a role in resistance towards treatment with chemotherapeutic agents (Wang *et al.*, 2005; Longley *et al.*, 2006). Therefore, c-FLIP is an attractive target in cancer therapy.

4.2 Sensitization of HTLV-1 infected T cells towards death receptor-mediated apoptosis by Rocaglamide

Plant-derived substances play a major role in treatment of human diseases in Traditional Chinese Medicine. Plant extracts have been used in treatment of various diseases for over 3.500 years, It became clear in the 1950s that plant-derived compounds can also play an important role in treatment of cancers. With a random screening approach of several plant extracts, the plant *genus Aglaia* of the family *Meliaceae* has been found to be a source of potential pharmaceutic agents (Fig. 9; King *et al*., 1982). The active compounds isolated from leaves and flowers of this plant were derivatives of cyclopenta[b]benzofuran and were named rocaglamide derivatives (King *et al*., 1982).

The therapeutic potential of rocaglamide derivatives in anti-cancer treatment has been demonstrated in xenograft mouse models. Three of the lead rocaglamide derivatives, rocaglamide (Fig 1.10 nr. 1), silvestrol (Fig. 1.10 nr. 8), and 4'-demethoxy-3',4'-methylenedioxy-methyl rocaglate (Fig. 1.10 nr. 30) were tested in *in vivo* models using the human prostate cancer cell line PC3 (Hwang *et al*., 2004) and the human breast cancer cell line BC1 (Lee *et al*., 1998), respectively. Both studies showed that the growth of both cancer cells was delayed after treatment without signs of toxicity. In another study aglaiastatin exhibited tumor selectivity *in vitro* and had high anti-tumor activity against the SW480 and HT29/H11 colon carcinoma cell lines but not against the normal intestinal epithelial cell line IEC18 (Hausott *et al*., 2004). The tumor selectivity *in vitro* was also observed for the rocaglamide derivative rocaglaol (Fig. 1.10 nr. 27). The inhibition of cell proliferation in three tumor cell lines, Lu1, LNCaP, and MCF-7, was over three-hundred-fold higher as in normal human umbilical vein endothelial cells (HUVEC) indicating a potential tumor selective property (Kim *et al*., 2006).

Recently, studies of our group demonstrated that rocaglamide derivatives do not only excert cytostatic activity but also have a cytotoxic effect, by inducing apoptosis in leukemia cell lines and leukemia cells derived from patients. Importantly, rocaglamide derivatives do not show substantial toxicity against normal peripheral blood lymphocytes isolated from healthy donors (Zhu *et al.,* 2007, Fig. 3.9; 3.10).

In this study we further investigated the mechanism of action of Rocaglamide derivatives. Therefore, the effect of one of the derivatives, Roc-AR was tested in CD95L and TRAIL resistant HTLV-1 infected T cells. HTLV-1 infected T cells were sensitive towards apoptosis only at a low percentage at a dosis of 100 nM Roc-AR (Fig. 3.22). However, treatment by Roc-AR with CD95L or TRAIL in combination resulted in a significant increase in apoptosis, which was demonstrated by DNA fragmentation, increased activation of caspase-8, the main caspase involved in receptor-mediated apoptosis, and the activation of the executing caspase-3 (Fig. 3.7; 3.8; 3.9). The effect of Roc-AR in combination with CD95L or TRAIL was rather synergistic than additive.

4.3 Molecular mechanims of Roc-induced sensitization of receptor-mediated apoptosis

Previous findings have shown that certain drugs can result in the upregulation of CD95 or TRAIL receptor expression and thereby sensitize cancer cells towards apoptosis. For example, it was shown that treatment of hepatoma cell lines with the proteasome inhibitor MG132 lead to upregulation of TRAIL-R2 (Ganten *et al*., 2005). Several anti-cancer drugs cause up-regulation of CD95 (Woo *et al*., 2004). Therefore, the receptor expression levels of Roc-AR-treated cells were examined. However, the surface expression of the death receptors (CD95, TRAIL-R1/R2) and decoy receptors (Dc-R1 and Dc-R2) were not affected by Roc-AR treatment (Fig. 3.11, 3.12). As receptor surface expression was not altered by Roc-AR treatment, it was likely that Roc-AR-induced sensitization does not occur at the receptor level. Therefore we analyzed the apoptotic signaling pathway downstream of the receptors.

As shown in our previous studies, resistance of HTLV-1 infected T cells to CD95-induced apoptosis was mediated by a high expression of c-FLIP protein (Krueger *et al*., 2006; Fig. 4.2A). In this study, treatment with Roc-AR resulted in a strong decrease in c-FLIP expression as early as 2 hr after exposure to Roc-AR (Fig. 3.13A; Fig. 4.2B). In contrast, the expression level of procaspase-8 was not altered after treatment with Roc-AR. As c-FLIP is found to be the major inhibitory protein at the DISC level, downregulating c-FLIP expression is therefore considered to be the major event responsible for the sensitization of the infected T cell lines. However, Roc-AR is also affecting other anti-apoptotic proteins such as Bcl-x_L and Mcl-1 which might also play a role in the sensitization towards CD95- and TRAIL-induced apoptosis (data not shown).

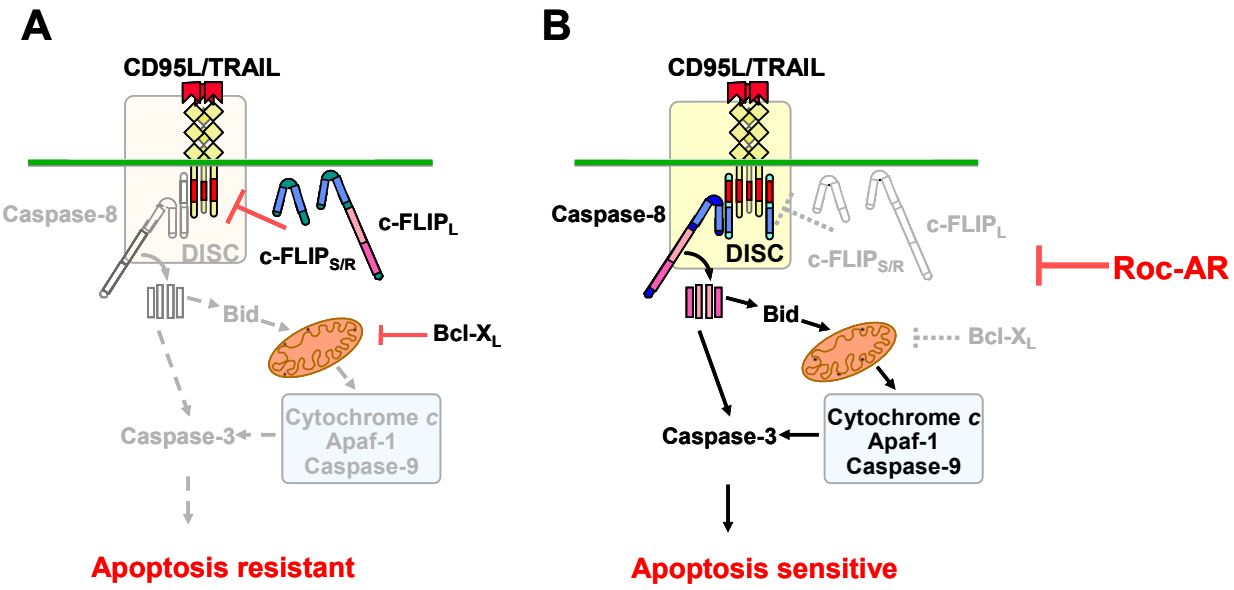


Figure 4.2: Mechanism of sensitization by Roc-AR of HTLV-1 infected T cells towards CD95-induced apoptosis.

A) HTLV-1 infected T cells are resistant towards CD95L- and TRAIL-induced apoptosis by an increased expression of c-FLIP and Bcl-X_L. B) Roc-AR decreases c-FLIP and Bcl-X_L expression and thereby sensitizes towards CD95L- and TRAIL-induced apoptosis.

4.4 Molecular mechanims of Roc-mediated suppression of c-FLIP expression

Next, the mechanism of the decrease in c-FLIP protein by Roc-AR was analyzed. Because our group previously showed that the Tax protein induced an increased expression of c-FLIP in HTLV-1 infected T cells (Krueger *et al*., 2006), the expression level of the Tax protein was analyzed after Roc-AR treatment. Roc-AR did not alter the Tax protein level for up to 32 hr of exposure to Roc-AR, whereas c-FLIP protein decreased already after 2 hr. Therefore, the Roc-mediated decrease in c-FLIP protein expression is independent of Tax protein expression levels, although the possibility that Roc-AR might interfere with Tax function cannot be excluded.

Tax was reported to induce the transcription factors NFAT and NF-κB, which were both reported to be involved in the regulation of c-FLIP expression (Micheau *et al*., 2001; Kreuz *et al*., 2001). Previous studies showed that Rocaglamide derivatives can inhibit NF-κB and NFAT activity (Baumann *et al*., 2002; Proksch *et al*., 2005). Therefore, Roc-

AR-mediated decrease in c-FLIP expression might be due to a Roc-mediated inhibition of NFAT and NF-κB activity. It was reported that NFAT was constitutively activated in HTLV-1 infected T cells (Good *et al*., 1996). However, a constitutive nuclear localization of NFAT could not be detected in the HTLV-1 infected T cell lines (Fig. 3.14A). In addition, cyclosporin A, a NFAT inhibitor, could not inhibit the high expression of c-FLIP in this study (Fig. 3.14B). This indicates that the decrease in c-FLIP protein expression is unlikely to be mediated by inhibition of NFAT activity.

NF-κB was reported to be activated in ATL patients and also in various types of other malignancies, most frequently in hematopoietic malignancies such as chronic lymphocytic leukemia (Delic *et al*., 1998), multiple myeloma (Feinman., *et al*., 1999), Hodgkin disease (Bargou., *et al*., 1997), acute lymphoblastic leukemia (Kordes *et al* 2001) and cutaneous T-cell lymphoma (Izban *et al*., 2000). The NF-κB inhibitor Bay 11-7082 was shown to down-regulate the expression of the anti-apoptotic protein Bcl-x_L (Fujii et al., 2002). Inhibition of the proteasome by PS-341 (bortezomib) inhibited the degradation of IκBα and thereby inhibited NF-κB. Both approaches lead to the induction of apoptosis in HTLV-1 infected T cells *in vitro* (Tan *et al*., 2002; Satou *et al*., 2004). Therefore, NF-κB is an attractive target for therapy. The constitutive activation of NF-κB was confirmed in the investigated HTLV-1 infected T cell lines (3.16A). In a previous study, the group of Proksch *et al,* claimed that Rocaglamide derivatives were potent NF-κB inhibitors (Baumann *et al*., 2002). However, our studies showed that NF-κB activity is not affected by Roc-AR. First, Roc-AR had no effect on the nuclear localization of the NF-κB subunits p50 and Rel A (3.16B), second, the NF-κB inhibitor A-4-quinazoline, the activity of a luciferase reporter containing 4x NF-κB consensus sequence could not be inhibited by Roc-AR (data not shown). In the study of Baumann *et al*., the effect of Rocaglamide derivative Roc-B on inhibition of IκB degradation was analyzed. However, our lab did not find any inhibitory effect on NF-κB activity for more than ten rocaglamide derivatives tested including Roc-B (diploma thesis, Zhu). There are also some critical data in the study by Baumann *et al*. First, inhibition of TNF- and PMA-induced IκB degradation by Roc-B was only observed at 30 min whereas other time points were not affected. This cannot explain the sustained NF-κB inhibition shown in their study. Second, Baumann *et al*, showed that NF-κB inhibition by Roc-B could only be seen in the Jurkat cell line, stably transfected with a NF-κB reporter construct, but not by

transient transfection of the NF-κB reporter plasmid into other cell lines. The authors explained such discrepancy by the hypothesis that NF-κB signal transduction cascades differ between T cells and other cell types. However, no evidence indicates that the kinases involved in signal transduction from the cell surface to the IKK complex are different between these cells. Furthermore, our lab could not detect an NF-κB inhibitory effect on Jurkat cells by transient transfection studies (data not shown).

In contrast to Roc-AR, treatment of HTLV-1 infected T cells with A-4-quinazoline led to an inhibition of c-FLIP mRNA expression. This indicated that the decrease in c-FLIP expression after Roc-AR treatment is not regulated at the transcriptional level (Fig. 3.17). After exclusion of transcriptional inhibition of c-FLIP by Roc-AR, the effect of Roc-AR on protein synthesis was examined and compared with the well-known protein translation inhibitor cycloheximide (CHX). Roc-AR inhibited protein synthesis almost completely at a concentration of 100 nM for the analyzed period (Fig. 3.18). Interestingly, Roc-AR had no effect on protein translation in an *in vitro* system (Fig. 3.19) indicating that Roc-AR does not directly affect the translation machinery and instead, affects a signaling pathway required for translation.

