AMERICAN UNIVERSITY OF BEIRUT

EFFICACY AND MECHANISM OF ACTION OF THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR) INHIBITOR RAD001 IN T-CELL LYMPHOMAS AND HTLV-I-ASSOCIATED ADULT T-CELL LEUKEMIA/LYMPHOMA

by
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Title: Efficacy and Mechanism of Action of the Mammalian Target of Rapamycin (mTOR) Inhibitor RAD001 in T-cell Lymphomas and HTLV-I-Associated Adult T-cell Leukemia/Lymphoma.

HTLV-I associated adult T-cell leukemia/lymphoma (ATL) and HTLV-I-negative peripheral T-cell lymphomas carry poor prognosis mainly due to acquired resistance to chemotherapy. The mammalian Target of Rapamycin (mTOR) is a serine/threonine kinase and a key regulator of cell growth and survival in many tumor cells. We show that the mTOR inhibitor RAD001 (Everolimus) resulted in a cytostatic inhibitory effect on the growth of primary ATL cells from two patients, as well as several HTLV-I-positive and -negative malignant T-cell lines. However, treatment with hundred-fold higher RAD001 concentrations did not affect the proliferation of normal resting and activated lymphocytes. RAD001 induced oncoprotein Tax degradation and senescence in ATL cells only, as shown by enhanced staining of β-galactosidase and upregulated expression of p21 protein levels. Short term RAD001 treatment caused a G_0/G_1 cell cycle arrest but no apoptosis induction in all tested malignant T-cells. However, prolonged treatment with RAD001 induced apoptosis in senescent ATL cells and growth arrested HTLV-I-negative malignant T-cells. This was accompanied by an increase in phosphorylated p53 in HTLV-I-negative cells only, an increase in pro-apoptotic Bax, PUMA-α, and DNA damage repair γH2AX proteins, and a down regulation of Bcl-x_L proteins. Overall RAD001 abrogates mTOR signaling, inhibits the growth of ATL and HTLV-I-negative malignant T-lymphocytes, and induces senescence in ATL cells and apoptosis in all malignant T-cells after prolonged treatment. These results support a potential therapeutic role for mTOR inhibitors in ATL and HTLV-I-negative peripheral T-cell lymphomas.

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ABBREVIATIONS

mTOR	mammalian Target of Rapamycin
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol 3-kinase related protein kinase
TOR	target of Rapamycin
HEAT	Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and TOR1
ATR	Rad3- related kinase
ATM	ataxia-telangiectasia mutated
FAT	focal adhesion targeting
FKBP12	FK506-binding protein
FRB	FKBP12 Rapamycin Binding
NRD	negative regulatory domain
FATC	focal adhesion targeting C-terminal domain
mTORC1	mTOR Complex 1
mTORC2	mTOR Complex 2
Raptor	regulatory associated protein of mTOR
PRAS40	proline-rich Akt substrate 40 kDa
Rictor	Rapamycin insensitive companion of mTOR
PKCα	Protein kinase C alpha
PKB	protein kinase B
EGF	epidermal growth factor
PI(3,4)P2	phosphatidylinositol-3,4-bisphosphate
PI(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate

PTEN	phosphatase and tensin homologue deleted on chromosome ten
PH	pleckstrin homology
ILK	integrin-linked kinase
TSC	tubersclerosis complex
GTP	guanine triphosphate
Rheb	*Ras h*omolog *e*nriched in *b*rain
GEF	guanine nucleotide exchange factor
GDI	guanine nucleotide dissociation factor
S6K	p70 ribosomal S6 kinase
eIF4	eukaryotic initiation factor 4
4EBP1	eIF4E binding protein 1
RP	ribosomal protein
eEF2K	Eukaryotic Elongation Factor-2 kinase
5'TOP	5'terminal oligopyrimidine
eEF2K	Eukaryotic Elongation Factor-2 Kinase
MAPK	Mitogen-Activated Protein kinase
PLD	phosphplipase D
PA	phosphatidic acid
AMPK	Adenosine Monophosphate-Activated Protein Kinase
VEGF	Vascular Endothelial Growth Factor
HIFα	Hypoxia Inducing Factor α
CCI-779	cell cycle inhibitor-779
IL	Interleukin
PDGF	Platelet Derived Growth Factor
FDA	Food and Drug Administration

PTLD	Post-Transplant Lymphoproliferative Disorders
Rb	Retinoblastoma protein
NCI	National Cancer Institute
AML	acute myeloid leukemia
CLL	chronic lymphoblastic leukemia
CDK	cyclin-dependent kinase
Skp2	S phase kinase protein 2
ASK1	Apoptosis Signal-regulating Kinase1
GC	glucocorticoid
ALL	acute lymphoblastic leukemia
MDS	Myelodyplastic Syndromes
CML	chronic myeloblastic leukemia
ATL	Adult T-cell Leukemia
HTLV-I	Human T-cell Lymphotropic Virus type- I
3-BrOP	3-bromo-2-oxoproprionate-1-propyl ester
ATP	adenosine triphosphate
5-FU	5-fluoroacil
HCC	hepatocellular carcinoma
AP-1	Activating Protein-1
NFκB	Nuclear Factor kappa B
MCL	mantle cell lymphoma
EC	endometrial carcinoma
GMB	glioblastoma multiforme
SCLC	small cell lung carcinoma
RCC	renal cell carcinoma

IFN	interferon
NHL	non-Hodgkin's lymphoma
MTC	medullary thyroid carcinoma
PBMC	Primary Blood Mononuclear Cells
LC3	protein 1 light chain 3
PARP	poly (ADP-ribose) polymerase
VEGFR	vascular endothelial growth factor receptor
NSCLC	Non-small cell lung cancer
GIST	gastrointestinal stromal tumors
NLS	nuclear localization signal
ATF/CREB	activating transcription factor/cyclic AMP response element binding protein
SRF	serum response factor
CBP	CREB binding protein
NLS	nuclear localization signal
IKKγ	inhibitor of IκB
FBS	fetal bovine serum
DMSO	dimethyl sulfoxide
PBS	phosphate-buffered saline
PHA	Phytohemagglutinin
OD	optical density
ELISA	enzyme-linked immuno-sorbent assay
PI	propidium iodide
R123	rhodamine 123
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester

APS	ammonium persulfate
TEMED	N,N,N’,N’-Tetramethylethylenediamine
ROS	Reactive oxygen species
SA-β-Gal	senescence associated-β-galactosidase
PTCL	peripheral T-cell lymphomas

CHAPTER I

INTRODUCTION

A. mTOR Signaling

1. Overview

The story of the drug Rapamycin and its target, the mammalian target of Rapamycin (mTOR), literally started from the soil. The macrolytic lactone Rapamycin was first discovered in soil samples of south pacific island of Rapa Nui in 1970 as a natural product of the plant *Streptomyces hygroscopius*[1], and was shown to have significant antifungal, immunosuppressive, and anti-tumor properties. Subsequently, a great deal of knowledge about the mTOR protein stemmed from the discovery of its inhibitor, Rapamycin, and its mechanism of action.

mTOR, a 290 kDa Serine/Threonine kinase of the phosphatidylinositol 3-kinase (PI3K) related protein kinase (PIKK) family, is a master regulator of protein synthesis and a key biological enzyme necessary for cell growth and survival. Rapamycin and other immunosuppressive agents were found to induce their pharmacological action by a novel mechanism, whereby binding to the target necessitates the formation of immunophilin complexes (aggregation of abundant cellular proteins) [2, 3]. Over the past decade, a lot of research has been dedicated to elucidate the signaling pathways of the target of Rapamycin (TOR) and elucidating the interesting effects of this signaling molecule. Such studies entail a huge scientific significance because mTOR is deregulated in a wide range of human

disorders such as Type II diabetes and tumors for which Rapamycin and its analogues are being tested for their anticancer activities. Some of the Rapamycins are now in clinical trials such as in renal and breast cancer. Moreover, the finding that Rapamycin affects the transcription of almost 5% of all genes in the genome implies that mTOR has a great impact on cellular functions, namely transcription, translation, cell size, mRNA turnover, protein stability, ribosomal biogenesis, vesicular trafficking, cytoskeletal organization, and autophagy[4, 5].

2. mTOR Structure

mTOR exits in 1.5-2 MDa multi protein complexes[6]. The large size and the multidomain structure of this molecule suggest its ability to interact with several proteins, though only a few are characterized. From N- to C- terminus, mTOR contains: 20 Huntingtin Elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats which are protein–protein interaction domains consisting of two tandem α-helices that are also found in the DNA damage-sensing kinases Rad3- related kinase (ATR) and ataxia-telangiectasia mutated (ATM)[7]; a focal adhesion targeting (FAT) domain, found in all PIKK family members involved in protein interaction[8]; a binding domain that interacts with a complex of Rapamycin and FK506-binding protein, namely FKBP12 Rapamycin Binding (FRB) domain[9]; the kinase domain; a putative negative regulatory domain (NRD)[10]; and another C-terminus FAT domain: the focal adhesion targeting C-terminal domain (FATC) required for PIKK kinase activity (Figure 1).

Some sequence studies showed that the FAT domain could be of importance as a structural scaffold or as a protein-binding domain, or both. Moreover, FAT and FATC

domains only occur in combination, suggesting that they interact with each other. It is possible that they fold together in a configuration that ensures proper function of the kinase domain, which is wedged in between the FAT and FATC domains. Furthermore, the small size of FATC (~35 amino acids) hinders it from folding independently, but because it is more conserved than the FAT domain (34% *versus* 16% average identity), it could be more important for catalytic activity than the FAT domain[8].

mTOR can exist in one of two functionally distinct complexes: mTOR Complex 1 (mTORC1) or mTOR Complex 2 (mTORC2). mTORC1 consists of mTOR, regulatory associated protein of mTOR (Raptor), mLST8, and the proline-rich Akt substrate 40 kDa (PRAS40). mTORC1 can be inhibited by Rapamycin, and it plays an important role in translational regulation and cell growth control. mTORC2, on the other hand, consists of mTOR complexed with Rapamycin insensitive companion of mTOR (Rictor), Sin1, and mLST8. mTORC2 was originally thought to be insensitive to Rapamycin action, until prolonged treatment with the macrolide drug lead to indirect inhibition of mTORC2[11]. The function of mTORC2 is not as extensively understood as that of mTORC1, but it can respond to mitogenic signals by phosphorylating and activating Protein kinase C alpha (PKCα), a Serine/Threonine kinase, thus inducing the assembly of the actin cytoskeleton[12, 13]. mTORC2 also contributes to tumorigenesis since it can phosphorylate the Serine/Threonine kinase Akt, also called protein kinase B (PKB), thus signaling antiapoptotic signals and promoting cell proliferation and survival[13].

mTOR can receive input signals from multiple pathways, which can be divided into two broad categories: the canonical Akt-dependent pathway of mTOR regulation, and the non-canonical Akt-independent pathway.

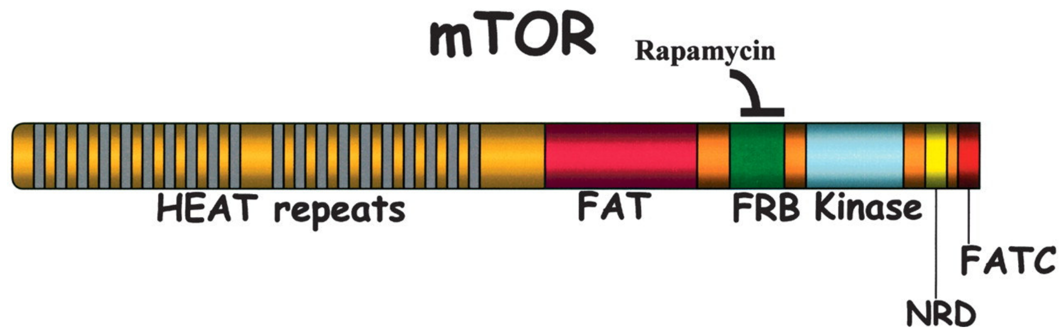

Fig.1. The primary structure of mTOR
Source: Hay, N., and Sonenberg, N. 2004. Upstream and downstream of mTOR. *Genes Dev*.18(16):1926-1945.

3. Akt-Dependent mTOR Regulation: Canonical Pathway

The prototypic pathway of mTOR signaling involves activation of the PI3K/Akt axis by mitogenic signals such as oncogene activation (i.e. Ras), activation of G protein coupled receptors, or binding of growth factors such as insulin and epidermal growth factor (EGF) to receptor tyrosine kinases (Figure 2). Activated PI3K would phosphorylate phosphoinositides at the D3 position, yielding lipid second messengers such as phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3). This phosphorylation is negatively regulated by the action of the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) which dephosphorylates phosphoinositides at the D3 position. PTEN mutations, silencing, or deletions result in a constitutive activation of mTOR signaling and are, thus, implicated in a variety of hematological disorders[14] and tumors such as glioblastomas, lung carcinomas, melanomas, prostate cancers[15]. PI(3,4,5)P3 in return recognizes the pleckstrin homology (PH) domain of Akt and recruits it to the membrane, thus promoting the

activation of another Serine/Threonine kinase, PDK-1. PDK-1 phosphorylates T308 located within the activation loop of Akt[16]. Another phosphorylation site is the S473 located within the C-terminal hydrophobic motif of Akt, which can be phosphorylated not only by PDK-1, but also by integrin-linked kinase (ILK), an ILK-associated kinase, a DNA-dependent protein kinase[17, 18], Akt itself[19], and most importantly mTORC2, supporting a dual role of mTORC2 as an effector and a target of Akt signaling[11].

Akt acts on a wide array of downstream signaling molecules such as forkhead transcription factors, GSK3β, Bad, and the cell cycle inhibitor p27^{kip1}. Of particular importance in mTORC1 activation is the phosphorylation of tubersclerosis complex (TSC)2 and PRAS40[20]. One way by which Akt indirectly activates mTORC1 is by phosphorylating TSC2. With the help of genetic studies in *Drosophila*, the discovery of TSC1 (Hemartin) and TSC2 (Tuberin) gene products was a turning point in mTOR research. These genes are tumor suppressors whose mutation produced overgrown cells and organs in *Drosophila*. Mutations in either genes result in the formation of a distinguished benign tumor termed hemartoma in different organs, thus leading to the disease TSC, a dominantly inherited disorder[21]. Moreover, overexpression, downregulation, or RNA interference experiments done on mammalian cells showed that cells deficit of TSC1 or TSC2 had elevated mTORC1 activity, resulting in an enhanced proliferation rate and an enlarged size of the mutant cells[22]. Consequently, the TSC1/TSC2 tumor suppressor complex was identified as a negative regulator upstream of mTOR[23]. The domains of TSC1 include a transmembrane domain and a coiled-coil domain, the latter which mediates the binding of TSC1 to TSC2. TSC2 functions as a guanine triphosphate (GTP)ase activating protein for the small G protein *Ras* *h*omolog *e*nriched in *b*rain (*Rheb*),

a target first identified by genetic screening and biochemical studies in *Drosophila*[24]. Mammalian TSC2 activity increases the rate of GTP hydrolysis on *Rheb* leading to its inactivation. Since *Rheb* is a positive regulator of mTORC1 activity *in vitro* and *in vivo*, inhibition of TSC2 by Akt results in a hyperactivated GTP-bound *Rheb* and consequently increased mTORC1 activity[25]. Whether *Rheb* is the direct upstream regulator of mTORC1 is still a subject of extensive research, whereby it was found in *S.pombe* that GTP bound *Rheb* enables its binding to TOR2[26]. This was in contrast to mammalian cells where the binding of *Rheb* to mTORC1 was found to be independent of any guanine nucleotide, although GTP-bound form of *Rheb* is necessary for the activation of mTORC1[27]. This might suggest that in mammalian cells, *Rheb* might be involved in positive or negative regulation of mTORC2. Another unsolved mystery is the identification of the guanine nucleotide exchange factor (GEF) of the guanine nucleotide dissociation factor (GDI) specific for *Rheb*. While some studies indicate the absence of *Rheb* GEF or GDI which explains *Rheb*'s low basal levels of GTPase activity[28], others report the existence of several GDIs or GEFs. However, it is established that the heterodimeric complex consisting of TSC1 and TSC2 inhibits the activity of mTORC1 by increasing the rate of GTP hydrolysis of its positive regulator *Rheb*.

Akt activation by mitogenic signals does not necessitate the activation of TSC2. In fact, another substrate for Akt is the PRAS40 which activates mTORC1 independently of TSC2[29]. This is possible because phosphorylation of PRAS40 by Akt attenuates the inhibitory effect of PRAS40 on mTORC1 (which occurs independently of TSC2). Therefore, Akt-dependent activation of the mTOR signaling pathway can occur in TSC2-dependent and -independent mechanisms. Once activated and through its Raptor

component, mTORC1 binds to and phosphorylates its downstream substrates, two of which are major translational regulators: p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4 (eIF4) E binding protein 1 (4EBP1). This explains the best studied function of mTOR in translational regulation.

mTORC1 can directly phosphorylate 4EBP1 on T37 and T41, causing its dissociation from eIF4E. Free eIF4E can assemble the large protein complex eIF4F at the 5' cap of mRNAs, thus, activating their translation (Figure 2). This is blocked by the binding of hypophosphorylated 4EBPs to eIF4E, preventing the association of eIF4E and eIF4G which would otherwise lead to translational activation. Furthermore, mTORC1 phosphorylates S6K's hydrophobic motif at T389. Activated S6K will then phosphorylate the ribosomal protein (RP) S6, which regulates cell size but not translation[30]. S6K can promote translation by phosphorylating other substrates that play a role in protein synthesis, such as eIF4B and Eukaryotic Elongation Factor-2 kinase (eEF2K) [31].

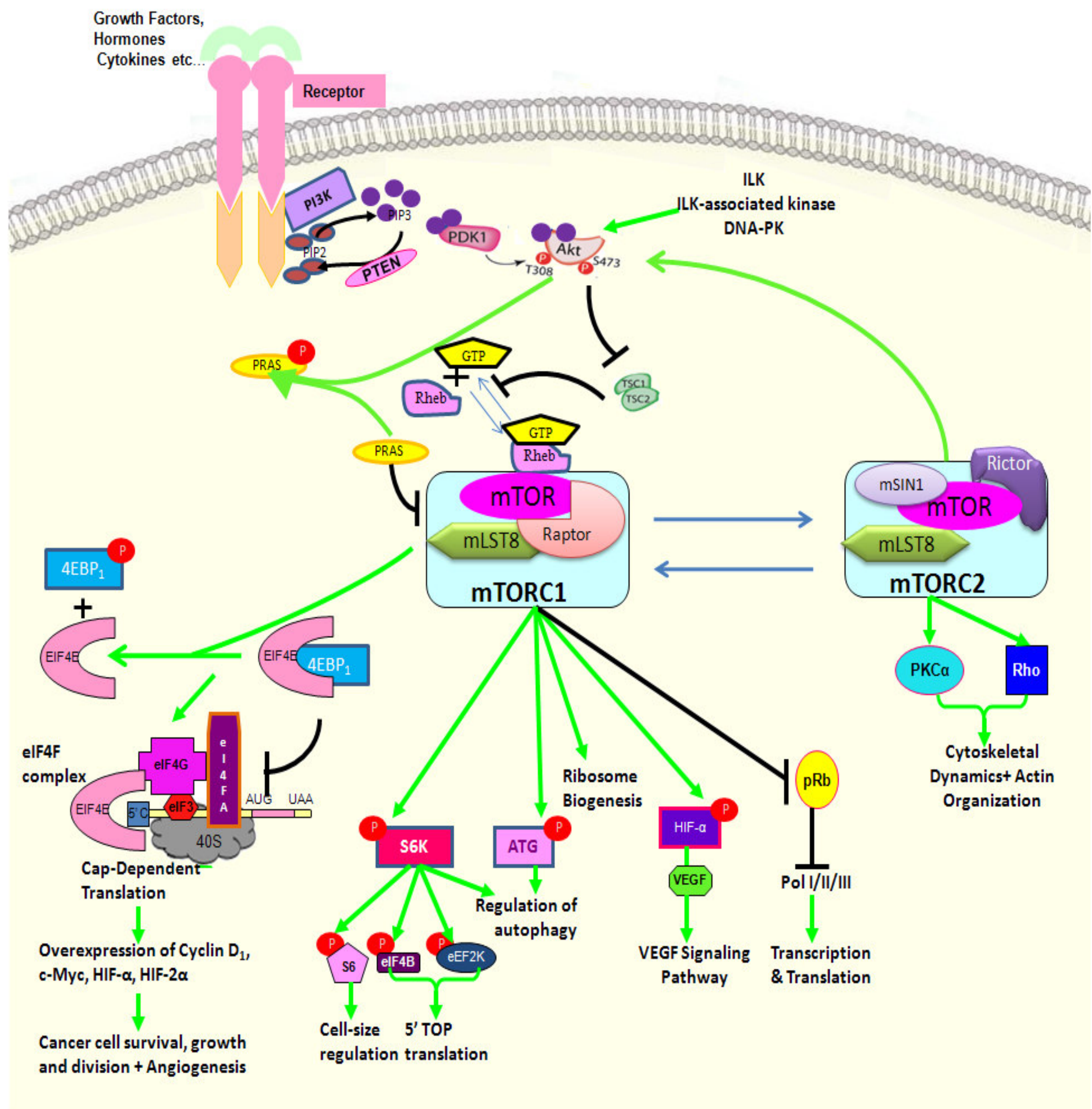

Fig. 2. Akt-dependent mTOR regulation: canonical pathway.

4. Akt-Independent mTOR Regulation: Non-Canonical Stress Signaling Pathway

A cell controls its growth and regulates its cell cycle progression and cell death mechanisms by integrating multiple stimuli that affect several cellular signaling pathways. Of particular importance is the Akt-independent mTOR signaling pathway which changes its activity in response to a constantly growing list of stress signals (Figure 3). Unlike the Akt-dependent mTOR signaling pathway, this non-canonical pathway does not involve downstream induction of the PI3K/Akt axis, and is mostly turned on by stress signals. Such extra-cellular and intra-cellular cues that modulate mTOR involve Mitogen-Activated Protein Kinase (MAPK) signaling, energy and nutrient sensing pathways, hypoxia, DNA damage, reactive oxygen species signaling, phospholipase D (PLD) and phosphatidic acid (PA) signaling, and a plethora of other inducers which are less studied or investigated[32] (Figure 3).

5. mTOR and Cancer

Several mutations in the mTOR signaling pathways have been implicated in common diseases and cancers, and are summarized in Table 1. A variety of intermediates in mTOR signaling pathway are linked to cancers due to activating mutations and amplification of oncogenes and loss of tumor suppressor genes. For example, activating mutations in the p110α subunit of PI3K have been reported in 40% of ovarian[33] and 35% of breast cancer patients[34]. Aberrantly high PI3K activity has been observed in a wide panel of human cancers, with major implications in cell transformation, tumor progression, and treatment resistance. Moreover, loss of PTEN tumor suppressor activity is implicated in a large fraction of advanced human cancers[35-37]. In addition, inherited mutations in

PTEN lead to the development of hamartoma tumor-predisposition syndromes such as Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, and Lhermitte-Duclos disease[38]. Both inherited mutations and loss of activity in PTEN result in constitutive activation of Akt and eventually aberrant mTOR signaling.

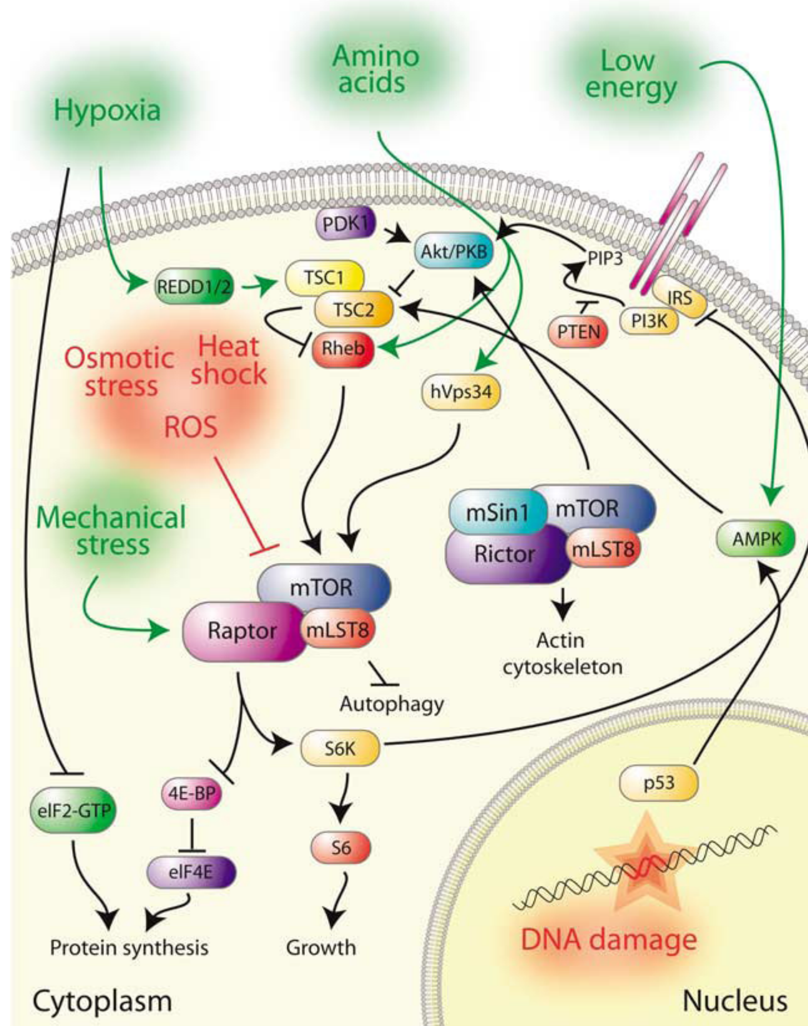

Fig. 3. mTOR signaling and stress

Source: Reiling, JH., and Sabatini, DM. 2006. Stress and mTORture signaling. *Oncogene* 25(48):6373–6383.

Furthermore, inactivation of LKB1 function is associated with Peutz –Jeghers cancer prone syndrome which is characterized by hamartomas in the gastrointestinal tract[39]. In energy-depleted conditions, LKB1 inhibits mTOR activity by activating Adenosine Monophosphate-Activated Protein Kinase (AMPK), which explains the increase in mTOR signaling in Peutz –Jeghers syndrome. Another mTOR signaling intermediate found to be mutated in TSC is the TSC2 protein, which inhibits mTOR under hypoxic and energy deprived conditions[25, 40]. Other mTOR kinase substrates and associated proteins have also been reported to be modulated in tumors. These include overexpression of S6K1 in some breast carcinomas[41] and early ovarian transformation events[42]. Also of great importance is the amplification of eIF4E, the positively regulated substrate of mTOR, in a wide array of cancers such as thyroid, colon, bladder, breast, among others[42-45].

The mTOR/Rictor complex is directly involved in the phosphorylation of Akt/PKB on S473 *in vitro*, thus, facilitating another phosphorylation step by PDK1. Knowing that deregulation of PKB is implicated in the pathogenesis of cancer and diabetes, Rictor-mTOR may serve as a drug target in tumors that have lost the expression of the tumor suppressor PTEN that opposes Akt/PKB activation[13]. The importance of this area of investigation can be further highlighted by the fact that mTOR signaling is implicated in tumor as well as endothelial cell proliferation. In fact, studies have shown that tumor Vascular Endothelial Growth Factor (VEGF) production is dependent on Hypoxia Inducing Factor α (HIFα) activity and mTORC1-dependent translation in cancer cells. This suggests that mTOR signaling controls to a certain extent hypoxia-induced production of VEGF by tumor and stromal cells and VEGF stimulation of endothelial cell proliferation and survival, leading to tumor angiogenesis[46].

These and other mutations in the PI3K/Akt/mTOR pathway do not only provide a growth advantage during carcinogenesis but may also contribute to chemotherapy resistance. If the growth and survival of tumor cells stems from PI3K/Akt/mTOR pathway aberrations, then, there is a high possibility that the resulting tumors would be sensitive to mTOR inhibitors.

Table 1. Mutations in mTOR signaling and link to common diseases

Disease[a]	Linked genetic mutation and clinical pathology[b]	Predicted functional link to mTOR signaling[c]
Tumor-prone syndromes		
TSC (tuberous sclerosis complex)	*TSC1* or *TSC2*; harmatomas in multiple organs	TSC1 and 2 negatively regulate Rheb
LAM (lymphangioleiomyomatosis)	*TSC2;* abnormal proliferation of smooth-muscle-like cells in the lung	TSC1 and 2 negatively regulate Rheb
Cowden's disease	*PTEN;* harmatomatous tumor syndrome	Might promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
Proteus syndrome	*PTEN;* harmatomatous tumor syndrome	Might promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
Lhermitte–Duclos disease	*PTEN;* harmatomatous tumor syndrome	Might promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
PJS (Peutz–Jeghers syndrome)	*STK11/LKB1;* gastrointestinal harmatoma tumor syndrome	STK11 activates AMPK, a positive regulator TSC2
HCM (familial hypertrophic cardiomyopathy)	*AMPK*; myocardial hypertrophy	AMPK promotes TSC2 function
Cancer		
Prostate	*PTEN*	PTEN loss promotes AKT activation
Breast	*PTEN*; PtdIns3K, *AKT* or *Her2/neu* amplification or hyperactivation	PTEN loss or gene amplifications promote AKT activation
Lung	*PTEN; HER* amplification	PTEN loss or gene amplifications promote AKT activation
Bladder	*PTEN*	Promotes AKT activation
Melanoma	*PTEN*	Promotes AKT activation
Renal-cell carcinoma	*PTEN*	Promotes AKT activation
Ovarian	*PTEN*; PtdIns3K, *AKT* or *Her2/neu* amplification or hyperactivation	PTEN loss or gene amplifications promote AKT activation
Endometrial	*PTEN*	Promotes AKT activation
Thyroid	*PTEN*; PtdIns3K, *AKT* or *Her2/neu* amplification or hyperactivation	PTEN loss or gene amplifications promote AKT activation
Brain (glioblastoma)	*PTEN*	Promotes AKT activation
CML (chronic myeloid leukemia)	*BCR–ABL* translocation	Promotes AKT activation

[a] List is not comprehensive, but rather attempts to indicate diseases commonly linked to genes involved in mTOR signaling. [b] Listed only are the linked mutations that might affect mTOR signaling. [c] Best prediction based on existing data.

Source: Guertin, D.A., and Sabatini, D.M. 2005. An expanding role of mTOR in cancer. *Trends in Molecular Medicine* 11(8):353-361.

B. mTOR Inhibitors

1. Overview

The fact that mTOR signaling is upregulated in several cancers has prompted the development of drugs targeting mTOR. In fact, the anti-proliferative effects of mTOR inhibitors such as Rapamycin or Sirolimus and derivatives cell cycle inhibitor-779 (CCI-779, Temsirolimus), RAD001 (Everolimus), and AP23573 (Deforolimus) were evaluated in numerous *in vitro* and *in vivo* models (Table 2). These agents block tumor and endothelial cell proliferation by inducing G_1 cell cycle arrest and inducing apoptosis in selected tumor models, have limited toxicity to normal tissues, and are currently in cancer clinical trials. mTOR inhibitors induce their anti-proliferative activities in picomolar to nanomolar concentrations, and they enhance the cytotoxicity of other chemotherapeutic agents and radiation in combinational therapy options.

In general, mTOR inhibitors work by forming an inhibitory complex *via* their methoxy group with their intracellular receptor FKBP12, which binds a region in the C-terminus of mTORC1 known as FRB, thus, inhibiting the mTORC1 activity (Figure 4). Because FKBP12-Rapamycin does not bind to mTORC2[13, 47], Rapamycin was thought to inhibit only mTORC1. However, more recent findings suggest that in some cell lines Rapamycin suppresses the assembly and function of mTORC2, and-upon prolonged treatment-reduces its levels below those needed to maintain Akt/PKB signaling[11]. This property of Rapamycin contributes to the *in vitro* and *in vivo* effects of the drug, such as induction of apoptosis in cells that exhibit Rapamycin-sensitive Akt/PKB phosphorylation. Interestingly, in cells derived from the hematological system, Rapamycin prevented the proper assembly of mTORC2 with Rictor and mSin1.

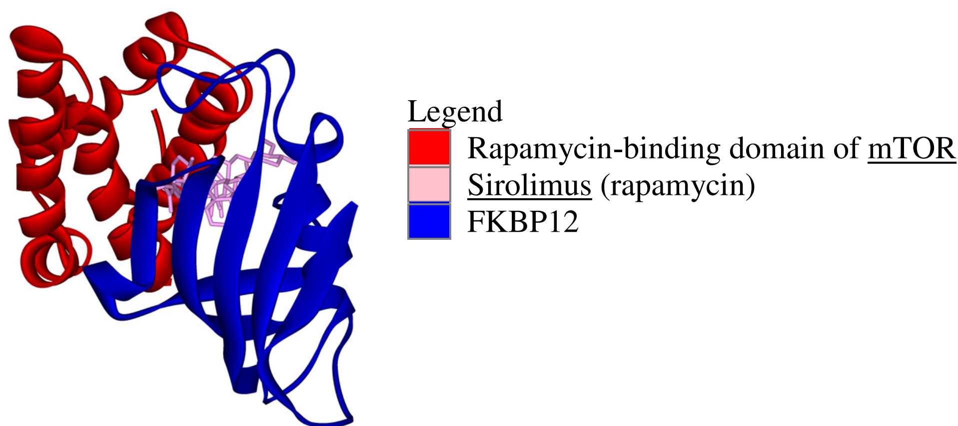

Fig. 4. Ribbon diagram of human immunophillin immunosuppressant FKBP12 in complex with sirolimus (rapamycin) interacting with the rapamycin-binding domain of FKBP12-rapamycin complex-associated protein (mTOR).

Source: Choi, J., Chen, J., Schreiber, S.L., Clardy, J. 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science* 273(5272):239-242.

Table 2. Rapalogs in clinical trials for cancer therapy

Compound	Structure	Manufacturer	Trial Status	Formulation
CCI-779 Temsirolimus		Wyeth	Phase III	Oral and intravenous
RAD001 Everolimus		Novartis	Phase III	Oral
AP23573 Deforolimus		Merck	Phase III	Oral and intravenous

Source: Fasolo, A., and Sessa, C. 2008. mTOR inhibitors in the treatment of cancer. *Expert Opin. Investig. Drugs* 17(11):1717-1734.

2. Rapamycin

After the discovery of the natural antibiotic Rapamycin, also known as Sirolimus, it was later chemically synthesized in1972 by Ayerst Research laboratories in Montreal. Initially, the macrolytic lactone was used as an antifungal agent against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. The white crystalline solid form is insoluble in aqueous solutions, but soluble in organic solvents. Rapamycin can form an intracellular complex with FK506-binding protein, a 12 kDa polypeptide immunophilin with a peptidyl-prolyl cis-trans isomerase activity. This complex can bind mTOR by recognizing the FRB domain at the N-terminus of mTOR's kinase domain[48, 49] (Figure 4). This binding leads to the inhibition of downstream mTOR effectors and to subsequent de-sensitization of T-cells to proliferation signals induced by antigens, mitogenic lectins, alloantigens and crosslinking of T-cell surface markers with monoclonal antibodies. It can also inhibit proliferative responses induced by cytokines such as Interleukin (IL)-1, IL-2, IL-3, IL-4 and IL-6, insulin-like growth factor, platelet derived growth factor (PDGF), and colony stimulating factor.

a. Rapamycin as an Immunosuppressive Agent:

Rapamycin has been extensively investigated as an immunoppressant in pre-clinical and clinical studies. With an IC_{50} ranging from 0.1 to 300 nM, Rapamycin has enhanced cyclosporin-induced prevention of renal graft rejection, decreased the cytotoxicity of cyclosporin and showed a significant level of synergy *in vitro* and *in vivo*. This drug also reversed lesions caused by immune injury following aortic allografts in non-human primate models. Rapamycin was approved in 1999 by the USA Food and Drug

Administration (FDA) to be used in combination with cyclosporine and steroids to prevent acute organ rejection. A year later, the European Agency replaced calcineurin antagonists with Rapamycin treatment as a long-term therapy to prevent graft rejection.

Organ transplant patients under immunosuppressive therapies are at high risk of developing several malignancies such Post-Transplant Lymphoproliferative Disorders (PTLD) and skin cancers. However, experimental models showed that Rapamycin decreased the risk of developing PTLD, skin carcinomas and Karposi's sarcoma, and successfully treated such malignancies while maintaining organ transplant viability. This suggests the efficacy of using this mTOR inhibitor in resuming immunosuppression and preventing the recurrence of malignancies in successfully treated patients.

In addition to its immunosupressive capacity, Rapamycin is now used as an impregnated stent to prevent coronary restenosis in a localized fashion. The efficacy of the treatment is due to Rapamycin's capacity to inhibit the proliferation of vascular smooth muscle cells after muscular injury, by binding to FKBP12, blocking P70S6K activity and retinoblastoma protein (Rb) phosphorylation, and preventing the downregulation of p27. PDGF-induced migration of human vascular smooth cells was also blocked *in vitro* with Rapamycin treatment, while preserving the cells' ability to bind collagen and the integrity of cytoskeletal components.

b. <u>Rapamycin as an Anti-Cancer Agent</u>

Screening the effect of Rapamycin on 60 murine and human tumor cell lines at the Developmental Therapeutic Branch of the National Cancer Institute (NCI) (COMPARE program) revealed significant noncytotoxic growth inhibitory effects in tissue culture and in

xenograft models. With an average GI_{50} of 8.2 nM (GI_{50} is the unit used by NCI to measure concentrations that cause 50% growth inhibition), sensitivity to Rapamycin was generally at doses below 2000 ng/ml in leukemia, ovarian, breast, central nervous system and small cell lung cancer cell lines[50].

i. Pre-clinical and Clinical Studies

Pre-clinical data show that growth-suppression by Rapamycin induces cell cycle arrest in a variety of hematological and solid tumor systems. In acute myeloid leukemia (AML), a clonal disorder characterized by accumulation of malignant hematopoietic progenitor cells with impaired differentiation program, patients under chemotherapy undergo high relapse rates often resulting in death. Rapamycin-induced mTOR inhibition was thus investigated as a potent and less toxic drug for the treatment of this disease. In a series of 23 AML cases, results showed strong growth inhibition of the most immature AML cell lines by inducing cell cycle arrest in the G_0/G_1 phase, accompanied by Rapamycin-sensitive phosphorylation of 4E-BP1 and p70S6K, and an impairment of the clonogenic properties of fresh AML cells while sparing normal hematopoietic progenitors. Clinically, 4 out of 9 patients with either refractory/relapsed *de novo* AML or secondary AML significantly responded to Rapamycin treatment. This suggested that Rapamycin was capable of targeting the clonogenic compartment of the AML leukemic clone where mTOR is aberrantly regulated[51].