It has been shown that the PI3K/Akt signaling pathway was activated in HTLV-1 infected T cells. HTLV-1 Tax stimulates PI3K/Akt and the downstream target mTOR, leading to the activation of pro-survival signals in HTLV-1 infected T cells (Ikezoe *et al*., 2006). The effects observed with Roc-AR treatment could have been mediated by interfering with this pathway. Therefore the phosphorylation status of mTOR and the downstream target S6 after Roc-AR treatment was tested and compared with rapamycin, a well known mTOR inhibitor. Rapamycin blocked phosphorylation of mTOR and the downstream kinase S6 (Fig. 3.20A). Further, rapamycin inhibited the proliferation of HTLV-1 infected T cells to some extent (data not shown). However, the phosphorylation status of mTOR was not altered or even seemed to be increased after Roc-AR treatment (Fig. 3.20B). These results demonstrate that Roc-AR does not block the mTOR signaling pathway.

Besides the PI3K/Akt/mTOR pathway, protein translation is also known to be regulated by the RAS-activated ERK signaling pathway. The ERK pathway has been implicated in several processes including the regulation of survival, angiogenesis and cell cycle (McKay and Morrison, 2007). The ERK pathway regulates protein translation *via* the phosphorylation of the kinase Mek1/2 which in turn phosphorylates the kinase Mnk1/2.

Mnk1/2 phosphorylates eIF4E which then has a higher affinity for the mRNA 5' cap structure and thereby stimulates translation. Stimuli that increased the rate of protein synthesis generally increased the state of phosphorylation of eIF4E, and it was generally thought, that phosphorylation would activate eIF4E by increasing its affinity for capped mRNA (Raught and Gingras, 1999). Others suggested that the phosphorylation of eIF4E releases the binding from the 5' cap structure once translation has been initiated to allow further binding of new ribosomes (Tuxworth *et al*., 2004). It has also been suggested that phosphorylation of eIF4E occurs later in the initiation process after recognition of the start codon. Phosphorylation of eIF4E could function to enhance the release of factors from the cap-structure, rendering the cap-binding factors available for the translation of different mRNAs. Our study showed that treatment with Roc-AR resulted in an inhibition of phosphorylation of MEK1/2, ERK1/2, Mnk1 and eIF4E which was detected as early as 30 min upon exposure to Roc-AR (Fig. 3.21; Fig. 4.3). This indicates that Roc-AR affects protein translation by blocking the activity of the ERK pathway.

Growth factor stimulation leads to activation of the ERK pathway which enhances synthesis of cyclin D1 and D2 and stimulates entry into the cell cycle (Vinals *et al*., 1999). Conversely, stresses that reduce eIF4E availability and inhibit protein translation, leading to inhibition of the rate of synthesis of cyclin D1 and D2, and prevent cell cycle progression (Brewer *et al*., 1999; Hashemolhosseini *et al*., 1998). HTLV-1 infected T cells were reported to have an accelerated progression into S phase (Lemoine *et al*., 2001). Furthermore, it was reported that cyclin D2 and to a lesser extend cyclin D1, were upregulated in HTLV-1 infected T cells. This was also confirmed by our study (Fig. 3.23).

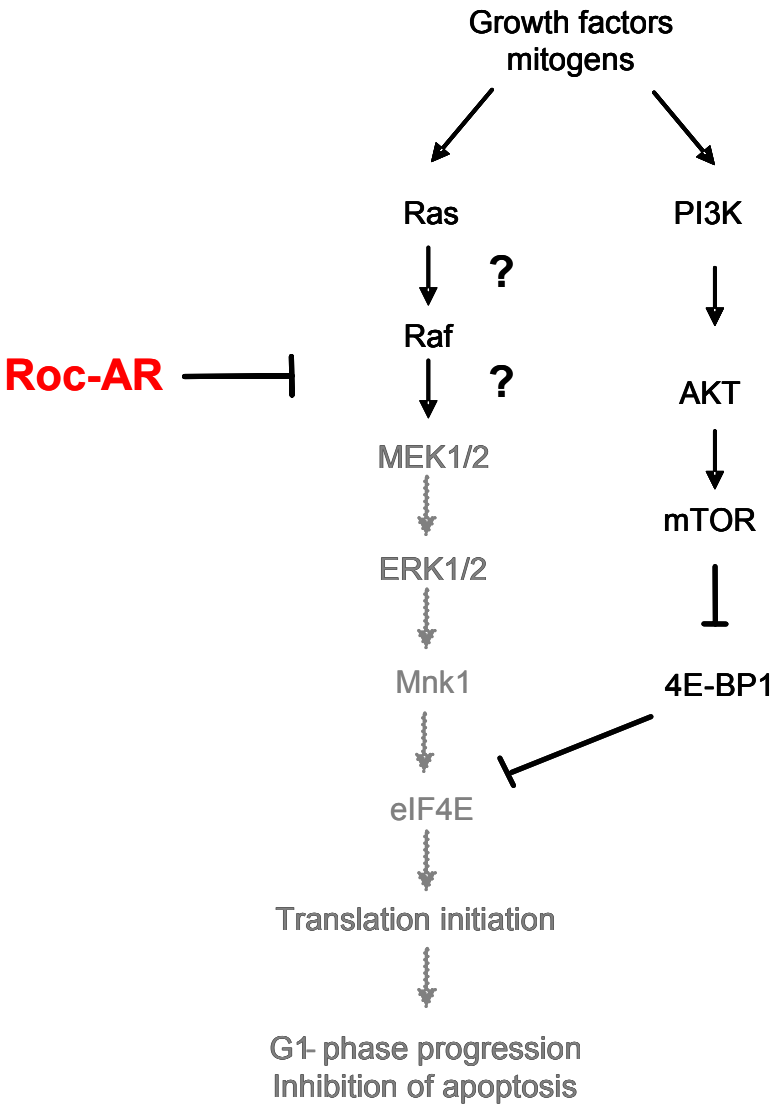


Figure 4.3: Mechanism of Roc-AR induced cell cycle arrest and sensitization towards death receptor induced apoptosis.

ERK phosphorylates the kinase Mnk1, which on its turn phosphorylates eIF4E. Phosphorylation of eIF4E increases its affinity for the 5' cap structure and thereby stimulates translation. This leads to stimulation of cell proliferation and inhibition of apoptosis by upregulation of proteins as Cyclin D and c-FLIP. Treatment with Roc-AR results in a dephosphorylation of ERK, Mnk1 and eIF4E and blocks thereby cap-dependent translation. This leads to a decrease in Cyclin D and c-FLIP expression and induces thereby a cell cycle arrest in G1-phase and sensitization towards death receptor induced apoptosis.

Cyclin D1 and D2 are critical regulators of G1-S cell cycle progression in T cells, and dysregulation of cyclin D expression contributes to the aberrant cell growth of HTLV-1 infected T cells. This study showed that Roc-AR decreased expression of cyclin D1 and D2, whereas the other cyclins remained unchanged (Fig. 3.23). This correlated with a G1 cell cycle arrest in all four HTLV-1 infected T cell lines (Fig. 3.22). The ERK pathway is crucial for cyclin D1 and D2 production and therefore provides a molecular link between this pathway and cell cycle control (Lavoie *et al*., 1999; Meloche and Pouyssegur, 2007). This is in agreement with our findings, which showed that the inhibition of this pathway leads to inhibition of cyclin D1 and D2 expression. Thus, inhibition of the ERK pathway by Roc-AR might be one of the important mechanisms by which Roc-AR inhibits c-FLIP expression and sensitizes towards death receptor-mediated apoptosis and induces a G1 arrest by inhibiting cyclin D1 and D2 expression.

4.5 Targeting the Raf-MEK-ERK pathway in cancer therapy

The ERK signaling pathway is activated by several growth factor receptors e.g. the epidermal growth factor receptor (EGFR). Binding of its ligand causes the receptor to dimerize with a neighboring EGFR. The EGFR dimer serves as a docking place for the Grb2-SOS complex, which in turn activates Ras. Activated Ras binds Raf, which is activated through a process of phosphorylation at activation sites and dephosphorylation at inhibitory sites. Activated Raf subsequently phosphorylates and activates MEK1/2. Activated MEK1/2 in turn phosphorylates and activates ERK1/2 (McKay and Morrison, 2007) (Fig. 4.4). The ERK pathway is aberrantly activated in cancer, particular by upstream activation of EGFR, the Ras small guanosine triphosphatases (GTPases) and Raf serine/threonine kinase isoforms (A-Raf-1, B-Raf, and C-raf). Mutations in the cytoplasmic kinase domain or truncations in the extracellular domain of EGFR are found in non-small cell carcinoma (NSCLC) (10%) and glioblastomas (20%) (Roberts and Der, 2007). Mutations in Ras are found in pancreas cancer (90%), papillary thyroid cancer (60%), colon cancer (50%) and NSCLC (30%) (Downward, 2003; Roberts and Der, 2007). Furthermore, B-Raf mutations are also found in a variety of solid tumor types, including melanoma (70%), papillary thyroid cancer (50%) and colon cancer (10%) (Brose *et al*, 2002; Roberts and Der, 2007).

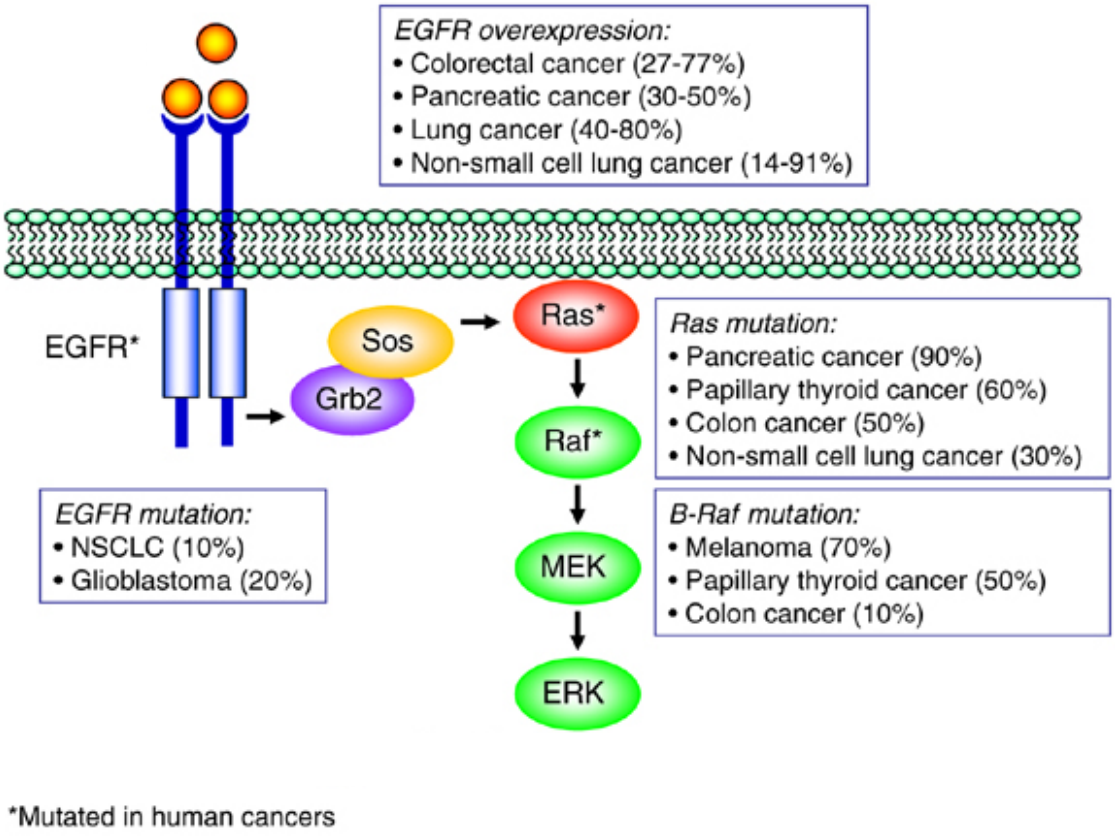


Figure 4.4: Oncogene activation of the ERK pathway.
Mutations in B-Raf, Ras, and the cytoplasmic kinase domain or extracellular domain of EGFR cause persistent activation of the ERK pathway in human cancers.