In vitro studies showed that Rapamycin inhibition was far more prominent in long term cultures (7 days) than in short term cultures (2 days), which could be enhanced by co-treatment with Etoposide. Importantly, Etoposide toxicity on $CD34^+$ cells from healthy

donors was not enhanced by addition of Rapamycin[52]. Rapamycin-induced cell cycle arrest was also investigated in Chronic Lymphoblastic Leukemia (CLL) cells, where mTOR inhibition prevented RB phosphorylation, inhibited cyclin-dependent kinase (CDK) 2 activity by preventing up-regulation of cyclin E, cyclin A, and survivin, and led to G_1 cell cycle arrest[53]. In addition, the mTOR inhibitor showed anti-tumorigenic properties in breast cancer cell lines by down-regulating the expression of S phase kinase protein 2 (Skp2), an important oncogene in the development and progression of breast cancer, by interfering with gene transcription as well as by increasing its rate of protein degradation[54].

Rapamycin not only induced G_1 cell cycle arrest, but also cell death, mainly apoptosis. For instance, Rapamycin was found to induce apoptosis as a cellular stress response in a panel of cells lacking p53. mTOR inhibition in p53-mutant human rhabdomyosarcoma cells led to suppression of protein phosphatase 5, an upstream negative regulator of the Apoptosis Signal-regulating Kinase1 (ASK1), thus, preventing its physical interaction with the ASK1. The resulting rapid and sustained activation of the ASK1 signaling pathway lead to selective Rapamycin-induced apoptosis of these cells lacking functional p53[55].

Nucleophosmin-anaplastic lymphoma kinase, an oncogene product involved in hematopoietic and non-hematopoietic malignancies, is characterized by resistance to glucocorticoid (GC) therapy especially in acute lymphoblastic leukemia (ALL) patients. Interestingly, Rapamycin was capable of reversing GC resistance by synergistically inhibiting mTOR signaling pathway, enhancing G_1-cell cycle arrest and promoting apoptotic cell death[56].

In a study that highlights the aberrant Akt/mTOR pathway as an important therapeutic target for high-risk Myelodysplastic Syndromes (MDS) patients, Rapamycin increased apoptotic cell death of $CD33^+$ cells from such patients, and negatively affected the clonogenic ability of high-risk MDS $CD34^+$ precursors[57]. Interestignly, Rapamycin made it to clinical trials in MDS patients who overxpress mTOR and VEGF, molecules previously shown to be targeted by this drug. Rapamycin treatment also showed activity in patients with more advanced MDS, thus making Rapamycin an interesting therapeutic target[58]. In chronic myeloblastic leukemia (CML), a stem cell disease characterized by the t(9;22) and the related oncogene *BCR/ABL,* Rapamycin was shown to be a potent inhibitor of *in vitro* growth of primary CML cells and of BCR/ABL-transformed cell lines, by down-regulating the expression of VEGF. Interestingly, similar results were observed *in vivo.* Moreover, Rapamycin inhibited the growth of Ba/F3 cells exhibiting various imatinib-resistant mutants of BCR/ABL, some of which are resistant to most currently available BCR/ABL kinase inhibitors[59].

ii. Combination Treatments

The anti-proliferative effects of Rapamycin were further investigated in combination with established chemotherapeutic agents and radiation. Results showed that in pre-clinical models, Rapamycin-induced mTOR inhibition had at least an additive effect in combination treatments. In Adult T-cell Leukemia (ATL), Rapamycin and the PI3K inhibitor LY294002 combination treatment induced growth arrest of Human T-Lymphotropic Virus type-I (HTLV-I)-infected cells, which was associated with de-phosphorylation of p70S6K and 4E-BP-1, down-regulation of c-Myc and cyclin D_1 protein

levels in these cells. Such longitudinal inhibition of PI3K/Akt/mTOR signaling is a promising treatment strategy for individuals with ATL[60].

Rapamycin was also investigated for a synergistic cytotoxic effect in leukemia and lymphoma in combination with the cell permeable glycolytic inhibitor 3-bromo-2-oxoproprionate-1-propyl ester (3-BrOP). Cancer cells are known for being highly hypoxic, and they exhibit increased dependency on glycolytic pathway for adenosine triphosphate (ATP) generation where glucose uptake and lactate production are accelerated without any change in oxygen consumption. This fundamental change in energy metabolism, termed the Warburg effect, creates a discrepancy between normal and cancer cells, which led to biochemically-based therapeutic approaches to preferentially kill cancer cells through glycolysis inhibition. This approach was combined with mTOR inhibition, since mTOR plays important roles in regulating nutrient metabolism and promoting the growth and survival of cancer cells exhibiting increased glycolysis for ATP generation (probably because mTOR increases the rate of HIF-1 protein translation). The results showed that simultaneous targeting of mTOR and glycolysis by Rapamycin and 3-BrOP, respectively, in HL60 cells, had a severe impact on the metabolism and survival of malignant cells as evidenced by suppressed glucose uptake and severely depleted cellular ATP pools, leading to enhanced apoptosis[52]. Synergy was also reported when Rapamycin treatment was combined with UCN-01, PKC and Chk1 inhibitor, resulting in significant pro-apoptotic effects in U937 monocytic leukemia cells. This phenomenon is possibly due to the inhibition of both Raf-1/MEK/ERK and Akt cytoprotective signaling pathways, as well as JNK activation[61].

Interestingly, combination of Rapamycin and the chemotherapeutic agent 5-fluoroacil (5-FU) was shown to overcome tumor growth in hepatocellular carcinoma (HCC). Results showed that combination treatment not only induced apoptosis but also cellular senescence, whereby the senescent cells showed less clonogenic potential than cells treated with 5-FU alone. Senescence-associated downregulation of Activating Protein-1 (AP-1) and Nuclear Factor kappa B (NFκB) transcriptional activities was responsible for the capacity of Rapamycin as a chemotherapeutic adjuvant in the treatment of HCC. Based on such results, it would be interesting to investigate whether a clinically prevailing senescent effect in HCC patients would maintain tumor growth inhibition[62].

Rapamycin was effective in completely blocking sorafenib-induced phosphorylation of mTOR targets and cyclin B_1 expression in HCC-derived tumors. This synergistic effect was accompanied by significant growth inhibition, increased apoptosis, and inhibition of VEGF Receptor-2 phosphorylation in a mouse HCC xenograft model, providing a rationale for clinical investigation of sorafenib in combination with mTOR inhibitors in HCC patients[63]. Moreover, oral treatment with Rapamycin induced regression of astrocytomas in patients with TSC. All lesions exhibited regression and, in one case necrosis[64].

iii. Rapamycin Resistance

A recent report reveals the mechanism of Rapamycin-induced mTOR suppression, and sheds light on its major therapeutic drawbacks[65]. This report shows that mTORC2 is inhibited by much higher concentrations of Rapamycin than mTORC1. This is consistent

with the fact that suppression of cell proliferation, which is more likely due to mTORC2 inhibition, is achieved by higher concentrations of Rapamycin, unlike S6K kinase phosphorylation, a direct downstream effector of mTORC1. Since such high doses are not tolerated by humans, these findings suggest the need for new strategies to enhance the effect of Rapamycin-based therapy in cancer. Moreover, a Rapamycin phase-I clinical trial in patients undergoing surgery after recurrence of PTEN-null glioblastoma, showed reduction of cell proliferation rate in only half of the patients recruited. This lack of response was probably due to the drug not being able to penetrate the tumor, since cells from tumors resistant to Rapamycin in patients were sensitive to Rapamycin in tissue culture.

The common resistance to Rapamycin therapy could be explained by the export of Rapamycin from cells by mediators of multidrug resistance such as ATP-binding cassette type transporters. In AML for example, the 170-kDa P-glycoprotein are highly expressed and account for discrepancy in response of AML patients to Rapamycin treatment, and in concentrations required to achieve IC_{50} on AML blast in clonogenic assay. Another limitation of Rapamycin that could also account for drug resistance is the upregulation of members of a family of Serine/Threonine kinases induced by growth factors and cytokines for regulating hematopoietic cell growth and differentiation.

Despite being approved as an immunosuppressant and demonstrating anti-proliferative effects in a panel of tumor models in phase I, phase II, and phase III clinical trials, Rapamycin was shown to be a poor candidate for parenteral adminstration because of its reduced aqueous solubility and its chemical instability in solution. Consequently, ester analogues with effects similar to Rapamycin but with more favourable pharmaceutical

properties were developed in the cell screening studies. Those are: CCI-779 (Temsirolimus, Wyeth-Ayerst Research; designed for intravenous injection), RAD001 (Everolimus, Novartis; available for oral administration and is approved in Europe as an immunosuppressive agent for solid organ transplantation), and AP23573 (Deforolimus, ARIAD Pharmaceuticals; completed preclinical trials and scheduled for clinical trials in late 2004). We will briefly describe CCI-779 and Deforolimus, followed by a more detailed description of RAD001, which is the drug used in our studies as a potential treatment for ATL and T-cell malignancies.

3. CCI-779 (Temsirolimus)

CCI-779 (Temsirolimus) was designed by Wyeth Ayerst investigators as a soluble 42-[2, 2-bis (hydroxymethyl)]-propionic ester analog of sirolimus which makes it a more available intravenous formulation. This drug has shown significant anti-tumor activity *in vivo* and *in vitro*, in a wide range of cancer types, including: glioblastoma, melanoma, prostate, breast, head and neck, renal cell and pancreatic cancers.

a. Pre-clinical and clinical studies

In vitro, CCI-779 displayed an anti-tumor activity comparable to that of Rapamycin, in a panel of cancer cell lines, in particular those with PTEN deletions. For example, breast cancer cell lines show different sensitivities to the mTOR inhibitor, and those that show the highest sensitivities are the ones harboring PTEN deletions, Her2/neu expression, or estrogen-receptor positive breast cancer cell lines. In other words, PTEN mutation and/or HER-2 overexpression in breast cancer may predict response to mTOR

inhibitors. This sensitivity was associated with decreased cyclin-D and c-myc expression levels, and correlated with cell lines expressing high levels of active Akt, which is assumed to be the common target for all the sensitive cell lines. Moreover, the anti-tumor effects of CCI-779 in prostate and pediatric brain tumor models was additive when administered with chemotherapeutic agents such as cisplatin.

In phase I clinical trials, CCI-779 was intermittently administered to minimize any possible immunosuppressive action while retaining its anti-tumor action. This drug showed promising results in previously treated patients with neuroendocrine tumors, lung, renal, and breast carcinomas. However, phase II trials showed that, as a single agent, CCI-779 was more effective at lower doses with a less toxic profile. More objective responses were noted in mantle cell lymphoma (MCL), endometrial carcinoma (EC) and glioblastoma multiforme (GMB), compared to less pronounced effects in small cell lung carcinoma (SCLC), metastatic breast cancer, and renal cell carcinoma (RCC). For example, in GMB, responses were mainly in patients with a mutated or deleted PTEN, or an altered form of EGF receptor which comprise approximately 40% of patients. However, myeloma cells which express wild type PTEN were at least 1000 fold less sensitive. In addition CCl-779 inhibited the growth of PTEN-deficient but not PTEN-expressing breast tumor cells in a nude mouse xenograft model, which further confirms the PTEN-dependent sensitivity in tumor cells exposed to CCI-779[66]. The t(11; 14) (q13; 32) translocation in MCL patients resulted in overexpressed cyclin D, which is a target of CCI-779. In fact, MCL was the first hematologic malignancy in which mTOR inhibition was explored as a treatment strategy. Phase II studies showed that the effect of CCI-779 treatment would be inactivation of CDK4/cyclin D complexes required for Rb phosphorylation and G_1 cell

cycle arrest, and thus, antitumor activity against relapsed, refractory disease. These results were further evaluated in a phase III study to compare two dose regimens of CCI-779 with the investigator's choice of single-agent therapy in relapsed or refractory MCL, and results were improved progression-free survival and objective response rate[67]. The anti-tumor activity could also be correlated with Akt hyperactivation, which has been reported in non-Hodgkin's lymphoma (NHL), a model in which tumors regressed with the administration of CCI-779[68].

b. Combination Treatments

CCI-779 phase II trials are still being investigated in SCLC, melanoma, prostate and pancreatic cancers, especially in combination with other agents such as tyrosine kinase inhibitors, cytotoxic agents like docetaxel, gemcitabine and others, although combination with 5-FU increased toxicity of the chemotherapeutic drug used as a single agent[50]. Moreover, patients with advanced leukemias, namely AML, CLL, and CML, are also being investigated for objective responses to the mTOR inhibitor[69].

Interestingly, in AML, the anti-tumor activity of CCI-779 was not only induced by suppression of mTORC1, the regularly thought biochemical action of Rapamycin inhibitors, but also *via* inhibition of mTORC2 and consequently blocking Akt phosphorylation on both S473 and T308. This unexpected finding suggested that AML could respond favorably to mTOR inhibition without exacerbating tumor aggressiveness, as is the case in some solid tumors in which mTOR inhibition resulted in Akt hyperphosphorylation through mTORC2 and p70S6K[70].

CCI-779 showed promising results in phase II clinical studies in RCC and mantle cell lymphoma (MCL), which prompted phase III trials on patients with these two diseases[71,72]. While no synergistic effect was observed in RCC patients treated with interferon (IFN)–α and CCI-779, the use of the mTOR inhibitor as a single agent led to favorable responses in RCC patients. Finally, the use of CCI-779 as an initial treatment for patients with advanced poor-prognosis RCC patients was approved in May 2007 by the FDA[72].

4. RAD001 (Everolimus)

RAD001 (Everolimus), 40-O-(2-hydroxyethyl)-Rapamycin, is an orally administered Rapamycin analogue. Although its *in vitro* immunosuppressive activity is three times less than that of Rapamycin, it has favorable pharmacokinetic properties when used *in vivo* due to its increased polarity that make it orally bioavailable. After being approved as an immunosuppressive agent in solid organ transplantations, RAD001 is now being used in combination with cyclosporine and corticosteroids for the prophylaxis of acute rejection in kidney and heart transplant patients. In fact, combination therapy with cyclosporine showed significant synergism, while decreasing cyclosporine toxicity in several tissue types.

a. Pre-clinical Studies

The anti-tumorigenic activity of RAD001 was first investigated in a panel of 24 human cancer cell lines, few of which displayed intrinsic resistance, with a median IC_{50} value of 8.8 nM, and a range of 0.7 - 4125 nM. The levels of Akt phosphorylation at

Ser473 and S6 phosphorylation at Ser240 and 244 were found to correlate with its antineoplastic activity. In general, no correlation was found with the degree of inhibition of p70S6K1 or 4EBP1 activation pathways, or even PTEN expression status. However, RAD001 antineoplastic effect and p70S6K inhibition shown in melanoma, lung, pancreas and colon carcinoma, revealed that inhibition of p70S6K might help to determine the biologically active doses of RAD001. RAD001 was also found to inhibit growth factor-stimulated proliferation of hematopoietic and non-hematopoietic cells, including vascular smooth cells.

i. Growth Inhibition

The *in vitro* effect of RAD001 in six different PTLD patients associated with Epstein–Barr virus and lymphoblastoid B cell lines was investigated. The inhibitory effect of RAD001 was shown to be associated with an increase in the number of cells in the G_0/G_1 phase of the cell cycle, implying a delay in cell cycle progression at this early phase. These results were confirmed by an *in vivo* mouse xenograft model[73].

Moreover the anti-proliferative effects of RAD001 were investigated against three MCL cell lines. Not only was growth inhibition associated with G_1 cell cycle arrest and inhibition of mTOR downstream target 4E-BP1, but synergism was also reported for RAD001 treatment in combination with secondary agents such as doxorubicin, vincristine or rituximab, as well as paclitaxel, vorinostat and bortezomib[74].

RAD001 treatment of human Medullary Thyroid Carcinoma (MTC) cell-line TT and in cells derived from human MTCs reduced cell viability in a dose- and time-dependent fashion, and diminished mTOR and p70S6K phosphorylation in an Akt-independent

manner. RAD001 induced G_0/G_1 cell-cycle arrest in TT-cells, but had no effect on apoptosis[75].

Growth inhibition was also induced by RAD001 in ovarian and breast cancer cell lines in a dose-dependent manner, with high sensitivity displayed in SK-OV-3 ovarian cancer cells. Combination treatment with RAD001 and tamoxifen *in vitro* resulted in an additive but not synergistic pro-apoptotic effect on ovarian cancer cells and an anti-tumor effect on MCF-7 breast cancer cells[76].

Further *in vivo* investigations showed that RAD001 had a concentration-dependent anti-tumor activity in a syngenic rat pancreas carcinoma model with an intermittent dosing schedule. This study also demonstrated a correlation between the anti-tumor efficacy of intermittent RAD001 treatment schedules and prolonged S6K inactivation in Primary Blood Mononuclear Cells (PBMC), further suggesting that PBMC S6K activity levels could be used for assessing RAD001 treatment schedules in cancer patients[77].

ii. Apoptosis

RAD001 in combination with the synthetic histone deacetylase inhibitor MS-275 had an additive growth inhibitory and apoptotic effect in an *in vitro* and *in vivo* model of AML without adverse effects[78]. Similar to the effects seen in CCI-779 treatment, RAD001 induced dual inhibition of mTORC1 and mTORC2 in AML cells *in vivo* and *in vitro*, thus making AML an effective target of both drugs[79].

iii. Autophagy

Apart from exerting its anti-proliferative effects *via* the induction of the apoptotic pathway, recent findings evaluated RAD001-induced autophagy in an *in vivo* model of childhood ALL. In fact, induction of autophagy was associated with increased Beclin 1 expression, the conversion of the soluble form of microtubule-associated protein 1 light chain 3 (LC3) to the autophagic vesicle-associated form LC3-II, and the occurrence of lysosomes/autophagosomes. This was accompanied with a significantly reduced leukemic mass and increased survival in treated mice. These results argue for the inclusion of mTOR inhibitors in future chemotherapy protocols of ALL[80].