Since the discovery that activating mutations of the ERK pathway are associated with human cancer, this pathway has been the focus of intense drug discovery effort. Inhibitors were developed several years ago against MEK1/2. PD98059 (Dudley *et al.*, 1995) and UO126 (Favata *et al.*, 1998) are fairly specific, non-ATP-competitive MEK1/2 inhibitors that have been widely used for research purposes. The inhibtor PD184352 has proven to be effective in inhibiting the growth of tumors in immunodeficient mice and has been used in a large study in colon carcinomas (Sebolt-Leopold *et al.*, 1999). This drug has recently undergone Phase I clinical trials. It seems to be well tolerated at doses that inhibit ERK activation in patients' peripheral-blood mononuclear cells.

A variety of agents have been discovered that interfere with Raf activity, including

antisense oligonucleotides and small molecule inhibitors (Crooke, 2000; Lyons *et al*., 2001). These inhibitors block the expression of Raf protein, the Ras/Raf interaction, or the Raf kinase activity. Sorafenib (BAY 43-9006) is the first oral multi-kinase inhibitor that targets the Raf kinases and recently entered phase III clinical trials (Lyons *et al*., 2001). Pre-clinical studies showed that the drug has anti-tumor activity *in vivo* in xenograft models, and Phase I and II clinical trials revealed minor side effects such as diarrhea and skin irritation resulting from affecting cells with rapid turnover (*i.e.* in gut and skin). However, it has since been reported that Sorafenib might not be as specific as first thought and that it also interferes in other signaling pathways. Therefore the development of more specific inhibitors is necessary (Sebolt-Leopold and Herrera, 2004). Charaterization of the three-dimensional structures of the kinases Raf, MEK1/2 and ERK1/2 will help to develop specific inhibitors. Also, detailed structural information on the binding modes of such inhibitors will significantly enhance the drug design process.

In our study, we could show that Roc-AR blocked the phosphorylation of and thereby inactivated MEK1/2, ERK, Mnk1 and eIF4E. The remaining question still to be answered is what is the direct target of Roc-AR? As dephosphorylation of MEK1/2 and ERK1/2 is observed, it could be that Rocaglamide derivatives directly target these kinases. Another possible target could be the p38 MAPK. In a previous study in our lab, it was shown that treatment of leukemia cells with Rocaglamide derivatives led to the activation of the p38 MAPK pathway (Zhu *et al*., 2007). The pro-apoptotic p38 MAPK pathway is activated by cellular stress, including UV light, arsenite, osmotic shock, and inflammatory cytokines (Ono *et al*., 2000). Recent findings indicate a requirement for a balance between MEK1/2, ERK1/2 and p38 signaling pathways to ensure appropriate regulation of cell survival (Ballif *et al*., 2001; Xia *et al*., 1995). It has been reported previously that activation of p38 MAPK inhibits the ERK1/2 signaling pathway *via* PP1/PP2A-dependent dephosphorylation of MEK1/2 (Westermarck *et al*., 2001). Furthermore, it was shown that inhibition of the ERK signaling pathway by p38 MAPK activation leads to initiation of apoptosis (Song-Ping *et al*., 2003). Therefore, inhibition of the ERK pathway could be regulated by a Roc-AR-mediated activation of p38 MAPK.

Another possible target could be one of the upstream Raf kinases. Multiple signals converge to regulate Raf activation (Kolch, 2005). As shown in (Fig. 4.5), the major

activators of Raf kinases are the Ras small GTPases. Ras facilitates the plasma membrane association of Raf normally found in the cytosol. Additional signaling activities, including phosphorylation (of *e.g.*, p21-activated protein kinases (PAK) serine/threonine and Src family tyrosine kinases) and dephosphorylation (of *e.g.* protein phosphatase 2A) events are required to fully activate Raf kinase function. Raf function is also regulated by interaction with other proteins, including 14-3-3 proteins and the chaperones, heat shock protein 50 and 90 (Hsp50/90). Another level of regulation of Raf involves the scaffold protein kinase suppressor of Ras 1 (KSR-1) that helps to regulate the activity, specificity and regulation of MEK/ERK signaling (Kolch, 2005). These multiple regulatory events suggest several possible targets for blocking the ERK pathway by Roc-AR.

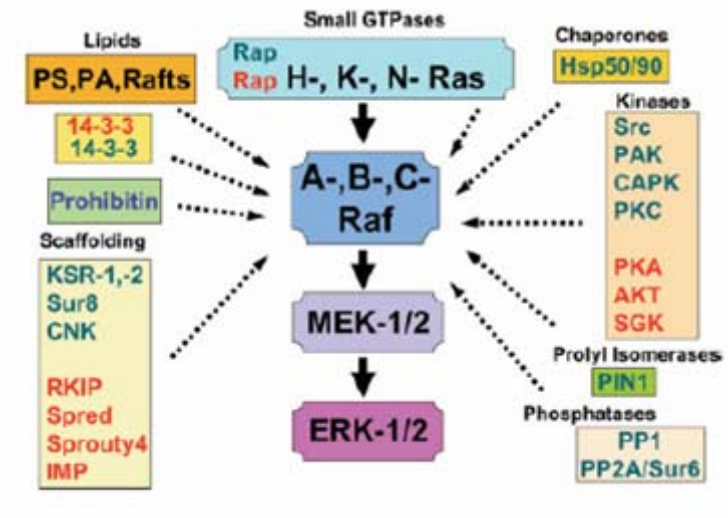


Figure 4.5: The Raf kinases are pivotal molecules within the ERK pathway, which regulate cellular proliferation and survival.

Raf function is regulated by both positive and negative signal imputs. In particular, scaffold proteins, small GTPases, chaperones, kinases and phosphatases.

Inhibitors of the ERK pathway can be classified as "cytostatic" rather than “cytotoxic” anticancer drugs (Downward, 2003). Such cytostatic agents may selectively inhibit the abnormal activation of their corresponding target molecule and suppress tumor cell growth without killing the tumor cells. This was observed with *e.g.* the MEK1/2 inhibitor

PD98059. Although treatment with this inhibitor completely suppressed the growth of tumor cells, it showed only a modest effect on induction of apoptosis (Hoshino *et al*., 2001). Furthermore, since many inhibitors of this pathway are reversible, their removal would permit re-initiation of tumor cell proliferation. The majority of tumor cells after treatment with these inhibitors is in a "resting" state, but apoptosis is not initiated in the presence of such cytostatic inhibitors. However, a combination of MEK1/2 inhibitors and vincristine, for example, markedly enhanced the apoptosis-inducing activity of vincristine not only in tumor cells *in vitro* but also in human colon tumor xenografts *in vivo* (Sebolt-Leopold and Herrera, 2004; Watanabe., *et al* 2002). Roc-AR displays not only cytostatic but also cytotoxic activity, which makes Roc-AR even more attractive as a therapeutic agent (Zhu, *et al*., 2007). This cytotoxic effect was also observed in this study, although at low amounts of Roc-AR, and the effect in HTLV-1 infected T cells was rather cytostatic. However, in combination with CD95L or TRAIL this cytotoxic effect is enhanced in a synergistic way. Therefore, as a therapeutic approach, since CD95 might be too toxic, combination therapy of Roc-AR with TRAIL is suggested. As discussed above, specific inhibition of the ERK pathway enhances the anticancer effect of a wide variety of cytotoxic chemotherapeutic agents (Dent and Grant, 2002). As a large number of ERK pathway inhibitors already entered clinical trials, there is considerable optimism that these inhibitors or new inhibitors such as Roc-AR in combination with targeting death receptor-mediated apoptosis will provide a new avenue of treatment.

LITERATURE

ACEHAN, D., JIANG, X., MORGAN, D. G., HEUSER, J. E., WANG, X. & AKEY, C. W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* **9,** 423-32.

ALBRECHT, B. & LAIRMORE, M. D. (2002). Critical role of human T-lymphotropic virus type 1 accessory proteins in viral replication and pathogenesis. *Microbiol Mol Biol Rev* **66,** 396-406, table of contents.

ARNOLD, R., LIOU, J., DREXLER, H. C., WEISS, A. & KIEFER, F. (2001). Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NFkappaB into an inhibitor of NFkappaB. *J Biol Chem* **276,** 14675-84.

ASHKENAZI, A. & DIXIT, V. M. (1999). Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* **11,** 255-60.

ASQUITH, B. & BANGHAM, C. R. (2000). The role of cytotoxic T lymphocytes in human T-cell lymphotropic virus type 1 infection. *J Theor Biol* **207,** 65-79.

ASQUITH, B., MOSLEY, A. J., BARFIELD, A., MARSHALL, S. E., HEAPS, A., GOON, P., HANON, E., TANAKA, Y., TAYLOR, G. P. & BANGHAM, C. R. (2005). A functional CD8+ cell assay reveals individual variation in CD8+ cell antiviral efficacy and explains differences in human T-lymphotropic virus type 1 proviral load. *J Gen Virol* **86,** 1515-23.

AWADA, A., HENDLISZ, A., GIL, T., BARTHOLOMEUS, S., MANO, M., DE VALERIOLA, D., STRUMBERG, D., BRENDEL, E., HAASE, C. G., SCHWARTZ, B. & PICCART, M. (2005). Phase I safety and pharmacokinetics of BAY 43-9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. *Br J Cancer* **92,** 1855-61.

AZIMI, N., BROWN, K., BAMFORD, R. N., TAGAYA, Y., SIEBENLIST, U. & WALDMANN, T. A. (1998). Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF-kappaB site. *Proc Natl Acad Sci U S A* **95,** 2452-7.

AZRAN, I., SCHAVINSKY-KHRAPUNSKY, Y. & ABOUD, M. (2004). Role of Tax protein in human T-cell leukemia virus type-I leukemogenicity. *Retrovirology* **1,** 20.

BALDWIN, A. S., JR. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14,** 649-83.

BALLARD, D. W., BOHNLEIN, E., LOWENTHAL, J. W., WANO, Y., FRANZA, B. R. & GREENE, W. C. (1988). HTLV-I tax induces cellular proteins that activate the kappa B element in the IL-2 receptor alpha gene. *Science* **241,** 1652-5.

BANGHAM, C. R. (2000). The immune response to HTLV-I. *Curr Opin Immunol* **12,** 397-402.

BANGHAM, C. R. (2003). The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. *J Gen Virol* **84,** 3177-89.

BARGOU, R. C., EMMERICH, F., KRAPPMANN, D., BOMMERT, K., MAPARA, M. Y., ARNOLD, W., ROYER, H. D., GRINSTEIN, E., GREINER, A., SCHEIDEREIT, C. & DORKEN, B. (1997). Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* **100,** 2961-9.

BAUMANN, B., BOHNENSTENGEL, F., SIEGMUND, D., WAJANT, H., WEBER, C., HERR, I., DEBATIN, K. M., PROKSCH, P. & WIRTH, T. (2002). Rocaglamide derivatives are potent inhibitors of NF-kappa B activation in T-cells. *J Biol Chem* **277,** 44791-800.

BAYSSAS, M., GOUVEIA, J., DE VASSAL, F., MISSET, J. L., SCHWARZENBERG, L., RIBAUD, P., MUSSET, M., JASMIN, C., HAYAT, M. & MATHE, G. (1980). Vindesine: a new vinca alkaloid. *Recent Results Cancer Res* **74,** 91-7.

BEG, A. A., RUBEN, S. M., SCHEINMAN, R. I., HASKILL, S., ROSEN, C. A. & BALDWIN, A. S., JR. (1992). I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. *Genes Dev* **6,** 1899-913.

BERAUD, C., SUN, S. C., GANCHI, P., BALLARD, D. W. & GREENE, W. C. (1994). Human T-cell leukemia virus type I Tax associates with and is negatively regulated by the NF-kappa B2 p100 gene product: implications for viral latency. *Mol Cell Biol* **14,** 1374-82.

BLANCHARD, H., KODANDAPANI, L., MITTL, P. R., MARCO, S. D., KREBS, J. F., WU, J. C., TOMASELLI, K. J. & GRUTTER, M. G. (1999). The three-dimensional structure of caspase-8: an initiator enzyme in apoptosis. *Structure* **7,** 1125-33.

BOHNENSTENGEL, F. I., STEUBE, K. G., MEYER, C., QUENTMEIER, H., NUGROHO, B. W. & PROKSCH, P. (1999). 1H-cyclopenta[b]benzofuran lignans from Aglaia species inhibit cell proliferation and alter cell cycle distribution in human monocytic leukemia cell lines. *Z Naturforsch [C]* **54,** 1075-83.

BOLDIN, M. P., VARFOLOMEEV, E. E., PANCER, Z., METT, I. L., CAMONIS, J. H. & WALLACH, D. (1995). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J Biol Chem* **270,** 7795-8.

BRADER, G., VAJRODAYA, S., GREGER, H., BACHER, M., KALCHHAUSER, H. & HOFER, O. (1998). Bisamides, lignans, triterpenes, and insecticidal Cyclopenta[b]benzofurans from Aglaia species. *J Nat Prod* **61,** 1482-90.

BRADY, J., JEANG, K. T., DUVALL, J. & KHOURY, G. (1987). Identification of p40x-responsive regulatory sequences within the human T-cell leukemia virus type I long terminal repeat. *J Virol* **61,** 2175-81.

BRAUWEILER, A., GARRUS, J. E., REED, J. C. & NYBORG, J. K. (1997). Repression of bax gene expression by the HTLV-1 Tax protein: implications for suppression of apoptosis in virally infected cells. *Virology* **231,** 135-40.

BRENNER, D., GOLKS, A., KIEFER, F., KRAMMER, P. H. & ARNOLD, R. (2005). Activation or suppression of NFkappaB by HPK1 determines sensitivity to activation-induced cell death. *Embo J* **24,** 4279-90.

BREWER, J. W., HENDERSHOT, L. M., SHERR, C. J. & DIEHL, J. A. (1999). Mammalian unfolded protein response inhibits cyclin D1 translation and cell-cycle progression. *Proc Natl Acad Sci U S A* **96,** 8505-10.

BROCKMAN, J. A., SCHERER, D. C., MCKINSEY, T. A., HALL, S. M., QI, X., LEE, W. Y. & BALLARD, D. W. (1995). Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. *Mol Cell Biol* **15,** 2809-18.

BROSE, M. S., VOLPE, P., FELDMAN, M., KUMAR, M., RISHI, I., GERRERO, R., EINHORN, E., HERLYN, M., MINNA, J., NICHOLSON, A., ROTH, J. A., ALBELDA, S. M., DAVIES, H., COX, C., BRIGNELL, G., STEPHENS, P., FUTREAL, P. A., WOOSTER, R., STRATTON, M. R. & WEBER, B. L. (2002). BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res* **62,** 6997-7000.

BROWN, K., GERSTBERGER, S., CARLSON, L., FRANZOSO, G. & SIEBENLIST, U. (1995). Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* **267,** 1485-8.

CARTER, R. S., GEYER, B. C., XIE, M., ACEVEDO-SUAREZ, C. A. & BALLARD, D. W. (2001). Persistent activation of NF-kappa B by the tax transforming protein involves chronic phosphorylation of IkappaB kinase subunits IKKbeta and IKKgamma. *J Biol Chem* **276,** 24445-8.

CARVALHO, E. M. & DA FONSECA PORTO, A. (2004). Epidemiological and clinical interaction between HTLV-1 and Strongyloides stercoralis. *Parasite Immunol* **26,** 487-97.

CHAI, J. & TARNAWSKI, A. S. (2002). Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. *J Physiol Pharmacol* **53,** 147-57.

CHAO, D. T. & KORSMEYER, S. J. (1998). BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16,** 395-419.

CHAUDHARY, P. M., EBY, M. T., JASMIN, A., KUMAR, A., LIU, L. & HOOD, L. (2000). Activation of the NF-kappaB pathway by caspase 8 and its homologs. *Oncogene* **19,** 4451-60.

CHEN, Y. C., WANG, C. H., SU, I. J., HU, C. Y., CHOU, M. J., LEE, T. H., LIN, D. T., CHUNG, T. Y., LIU, C. H. & YANG, C. S. (1989). Infection of human T-cell leukemia virus type I and development of human T-cell leukemia lymphoma in patients with hematologic neoplasms: a possible linkage to blood transfusion. *Blood* **74,** 388-94.

CHEN, Z. J., PARENT, L. & MANIATIS, T. (1996). Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. *Cell* **84,** 853-62.

CHENG, M., SEXL, V., SHERR, C. J. & ROUSSEL, M. F. (1998). Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). *Proc Natl Acad Sci U S A* **95,** 1091-6.

CHINNAIYAN, A. M., O'ROURKE, K., TEWARI, M. & DIXIT, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81,** 505-12.

CHU, Z. L., DIDONATO, J. A., HAWIGER, J. & BALLARD, D. W. (1998). The tax oncoprotein of human T-cell leukemia virus type 1 associates with and persistently activates IkappaB kinases containing IKKalpha and IKKbeta. *J Biol Chem* **273,** 15891-4.

CLARK, J. W., EDER, J. P., RYAN, D., LATHIA, C. & LENZ, H. J. (2005). Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. *Clin Cancer Res* **11,** 5472-80.

CLEMENS, M. J. (2001). Translational regulation in cell stress and apoptosis. Roles of the eIF4E binding proteins. *J Cell Mol Med* **5,** 221-39.

COHEN, G. M. (1997). Caspases: the executioners of apoptosis. *Biochem J* **326 (Pt 1),** 1-16.

COPELAND, K. F., HAAKSMA, A. G., GOUDSMIT, J., KRAMMER, P. H. & HEENEY, J. L. (1994). Inhibition of apoptosis in T cells expressing human T cell leukemia virus type I Tax. *AIDS Res Hum Retroviruses* **10,** 1259-68.

CRENON, I., BERAUD, C., SIMARD, P., MONTAGNE, J., VESCHAMBRE, P. & JALINOT, P. (1993). The transcriptionally active factors mediating the effect of the HTLV-I Tax transactivator on the IL-2R alpha kappa B enhancer include the product of the Rel A proto-oncogene. *Oncogene* **8,** 867-75.

DE THE, G. & BOMFORD, R. (1993). An HTLV-I vaccine: why, how, for whom? *AIDS Res Hum Retroviruses* **9,** 381-6.

DELIC, J., MASDEHORS, P., OMURA, S., COSSET, J. M., DUMONT, J., BINET, J. L. & MAGDELENAT, H. (1998). The proteasome inhibitor lactacystin induces apoptosis and sensitizes chemo- and radioresistant human chronic lymphocytic leukaemia lymphocytes to TNF-alpha-initiated apoptosis. *Br J Cancer* **77,** 1103-7.

DENT, P. & GRANT, S. (2001). Pharmacologic interruption of the mitogen-activated extracellular-regulated kinase/mitogen-activated protein kinase signal transduction pathway: potential role in promoting cytotoxic drug action. *Clin Cancer Res* **7,** 775-83.

DIDONATO, J., MERCURIO, F., ROSETTE, C., WU-LI, J., SUYANG, H., GHOSH, S. & KARIN, M. (1996). Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* **16,** 1295-304.

DOWNWARD, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* **3,** 11-22.

DUA, K., WILLIAMS, T. M. & BERETTA, L. (2001). Translational control of the proteome: relevance to cancer. *Proteomics* **1,** 1191-9.

DUDLEY, D. T., PANG, L., DECKER, S. J., BRIDGES, A. J. & SALTIEL, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A* **92,** 7686-9.

DUTTON, A., O'NEIL, J. D., MILNER, A. E., REYNOLDS, G. M., STARCZYNSKI, J., CROCKER, J., YOUNG, L. S. & MURRAY, P. G. (2004). Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci U S A* **101,** 6611-6.

EDLICH, R. F., ARNETTE, J. A. & WILLIAMS, F. M. (2000). Global epidemic of human T-cell lymphotropic virus type-I (HTLV-I). *J Emerg Med* **18,** 109-19.

ETOH, K., TAMIYA, S., YAMAGUCHI, K., OKAYAMA, A., TSUBOUCHI, H., IDETA, T., MUELLER, N., TAKATSUKI, K. & MATSUOKA, M. (1997). Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo. *Cancer Res* **57,** 4862-7.

FADOK, V. A., VOELKER, D. R., CAMPBELL, P. A., COHEN, J. J., BRATTON, D. L. & HENSON, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148,** 2207-16.

FAVATA, M. F., HORIUCHI, K. Y., MANOS, E. J., DAULERIO, A. J., STRADLEY, D. A., FEESER, W. S., VAN DYK, D. E., PITTS, W. J., EARL, R. A., HOBBS, F., COPELAND, R. A., MAGOLDA, R. L., SCHERLE, P. A. & TRZASKOS, J. M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* **273,** 18623-32.

FEINMAN, R., KOURY, J., THAMES, M., BARLOGIE, B., EPSTEIN, J. & SIEGEL, D. S. (1999). Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. *Blood* **93,** 3044-52.

FELBER, B. K., PASKALIS, H., KLEINMAN-EWING, C., WONG-STAAL, F. & PAVLAKIS, G. N. (1985). The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science* **229,** 675-9.

FERNANDES-ALNEMRI, T., ARMSTRONG, R. C., KREBS, J., SRINIVASULA, S. M., WANG, L., BULLRICH, F., FRITZ, L. C., TRAPANI, J. A., TOMASELLI, K. J., LITWACK, G. & ALNEMRI, E. S. (1996). In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci U S A* **93,** 7464-9.

FLYNN, A. & PROUD, G. (1996). Insulin-stimulated phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway. *FEBS Lett* **389,** 162-6.

FRANCHINI, G. (1995). Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection. *Blood* **86,** 3619-39.

FRANKLIN, A. A. & NYBORG, J. K. (1995). Mechanisms of Tax Regulation of Human T Cell Leukemia Virus Type I Gene Expression. *J Biomed Sci* **2,** 17-29.

FUJII, M., CHUHJO, T., MINAMINO, T., MASAAKI, N., MIYAMOTO, K. & SEIKI, M. (1995). Identification of the Tax interaction region of serum response factor that mediates the aberrant induction of immediate early genes through CArG boxes by HTLV-I Tax. *Oncogene* **11,** 7-14.

FUJII, M., NIKI, T., MORI, T., MATSUDA, T., MATSUI, M., NOMURA, N. & SEIKI, M. (1991). HTLV-1 Tax induces expression of various immediate early serum responsive genes. *Oncogene* **6,** 1023-9.

FUJII, M., TSUCHIYA, H., CHUHJO, T., AKIZAWA, T. & SEIKI, M. (1992). Interaction of HTLV-1 Tax1 with p67SRF causes the aberrant induction of cellular immediate early genes through CArG boxes. *Genes Dev* **6,** 2066-76.

FUKUNAGA, R. & HUNTER, T. (1997). MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *Embo J* **16,** 1921-33.

GAZZOLO, L. & DUC DODON, M. (1987). Direct activation of resting T lymphocytes by human T-lymphotropic virus type I. *Nature* **326,** 714-7.

GELEZIUNAS, R., FERRELL, S., LIN, X., MU, Y., CUNNINGHAM, E. T., JR., GRANT, M., CONNELLY, M. A., HAMBOR, J. E., MARCU, K. B. & GREENE, W. C. (1998). Human T-cell leukemia virus type 1 Tax induction of NF-kappaB involves activation of the IkappaB kinase alpha (IKKalpha) and IKKbeta cellular kinases. *Mol Cell Biol* **18,** 5157-65.

GESSAIN, A., BARIN, F., VERNANT, J. C., GOUT, O., MAURS, L., CALENDER, A. & DE THE, G. (1985). Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **2,** 407-10.

GLUCKSMANN, A. (1965). Cell death in normal development. *Arch Biol (Liege)* **76,** 419-37.

GOLKS, A., BRENNER, D., FRITSCH, C., KRAMMER, P. H. & LAVRIK, I. N. (2005). c-FLIPR, a new regulator of death receptor-induced apoptosis. *J Biol Chem* **280,** 14507-13.

GOOD, L., MAGGIRWAR, S. B. & SUN, S. C. (1996). Activation of the IL-2 gene promoter by HTLV-I tax involves induction of NF-AT complexes bound to the CD28-responsive element. *Embo J* **15,** 3744-50.

GRASSMANN, R., BERCHTOLD, S., RADANT, I., ALT, M., FLECKENSTEIN, B., SODROSKI, J. G., HASELTINE, W. A. & RAMSTEDT, U. (1992). Role of human T-cell leukemia virus type 1 X region proteins in immortalization of primary human lymphocytes in culture. *J Virol* **66,** 4570-5.

GRASSMANN, R., DENGLER, C., MULLER-FLECKENSTEIN, I., FLECKENSTEIN, B., MCGUIRE, K., DOKHELAR, M. C., SODROSKI, J. G. & HASELTINE, W. A. (1989). Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc Natl Acad Sci U S A* **86,** 3351-5.

GRIFFITH, T. S., BRUNNER, T., FLETCHER, S. M., GREEN, D. R. & FERGUSON, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270,** 1189-92.

GUY, A. L. & TAYLOR, J. H. (1978). Actinomycin D inhibits initiation of DNA replication in mammalian cells. *Proc Natl Acad Sci U S A* **75,** 6088-92.