RAD001 increased the median survival, caused a G_0/G_1 cell cycle arrest and dephosphorylation of the Rb protein, and reduced levels of CDK 4 and CDK 6 in a mouse model of childhood ALL. Ultrastructural evidence and poly (ADP-ribose) polymersae (PARP) cleavage demonstrated limited apoptosis and enhanced autophagy when animals were treated with RAD001 alone. However, apoptosis was more prominent in the combination approach, supporting further clinical development of mTOR inhibitors for the treatment of patients with ALL[80].

iv. Anti-angiogenesis

In vivo investigation of RAD001's anti-angiogenic activity showed that RAD001 inhibits the proliferation of human vascular endothelial cells. *In vivo* xenograft experiments showed that RAD001 not only exhibits anti-tumor activity, but also inhibits VEGF-dependent angiogenic response at well-tolerated doses. The effects of RAD001 on tumor vasculature were distinct from those induced by pan-VEGF receptor (VEGFR)

inhibitor vatalanib. Therefore, the anticancer activity of RAD001 targets both tumor and stroma of solid tumors with a mechanism distinct from agents targeting the VEGFR signaling pathway. Such results have vast implications pertaining to RAD001 as an effective drug in monotherapy as well as in combination with VEGFR inhibitors[81].

b. Clinical Trials and Combination Treatments

Based on pre-clinical data, RAD001 is undergoing phase II clinical trials in children with solid and brain tumors, particularly rhabdomyosarcomas and non-rabdomyosarcomatous soft tissue sarcomas.

In hematological malignancies, a phase I clinical study conducted on MDS patients showed RAD001 tolerance up to a daily dose of 10 mg. 30% of such patients develop AML, and this evolution is characterized by the progression of clonal cells with high levels of Akt expression and, consequently, increased survival potential and decreased apoptosis. Like Rapamycin, RAD001 could be promising in the treatment of AML and MDS, as a single agent or in combination with other anti-cancer agents[82].

Phase I studies of RAD001 in combination with gefitinib in patients with non–small cell lung cancer (NSCLC) and acquired resistance to erlotinib or gefitinib, revealed reduced toxicities at doses considerably less than those demonstrated by phase II single agent trials. Combination studies of RAD001 and other therapeutic agents directed against other components of the phosphatidylinositol 3-kinase/Akt/mTOR pathway are highly warranted[83].

Patients with relapsed/refractory AML, MDS, B-CLL, NK/T-cell leukemia/lymphoma or MCL were also enrolled in a phase I/II clinical study of RAD001.

Preliminary data seem promising, with some patients exhibiting significant reduction in tumor extent and a diminished requirement for transfusion[82].

Knowing that 40-50% of endometrial cancers have PTEN mutations and, thus, have hyperactivated Akt and mTOR, a phase II clinical trial was launched to evaluate the effect of RAD001-induced mTOR inhibition in patients with progressive or recurrent endometrial cancer.

Similarly, a phase I/II study was initiated to investigate the combination of RAD001 and imatinib mesylate in patients with gastrointestinal stromal tumors (GIST). This combination treatment had previously shown promising preclinical results: synergism, proliferation inhibition, and induction of apoptosis in primary GIST-cell lines refractory to imatinib[69].

Promising phase II clinical data prompted the launching of multiple phase III studies of RAD001, including a clinical study conducted on heavily pre-treated B-CLL patients. RAD001 exhibited modest effectiveness. Combination strategies based with RAD001 and Rituximabin are also being considered in hematological malignancies.
A phase III clinical study was conducted on 15 newly diagnosed localized prostate cancer patients receiving weekly (30, 50 or 70 mg) or daily (5 or 10 mg) doses of RAD001. At the molecular level, treatment was associated with changes in expression of phosphorylated S6K, Akt and 4E-BP1[84]. In a randomized phase III study, RAD001 was associated with a reduction in the risk of progression or death compared with placebo in patients with metastatic RCC. In those patients, disease had progressed after treatment with VEGF-targeted therapies that were not curative and lacked any possible therapeutic option (VEGF receptor tyrosine kinase inhibitors: sunitinib and sorafenib, and VEGF ligand antibodies:

bevacizumab) [81]. The two separate phase III clinical trials with CCI-779 and RAD001 established mTOR inhibitors as valid therapeutic approach to RCC. However, while CCI-779 requires weekly intravenous administration, RAD001 is administered orally, which could be viewed as a distinct advantage. Another distinction between these two trials are that the patient population for the CCI-779 study was comprised of previously untreated, poor-prognosis RCC, compared to previously treated patients resistant to sunitinib or sorafenib in the RAD001 clinical study [85].

5. AP23573 (Deforolimus)

AP23573 is a non-prodrug Rapamycin analogue which was developed by Ariad Pharmaceuticals. This most recent rapalog is a phosphorus-containing compound synthesized with the aid of computational modeling studies. Rapamycin had undergone a modification of C-43 secondary alcohol moiety of the cyclohexyl group with substituted phosphonate and phosphinate groups, while retaining the high-affinity binding to FKBP and mTOR[50]. Owing to its stability in organic solvents, aqueous solutions at a variety of pHs, and in plasma and whole blood, both *in vitro* and *in vivo*, AP23573 became a potent target of mTOR to be investigated. It has demonstrated its *in vitro* anti-proliferative activity in a variety of PTEN-deficient tumor cell lines, including glioblastoma, prostate, breast, pancreas, lung and colon[69]. *In vivo* activity of this most recent Rapamycin analogue was significant in PBMC *via* assessment of decreased phosphorylation levels of mTOR messenger proteins[86].

In the phase I study by Rivera *et al.*, 32 patients with different malignancies received AP23573. Significant pharmakodynamic effects were significant in 85% of

patients, thus confirming good tissue penetration of the mTOR inhibitor. AP23573 exerted a pharmacodynamic effect in more than 85% of examined patients, showing a decrease of phosphorylated S6K expression in the skin, with the stable expression of total p70S6K, which confirmed good tissue penetration of the drug[87].

Moreover, preclinical studies have shown 40% growth reduction of glioblastoma cells treated with AP23573, which prompted a phase I clinical trial to evaluate the effect of this Rapamycin analogue on patients with relapsed or refractory glioblastoma. Another clinical trial has already been initiated involving patients with soft-tissue and bone sarcomas, including leiomyosarcoma, liposarcoma and osteosarcoma, and metastases[69].

Promising data from phase I clinical trials expanded the clinical development of AP23573. On one hand, preliminary data from phase II study of 25 patients with advanced sarcomas, including bone sarcomas, leiomyosarcoma or liposarcoma, was recently reported. AP23573 was administered at doses used in phase I clinical trial and the overall effects of treatment prompted continuation of the study.

Moreover, a phase II clinical trial with AP23573 in 51 patients with relapsed or refractory hematological malignancies such as ALL, CLL, MCL, and T-cell lymphomas showed anti-tumor activity in 41% of the cases with an acceptable side-effect profile. Concomitantly, another phase II clinical trial reported safety and efficacy of AP23573 treatment in relapsed or refractory hematological malignancies. Treatment efficacy, safety, time to disease progression, progression-free survival, and the duration of response showed that AP23573 was well-tolerated and efficient in patients with heavily pretreated hematologic malignancies[69, 88].

In summary, mTOR inhibitors are promising in the treatment of leukemias and other hematological malignancies, and are currently evaluated in several clinical trials (Table 3).

Table 3. mTOR inhibitors in clinical trials for leukemia and other hematological malignancies.

Disease	Phase	Location(s)	Clinical trials.gov#	Additional information
ALL	II	COG Transplant Centres	NCT00795886	Randomized trial comparing sirolimus plus standard GVHD ppx *versus* standard GVHD ppx alone after stem cell transplant for ALL*
ALL/NHL	I	Philadelphia, PA	NCT00068302	Sirolimus for relapsed/refractory ALL or NHL
ALL/NHL CML**	I/II	Philadelphia, PA	NCT00776373	Sirolimus plus etoposide and cytarabine for relapsed/refractory lymphoid malignancies
AML	I/II	Philadelphia, PA	NCT00780104	Sirolimus plus MEC chemotherapy for high risk AML
AML	I	Melbourne, Australia	NCT00636922	Everolimus plus cytarabine in elderly with AML
AML	I	Paris, France	NCT00544999	Everolimus plus cytarabine and daunorubicin in relapsed AML
AML	II	Rome, Italy	NCT00775593	Temsirolimus and clofarabine for relapsed or refractory AML
AML	I/II	Bavaria, Germany	NCT00762632	Everolimus plus nilotinib for c-kit+ CML
CLL/B-NHL	II	Houston, TX	NCT00290472	Temsirolimus for relapsed/refractory CLL or B cell NHL
CML**	I	Multiple centres in U.S., China, and Singapore	NCT00101088	Temsirolimus and imatinib for CML accelerated phase
NHL	I	Ontario, Canada	NCT00659568	Temsirolimus for advanced lymphoma
NHL	I	Cleveland, OH	NCT00671112	Everolimus plus bortezomib for relapsed refractory MCL and other NHL
NHL	II	Multiple centres U.S.	NCT00436618	Everolimus for refractory or advanced NHL
NHL	I	Tokyo, Japan	NCT00622258	Everolimus for refractory or relapsed NHL
NHL/HL	I/II	Multiple centres U.S.	NCT00704054	Deforolimus for relapsed/refractory NHL HD
MCL	II	Munich, Germany	NCT00727207	Everolimus for relapsed/refractory MCL
NHL/HL MM	I/II	Multiple centres U.S.	NCT00474929	Everolimus and Sorafenib for relapsed or refractory NHL, HD, or MM
MM	I	New York, NY	NCT00317798	Sirolimus and ATG for relapsed MM
MM	I	Multiple centres U.S.	NCT00729638	Everolimus and lenalidomide for relapsed MM
Advanced malignancies	I	Houston, TX	NCT00678233	Temsirolimus plus IMC-A12 (anti-IGF-1R ab) for locally advanced or metastatic malignancy, including haematological
Advanced malignancies	I	San Antonio, TX	NCT00060645	Deforolimis for relapsed/refractory malignancies, including NHL, HD, and MM

*A number of clinical trials are on-going using sirolimus post-stem cell transplant as part of GVHD prophylaxis in patients with haematological maligancies. ASCT0431 is the only one that randomizes patients to sirolimus versus no sirolimus with the hypothesis that sirolimus will improve survival *via* a direct action of sirolimus on ALL blasts. **CML in late accelerated phase or blast crisis. AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; NHL, Non-Hodgkin lymphoma; HL, Hodgkin lymphoma; MM, multiple myeloma; GVHD, graft versus host disease; ppx, prophylaxis; ATG, antithymocyte globulin; MEC, mitoxantrone, cytarabine, etoposide.
Source: Teachey, D., and Brown, V. 2009. Mammalian target of rapamycin inhibitors and their potential role in therapy in leukaemia and other haematological malignancies. *British Journal of Hematology* 145(5):569-580.

C. HTLV-I and Adult T-Cell Leukemia

It has been 30 years since a new leukemia termed ATL was described in Japan[89] based on the characteristic clinical features and geographical distribution of the patients' birth places[90]. ATL is an aggressive malignancy of mature activated T-cells associated with HTLV-I[91], the first retrovirus directly associated with a human malignancy[92, 93]. At present, an estimated 10-20 million people worldwide are infected with HTLV-I, in regions where HTLV-I is endemic such as southern Japan, the Caribbean, subsaharan Africa, Brazil, and northern Iran[94 95]. The infectivity of HTLV-1 is tightly cell-associated, and is mediated through a virological synapse. HTLV-I might be transmitted intravenously or by sexual intercourse, still vertical transmission through breast feeding is needed for development of ATL[95].

ATL develops in a small proportion of HTLV-I infected people (3-5%) after a very long latency period (approximately 50 to 60 years after infection) [96]. ATL has four subtypes: acute, lymphoma-type, chronic, and smouldering. The first two types show aggressive clinical courses, whereas the last two types progress more slowly[97]. Clinical features of ATL include leukemic cells with multi-lobulated nuclei called "flower cells" which infiltrate various tissues such as lymph nodes, spleen, liver, skin, and lungs[97]. ATL carries a poor prognosis because of resistance to chemotherapy and severe immunosuppression, predisposing patients to opportunistic infections. Despite important advances in the treatment of ATL with the combination of zidovudine and IFN, which improved the response rate in patients and prolonged their survival[98], unfortunately, most of the patients relapsed thus underlining the need to search for alternative or complementary therapies.

Oligoclonal expansions of activated T-cells that have been infected with HTLV-I (at least early after infection) result from the expression of a viral gene known as the transactivator Tax, which is critical for increasing viral gene expression. Current data support the view that the viral Tax protein is needed to initiate but not to maintain cellular transformation because it is the main *in vivo* target of the host's cytotoxic T-lymphocytes (Figure 5). This serves to explain the observation that in the late stages of *in vivo* leukaemogenesis, ATL cells that lack Tax expression are selected to emerge[99]. The transformation of infected cells is related to the ability of Tax to deregulate the transcription of genes involved in cellular proliferation, DNA repair mechanisms, cell-cycle control, and apoptosis[100].

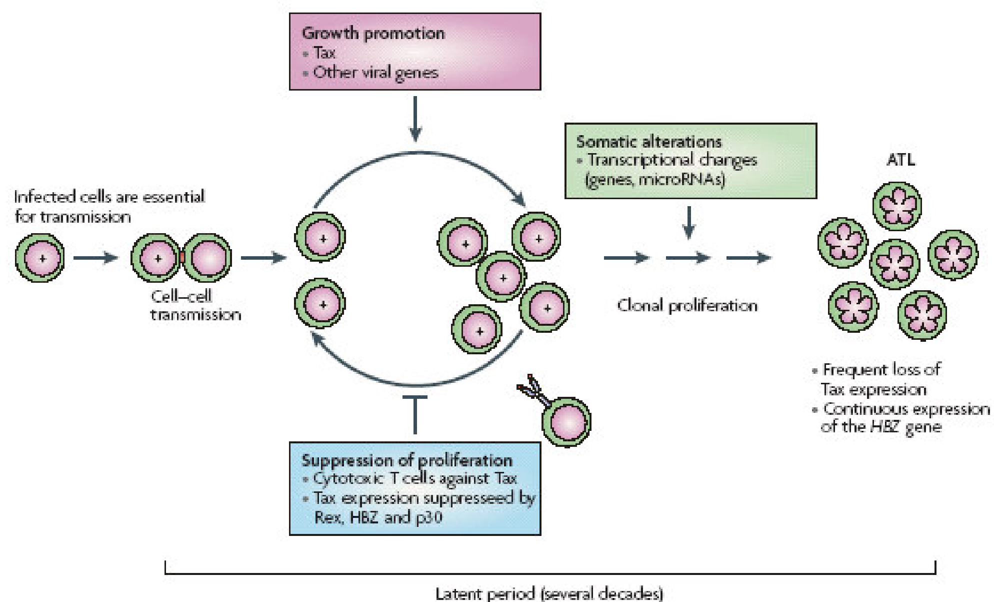

Fig. 5. The natural history of HTLV-1 infection.
Source: Matsuoka, M., & Jeang, K. T. 2007. Human T-cell leukaemia virus type I (HTLV-I) infectivity and cellular transformation. *Nature Reviews Cancer* 7(4): 270-280.

D. The Oncoprotein Tax

1. Overview

Tax is a 40-kDa nuclear phosphoprotein, which is encoded by the pX region of the viral genome[101]. It is predominantly present in the nucleus and can shuttle into the cytoplasm using a nuclear export signal[102]. Moreover, it has been shown that Tax contains a nuclear localization signal (NLS) found in its N-terminal 48 residues (Figure 6)[103]. Several lines of evidence link the virally encoded Tax to cellular transformation[104,105]. Tax has been shown to immortalize T-lymphocytes[106] and to transform rat fibroblasts[105]. Tax-transformed fibroblasts and lymphoid cells induce tumors *in vivo* when injected into nude mice[105, 107]. Moreover, overexpression of HTLV-I Tax in transgenic mice results in the formation of mesenchymal tumors[108], salivary and lacrimal gland exocrinopathy[109], lymphodenopathy or splenomegaly[110], lymphoma and leukemia[109]. Tax does not bind to DNA by itself but to transcription factors that bind specific enhancers, thus it stimulates transcription from the HTLV-I long terminal repeat[111] and from the promoters of specific cellular genes[112] by recruiting cellular transcription factors. Stimulation of gene expression by Tax involves members of the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB), NFκB, and the serum response factor (SRF) as well as the two related transcriptional co-activators CREB binding protein (CBP) and p300.

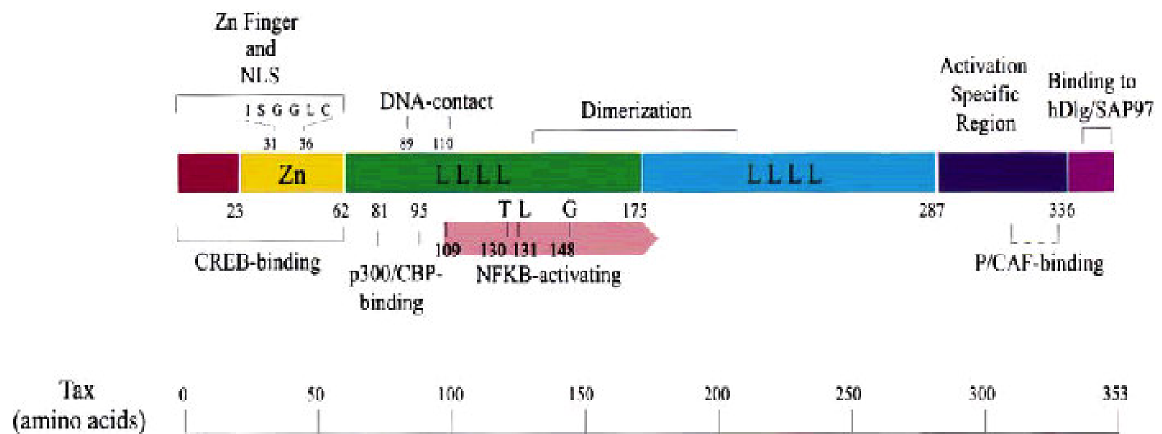

Fig. 6. The various functional domains found in Tax.

Source: Jeang KT. 2001. Functional activities of the human T-cell leukemia virus type I Tax oncoprotein: cellular signaling through NF kappa B. *Cytokine Growth Factor Reviews* 12: 207-217.

2. Cellular Functions of Tax

a. Cell Survival

HTLV-I uses Tax to engage two cellular pathways for suppressing apoptosis (Figure 7). First, Akt is activated through its phosphorylation at Ser 473 *via* PI3K. Tax promotes Akt phosphorylation by directly binding PI3K[113], resulting in survival and increased proliferation of virus-infected cells. When Tax-expressing cells are treated with PI3K inhibitors to prevent Akt phosphorylation, cell death occurs[60].