GUZMAN, M. L., NEERING, S. J., UPCHURCH, D., GRIMES, B., HOWARD, D. S., RIZZIERI, D. A., LUGER, S. M. & JORDAN, C. T. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* **98,** 2301-7.

HALLER, K., WU, Y., DEROW, E., SCHMITT, I., JEANG, K. T. & GRASSMANN, R. (2002). Physical interaction of human T-cell leukemia virus type 1 Tax with cyclin-dependent kinase 4 stimulates the phosphorylation of retinoblastoma protein. *Mol Cell Biol* **22,** 3327-38.

HANON, E., HALL, S., TAYLOR, G. P., SAITO, M., DAVIS, R., TANAKA, Y., USUKU, K., OSAME, M., WEBER, J. N. & BANGHAM, C. R. (2000). Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. *Blood* **95,** 1386-92.

HARTWELL, L. H., CULOTTI, J., PRINGLE, J. R. & REID, B. J. (1974). Genetic control of the cell division cycle in yeast. *Science* **183,** 46-51.

HASHEMOLHOSSEINI, S., NAGAMINE, Y., MORLEY, S. J., DESRIVIERES, S., MERCEP, L. & FERRARI, S. (1998). Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J Biol Chem* **273,** 14424-9.

HAUSOTT, B., GREGER, H. & MARIAN, B. (2004). Flavaglines: a group of efficient growth inhibitors block cell cycle progression and induce apoptosis in colorectal cancer cells. *Int J Cancer* **109,** 933-40.

HEGER, P., ROSORIUS, O., HAUBER, J. & STAUBER, R. H. (1999). Titration of cellular export factors, but not heteromultimerization, is the molecular mechanism of trans-dominant HTLV-1 rex mutants. *Oncogene* **18,** 4080-90.

HENGARTNER, M. O. (2001). Apoptosis: corralling the corpses. *Cell* **104,** 325-8.

HIMES, S. R., COLES, L. S., KATSIKEROS, R., LANG, R. K. & SHANNON, M. F. (1993). HTLV-1 tax activation of the GM-CSF and G-CSF promoters requires the interaction of NF-kB with other transcription factor families. *Oncogene* **8,** 3189-97.

HOSHINO, R., TANIMURA, S., WATANABE, K., KATAOKA, T. & KOHNO, M. (2001). Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). *J Biol Chem* **276,** 2686-92.

HUANG, Y., OHTANI, K., IWANAGA, R., MATSUMURA, Y. & NAKAMURA, M. (2001). Direct trans-activation of the human cyclin D2 gene by the oncogene product Tax of human T-cell leukemia virus type I. *Oncogene* **20,** 1094-102.

HWANG, B. Y., SU, B. N., CHAI, H., MI, Q., KARDONO, L. B., AFRIASTINI, J. J., RISWAN, S., SANTARSIERO, B. D., MESECAR, A. D., WILD, R., FAIRCHILD, C. R., VITE, G. D., ROSE, W. C., FARNSWORTH, N. R., CORDELL, G. A., PEZZUTO, J. M., SWANSON, S. M. & KINGHORN, A. D. (2004a). Silvestrol and episilvestrol, potential anticancer rocaglate derivatives from Aglaia silvestris. *J Org Chem* **69,** 3350-8.

HWANG, Y. H., CHOI, J. Y., KIM, S., CHUNG, E. S., KIM, T., KOH, S. S., LEE, B., BAE, S. H., KIM, J. & PARK, Y. M. (2004b). Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma. *Hepatol Res* **29,** 113-121.

IKEZOE, T., NISHIOKA, C., BANDOBASHI, K., YANG, Y., KUWAYAMA, Y., ADACHI, Y., TAKEUCHI, T., KOEFFLER, H. P. & TAGUCHI, H. (2006). Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells. *Leuk Res*.

IRMLER, M., THOME, M., HAHNE, M., SCHNEIDER, P., HOFMANN, K., STEINER, V., BODMER, J. L., SCHROTER, M., BURNS, K., MATTMANN, C., RIMOLDI, D., FRENCH, L. E. & TSCHOPP, J. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature* **388,** 190-5.

ITOH, N. & NAGATA, S. (1993). A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J Biol Chem* **268,** 10932-7.

ITOH, N., YONEHARA, S., ISHII, A., YONEHARA, M., MIZUSHIMA, S., SAMESHIMA, M., HASE, A., SETO, Y. & NAGATA, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66,** 233-43.

IZBAN, K. F., ERGIN, M., QIN, J. Z., MARTINEZ, R. L., POOLEY, R. J., SAEED, S. & ALKAN, S. (2000). Constitutive expression of NF-kappa B is a characteristic feature of mycosis fungoides: implications for apoptosis resistance and pathogenesis. *Hum Pathol* **31,** 1482-90.

JACOBSON, S., SHIDA, H., MCFARLIN, D. E., FAUCI, A. S. & KOENIG, S. (1990). Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature* **348,** 245-8.

JEANG, K. T., BOROS, I., BRADY, J., RADONOVICH, M. & KHOURY, G. (1988). Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40x-responsive 21-base-pair sequence. *J Virol* **62,** 4499-509.

JEFFERY, K. J., USUKU, K., HALL, S. E., MATSUMOTO, W., TAYLOR, G. P., PROCTER, J., BUNCE, M., OGG, G. S., WELSH, K. I., WEBER, J. N., LLOYD, A. L., NOWAK, M. A., NAGAI, M., KODAMA, D., IZUMO, S., OSAME, M. & BANGHAM, C. R. (1999). HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. *Proc Natl Acad Sci U S A* **96,** 3848-53.

JEONG, S. J., PISE-MASISON, C. A., RADONOVICH, M. F., PARK, H. U. & BRADY, J. N. (2005). Activated AKT regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells. *Oncogene* **24,** 6719-28.

KANNAGI, M., HARASHIMA, N., KURIHARA, K., OHASHI, T., UTSUNOMIYA, A., TANOSAKI, R., MASUDA, M., TOMONAGA, M. & OKAMURA, J. (2005). Tumor immunity against adult T-cell leukemia. *Cancer Sci* **96,** 249-55.

KANNO, T., BROWN, K., FRANZOSO, G. & SIEBENLIST, U. (1994). Kinetic analysis of human T-cell leukemia virus type I Tax-mediated activation of NF-kappa B. *Mol Cell Biol* **14,** 6443-51.

KARIN, M. & BEN-NERIAH, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18,** 621-63.

KARIN, M. & LIN, A. (2002). NF-kappaB at the crossroads of life and death. *Nat Immunol* **3,** 221-7.

KATAOKA, T., BUDD, R. C., HOLLER, N., THOME, M., MARTINON, F., IRMLER, M., BURNS, K., HAHNE, M., KENNEDY, N., KOVACSOVICS, M. & TSCHOPP, J. (2000). The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol* **10,** 640-8.

KATAOKA, T. & TSCHOPP, J. (2004). N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. *Mol Cell Biol* **24,** 2627-36.

KAYAGAKI, N., KAWASAKI, A., EBATA, T., OHMOTO, H., IKEDA, S., INOUE, S., YOSHINO, K., OKUMURA, K. & YAGITA, H. (1995). Metalloproteinase-mediated release of human Fas ligand. *J Exp Med* **182,** 1777-83.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.

KIM, S., CHIN, Y. W., SU, B. N., RISWAN, S., KARDONO, L. B., AFRIASTINI, J. J., CHAI, H., FARNSWORTH, N. R., CORDELL, G. A., SWANSON, S. M. & KINGHORN, A. D. (2006a). Cytotoxic flavaglines and bisamides from Aglaia edulis. *J Nat Prod* **69,** 1769-75.

KIM, S., SALIM, A. A., SWANSON, S. M. & KINGHORN, A. D. (2006b). Potential of cyclopenta[b]benzofurans from Aglaia species in cancer chemotherapy. *Anticancer Agents Med Chem* **6,** 319-45.

KIMATA, J. T. & RATNER, L. (1991). Temporal regulation of viral and cellular gene expression during human T-lymphotropic virus type I-mediated lymphocyte immortalization. *J Virol* **65,** 4398-407.

KINOSHITA, T., SHIMOYAMA, M., TOBINAI, K., ITO, M., ITO, S., IKEDA, S., TAJIMA, K., SHIMOTOHNO, K. & SUGIMURA, T. (1989). Detection of mRNA for the tax1/rex1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc Natl Acad Sci U S A* **86,** 5620-4.

KISCHKEL, F. C., HELLBARDT, S., BEHRMANN, I., GERMER, M., PAWLITA, M., KRAMMER, P. H. & PETER, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14,** 5579-88.

KISCHKEL, F. C., LAWRENCE, D. A., TINEL, A., LEBLANC, H., VIRMANI, A., SCHOW, P., GAZDAR, A., BLENIS, J., ARNOTT, D. & ASHKENAZI, A. (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J Biol Chem* **276,** 46639-46.

KISHI, S., SAIJYO, S., ARAI, M., KARASAWA, S., UEDA, S., KANNAGI, M., IWAKURA, Y., FUJII, M. & YONEHARA, S. (1997). Resistance to fas-mediated apoptosis of peripheral T cells in human T lymphocyte virus type I (HTLV-I) transgenic mice with autoimmune arthropathy. *J Exp Med* **186,** 57-64.

KLEIJN, M., SCHEPER, G. C., VOORMA, H. O. & THOMAS, A. A. (1998). Regulation of translation initiation factors by signal transduction. *Eur J Biochem* **253,** 531-44.

KLIPPEL, A., ESCOBEDO, M. A., WACHOWICZ, M. S., APELL, G., BROWN, T. W., GIEDLIN, M. A., KAVANAUGH, W. M. & WILLIAMS, L. T. (1998). Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. *Mol Cell Biol* **18,** 5699-711.

KOLCH, W., KOTWALIWALE, A., VASS, K. & JANOSCH, P. (2002). The role of Raf kinases in malignant transformation. *Expert Rev Mol Med* **2002,** 1-18.

KOMURO, A., HAYAMI, M., FUJII, H., MIYAHARA, S. & HIRAYAMA, M. (1983). Vertical transmission of adult T-cell leukaemia virus. *Lancet* **1,** 240.

KORNER, A. (1966). Effect of cycloheximide on protein biosynthesis in rat liver. *Biochem J* **101,** 627-631.

KOZAKO, T., ARIMA, N., TOJI, S., MASAMOTO, I., AKIMOTO, M., HAMADA, H., CHE, X. F., FUJIWARA, H., MATSUSHITA, K., TOKUNAGA, M., HARAGUCHI, K., UOZUMI, K., SUZUKI, S., TAKEZAKI, T. & SONODA, S. (2006). Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8+ T cell in adult T cell leukemia patients. *J Immunol* **177,** 5718-26.

KRAMMER, P. H. (2000). CD95's deadly mission in the immune system. *Nature* **407,** 789-95.

KRAMMER, P. H., DHEIN, J., WALCZAK, H., BEHRMANN, I., MARIANI, S., MATIBA, B., FATH, M., DANIEL, P. T., KNIPPING, E., WESTENDORP, M. O. & ET AL. (1994). The role of APO-1-mediated apoptosis in the immune system. *Immunol Rev* **142,** 175-91.

KREUZ, S., SIEGMUND, D., SCHEURICH, P. & WAJANT, H. (2001). NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol* **21,** 3964-73.

KRUEGER, A., BAUMANN, S., KRAMMER, P. H. & KIRCHHOFF, S. (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* **21,** 8247-54.

KRUEGER, A., FAS, S. C., GIAISI, M., BLEUMINK, M., MERLING, A., STUMPF, C., BAUMANN, S., HOLTKOTTE, D., BOSCH, V., KRAMMER, P. H. & LI-WEBER, M. (2006). HTLV-1 Tax protects against CD95-mediated apoptosis by induction of the cellular FLICE-inhibitory protein (c-FLIP). *Blood* **107,** 3933-9.

KUPSCH, P., HENNING, B. F., PASSARGE, K., RICHLY, H., WIESEMANN, K., HILGER, R. A., SCHEULEN, M. E., CHRISTENSEN, O., BRENDEL, E., SCHWARTZ, B., HOFSTRA, E., VOIGTMANN, R., SEEBER, S. & STRUMBERG, D. (2005). Results of a phase I trial of sorafenib (BAY 43-9006) in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer. *Clin Colorectal Cancer* **5,** 188-96.

KYRIAKIS, J. M. & AVRUCH, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* **271,** 24313-6.

LAVOIE, J. N., L'ALLEMAIN, G., BRUNET, A., MULLER, R. & POUYSSEGUR, J. (1996). Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271,** 20608-16.

LEE, S. K., CUI, B., MEHTA, R. R., KINGHORN, A. D. & PEZZUTO, J. M. (1998). Cytostatic mechanism and antitumor potential of novel 1H-cyclopenta[b]benzofuran lignans isolated from Aglaia elliptica. *Chem Biol Interact* **115,** 215-28.

LEMOINE, F. J. & MARRIOTT, S. J. (2001). Accelerated G(1) phase progression induced by the human T cell leukemia virus type I (HTLV-I) Tax oncoprotein. *J Biol Chem* **276,** 31851-7.

LEONARDO, M.J., Molecular regulation of T lymphocyte homeostasis in the healthy and diseased immune system. (2003) *Immunol Res* **27,** 387-98.

LEUNG, K. & NABEL, G. J. (1988). HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-kappa B-like factor. *Nature* **333,** 776-8.

LI, Q. & VERMA, I. M. (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2,** 725-34.

LI, S., TAKASU, T., PERLMAN, D. M., PETERSON, M. S., BURRICHTER, D., AVDULOV, S., BITTERMAN, P. B. & POLUNOVSKY, V. A. (2003). Translation factor eIF4E rescues

cells from Myc-dependent apoptosis by inhibiting cytochrome c release. *J Biol Chem* **278,** 3015-22.

LI, X. H., FANG, X. & GAYNOR, R. B. (2001). Role of IKKgamma/nemo in assembly of the Ikappa B kinase complex. *J Biol Chem* **276,** 4494-500.

LIU, L., CAO, Y., CHEN, C., ZHANG, X., MCNABOLA, A., WILKIE, D., WILHELM, S., LYNCH, M. & CARTER, C. (2006). Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* **66,** 11851-8.

LIU, Y., WANG, Y., YAMAKUCHI, M., MASUDA, S., TOKIOKA, T., YAMAOKA, S., MARUYAMA, I. & KITAJIMA, I. (2001). Phosphoinositide-3 kinase-PKB/Akt pathway activation is involved in fibroblast Rat-1 transformation by human T-cell leukemia virus type I tax. *Oncogene* **20,** 2514-26.

LONGLEY, D. B., WILSON, T. R., MCEWAN, M., ALLEN, W. L., MCDERMOTT, U., GALLIGAN, L. & JOHNSTON, P. G. (2006). c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. *Oncogene* **25,** 838-48.

LOS, M., WESSELBORG, S. & SCHULZE-OSTHOFF, K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity* **10,** 629-39.

LOW, K. G., DORNER, L. F., FERNANDO, D. B., GROSSMAN, J., JEANG, K. T. & COMB, M. J. (1997). Human T-cell leukemia virus type 1 Tax releases cell cycle arrest induced by p16INK4a. *J Virol* **71,** 1956-62.

MARIANI, S. M., MATIBA, B., BAUMLER, C. & KRAMMER, P. H. (1995). Regulation of cell surface APO-1/Fas (CD95) ligand expression by metalloproteases. *Eur J Immunol* **25,** 2303-7.

MARINER, J. M., LANTZ, V., WALDMANN, T. A. & AZIMI, N. (2001). Human T cell lymphotropic virus type I Tax activates IL-15R alpha gene expression through an NF-kappa B site. *J Immunol* **166,** 2602-9.

MARUYAMA, M., SHIBUYA, H., HARADA, H., HATAKEYAMA, M., SEIKI, M., FUJITA, T., INOUE, J., YOSHIDA, M. & TANIGUCHI, T. (1987). Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded p40x and T3/Ti complex triggering. *Cell* **48,** 343-50.

MATHAS, S., LIETZ, A., ANAGNOSTOPOULOS, I., HUMMEL, F., WIESNER, B., JANZ, M., JUNDT, F., HIRSCH, B., JOHRENS-LEDER, K., VORNLOCHER, H. P., BOMMERT, K., STEIN, H. & DORKEN, B. (2004). c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med* **199,** 1041-52.

MATSUDA, T., ALMASAN, A., TOMITA, M., UCHIHARA, J. N., MASUDA, M., OHSHIRO, K., TAKASU, N., YAGITA, H., OHTA, T. & MORI, N. (2005). Resistance to Apo2 ligand (Apo2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and constitutive expression of Apo2L/TRAIL in human T-cell leukemia virus type 1-infected T-cell lines. *J Virol* **79,** 1367-78.

MCKAY, M. M. & MORRISON, D. K. (2007). Integrating signals from RTKs to ERK/MAPK. *Oncogene* **26,** 3113-21.

MCKINSEY, T. A., BROCKMAN, J. A., SCHERER, D. C., AL-MURRANI, S. W., GREEN, P. L. & BALLARD, D. W. (1996). Inactivation of IkappaBbeta by the tax protein of human T-cell leukemia virus type 1: a potential mechanism for constitutive induction of NF-kappaB. *Mol Cell Biol* **16,** 2083-90.

MCPHILLIPS, F., MULLEN, P., MONIA, B. P., RITCHIE, A. A., DORR, F. A., SMYTH, J. F. & LANGDON, S. P. (2001). Association of c-Raf expression with survival and its

targeting with antisense oligonucleotides in ovarian cancer. *Br J Cancer* **85,** 1753-8.

MELOCHE, S. & POUYSSEGUR, J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene* **26,** 3227-39.

MERCURIO, F., ZHU, H., MURRAY, B. W., SHEVCHENKO, A., BENNETT, B. L., LI, J., YOUNG, D. B., BARBOSA, M., MANN, M., MANNING, A. & RAO, A. (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278,** 860-6.

MICHEAU, O., LENS, S., GAIDE, O., ALEVIZOPOULOS, K. & TSCHOPP, J. (2001). NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* **21,** 5299-305.

MITSIADES, N., MITSIADES, C. S., POULAKI, V., ANDERSON, K. C. & TREON, S. P. (2002). Intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human multiple myeloma cells. *Blood* **99,** 2162-71.

MITSUYA, H., MATIS, L. A., MEGSON, M., BUNN, P. A., MURRAY, C., MANN, D. L., GALLO, R. C. & BRODER, S. (1983). Generation of an HLA-restricted cytotoxic T cell line reactive against cultured tumor cells from a patient infected with human T cell leukemia/lymphoma virus. *J Exp Med* **158,** 994-9.

MITTL, P. R., DI MARCO, S., KREBS, J. F., BAI, X., KARANEWSKY, D. S., PRIESTLE, J. P., TOMASELLI, K. J. & GRUTTER, M. G. (1997). Structure of recombinant human CPP32 in complex with the tetrapeptide acetyl-Asp-Val-Ala-Asp fluoromethyl ketone. *J Biol Chem* **272,** 6539-47.

MOCHIZUKI, M., WATANABE, T., YAMAGUCHI, K., YOSHIMURA, K., NAKASHIMA, S., SHIRAO, M., ARAKI, S., TAKATSUKI, K., MORI, S. & MIYATA, N. (1992). Uveitis associated with human T-cell lymphotropic virus type I. *Am J Ophthalmol* **114,** 123-9.

MORI, N., FUJII, M., CHENG, G., IKEDA, S., YAMASAKI, Y., YAMADA, Y., TOMONAGA, M. & YAMAMOTO, N. (2001). Human T-cell leukemia virus type I tax protein induces the expression of anti-apoptotic gene Bcl-xL in human T-cells through nuclear factor-kappaB and c-AMP responsive element binding protein pathways. *Virus Genes* **22,** 279-87.

MORLEY, S. J. & MCKENDRICK, L. (1997). Involvement of stress-activated protein kinase and p38/RK mitogen-activated protein kinase signaling pathways in the enhanced phosphorylation of initiation factor 4E in NIH 3T3 cells. *J Biol Chem* **272,** 17887-93.

MOSLEY, A. J., ASQUITH, B. & BANGHAM, C. R. (2005). Cell-mediated immune response to human T-lymphotropic virus type I. *Viral Immunol* **18,** 293-305.

MUKHERJEE, R., BARTLETT, J. M., KRISHNA, N. S., UNDERWOOD, M. A. & EDWARDS, J. (2005). Raf-1 expression may influence progression to androgen insensitive prostate cancer. *Prostate* **64,** 101-7.

MUZIO, M., STOCKWELL, B. R., STENNICKE, H. R., SALVESEN, G. S. & DIXIT, V. M. (1998). An induced proximity model for caspase-8 activation. *J Biol Chem* **273,** 2926-30.

NAGAR, B., BORNMANN, W. G., PELLICENA, P., SCHINDLER, T., VEACH, D. R., MILLER, W. T., CLARKSON, B. & KURIYAN, J. (2002). Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* **62,** 4236-43.

NAGATA, S. & GOLSTEIN, P. (1995). The Fas death factor. *Science* **267,** 1449-56.

NAISMITH, J. H. & SPRANG, S. R. (1998). Modularity in the TNF-receptor family. *Trends Biochem Sci* **23,** 74-9.

NAKANO, H., SHINDO, M., SAKON, S., NISHINAKA, S., MIHARA, M., YAGITA, H. & OKUMURA, K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc Natl Acad Sci U S A* **95,** 3537-42.

NERENBERG, M., HINRICHS, S. H., REYNOLDS, R. K., KHOURY, G. & JAY, G. (1987). The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. *Science* **237,** 1324-9.

NICOLETTI, I., MIGLIORATI, G., PAGLIACCI, M. C., GRIGNANI, F. & RICCARDI, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139,** 271-9.

NICOT, C., TIE, F. & GIAM, C. Z. (1998). Cytoplasmic forms of human T-cell leukemia virus type 1 Tax induce NF-kappaB activation. *J Virol* **72,** 6777-84.

NISHIOKA, K., MARUYAMA, I., SATO, K., KITAJIMA, I., NAKAJIMA, Y. & OSAME, M. (1989). Chronic inflammatory arthropathy associated with HTLV-I. *Lancet* **1,** 441.

NORBURY, C. J. & ZHIVOTOVSKY, B. (2004). DNA damage-induced apoptosis. *Oncogene* **23,** 2797-808.

NURSE, P. (1975). Genetic control of cell size at cell division in yeast. *Nature* **256,** 547-51.

O'CONNELL, J., O'SULLIVAN, G. C., COLLINS, J. K. & SHANAHAN, F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J Exp Med* **184,** 1075-82.

OEHM, A., BEHRMANN, I., FALK, W., PAWLITA, M., MAIER, G., KLAS, C., LI-WEBER, M., RICHARDS, S., DHEIN, J., TRAUTH, B. C. & ET AL. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J Biol Chem* **267,** 10709-15.

OKA, H., CHATANI, Y., HOSHINO, R., OGAWA, O., KAKEHI, Y., TERACHI, T., OKADA, Y., KAWAICHI, M., KOHNO, M. & YOSHIDA, O. (1995). Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res* **55,** 4182-7.

OKADA, H. & MAK, T. W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* **4,** 592-603.

OKAMOTO, K., FUJISAWA, J., RETH, M. & YONEHARA, S. (2006). Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kappaB. *Genes Cells* **11,** 177-91.

OSAME, M., USUKU, K., IZUMO, S., IJICHI, N., AMITANI, H., IGATA, A., MATSUMOTO, M. & TARA, M. (1986). HTLV-I associated myelopathy, a new clinical entity. *Lancet* **1,** 1031-2.

OYARZO, M. P., MEDEIROS, L. J., ATWELL, C., FERETZAKI, M., LEVENTAKI, V., DRAKOS, E., AMIN, H. M. & RASSIDAKIS, G. Z. (2006). c-FLIP confers resistance to FAS-mediated apoptosis in anaplastic large-cell lymphoma. *Blood* **107,** 2544-7.

PANKA, D. J., MANO, T., SUHARA, T., WALSH, K. & MIER, J. W. (2001). Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J Biol Chem* **276,** 6893-6.

PETER, M. E., HEUFELDER, A. E. & HENGARTNER, M. O. (1997). Advances in apoptosis research. *Proc Natl Acad Sci U S A* **94,** 12736-7.

PETIT, P. X., LECOEUR, H., ZORN, E., DAUGUET, C., MIGNOTTE, B. & GOUGEON, M. L. (1995). Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* **130,** 157-67.

POIESZ, B. J., POIESZ, M. J. & CHOI, D. (2003). The human T-cell lymphoma/leukemia viruses. *Cancer Invest* **21,** 253-77.

POIESZ, B. J., RUSCETTI, F. W., GAZDAR, A. F., BUNN, P. A., MINNA, J. D. & GALLO, R. C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A* **77,** 7415-9.

POMERANTZ, J. L. & BALTIMORE, D. (2002). Two pathways to NF-kappaB. *Mol Cell* **10,** 693-5.