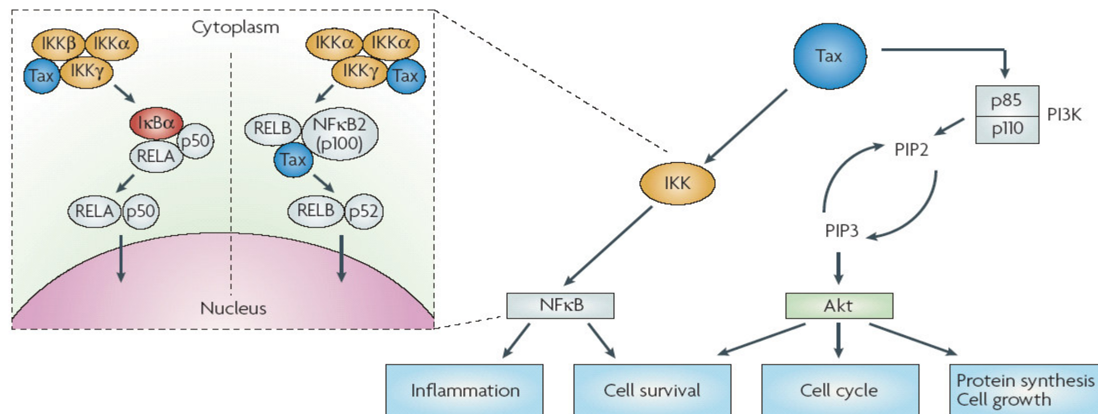

Fig. 7. Tax activates two survival pathways, NFκB and Akt, to promote cell survival and proliferation.

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

NFκB is a second major survival pathway engaged by HTLV-I (Figure 8). NFκB is activated in many human cancers including ATL[114]. Tax activation of NFκB occurs predominantly in the cytoplasm where it binds to inhibitor of IκB (IKKγ). This binding triggers the phosphorylation of IKKα and IKKβ, which form a complex with IKKγ. Subsequently, the IKKα-IKKβ-IKKγ complex phosphorylates IκBα, leading to its proteasome-mediated degradation. Upon removal of IκBα, cytoplasmic NFκB molecules are freed to migrate into the nucleus where they activate the transcription of NFκB-responsive genes[115]. Tax can also stimulate a second NFκB pathway through the IKKα-dependent processing of the NFκB p100 precursor protein to its active p52 form. This pathway is activated by Tax binding to IKKγ and p100 in an IKKα-IKKγ-p100 complex that lacks IKKβ[116].

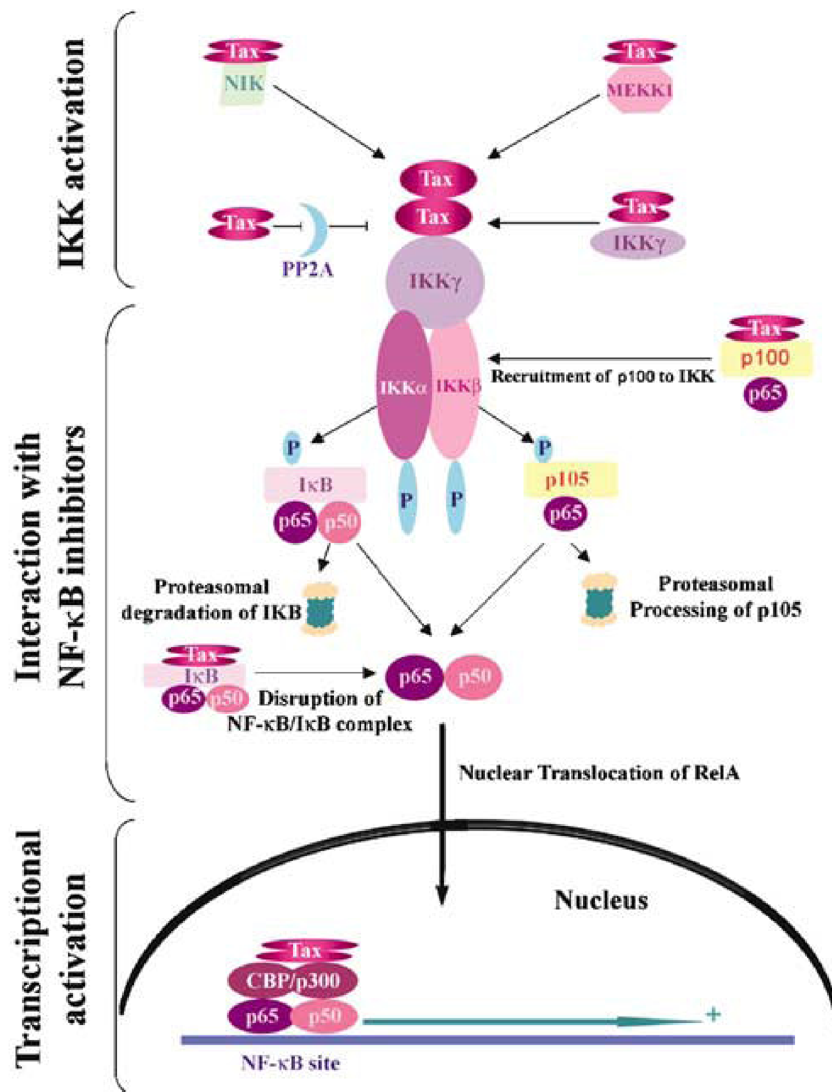

Fig. 8. Molecular mechanisms of Tax-induced activation of the NF-κB pathway.

Source: Kfoury, Y., Nasr, R., Hermine, O., de The´, H., Bazarbachi, B. 2005. Proapoptotic regimes for HTLV-I-transformed cells: targeting Tax and the NF-κB pathway. *Cell Death and Differentiation* (12): 871–877.

b. Cell Cycle Progression

Tax deregulates the normal cell cycle control in T-cells by targeting different regulators of cell cycle progression (Figure 9 and Table 4). In addition, Tax provides significant mitogenic activity especially at G_1-S-phase transition[117, 118], by provoking several cellular activities (Table 4). Although Tax does not interact directly with Rb, it forms a complex with $p16^{INK4a}$ and $p15^{INK4b}$, two cell cycle inhibitors that bind to CDK4 rendering it inactive, resulting in Rb phosophorylation[102]. This phosphorylation frees the E2F1 transcription factor, accelerating cell-cycle transition from G_1 to S[119, 120]. Tax can also interfere with the cell cycle progression through the transcriptional activation or repression of $p18^{INK4c}$, cyclins A, C, D2 and E, CDK2, $p21^{(CIP1/WAF1)}$, and E2F. Moreover, Tax interferes with cell cycle regulation at the post-translational level by forming a complex with cyclin D3, which induces its phosphorylation[102].

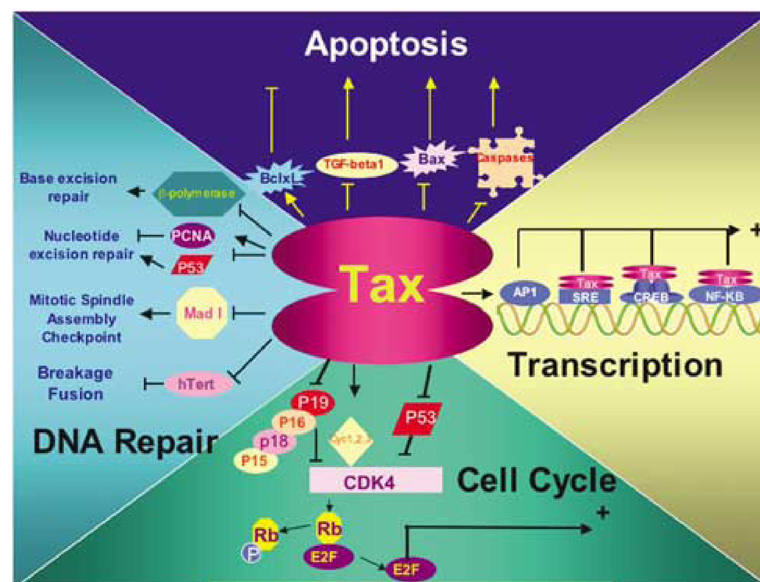

Fig. 9. Cellular pathways altered by the HTLV-I oncoprotein Tax
Source: Kfoury, Y., Nasr, R., Hermine, O., de The´, H., Bazarbachi, B. 2005. Proapoptotic regimes for HTLV-I-transformed cells: targeting Tax and the NF-κB pathway. *Cell Death and Differentiation* (12): 871–877.

Table 4. Cellular activities modulated by Tax

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

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

c. Multipolar Mitosis and Aneuploidy

Structurally damaged DNA and chromosomal numerical abnormalities (aneuploidy and/or polyploidy) are common in most human cancers. As a result, aneuploidy has been proposed to be a cause of transformation[121]. ATL has chromosomal instability, with cells that are usually aneuploid[122].

Aneuploidy can arise from multipolar mitosis which happens when more than two spindle poles emerge in one cell. Recent studies have shown that HTLV-I causes

multipolar mitosis[123, 124]. Tax causes amplification of centrosomes during interphase by affecting the centrosome-associated protein TAX1BP2, which normally blocks centriole replication. Moreover, to allow the emergence of aneuploid cells, Tax binds the cell's spindle assembly checkpoint protein MAD1[125], which normally functions to check for proper microtubule attachment and mitotic chromosome partitioning. The result is that missegregated chromosomes are captured in a small nuclear sack to create a micronucleus that is separate from the cell's main nucleus, or cells have a loss or gain of chromosome copies[126].

d. DNA Structural Damage

HTLV-I abrogates cellular checkpoints and DNA repair functions that monitor and edit DNA structural damage. The cellular processes attenuated by Tax in HTLV-I infected cells include the loss of the p53 checkpoint, decreased cellular DNA repair functions (nucleotide excision repair, base excision repair, or mismatch repair), and the repression of telomerase function[97] (Figure 9).

For example, Tax represses the expression of DNA polymerase-β, an enzyme involved in base excision repair. Furthermore, Tax-mediated inactivation of the p53 checkpoint results in a suppression of the nucleotide excision repair mechanism. Tax-inactivation of p53 is not accomplished by direct binding of Tax to p53, but rather by a unique mechanism that includes constitutive phosphorylation of p53 at S15 and S392[127]. Analysis of Tax mutants in lymphocytes demonstrates that Tax-induced p53 inhibition correlates with the ability of Tax to activate NFκB[127]. Moreover, by repressing the

expression of human telomerase hTert, Tax interferes with protective mechanisms used to prevent inappropriate breakages fusion. Tax has also been found to bind and thus impair the function Mad1, a mitotic spindle checkpoint constituent[102].

e. Apoptosis

Tax has the ability to protect HTLV-I-infected cells from both spontaneous and chemotherapy-induced apoptosis (Figure 10). This oncoprotein induces the expression of the antiapoptotic protein Bcl-x_L through the NF-κB and the CREB pathways[128], and represses the transcription of the proapoptotic bax gene[129]. Furthermore, Tax inhibits the caspase cascade in an NF-κB-dependent manner through the induction of the caspase inhibitors X-IAP, cIAP-1 and c-IAP-2[130]. Moreover, Tax-dependent activation of the NF-κB pathway inhibits p53 transactivation function, thus, interfering with G_1 arrest and p53-dependant apoptosis[131]. This explains why in ATL-derived cells, p53 is sometimes mutated but most frequently functionally inactive[132].

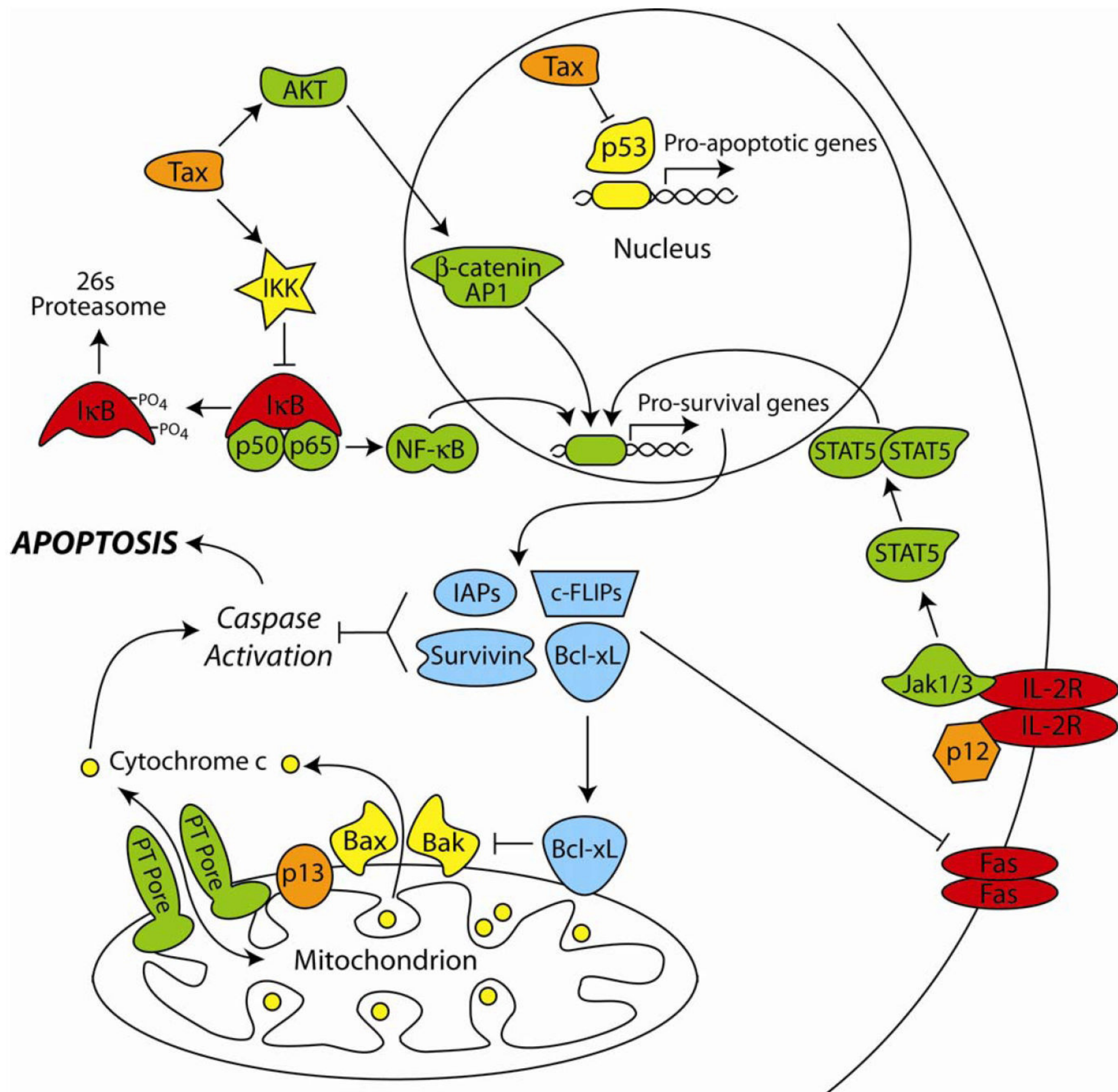

Fig. 10. Apoptotic regulatory pathways interrupted by HTLV-1 proteins.

Source: Taylor, J., & Nicot, C. 2008. HTLV-I and apoptosis: role in cellular transformation and recent advances in therapeutic approaches. *Apoptosis* June 13(6): 733–747.

E. Aim of the Study

The mTOR signaling pathway is fundamental for cell survival and is deregulated and activated in several tumors, therefore, it has been investigated as an attractive target for the development of cancer therapeutics. mTOR inhibitors are particularly beneficial in cancer models with aberrant expression and/or activation of PI3K/Akt/mTOR pathway signaling molecules, and/or aberrant cell cycle regulation or expression of anti-apoptotic markers. Clinical trials with mTOR inhibitors, including Rapamycin analogs CCI-779, RAD001 and AP23573, were well tolerated and showed favorable responses in relapsed or resistant solid tumors and some hematological malignancies.

Knowing that the oncoprotein Tax can activate the PI3K/Akt signalling pathway, we aim at using the ATL/lymphoma as a model to explore the effect of RAD001 on viability, and determine the extent of growth suppression in HTLV-I-positive and HTLV-I-negative T-cells. In addition, we will investigate the mechanisms of RAD001-induced growth suppression and cell death in ATL and peripheral T-cell lymphomas.

Specifically, we will study the following aims:

1) Effect of mTOR inhibition by RAD001 on cell survival by assessing the effect of treatment on:

a) Growth of ATL and malignant T-cell lines, normal resting and activated circulating T-lymphocytes, and on primary ATL cells from two patients

b) Mitochondial membrane integrity

c) Intracellular ROS levels

d) Cytochrome c levels

e) Ceramide levels

2) Effect of RAD001 treatment on cell cycle distribution upon:

a) Short-term RAD001 treatment

b) Long-term RAD001 treatment

3) Induction of cell death pathways upon RAD001 treatment:

a) Induction of senescence upon short-term treatment

b) Induction of apoptosis upon long-term treatment

4) Modulation of cell cycle regulators involved in cell death and survival upon RAD001 treatment.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

CEM, Jurkat, HuT-78 and Molt-4 are HTLV-I negative $CD4^+$ malignant T-cell lines, whereas, HuT-102, MT2, C81 and C91 are HTLV-I transformed $CD4^+$ T-cell lines. The HTLV-I transformed ATL cell lines are infected by the HTLV-I retrovirus and transformed by Tax oncoprotein. CEM cells are T-lymphoblastoid cell lines derived by Foley *et al*,[133] from peripheral blood of a 4-year-old Caucasian female with ALL. Jurkat cell lines were originally established by Schneider *et al.* from the peripheral blood of a 14-year-old with ALL in relapse[134]. HuT-78 cells are cutaneous T-lymphocytes obtained from a 53 year old Caucasian male patient with Sezary syndrome. Molt-4 cells, established by Minowada *et al*., are T-lymphoblastoid cell lines taken from a 19-year old relapsed ALL male patient who received prior multidrug chemotherapy[135]. HuT-102 cells were originally derived from the peripheral blood of a 26 year old black male patient with cutaneous T-cell lymphoma and were found to contain type C retrovirus particles[136]. MT2 cells are derived from bone marrow $CD4^+$ T-lymphocytes of a healthy donor after cocultivation with leukemic cells from an ATL patient. C81 cells harbor a defective provirus that selectively expresses Tax, and they are derived from umbilical cord T-cells transformed by HTLV-I. C91 cells are derived from human umbilical cord cells transformed by HTLV-I.

1. Cell Growth and Drugs' Treatment

All HTLV-I positive and negative cells were cultured in RPMI 1640 medium (Invitrogen, USA) containing 10% heat inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotics, 1% kanamycin solution, and 1% sodium pyruvate (Invitrogen). All cells were grown in a humidified incubator (95% air, 5% CO_2).

a. Cell Passaging

Cells were passaged or fed for maintenance and expanded before an experiment. To expand the number of cells, RPMI 1640 medium was added to increase volume of cells three fold. For experimentation, cell number was calculated using a hemocytometer according to the following formula: cells/ml = average number of cells x dilution factor x volume of suspension x 10^4. Cells were counted before experimentation by trypan blue dye exclusion using 0.4% trypan blue solution (SAF Bulk Chemicals, USA). For most experiments, cells were seeded at a density of 2 x 10^5 cells/ml. Tissue culture flasks were purchased in a variety of sizes from the Falcon division of Becton, Dickinson and Co. (Cockeyesville, MD).

b. Preparation of RAD001

RAD001 (10 mg powder) was obtained from Novartis (Switzerland) and stored at 4°C. 2 mg were weighed and dissolved in 100 μl of Dimethyl sulfoxide (DMSO), to get a final stock concentration of 20 mM. 10 μl aliquots were stored at -20^oC. In all assays, the

final concentration of DMSO never exceeds 0.1% and this level shows no effect on the proliferation of all tested cell lines[137].