PORTO, A. F., NEVA, F. A., BITTENCOURT, H., LISBOA, W., THOMPSON, R., ALCANTARA, L. & CARVALHO, E. M. (2001). HTLV-1 decreases Th2 type of immune response in patients with strongyloidiasis. *Parasite Immunol* **23,** 503-7.

POULAKI, V., MITSIADES, C. S., KOTOULA, V., TSELENI-BALAFOUTA, S., ASHKENAZI, A., KOUTRAS, D. A. & MITSIADES, N. (2002). Regulation of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in thyroid carcinoma cells. *Am J Pathol* **161,** 643-54.

POZZATTI, R., VOGEL, J. & JAY, G. (1990). The human T-lymphotropic virus type I tax gene can cooperate with the ras oncogene to induce neoplastic transformation of cells. *Mol Cell Biol* **10,** 413-7.

PROKSCH, P., GIAISI, M., TREIBER, M. K., PALFI, K., MERLING, A., SPRING, H., KRAMMER, P. H. & LI-WEBER, M. (2005). Rocaglamide derivatives are immunosuppressive phytochemicals that target NF-AT activity in T cells. *J Immunol* **174,** 7075-84.

RAUGHT, B. & GINGRAS, A. C. (1999). eIF4E activity is regulated at multiple levels. *Int J Biochem Cell Biol* **31,** 43-57.

RICHARDSON, J. H., HOLLSBERG, P., WINDHAGEN, A., CHILD, L. A., HAFLER, D. A. & LEVER, A. M. (1997). Variable immortalizing potential and frequent virus latency in blood-derived T-cell clones infected with human T-cell leukemia virus type I. *Blood* **89,** 3303-14.

RINKER-SCHAEFFER, C. W., AUSTIN, V., ZIMMER, S. & RHOADS, R. E. (1992). Ras transformation of cloned rat embryo fibroblasts results in increased rates of protein synthesis and phosphorylation of eukaryotic initiation factor 4E. *J Biol Chem* **267,** 10659-64.

RIPPO, M. R., MORETTI, S., VESCOVI, S., TOMASETTI, M., ORECCHIA, S., AMICI, G., CATALANO, A. & PROCOPIO, A. (2004). FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. *Oncogene* **23,** 7753-60.

ROTONDA, J., NICHOLSON, D. W., FAZIL, K. M., GALLANT, M., GAREAU, Y., LABELLE, M., PETERSON, E. P., RASPER, D. M., RUEL, R., VAILLANCOURT, J. P., THORNBERRY, N. A. & BECKER, J. W. (1996). The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat Struct Biol* **3,** 619-25.

ROY, N., DEVERAUX, Q. L., TAKAHASHI, R., SALVESEN, G. S. & REED, J. C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J* **16,** 6914-25.

RUBEN, S., POTEAT, H., TAN, T. H., KAWAKAMI, K., ROEDER, R., HASELTINE, W. & ROSEN, C. A. (1988). Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-I tax gene product. *Science* **241,** 89-92.

SANTIAGO, F., CLARK, E., CHONG, S., MOLINA, C., MOZAFARI, F., MAHIEUX, R., FUJII, M., AZIMI, N. & KASHANCHI, F. (1999). Transcriptional up-regulation of the cyclin D2 gene and acquisition of new cyclin-dependent kinase partners in human T-cell leukemia virus type 1-infected cells. *J Virol* **73,** 9917-27.

SATOU, Y., NOSAKA, K., KOYA, Y., YASUNAGA, J. I., TOYOKUNI, S. & MATSUOKA, M. (2004). Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro. *Leukemia* **18,** 1357-63.

SAVILL, J., FADOK, V., HENSON, P. & HASLETT, C. (1993). Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* **14,** 131-6.

SCAFFIDI, C., FULDA, S., SRINIVASAN, A., FRIESEN, C., LI, F., TOMASELLI, K. J., DEBATIN, K. M., KRAMMER, P. H. & PETER, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17,** 1675-87.

SCAFFIDI, C., SCHMITZ, I., KRAMMER, P. H. & PETER, M. E. (1999). The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* **274,** 1541-8.

SCHULZE-OSTHOFF, K., FERRARI, D., LOS, M., WESSELBORG, S. & PETER, M. E. (1998). Apoptosis signaling by death receptors. *Eur J Biochem* **254,** 439-59.

SCOTT, M. L., FUJITA, T., LIOU, H. C., NOLAN, G. P. & BALTIMORE, D. (1993). The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev* **7,** 1266-76.

SEBOLT-LEOPOLD, J. S., DUDLEY, D. T., HERRERA, R., VAN BECELAERE, K., WILAND, A., GOWAN, R. C., TECLE, H., BARRETT, S. D., BRIDGES, A., PRZYBRANOWSKI, S., LEOPOLD, W. R. & SALTIEL, A. R. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med* **5,** 810-6.

SEBOLT-LEOPOLD, J. S. & HERRERA, R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* **4,** 937-47.

SEIKI, M., HATTORI, S., HIRAYAMA, Y. & YOSHIDA, M. (1983). Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc Natl Acad Sci U S A* **80,** 3618-22.

SEIKI, M., INOUE, J., TAKEDA, T. & YOSHIDA, M. (1986). Direct evidence that p40x of human T-cell leukemia virus type I is a trans-acting transcriptional activator. *Embo J* **5,** 561-5.

SENFTLEBEN, U., CAO, Y., XIAO, G., GRETEN, F. R., KRAHN, G., BONIZZI, G., CHEN, Y., HU, Y., FONG, A., SUN, S. C. & KARIN, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293,** 1495-9.

SHAULIAN, E. & KARIN, M. (2002). AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4,** E131-6.

SHERIDAN, J. P., MARSTERS, S. A., PITTI, R. M., GURNEY, A., SKUBATCH, M., BALDWIN, D., RAMAKRISHNAN, L., GRAY, C. L., BAKER, K., WOOD, W. I., GODDARD, A. D., GODOWSKI, P. & ASHKENAZI, A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277,** 818-21.

SHIMOYAMA, M. (1991). Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). *Br J Haematol* **79,** 428-37.

SHU, H. B., HALPIN, D. R. & GOEDDEL, D. V. (1997). Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* **6,** 751-63.

SHUH, M. & DERSE, D. (2000). Ternary complex factors and cofactors are essential for human T-cell leukemia virus type 1 tax transactivation of the serum response element. *J Virol* **74,** 11394-7.

SIEBENLIST, U., FRANZOSO, G. & BROWN, K. (1994). Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* **10,** 405-55.

SIEGEL, R. S., GARTENHAUS, R. B. & KUZEL, T. M. (2001). Human T-cell lymphotropic-I-associated leukemia/lymphoma. *Curr Treat Options Oncol* **2,** 291-300.

SMALLEY, K. S. & HERLYN, M. (2005). Targeting intracellular signaling pathways as a novel strategy in melanoma therapeutics. *Ann N Y Acad Sci* **1059,** 16-25.

SODROSKI, J., ROSEN, C., GOH, W. C. & HASELTINE, W. (1985). A transcriptional activator protein encoded by the x-lor region of the human T-cell leukemia virus. *Science* **228,** 1430-4.

SONODA, S., FUJIYOSHI, T. & YASHIKI, S. (1996). Immunogenetics of HTLV-I/II and associated diseases. *J Acquir Immune Defic Syndr Hum Retrovirol* **13 Suppl 1,** S119-23.

SPRICK, M. R., RIESER, E., STAHL, H., GROSSE-WILDE, A., WEIGAND, M. A. & WALCZAK, H. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *Embo J* **21,** 4520-30.

STELLER, H. (1995). Mechanisms and genes of cellular suicide. *Science* **267,** 1445-9.

STENNICKE, H. R. & SALVESEN, G. S. (1998). Properties of the caspases. *Biochim Biophys Acta* **1387,** 17-31.

STRAND, S., HOFMANN, W. J., HUG, H., MULLER, M., OTTO, G., STRAND, D., MARIANI, S. M., STREMMEL, W., KRAMMER, P. H. & GALLE, P. R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? *Nat Med* **2,** 1361-6.

SUDA, T., TAKAHASHI, T., GOLSTEIN, P. & NAGATA, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75,** 1169-78.

SUHARA, T., MANO, T., OLIVEIRA, B. E. & WALSH, K. (2001). Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis *via* regulation of FLICE-inhibitory protein (FLIP). *Circ Res* **89,** 13-9.

SUN, S. C., ELWOOD, J., BERAUD, C. & GREENE, W. C. (1994). Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the Rel A gene. *Mol Cell Biol* **14,** 7377-84.

SUZUKI, T., FUJISAWA, J. I., TOITA, M. & YOSHIDA, M. (1993). The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1. *Proc Natl Acad Sci U S A* **90,** 610-4.

TAGUCHI, H., KINOSHITA, K. I., TAKATSUKI, K., TOMONAGA, M., ARAKI, K., ARIMA, N., IKEDA, S., UOZUMI, K., KOHNO, H., KAWANO, F., KIKUCHI, H., TAKAHASHI, H., TAMURA, K., CHIYODA, S., TSUDA, H., NISHIMURA, H., HOSOKAWA, T., MATSUZAKI, H., MOMITA, S., YAMADA, O. & MIYOSHI, I. (1996). An intensive chemotherapy of adult T-cell leukemia/lymphoma: CHOP followed by etoposide, vindesine, ranimustine, and mitoxantrone with granulocyte colony-stimulating factor support. *J Acquir Immune Defic Syndr Hum Retrovirol* **12,** 182-6.

TAJIMA, K. (1988). Malignant lymphomas in Japan: epidemiological analysis of adult T-cell leukemia/lymphoma (ATL). *Cancer Metastasis Rev* **7,** 223-41.

TAKAHASHI, T., TANAKA, M., INAZAWA, J., ABE, T., SUDA, T. & NAGATA, S. (1994). Human Fas ligand: gene structure, chromosomal location and species specificity. *Int Immunol* **6,** 1567-74.

TAN, A., BITTERMAN, P., SONENBERG, N., PETERSON, M. & POLUNOVSKY, V. (2000). Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. *Oncogene* **19,** 1437-47.

TAN, C. & WALDMANN, T. A. (2002). Proteasome inhibitor PS-341, a potential therapeutic agent for adult T-cell leukemia. *Cancer Res* **62,** 1083-6.

TANAKA, M., ITAI, T., ADACHI, M. & NAGATA, S. (1998). Downregulation of Fas ligand by shedding. *Nat Med* **4,** 31-6.

TANIMURA, S., NOMURA, K., OZAKI, K., TSUJIMOTO, M., KONDO, T. & KOHNO, M. (2002). Prolonged nuclear retention of activated extracellular signal-regulated kinase 1/2 is required for hepatocyte growth factor-induced cell motility. *J Biol Chem* **277,** 28256-64.

TARTAGLIA, L. A., AYRES, T. M., WONG, G. H. & GOEDDEL, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74,** 845-53.

TERADA, K., KATAMINE, S., EGUCHI, K., MORIUCHI, R., KITA, M., SHIMADA, H., YAMASHITA, I., IWATA, K., TSUJI, Y., NAGATAKI, S. & ET AL. (1994). Prevalence of serum and salivary antibodies to HTLV-1 in Sjogren's syndrome. *Lancet* **344,** 1116-9.

THOMPSON, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456-62.

THORNBERRY, N. A. & LAZEBNIK, Y. (1998). Caspases: enemies within. *Science* **281,** 1312-6.

TOCHIKURA, T., IWAHASHI, M., MATSUMOTO, T., KOYANAGI, Y., HINUMA, Y. & YAMAMOTO, N. (1985). Effect of human serum anti-HTLV antibodies on viral antigen induction in vitro cultured peripheral lymphocytes from adult T-cell leukemia patients and healthy virus carriers. *Int J Cancer* **36,** 1-7.

TRAUTH, B. C., KLAS, C., PETERS, A. M., MATZKU, S., MOLLER, P., FALK, W., DEBATIN, K. M. & KRAMMER, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* **245,** 301-5.

TSANG, C. K., QI, H., LIU, L. F. & ZHENG, X. F. (2007). Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today* **12,** 112-24.

TSUKAHARA, T., KANNAGI, M., OHASHI, T., KATO, H., ARAI, M., NUNEZ, G., IWANAGA, Y., YAMAMOTO, N., OHTANI, K., NAKAMURA, M. & FUJII, M. (1999). Induction of Bcl-x(L) expression by human T-cell leukemia virus type 1 Tax through NF-kappaB in apoptosis-resistant T-cell transfectants with Tax. *J Virol* **73,** 7981-7.

UHLIK, M., GOOD, L., XIAO, G., HARHAJ, E. W., ZANDI, E., KARIN, M. & SUN, S. C. (1998). NF-kappaB-inducing kinase and IkappaB kinase participate in human T-cell leukemia virus I Tax-mediated NF-kappaB activation. *J Biol Chem* **273,** 21132-6.

URIARTE, S. M., JOSHI-BARVE, S., SONG, Z., SAHOO, R., GOBEJISHVILI, L., JALA, V. R., HARIBABU, B., MCCLAIN, C. & BARVE, S. (2005). Akt inhibition upregulates FasL, downregulates c-FLIPs and induces caspase-8-dependent cell death in Jurkat T lymphocytes. *Cell Death Differ* **12,** 233-42.

USUKU, K., SONODA, S., OSAME, M., YASHIKI, S., TAKAHASHI, K., MATSUMOTO, M., SAWADA, T., TSUJI, K., TARA, M. & IGATA, A. (1988). HLA haplotype-linked high immune responsiveness against HTLV-I in HTLV-I-associated myelopathy: comparison with adult T-cell leukemia/lymphoma. *Ann Neurol* **23 Suppl,** S143-50.

VAN PARIJS, L. & ABBAS, A. K. (1996). Role of Fas-mediated cell death in the regulation of immune responses. *Curr Opin Immunol* **8,** 355-61.

VAUX, D. L. & KORSMEYER, S. J. (1999). Cell death in development. *Cell* **96,** 245-54.

VERMA, I. M., STEVENSON, J. K., SCHWARZ, E. M., VAN ANTWERP, D. & MIYAMOTO, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9,** 2723-35.

VINALS, F., CHAMBARD, J. C. & POUYSSEGUR, J. (1999). p70 S6 kinase-mediated protein synthesis is a critical step for vascular endothelial cell proliferation. *J Biol Chem* **274,** 26776-82.

VINCENZ, C. & DIXIT, V. M. (1997). Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J Biol Chem* **272,** 6578-83.

VINE, A. M., HEAPS, A. G., KAFTANTZI, L., MOSLEY, A., ASQUITH, B., WITKOVER, A., THOMPSON, G., SAITO, M., GOON, P. K., CARR, L., MARTINEZ-MURILLO, F., TAYLOR, G. P. & BANGHAM, C. R. (2004). The role of CTLs in persistent viral infection: cytolytic gene expression in CD8+ lymphocytes distinguishes between individuals with a high or low proviral load of human T cell lymphotropic virus type 1. *J Immunol* **173,** 5121-9.

WALCZAK, H., MILLER, R. E., ARIAIL, K., GLINIAK, B., GRIFFITH, T. S., KUBIN, M., CHIN, W., JONES, J., WOODWARD, A., LE, T., SMITH, C., SMOLAK, P., GOODWIN, R. G., RAUCH, C. T., SCHUH, J. C. & LYNCH, D. H. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5,** 157-63.

WALDMANN, T. A., GOLDMAN, C. K., BONGIOVANNI, K. F., SHARROW, S. O., DAVEY, M. P., CEASE, K. B., GREENBERG, S. J. & LONGO, D. L. (1988). Therapy of patients with human T-cell lymphotrophic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2. *Blood* **72,** 1805-16.

WALDMANN, T. A., WHITE, J. D., CARRASQUILLO, J. A., REYNOLDS, J. C., PAIK, C. H., GANSOW, O. A., BRECHBIEL, M. W., JAFFE, E. S., FLEISHER, T. A., GOLDMAN, C. K., TOP, L. E., BAMFORD, R., ZAKNOEN, E., ROESSLER, E., KASTEN-SPORTES, C., ENGLAND, R., LITOU, H., JOHNSON, J. A., JACKSON-WHITE, T., MANNS, A., HANCHARD, B., JUNGHANS, R. P. & NELSON, D. L. (1995). Radioimmunotherapy of interleukin-2R alpha-expressing adult T-cell leukemia with Yttrium-90-labeled anti-Tac. *Blood* **86,** 4063-75.

WANG, J., CHUN, H. J., WONG, W., SPENCER, D. M. & LENARDO, M. J. (2001). Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A* **98,** 13884-8.

WANG, X., FLYNN, A., WASKIEWICZ, A. J., WEBB, B. L., VRIES, R. G., BAINES, I. A., COOPER, J. A. & PROUD, C. G. (1998). The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J Biol Chem* **273,** 9373-7.

WANG, Z., GOULET, R., 3RD, STANTON, K. J., SADARIA, M. & NAKSHATRI, H. (2005). Differential effect of anti-apoptotic genes Bcl-xL and c-FLIP on sensitivity of MCF-7 breast cancer cells to paclitaxel and docetaxel. *Anticancer Res* **25,** 2367-79.

WASKIEWICZ, A. J. & COOPER, J. A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr Opin Cell Biol* **7,** 798-805.

WASKIEWICZ, A. J., FLYNN, A., PROUD, C. G. & COOPER, J. A. (1997). Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *Embo J* **16,** 1909-20.

WATANABE, T., SEIKI, M. & YOSHIDA, M. (1984). HTLV type I (U. S. isolate) and ATLV (Japanese isolate) are the same species of human retrovirus. *Virology* **133,** 238-41.

WATANABE, T., YAMAGUCHI, K., TAKATSUKI, K., OSAME, M. & YOSHIDA, M. (1990). Constitutive expression of parathyroid hormone-related protein gene in human T cell leukemia virus type 1 (HTLV-1) carriers and adult T cell leukemia patients that can be trans-activated by HTLV-1 tax gene. *J Exp Med* **172,** 759-65.

WOO, S. H., PARK, I. C., PARK, M. J., AN, S., LEE, H. C., JIN, H. O., PARK, S. A., CHO, H., LEE, S. J., GWAK, H. S., HONG, Y. J., HONG, S. I. & RHEE, C. H. (2004). Arsenic trioxide sensitizes CD95/Fas-induced apoptosis through ROS-mediated upregulation of CD95/Fas by NF-kappaB activation. *Int J Cancer* **112,** 596-606.

WUCHTER, C., KRAPPMANN, D., CAI, Z., RUPPERT, V., SCHEIDEREIT, C., DORKEN, B., LUDWIG, W. D. & KARAWAJEW, L. (2001). In vitro susceptibility to TRAIL-induced apoptosis of acute leukemia cells in the context of TRAIL receptor gene expression and constitutive NF-kappa B activity. *Leukemia* **15,** 921-8.

WYLLIE, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555-6.

XIAO, G., HARHAJ, E. W. & SUN, S. C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* **7,** 401-9.

XU, X., HEIDENREICH, O., KITAJIMA, I., MCGUIRE, K., LI, Q., SU, B. & NERENBERG, M. (1996). Constitutively activated JNK is associated with HTLV-1 mediated tumorigenesis. *Oncogene* **13,** 135-42.

YAMAOKA, S., COURTOIS, G., BESSIA, C., WHITESIDE, ST., WEIL, R., AGOU, F., KIRK, HE., KAY, RJ., ISRAEL, A., Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**, 1231-40

YAMAGUCHI, K. (1994). Human T-lymphotropic virus type I in Japan. *Lancet* **343,** 213-6.

YASUNAGA, J., SAKAI, T., NOSAKA, K., ETOH, K., TAMIYA, S., KOGA, S., MITA, S., UCHINO, M., MITSUYA, H. & MATSUOKA, M. (2001). Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state. *Blood* **97,** 3177-83.

YEH, J. H., HSU, S. C., HAN, S. H. & LAI, M. Z. (1998). Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. *J Exp Med* **188,** 1795-802.

YONEHARA, S., ISHII, A. & YONEHARA, M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J Exp Med* **169,** 1747-56.

YOSHIDA, M. (2001). Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. *Annu Rev Immunol* **19,** 475-96.

YOSHIDA, M., MIYOSHI, I. & HINUMA, Y. (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci U S A* **79,** 2031-5.

YOSHIOKA, C., MURAKI, Y., FUKUDA, J., HANEJI, T. & KOBAYASHI, N. (1996). Identification of the Fas antigen in human gingiva. *J Dent Res* **75,** 1353-7.

ZAMZAMI, N., MARCHETTI, P., CASTEDO, M., ZANIN, C., VAYSSIERE, J. L., PETIT, P. X. & KROEMER, G. (1995). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* **181,** 1661-72.

ZANDI, E., ROTHWARF, DM., DELHASE, M., HAYAKAWA, M., KARIN, M., (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91,** 243-52

ZHA, J., HARADA, H., OSIPOV, K., JOCKEL, J., WAKSMAN, G. & KORSMEYER, S. J. (1997). BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. *J Biol Chem* **272,** 24101-4.

ZHANG, X., JIN, T. G., YANG, H., DEWOLF, W. C., KHOSRAVI-FAR, R. & OLUMI, A. F. (2004). Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* **64,** 7086-91.

ZHU, J.Y., LAVRIK, I.N., MAHLKNECHT, U., GIAISI, M., PROKSCH, P., KRAMMER, P.H., LI WEBER, M. (2007). The traditional Chinese herbal compound rocaglamide preferentially induces apoptosis in leukemia cells by modulation of mitogen-activated protein kinase activities. *Int J Cancer, in press*

ZOU, H., LI, Y., LIU, X. & WANG, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* **274,** 11549-56.

ABBREVIATIONS

4E-BP1	eIF4E-binding protein
Ab	antibody
AICD	Activation-Induced Cell Death
AIDS	Acquired Immunodeficiency Syndrome
ALPS	Autoimmune Lymphoproliferative Syndrome
Apaf-1	apoptosis protease-activating factor 1
APS	Ammonium peroxidisulfate
Asp	Aspartate
ATL	adult T-cell leukemia/lymphoma
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BH	Bcl-2 homology
bp	Base pare
caspase	cysteine aspartate-specific protease
CD	cluster of differentiation
CDK	Cyclin-dependent kinase
cDNA	complementary desoxy-ribose-nucleic acid
c-FLIP	cellular FLICE-inhibitory protein
CHX	Cycloheximide
CREB	cAMP responsive element binding factor
CsA	Cyclosporin A
DAG	Diacylglycerol
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DN	Dominant negative
DNA	desoxy-ribose-nucleic acid
DR	death receptor
DTT	Dithiotreitol

ECL	enhanced chemiluminescence
eIF	eukaryotic initiation factor
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FCS	fetal calf serum
FLICE	FADD-like ICE
FSC/SSC	Forward Scatter, sideward scatter
HAM	HTLV-1-associated myelopathy
hr	hour
HRP	horseradish peroxidase
HTLV	Human T-Cell Leukemia Virus
IAP	inhibitors of apoptotic proteins
IC_{50}	Inhibitory concentration 50%
ICE	Interleukin-1β converting enzyme
IEF	Isoelectric focusing
IgG	Immunoglobulin G class
IκB	Inhibitor of κB proteins
IKK	I-κB kinase
IL	Interleukine
JNK	c-Jun N-terminal Kinase
kD	Kilodalton
LTR	Long terminal repeat
LZ	Leucine-Zipper
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MEKK1	MAPK/ERK Kinase Kinase 1
min	Minute
Mnk1	MAP kinase interacting kinase 1
mRNA	Messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells

NF-κB	nuclear factor κB
NGF	Nerve growth factor
NIK	NFkB Inducing Kinase
O/N	Over night
ORF	Open reading frame
PAGE	Polyacrylamide gel-elektrophoresis
PCR	polymerase chain reaction
PI	Isoelectric point
PI3K	Phosphoinositide-3 kinase
PMA	phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
Roc-AR	Rocaglamide derivative AR (oxo-11,12-methylendioxy-rocglaol)
RT	Reverse Transcriptase
RT	room temperature
RT-PCR	Reverse Transkription Polymerase chain reaction
SDS	Natriumdodecylsulfate
TCR	T cell receptor
TEMED	Tetramethylethyl endiamin
TNF	Tumor Necrosis Factor
TNF-R	Tumor Necrosis Factor-Receptor
TRAIL	TNF-related apoptosis-inducing ligand
TSP	Tropical Spastic Paraparesis
U	Unit
UV	Ultraviolet
V	Volt
v-FLIP	Viral FLICE-inhibitory protein
WB	Western blot

PUBLICATIONS

Krueger, A., Fas, S.C., Giasi, M., **Bleumink, M.**, Merling, A., Stumpf, C., Baumann, S., Holtkotte, D., Bosch, V., Krammer, P.H., Li-Weber, M. (2006). HTLV-1 Tax protects against CD95-mediated apoptosis by induction of the cellular FLICE-inhibitory protein (c-FLIP). Blood 107(10):3933-9.

Bleumink, M. Krammer, P.H., Li-Weber, M. Sensitization of resistant HTLV-1 infected T cells towards death receptor mediated apoptosis by the small molecule inhibitor Rocaglamide. Manuscript in preparation