2. Isolation of Peripheral Blood Mononuclear Cells

For the normal PBMCs, 10 ml of blood from each of the three healthy donors was withdrawn and put in a tube containing heparin to avoid coagulation. Blood was then diluted (1/4 dilution) in 30 ml phosphate-buffered saline (PBS). 10 ml of blood was added to 5 ml of Ficoll paque (GE Healthcare), while avoiding mixing the blood with Ficoll. Ficoll paque divides the blood into 3 layers: Plasma (upper layer); lymphocytes (middle layer); red blood cells (lower layer). Tubes are centrifuged at 400 g (~1410 rpm) for 30 min at 20°C, using a centrifuge with no brake to avoid mixing of Ficoll with blood. The upper plasma phase is aspirated and discarded. The middle phase (lymphocytes + monocytes) is aspirated from the four tubes and divided into two new 15 ml tubes and diluted by adding 10 ml PBS into each tube and mixed by inverting. The suspension is centrifuged for 10 min at 900 rpm RT and washed with 10 ml 1X PBS and spin again. The pellet is resuspended in 4 ml of RPMI containing 20% FBS and 2% Penicillin-Streptomycin. Cells were counted (around 4 x 10^6 cells/ml) and placed in 25 cm^2 flasks containing 10 ml of RPMI. For activation, 50 μl of 2 % Phytohemagglutinin (PHA) /10 ml media were added (Gibco), and the activated cells were incubated with PHA for 24 h before starting the treatment.

PBMCs were also isolated from two ATL patients after obtaining their informed consent, and centrifuged over Ficoll-Hypaque. Before treatment, cells were grown for

three days in 10% Rec IL-2 (Roche, Mannheim, Germany), 20% heat inactivated FBS, and a ratio of 1:400 PHA, then frozen. When needed, cells were cultured in 20% heat inactivated FBS and 10% Rec IL-2.

B. Growth Assays

All cell lines were seeded into 96-well plates (Falcon, Cockeyesville, MD, USA) at a density of 2 x 10^5 cell/ml and treated with 0.1% DMSO or 2, 20, 200, and/or 2000 nM RAD001. Cell growth was assessed by the use of the CellTiter 96® non-radioactive cell proliferation assay kit as suggested by the manufacturer's recommendations (Promega Corp., Madison, WI, USA). Relative MTT dye uptake is assessed by optical density (OD) measurement at 595 nm using an enzyme-linked immuno-sorbent assay (ELISA) microplate reader. Results are expressed as growth relative to DMSO-treated controls and are derived from the mean of quadruplicate wells. In addition, cell count using trypan blue dye exclusion protocols (hemocytometer) was used to evaluate cell growth.

C. Long-term Treatment with RAD001

Cells were seeded at 2 x 10^5 cells/ml in 25 cm^2 flasks (Falcon), starting with 6 ml for control flasks and 5ml for RAD001-treated flasks. Every three days, cells were counted with trypan blue (% control) and replenished by splitting them 1 in 4 in fresh RPMI, while increasing the volume of the treated cells to obtain enough for cell cycle analysis and/or protein extraction. Once the cell count started to drop, cells were harvested to prepare the

samples for the appropriate procedure (propidium iodide (PI), TUNEL, and protein extraction).

D. Cell Cycle Analysis

Cells were cultured in 25 cm^2 flasks (Falcon) and collected at different time-points following RAD001 treatment. Cells were then washed twice with cold PBS, fixed in ice cold 100% ethanol, and stored for 24 h at -20°C. Subsequently, they were rinsed with PBS, incubated for one hour in PBS containing 50 units RNaseA (Roche, Mannheim, Germany) and then stained with PI (50 μg/ml) (Sigma). Cell cycle analysis was performed using a FACS scan flow cytometer (Becton Dickinson). Each sample was collected as 10,000 ungated events and the corresponding cell cycle distribution was determined using CellQuest software (Becton-Dickinson).

E. TUNEL Assay

The TUNEL assay is a measure of apoptosis through detection of DNA strand breaks (a late apoptotic event). DNA strand breaks contain free 3'-OH termini which may be conjugated to dUTP-fluorescein through the enzymatic action of terminal deoxynucleotidyl transferase. Fluorescein fluorescence is detected by flow cytometry and serves as a measure of apoptosis. In brief, cells are seeded (10 million cells/condition) and treated with 0.1% DMSO for control or 20 nM RAD001. Two extra control flasks, one for positive and one for negative controls, are prepared from any cell line used in the experiment. Cells are washed and fixed with 4% formaldehyde solution for 15 min at room temperature, and then the dry pellet is stored at -20°C for not more than one week. Cells

are then washed with 1X PBS and incubated with 250 μl permeabilization solution (0.1% triton X-100 in 0.1% sodium- citrate) on ice for 4 *min*. Only the positive control cells are then incubate in 200 μl of 1mg/ml DNase (prepared in 50 mM Tris pH 7.5) for 15 min at room temperature, then washed twice with 1X PBS. Other samples are washed once with 1X PBS, and pellets are re-suspended and incubated for 60 min at 37°C in an incubator in the dark in TUNEL reagents: 50 μl Label solution for negative control tube, 50 μl TUNEL reaction mixture for other samples (TUNEL reaction mixture: 50 μl enzyme + 450 μl label solution). Cells are then washed twice with 1X PBS, re-suspended in 1 ml PBS, and transferred into polystyrene falcon round bottom tubes for flow cytometry analysis (with the excitation wavelength set at 470–490 nm and the emission wavelength at 505 nm).

F. Rhodamine 123 Staining

Quantitation of MPT was determined by rhodamine (R123) (Sigma) retention. R123 is a cationic fluorescent dye that accumulates in active mitochondria with high membrane potentials. RAD001 treated cells and HPR treated cells (positive control) were washed twice with buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO4, 1 mM $CaCl_2$, 1 mM MgCl2, and 25 mM Hepes buffer (pH 7.4), and were then loaded with 5 μM R123 for 30 min in the buffer at 37°C. Subsequently, cells were washed twice with the former buffer. R123 was excited at 488 nm, and fluorescence emission at 525 nm was assessed using flow cytometry. The results were analyzed using CellQuest software (Becton-Dickinson).

G. ROS Measurement

Generation of intracellular ROS was measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) kit (Molecular Probes, Eugene, OR, USA). CM-H_2DCFDA is a cell-permeant indicator for ROS that is non-fluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell yielding the fluorescent derivative 2'-7'-dichlorofluorescin. Following indicated treatments, cells were spun down and resuspended in 500 μl RPMI containing 2% FBS and 10 μM H_2DCFDA for 20 min at 37^0C. Subsequenly, cells were washed twice with PBS and then analyzed with a FACS scan flow cytometer (Becton Dickinson, CA, USA) with excitation set at 488 nm and emission at 530 nm.

H. Measurement of Cytochrome c Release

Cytochrome c release was measured using the Cytochrome c ELISA Kit (Calbiochem, Darmstadt, Germany) according to manufacturer's recommendations. Subcellular fractionation was performed before solubilization. Cells were washed twice with PBS, and the pellet was suspended in 1ml of ice cold buffer A (20 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCL, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose. The cells were homogenized on ice by douncing three times in a Dounce homogenizer with a sandpaper-polished pestle. After centrifugation for 5 min at 4°C, the supernatants were centrifuged at 105,000 x g for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

I. Ceramide Measurement

Lipids were collected according to the method of Bligh and Dyer[138]. Ceramide was measured with a modified diacylglycerol kinase assay using external ceramide standards, as described[139]. Briefly, 80% of the lipid sample was dried under N_2. The dried lipid was solubilized in 20 μl of anoCtyl-$^{\beta}$-D glucoside/dioleoyl phosphatidylglycerol micellar solution (7.5%oCtyl-$^{\beta}$-D-glucoside, 25 mM dioleoyl phosphatidylglycerol) by several cycles of sonication in a bath sonicator followed by resting at room temperature for 15-20 min. The reaction buffer was prepared as a 2X solution, containing 100 mM imidazole HCL pH 6.6, 100 mM LiCl, 25 mM $MgCl_2$, 2 mM EGTA. To the lipid micelles, 50 μl of 2X reaction buffer, 0.2 μl of 1 M dithiothreitol, 5 μg of diglycerol kinase membranes, and dilution buffer were added (10 mM imidazole, pH 6.6, 1 mM diethylenetriaminepentaacetic acid, pH 7) to a final volume of 90 μl. The reaction started by adding 10 μl 2.5 mM [γ-^{32}P] ATP solution (specific activity of 75 000-200 000 cpm/nmol) and allowed to proceed at 25°C for 30 min. Bligh and Dyer lipid extraction was performed and a 1.5 ml aliquot of the organic phase was dried under N_2. Lipids were then resuspended in a volume of 50 μl methanol/chloroform (1:9, v/v) and 25 μl were spotted on a 20 cm silica gel thin layer chromatography plate. Plates were developed with chloroform:acetone:methanol:acetic acid:H_2O (50:20:15:10:5), air dried, and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide-phosphate, the phosphorylated products of diacylglycerol and ceramide, respectively, were identified by comparison to known standards. Spots were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted on a scintillation counter. Linear curves

of phosphorylation were produced over a concentration range of 0-960 pM of external standards (dioleoyl glycerol and CIII ceramide, Sigma). Ceramide levels were normalized to lipid phosphate levels. It is important to note that under these conditions, there was a total conversion of ceramide and diacylglycerol to their phosphorylated products, and there was no change in the specific activity of the diacylglycerol kinase enzyme.

J. Acridine Orange Staining

Cells were cultured in 25 cm^2 flasks (Falcon) and collected at different time-points post-RAD001 treatment. On the day of harvesting, cells were spun down for 10 min at 1500 rpm, washed twice with 1ml of 1X PBS, and spun again at 1500 rpm for 10 min. Pellet was resuspend in 1 ml PBS and transferred to polystyrene tubes. Acridine orange was added at a final concentration of 1 μg/ml and cells were incubated for 15 min. Green and red florescence emissions from 10^4 cells illuminated with blue (488 nm) excitation light were detected using FACS flow cytometer, and results were analyzed using CellQuest software (Becton-Dickinson).

K. Senescence-Associated β-Galactosidase Assay

Senescence was detected using Senescence β-Galactosidase Staining Kit (Cell Signalling Technology). Cells were seeded at 2 x 10^5 cells/ml, 10 ml per condition, and cultured in 25 cm^2 flasks (Falcon). Treatment was done with 0.1% DMSO (control) or 20 nM RAD001. Every 4 days, cells were counted by trypan blue exclusion and samples were replenished by centrifugation at 900 rpm for 5 min, then re-suspending the pellet in fresh

RPMI with 0.1% DMSO or 20 nM RAD001. On the day of harvesting (day 4, 8, 12, or 16 post-treatment), cells were spun at 1500 rpm for 10 min and washed once with 2 ml PBS. Cells were fixed at room temperature for 15 min with 1 ml Fixative Solution (prepared from 10X Fixative solution with distilled water). Cells were washed once with 1ml 1X PBS and pellet was re-suspended in a 6-well plate with 1 ml Staining Solution mix (930 μl Staining Solution, 10 μl Staining Supplement A, 10 μl Staining Supplement B, 50 μl 20 mg/ml X-gal in DMF prepared by dissolving 20 mg of the powder in 1ml DMF). Cells were incubated overnight at 37°C and viewed using a light microscope at 20X and 40X magnifications. For long-term storage of stained wells, Staining Solution was removed and cells were stored at 4°C with 4% formaldehyde. For mounting, cells were washed once with 1X PBS and around 180,000 cells per condition were cytospun at 1500 rpm for 5 min. To each slide, 10 μl of 70% glycerol was added with a coverslip on top, and pictures were taken using the confocal microscope.

L. Immunoblot Assays

1. Protein Extraction

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

2. Gel Casting

Denaturing polyacrylamide gels were cast in two layers according to the following procedure. Using the Bio-Rad electrophoresis cell, the gel casting stand was assembled according to manufacturer's instructions. A 12% separating gel was prepared by combining 3 ml of 30% acrylamide / 0.8% N'N'-bis-methylene-acrylamide solution, 2 ml of 4X separating gel buffer (1.6 M tris-HCl pH 8.8, 10% (w/v) SDS) and 2.5 ml ddH_2O. A 10% gel was prepared in the same way except 2.5 ml acrylamide solution and 3 ml ddH_2O were used. Before pouring the separating gel, 45 μl 10% ammonium persulfate (APS) (freshly prepared) and 12 μl N,N,N',N'-Tetramethylethylenediamine (TEMED, ICN Biomedicals) were added. The gel was poured to ~3 cm below the top of the smaller glass plate and distilled water was layered atop the gel to allow for a level upper surface. After polymerization of the separating gel (~10-15 min), the layered water was poured off. The stacking gel was then prepared by combining 1ml of 30% acrylamide / 0.8% N'N'-bis-methylene-acrylamide solution, 1.7 ml of 4X stacking gel buffer (0.5 M tris-HCl pH 6.8, 0.4% (w/v) SDS), and 4 ml ddH_2O. Before pouring the gel, 40 μl 10% APS (freshly prepared) and 10.6 μl TEMED were added. The gel solution was poured to the top of the smaller glass plate and a comb was inserted between the plates. After polymerization of the stacking gel (30-60 min), the comb was gently withdrawn and the wells were rinsed with 1x electrophoresis buffer (prepared from a 5X solution containing 120 mM tris base, 1M glycine, 0.5% (w/v) SDS, pH to 8.3).

3. Gel Running and Protein Transfer

After the gels were cast, cellular protein extracts (30-50 μg) were loaded and run in 1X electrophoresis buffer at 74 Volts for ~1.5 h using the Mini-PROTEAN II electrophoresis cell unit. After the run, gel sandwiches were disassembled and gels were set up for transfer in the Bio-Rad Trans-Blot® Electrophoretic Transfer Cell according to manufacturer's instructions. Proteins were transferred onto nitrocellulose membranes (Biorad) in transfer buffer [(50 mM tris base, 77 mM glycine, 0.04% SDS (w/v), 20% methanol (v/v)] under 30 Volts overnight at 4^oC while stirring.

4. Hybridization and Protein Detection

Hybridization conditions for PARP, p21, Bax, cyclin-D_1, Bcl-2, Bak (Santa Cruz Biotechnology, CA, USA), p53, phosphorylated p53, PUMA (Cell Signalling), Tax, Rb (oncogene research products) and GAPDH (Biogenesis, UK), Autophagy LC3 (Abgent, USA) polyclonal antibodies are similar. Briefly, membranes were incubated in TBS (10 mM tris-HCl pH 8.0, 150 mM NaCl) containing 5% dry milk and 0.05% tween 20 for 1 h at room temperature while shaking. They were then incubated with the primary antibody for either 2 h at room temperature or overnight at 4^oC. Membranes were then washed 3 times (10 min per wash) in TBS containing 0.05% tween 20 and placed in sealed bags with a the secondary antibody (Santa Cruz, horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat) at a dilution of 1:5000 for 1 h at room temperature while shaking. Finally, membranes were washed 3 times (10 min per wash) with TBS containing 0.05% tween 20 before protein detection.

Proteins were detected by enhanced chemiluminescence using the ECL system (Santa Cruz Biotechnology) in conjunction with horseradish peroxidase-conjugated secondary antibodies. Membranes were then exposed to x-ray films for varying time periods (10 sec-15 min). Equal protein loading and quality were verified using GAPDH constitutive protein expression levels.

M. Statistical Analysis

Statistical analysis was performed on raw data using SPSS v16.0. Dunnett's two-way analysis of variance (ANOVA) was used to compare the proliferation difference of treated to control cell lines, primary ATL cells, and normal PBMC. Statistical significance is assumed when the *p*-value is less than 0.05.

CHAPTER III

RESULTS

A. Effect of RAD001 on the Growth of Normal and Malignant T-Lymphocytes

1. HTLV-I Positive and Negative T-Cells

HTLV-I transformed (HuT-102, MT2, C91, and C81) and HTLV-I negative (CEM, Jurkat, HuT-78, and Molt-4) malignant T-cell lines were treated with RAD001 concentrations ranging from 2 to 2000 nM. RAD001 abrogated mTOR signaling in the tested cells as phopshorylation of the downstream substrate 4E-BP1 was inhibited (data not shown). RAD001 treatment at any of these concentrations up to 96 h resulted in cytostatic growth inhibition in all tested cell lines ranging from 34% to 74% (Figure 11). Furthermore, RAD001's effect on proliferation was dose-independent in all tested cells as 20 nM concentrations reduced cell viability similarly to 100-fold higher concentrations of RAD001 (2000 nM) up to 96 h (Figure 11). Similar cell growth results were observed when viability was assessed using trypan blue dye exclusion assay (data not shown). These RAD001 concentrations are pharmacologically achievable in humans[140, 141] and non-cytotoxic to cells as no significant necrosis was observed in the various cell lines after 6 h of RAD001 treatment (data not shown).

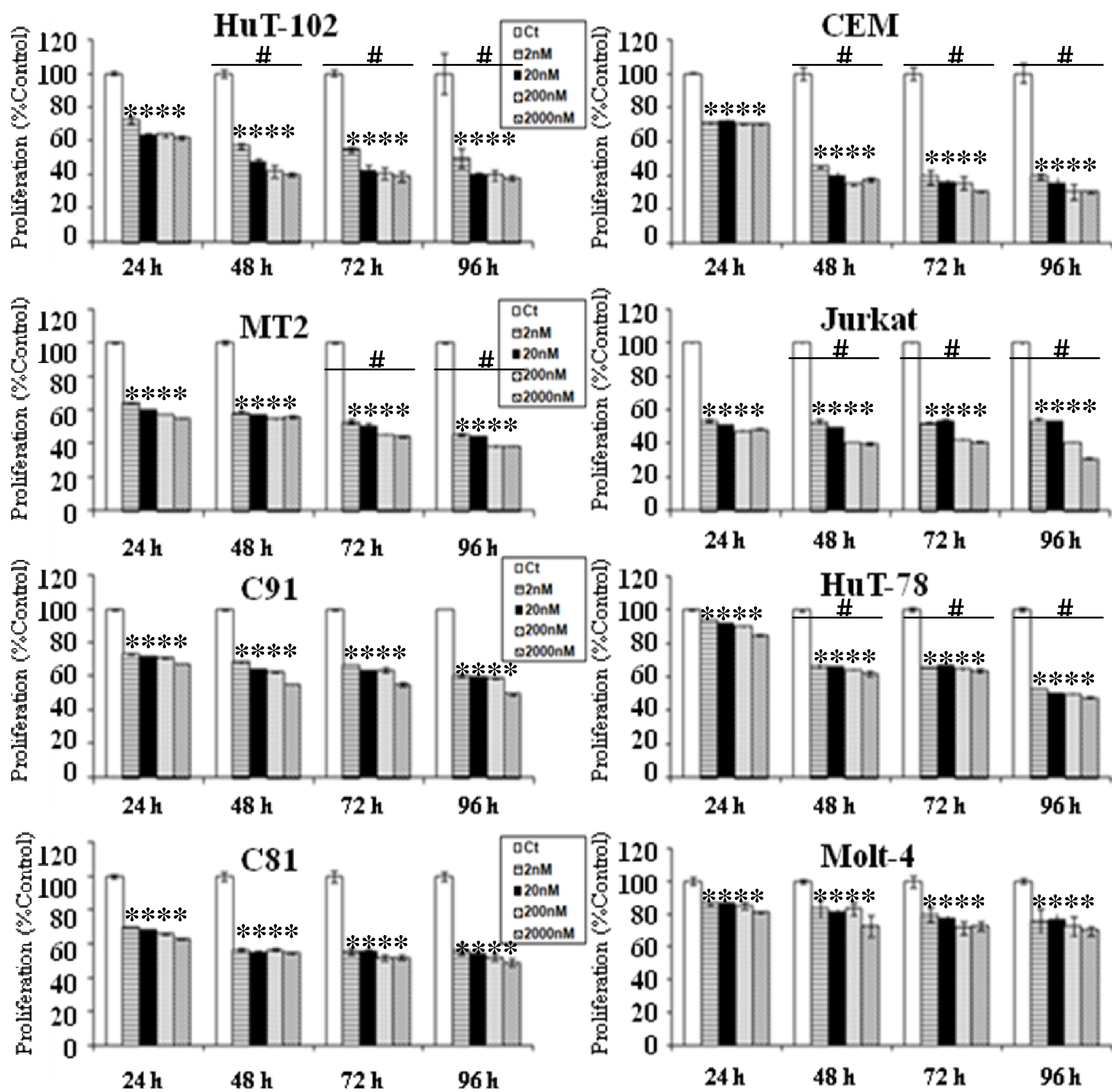

Fig. 11. HTLV-I positive and HTLV-I negative malignant T-cells are sensitive to RAD001 treatment. HTLV-I positive (HuT-102, MT2, C91 and C81) and HTLV-I negative (CEM, Jurkat, HuT-78 and Molt-4) human T-cell lines were treated with the indicated RAD001 concentrations for up to 96 h. Cell growth was assayed in quadruplicate wells with the CellTiter 96® nonradioactive cell proliferation kit, and cell viability is expressed as percentage of control (0.1% DMSO). The results of quadruplicate cultures ($n = 4$) are expressed as the mean values ± SD and are representative of at least three independent experiments. *, # significantly different from the untreated control or 24 h timepoint, respectively, using two-way ANOVA followed by Dunnett's test, $p < 0.05$.

2. Primary ATL Cells and Normal Resting and Activated Peripheral Blood Mononuclear Cells

We, then, examined the effect of RAD001 on the proliferation of ATL cells collected from two ATL patients. Patient one was newly diagnosed with ATL whereas patient two relapsed from previous chemotherapy treatments[142]. 20 nM and 2000 nM RAD001 concentrations killed primary ATL cells from patient one by 96 h, whereas both concentrations only decreased ATL cell viability by approximately 55% in patient two (Figure 12a). ATL cells from patient two, who had acquired resistance to chemotherapy, displayed a cytostatic growth inhibitory profile that is similar to that observed in HTLV-I positive cell lines, in response to RAD001 treatment. Interestingly, resting and PHA-stimulated normal Peripheral Blood Mononuclear Cells (PBMC) from three healthy donors were completely resistant to RAD001 treatment up to 2000 nM concentrations for 96 h (Figure 12b).

B. Mechanism of Growth Suppression Induced by Short-term RAD001 Treatment in Malignant T-Cells

1. RAD001 Causes G_1 Cell Cycle Arrest but no Apoptosis or Autophagy Induction

To explore the impact of mTOR blockage by RAD001 on the growth of HTLV-I positive and HTLV-I negative malignant T-lymphocytes, cell cycle changes were examined by flow cytometry analysis of PI-stained DNA. ATL-derived cells (HuT-102, MT2) and HTLV-I-negative malignant T-cells (CEM, Jurkat) were treated with 20 nM and 2000 nM concentrations of RAD001 up to 72 h. At both concentrations, RAD001 caused a G_1 cell cycle arrest in all tested cells and effects were more pronounced in the HTLV-I-negative cells CEM and Jurkat (Figure 13a and data not shown). Upon 20 nM RAD001 treatment

for 72 h, the percentage of cycling cells (S + G_2 + M) decreased by 55% in CEM and only by 34% in HuT-102 cells. RAD001 did not cause any increases in the pre-G_1 region, presumably representing apoptotic cells, in all tested cells even at a 2000 nM treatment up to 72 h (Figure 13a). Furthermore, we did not observe any PARP cleavage in proteins extracted from HuT-102, MT2, CEM, or Jurkat cells treated with 20 nM of RAD001 up to 72 h (Figure 13b and data not shown). Finally, HuT-102 and CEM cells treated with 20 nM RAD001 up to 72 h showed no increase in TUNEL positivity (Figure 13c) further ruling out apoptotic cell death.

Because mTOR inhibitors were shown to induce autophagy[143], we checked for the effect of RAD001 in malignant T-cell growth suppression. We did not detect any autophagy induction in RAD001-treated malignant T-cells, since we did not observe any LCIII protein cleavage, a marker of autophagy[144], in protein extracts of HuT-102, CEM or Jurkat treated with 20 nM RAD001 up to 3 days (data not shown). Furthermore, we did not observe any accumulation of the acidic vacuole organelles, another hallmark of autophagic cell death[145], upon vital acridine orange staining of HuT-102, MT2, CEM or Jurkat cells treated with 2000 nM RAD001 up to 8 days (data not shown).

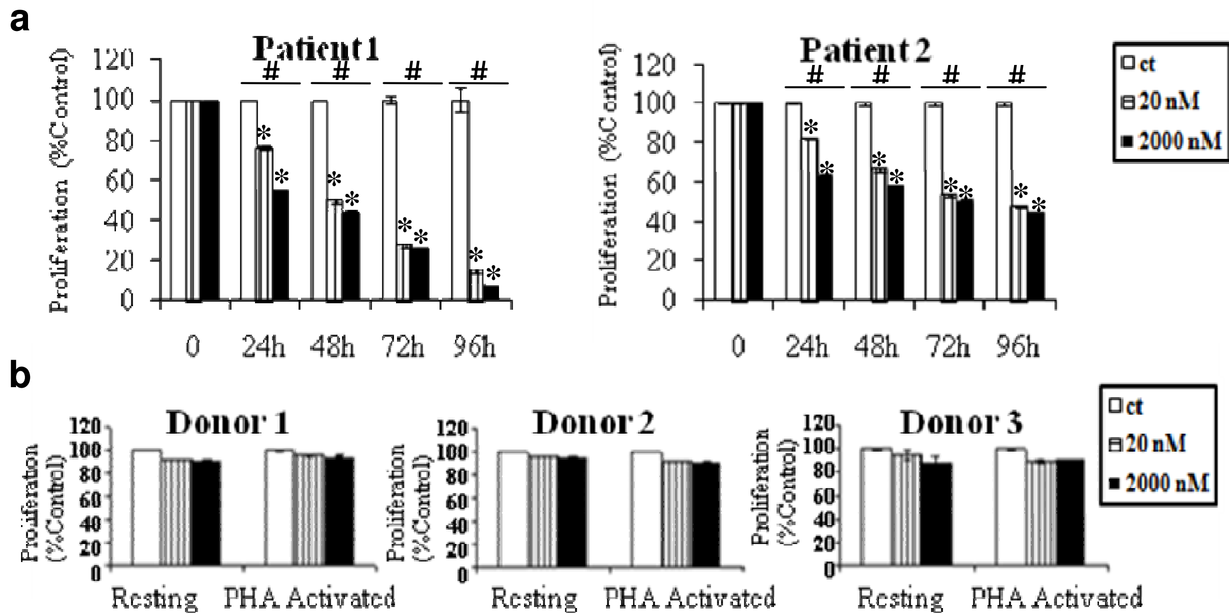

Fig. 12. RAD001 induces growth inhibition in primary ATL cells but not normal resting or activated T-lymphocytes. (a) ATL cells from two patients were cultured at 10^6 cells/ml with 20 nM and 2000 nM RAD001 up to 96 h. Cell growth was assessed in quadruplicate wells with the CellTiter 96® nonradioactive cell proliferation kit, and cell viability is expressed as percentage of control (0.1% DMSO). The results of quadruplicate cultures (n = 4) are expressed as the mean values ± SD and are representative of three independent experiments. (b) Peripheral blood mononuclear cells (PBMC) were collected from three healthy HTLV-I negative donors after informed consent. Activated PBMC were supplemented with 2% PHA. RAD001 was added at the indicated concentrations for up to 96 h. Cell growth was assessed in quadruplicate wells with the CellTiter 96® nonradioactive cell proliferation kit, and cell viability is expressed as percentage of control (0.1% DMSO). The results of quadruplicate cultures (n = 4) are expressed as the mean values ± SD and represent three independent experiments. *, # significantly different from the untreated control or 0 h timepoint, respectively, using two-way ANOVA followed by Dunnett's test, $p < 0.05$.

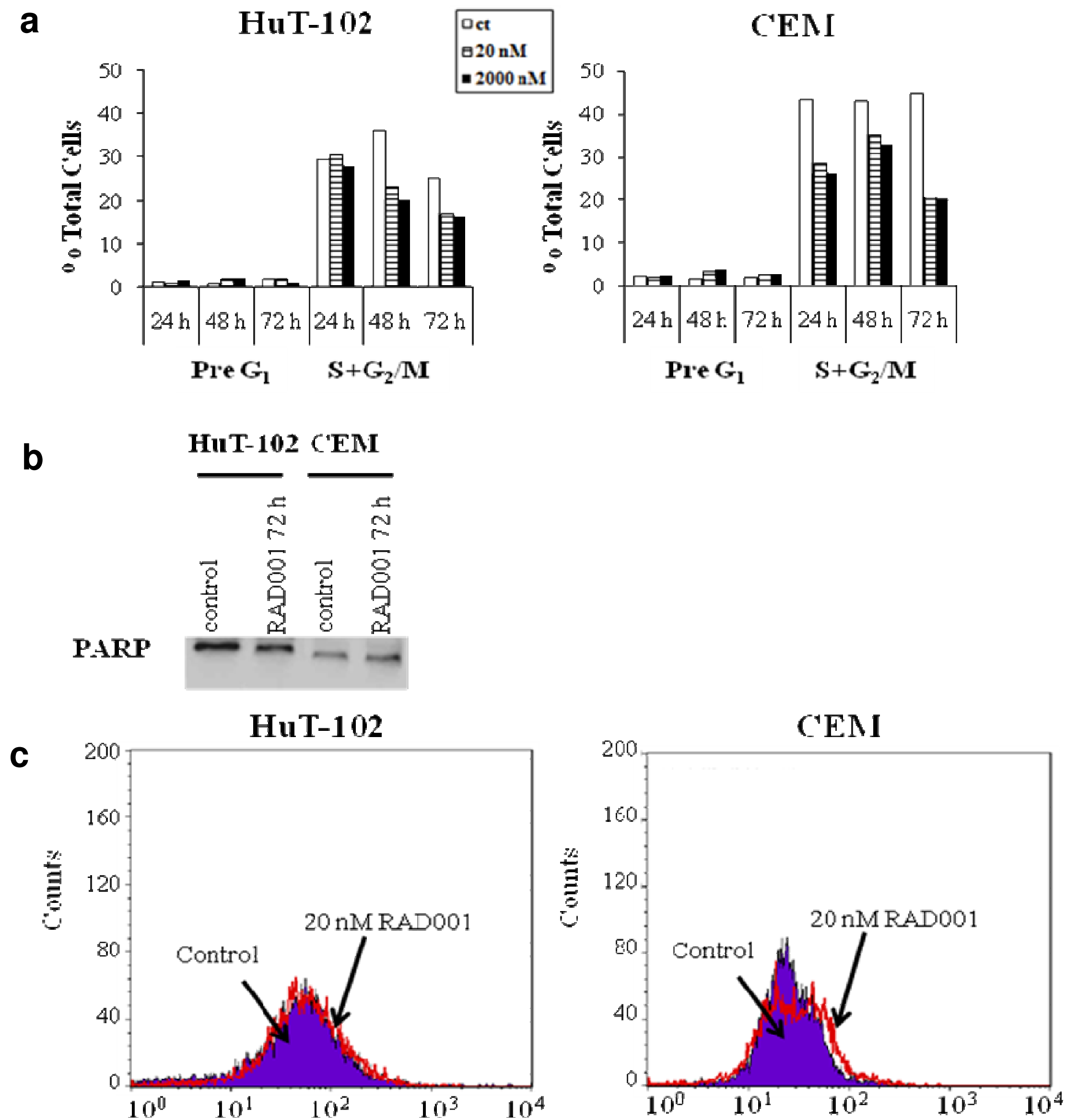

Fig. 13. RAD001 induces G_1 cell cycle arrest in HTLV-I positive and HTLV-I negative malignant T-cells. (a) Effects of RAD001 on the cell cycle distribution of HuT-102 and CEM cells. RAD001-treated cells were stained with propidium iodide (PI) (50 μg/ml) and the cell cycle analysis was performed using a FACScan flow cytometer. The pre-G_1 percentage represents apoptotic cells. Cycling cells, the sum of ($S+G_2/M$) phases, are a percentage of nonapoptotic cells. The results are representative of two independent experiments. (b) RAD001 does not cause PARP cleavage in either HuT-102 or CEM cells. HuT-102 and CEM cells were treated up to 72 h with 20 nM RAD001. Total SDS protein lysates (30 μg/lane) were prepared and immunoblotted against PARP antibody. (c) TUNEL analysis of HuT-102 and CEM cells treated for 72 h with RAD001 concentrations 20- and 2000 nM. Panels represent overlay of TUNEL positivity of treated cells (open graphs) over control cells (solid graphs). The results are representative of two independent experiments.

2. Effect of RAD001 on Dissipation of Mitochondrial Membrane Potential

We have investigated whether RAD001 treatment of malignant T-cells causes dissipation of mitochondrial membrane potential as measured by a decrease in R123 fluorescence. HTLV-I-positive (HuT-102, MT2) and HTLV-I-negative malignant T-cell lines (CEM, Jurkat) were treated with 20 and 2000 nM RAD001 for 24 and 48 h. The dissipation of mitochondrial membrane potential was moderate in RAD001-treated CEM and Jurkat cells at both concentrations, but not in HuT-102 or MT2 cells even up to 48 h (Figure 14 and data not shown).

3. Induction of Cytochrome c Release

We have also determined whether RAD001 treatment of malignant T-cells results in a release of cytochrome c. HuT-102 and CEM cell lines were treated with 20 nM RAD001 up to 24 h. Cytochrome c release was measured using the Cytochrome c ELISA Kit (EMD Biosciences, Darmstadt, Germany). Sub-cellular fractionation was performed before solubilization. Subsequently, apoptotic cytochrome c was measured in the cytosolic fraction according to the manufacturer's instructions. Cytochrome c levels were assayed by OD measurement at 450 nM using an ELISA microplate reader. No significant increase in cytochrome c release was detected in both cell lines compared to control values (Figure 15).

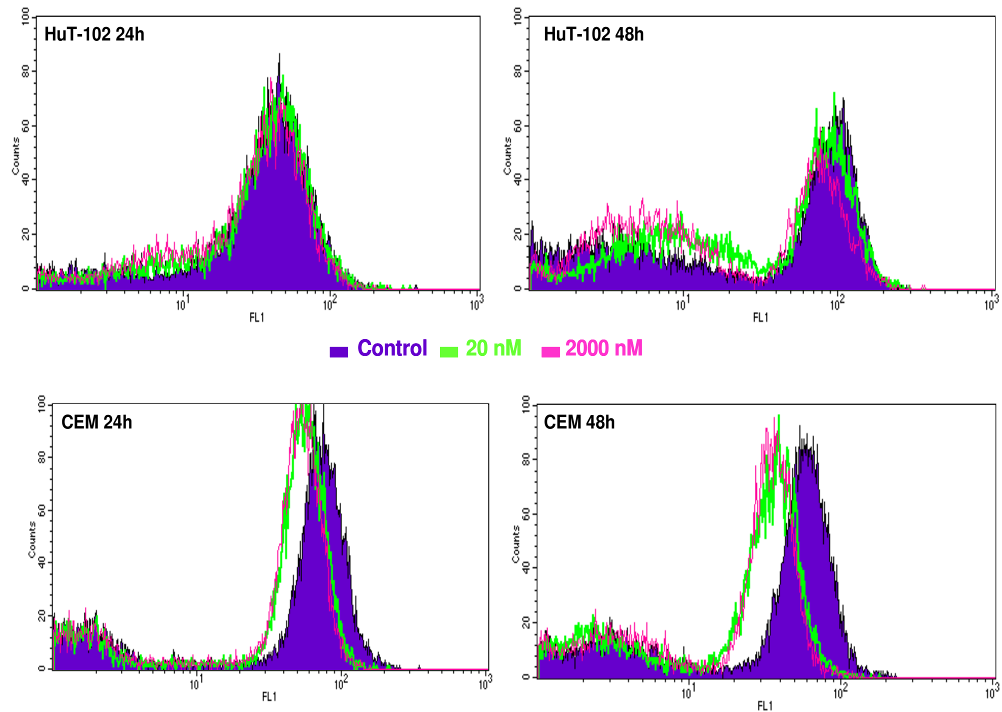

Fig. 14. RAD001 causes mitochondrial membrane potential dissipation in HTLV-I negative malignant T-cells only. HuT-102 and CEM cells were treated with RAD001 for 24 h and 48 h at the indicated concentrations. Quantification of mitochondrial membrane potential was determined by Rhodamine-123 (R123) retention. Fluorescence emission at 525 nM after excitation at 488 nM was quantified by flow cytometry using the CellQuest software. Panels represent overlay of R123 fluorescence of treated cells (open graphs) over control cells (solid graphs). Results are representative of two independent experiments.

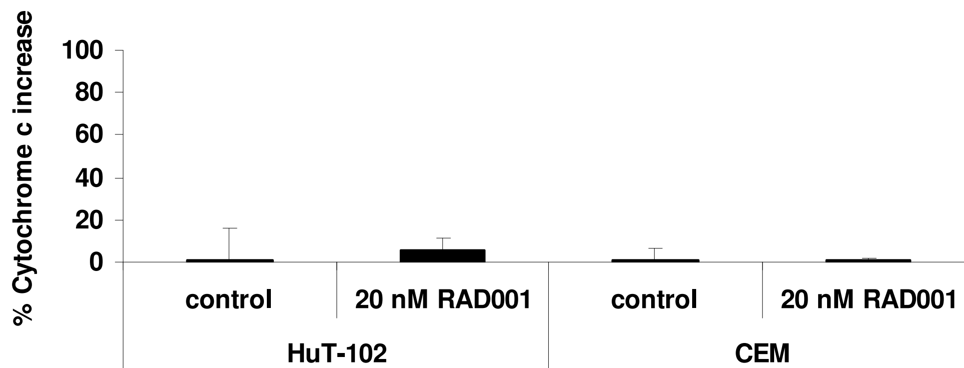

Fig. 15. RAD001 treatment does not induce cytochrome c release. HuT-102 and CEM cells were treated with 20 nM RAD001 for 12 h. Apoptotic cytochrome c levels were calculated from duplicate measurements and are expressed as percentage increase over control (ctrl) set as one. Each bar represents the mean ± range.

4. Effect of RAD001 on Reactive Oxygen Species Generation

To check whether the growth inhibitory effects of RAD001 treatment are mediated by reactive oxygen species (ROS) generation, we used the ROS kit: CM-H2DCFDA (Molecular Probes, Eugene, OR, USA) to detect ROS levels. HuT-102 and CEM cells were treated with 20 nM RAD001 for 30 min, 2 h, 6 h, 12 h, and 24 h. 0.1% DMSO was added to control cultures, and exposure of HuT-102 to 5 μM HPR for 6 h was used as a positive control for ROS production. Generation of intracellular ROS was measured using 20-70-dichlorofluorescin diacetate (H2DCFDA) upon oxidation to the fluorescent derivative 20-70-dichlorofluorescin by reactions with ROS. Samples were analyzed with a FACS scan flow cytometer (Becton Dickinson, San Jose, CA, USA) with excitation set at 488 nM and emission at 530 nM. No ROS was generated in either cell line when treated with RAD001 for the different time points (Figure 16). As expected, HPR treatment resulted in increased ROS levels.

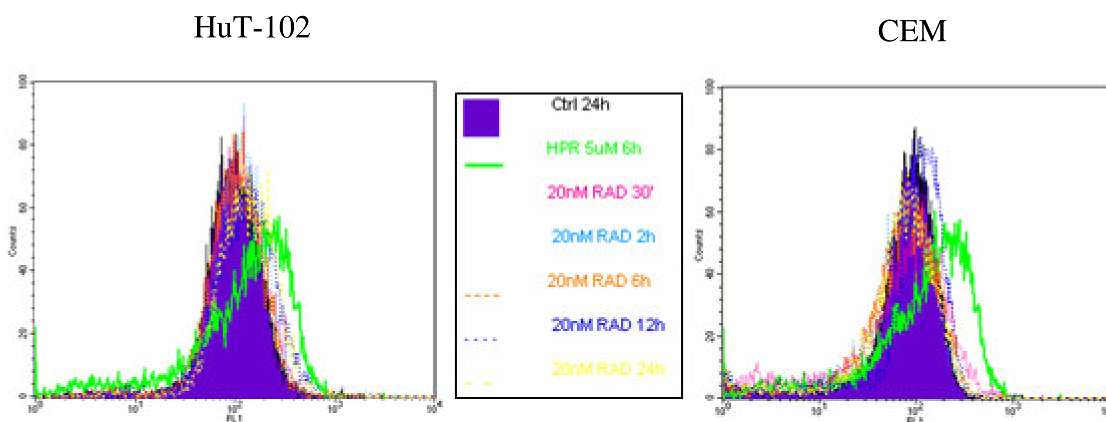

Fig. 16. RAD001 treatment does not induce reactive oxygen species (ROS) generation. HuT-102 and CEM cells were continuously exposed to 20 nM RAD001 and ROS levels were determined by the H2DCFDA assay for the indicated time points. The following results are representative of three independent experiments.

5. Short-term RAD001 Treatment Does not Cause Accumulation of Ceramide

Ceramide is a sphingolipid second messenger that is generally recognized to promote apoptosis in response to conditions associated with oxidative stress. To investigate whether RAD001-induced growth arrest occurs along with ceramide accumulation, levels of ceramide were measured in 20 nM RAD001-treated cells up to 6 days. No significant change in cellular ceramide levels was detected in HuT-102, C91, CEM, or Jurkat cells (Figure 17). However, CD437 treatment increased ceramide levels in treated cells and was used as a positive control (results not shown).

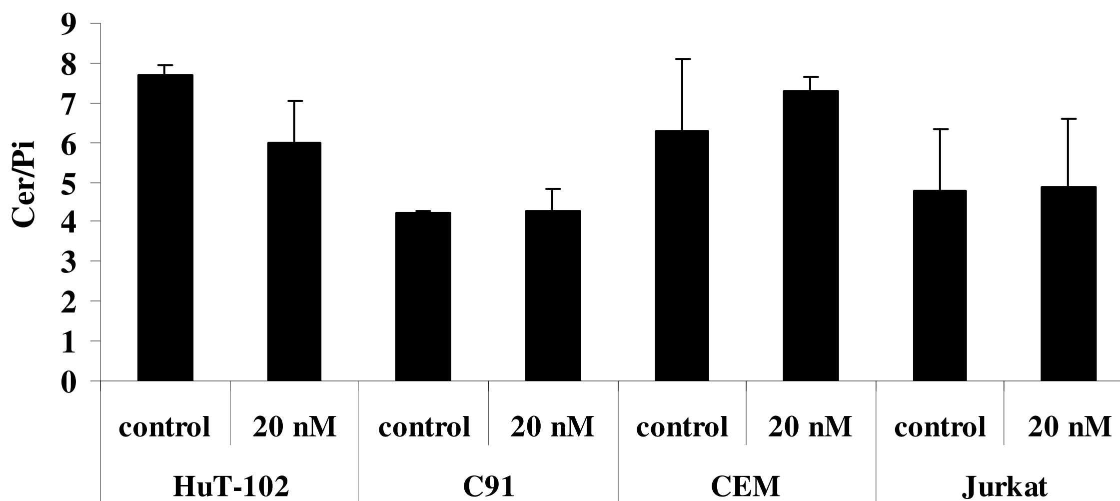

Fig. 17. RAD001 treatment does not induce ceramide accumulation. HTLV-I positive (HuT-102 and C91) and HTLV-I negative (CEM and Jurkat) malignant T-cell lines were treated with 20 nM RAD001 for 6 days. Ceramide levels were determined in control or treated cells using the DGK assay, as described in materials and methods, and normalized to total cellular lipid phosphate levels. Data points represent the mean ± range (n=2).

6. *Effect of RAD001 on Induction of Senescence and DNA Damage Response*

Senescence can act as a functional barrier to tumor progression probably by eliciting a DNA damage response that causes the cell division cycle to arrest[146]. Therefore, to investigate the potential role of senescence in the RAD001-induced cytostatic growth observed in ATL and the HTLV-I malignant T-cells, we tested for senescence associated-β-galactosidase (SA-β-Gal) positivity. HuT-102 cells were treated with 20 nM RAD001 for 4, 8, or 12 days, and they were positively stained with SA-β-Gal, a hallmark of senescent cells, as early as 4 days post-treatment (Figure 18). Indeed, HuT-102 cells treated with 20 nM RAD001 showed 56% and 82% SA-β-Gal positivity at 8 and 12 days, respectively (Figure 18). The senescent cells also assumed a characteristic enlarged, granular, and flattened appearance in culture (data not shown). HTLV-I transformed C91 cells were also tested for β-galactosidase positivity and by day 8 post-treatment with 20 nM RAD001, 72% of cells were SA-β-Gal positive, compared to 14% in control cells (Figure 18). However, MT2 cells were less sensitive to RAD001-induced senescence as treatment for 12 days with 20 nM RAD001 caused 27% SA-β-Gal positivity only, which was increased to 90% at day 16 (Figure 18). Conversely, treatment of HTLV-I negative cells CEM and Jurkat, with 20 nM RAD001 up to 12 days, did not induce SA-β-Gal positivity (Figure 18). Interestingly, Tax protein levels were degraded in RAD001-treated HuT-102 and MT2 cells (Figure 19a). We, then, checked for the expression of the CDK inhibitor p21, a major senescence player. RAD001-treated HuT-102 and MT2 cells showed upregulation of p21 starting day 1 while p21 protein levels remained undetectable in HTLV-I negative CEM and Jurkat cells (Figure 19a). Although p21 is a downstream effector of p53, no change in the expression pattern of total p53 proteins was observed in both ATL-derived and HTLV-I negative malignant

T-cells, but a significant increase in the phosphorylated form of p53 proteins was detected in CEM and Jurkat cells as early as day 1of RAD001 treatment (Figure 19b). RAD001 had no effect on phopshorylated p53 in HuT-102 and MT2 cells (Figure 19b). In addition, PUMA-α protein, a p53-dependent BH3-only member of Bcl-2 family of proteins, increased significantly in HuT-102 cells by day 1 post-treatment, and by day 6 in HTLV-I negative cell lines CEM and Jurkat (Figure 19a). Finally, the levels of γH_2AX DNA damage repair protein increased as early as 24 h following RAD001 treatment in all tested malignant T-cells (Figure 19a). All together, these results indicate that RAD001-induced senescence only in HTLV-I positive cells (HuT-102, C91, and MT2) but not in HTLV-I-negative cells (CEM and Jurkat). These results also show that growth arrest was probably mediated by DNA damage in all malignant T-cells and by Tax degradation and phosphorylation of p53 proteins in HTLV-I positive and negative cells, respectively.

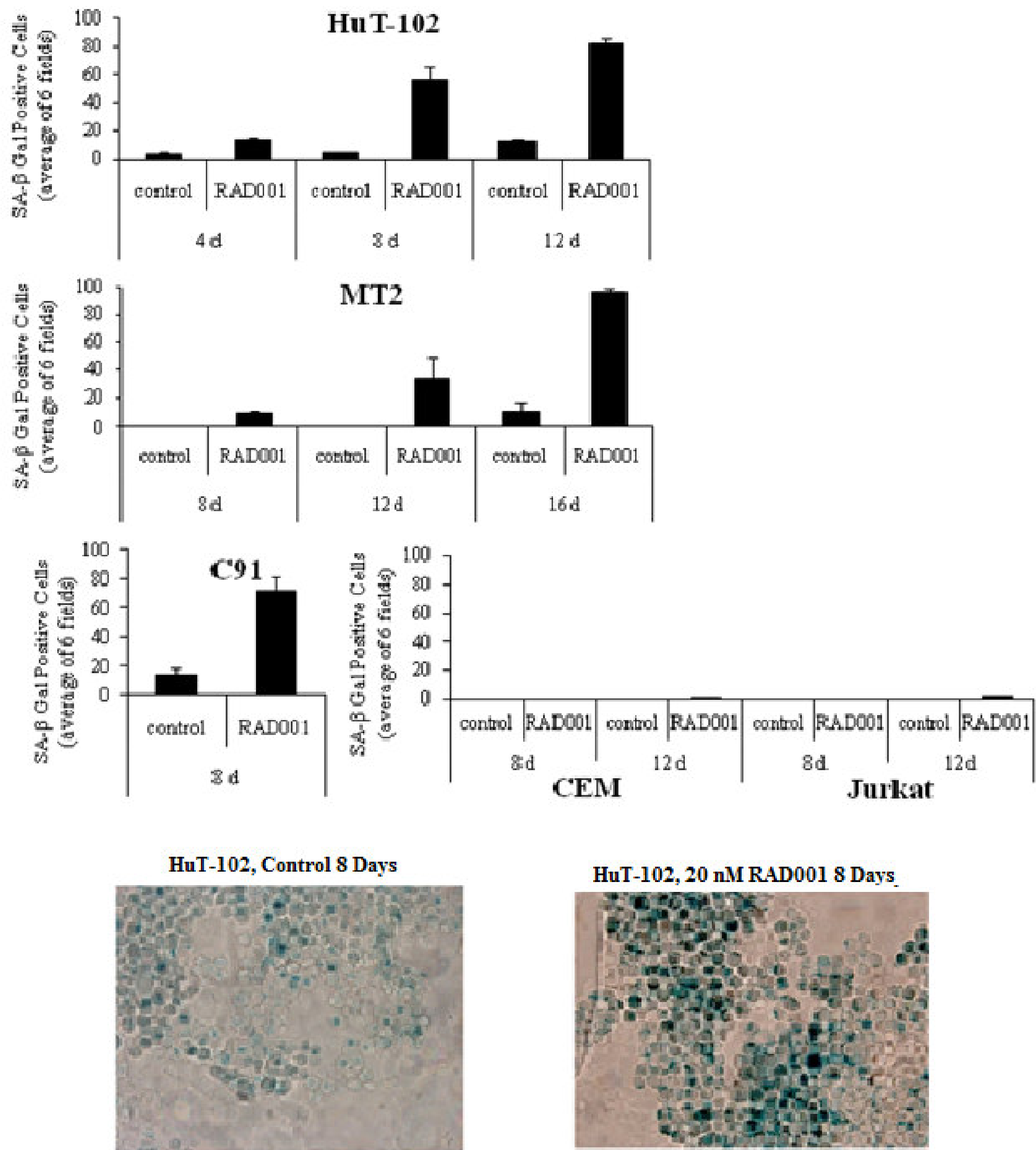

Fig. 18. RAD001 treatment induces senescence-associated β-galactosidase (SA-β-Gal) positivity in HTLV-I positive cells only. HTLV-I positive and -negative cells were treated with 0.1% DMSO (control cultures) or 20 nM RAD001, and cells were replenished every 4 days with fresh media (RPMI) containing 20 nM RAD001 or 0.1% DMSO. β-galactosidase staining and fixation were done at the indicated time points. Data show percentage of positive blue cells as determined by counting six random fields (100 cells/field), and is expressed as the mean values ± SD. Confocal microscopy of HuT-102 cells stained at day 8 post- RAD001 treatment shows that senescent cells express SA-β-gal.

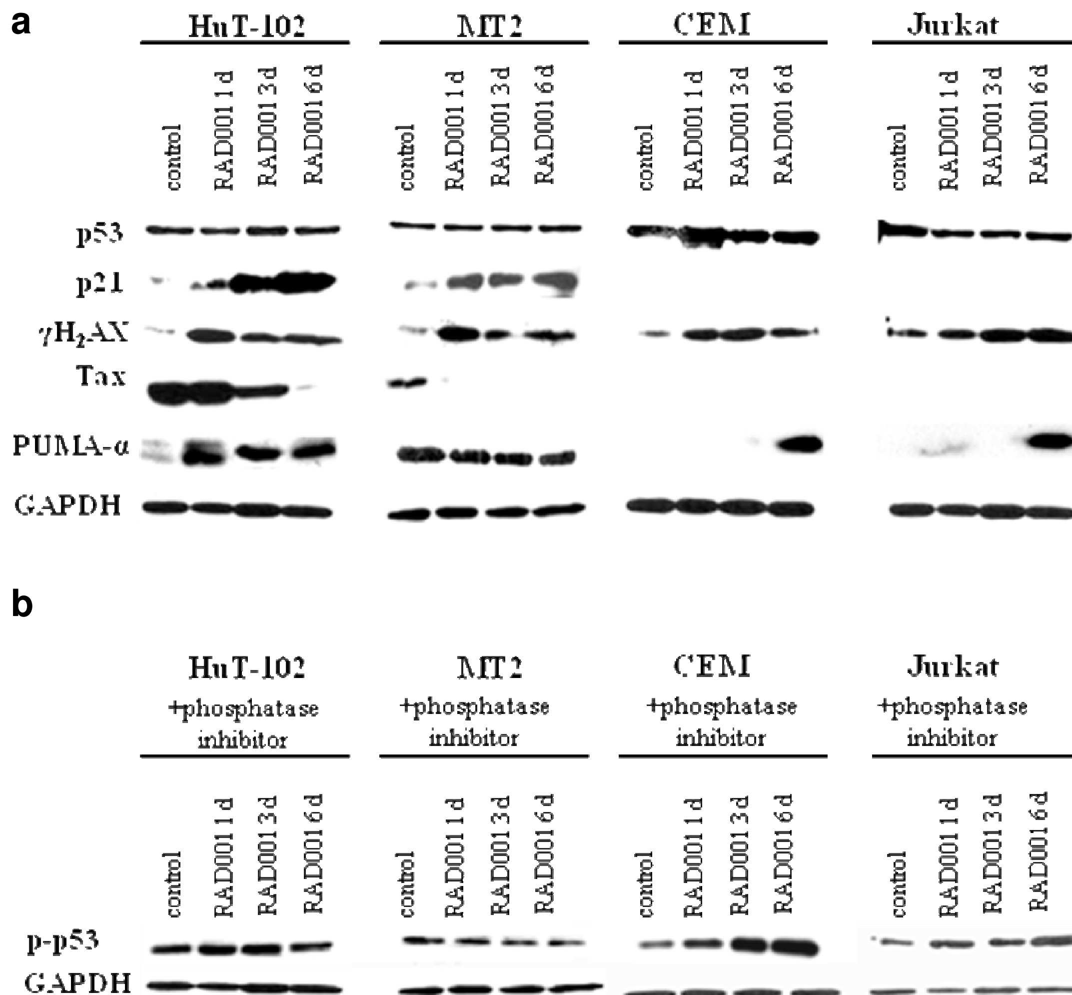

Fig. 19. RAD001 treatment modulates p21, DNA damage proteins, and p53 response in malignant T-cells. (a) RAD001 treatment induces changes in p21, γH2AX and PUMA-α protein expression in ATL and malignant T-cells. HTLV-I-positive cells (HuT-102, MT2) and HTLV-I-negative malignant T-cells (CEM, Jurkat) were treated with 20 nM RAD001 up to 6 days. Total SDS protein lysates (30 μg/lane) were prepared on days 1, 3 and 6 post-treatment, and immunoblotted against p53, p21, γH2AX, Tax, and PUMA-α antibodies. (b) RAD001 increases the expression of phosphorylated p53 in HTLV-I negative cells only. HuT-102, MT2, CEM, and Jurkat cells were treated with 20 nM RAD001 up to 6 days. Total SDS protein lysates (30 μg/lane) with added phosphatase inhibitor were prepared and immunoblotted against phosphorylated-p53 antibody. In (a) and (b), blots were re-probed with GAPDH to ensure equal protein loading. Results are representative of two independent experiments.

C. Effect of Long-term RAD001 Treatment on Apoptosis Induction in Malignant T-cells

1. Effect of RAD001 on Cell Viability and Cell Cycle Distribution

In order to investigate the mechanism of RAD001-induced cell death in senescent HTLV-I positive and growth arrested HTLV-I negative malignant T-cells, we treated HuT-102, MT2, CEM, and Jurkat cells with 20 nM RAD001 up to 21 days. Assessment of viability using trypan blue showed that HTLV-I-positive cells start dying by day 9, whereas HTLV-I-negative cells died at later time-points (Figure 20). Cells were harvested and analyzed using PI-stained DNA content, TUNEL, and PARP cleavage. Flow cytometric analysis showed a significant increase in pre-G_1 region representing apoptotic cells upon treatment with 20 nM RAD001 (23% increase in HuT-102 cells by day 9, and 52% increase in CEM cells by day 14) (Figure 21). Similar increases in pre-G_1 were detected in RAD001-treated MT2 and Jurkat cells (Figure 21). To confirm the induction of apoptosis in RAD001-treated malignant T-cells, we performed TUNEL assay. The number of TUNEL positive cells increased from 4 to 21% in HuT-102 cells and from 5 to 25% in CEM cells treated with 20 nM RAD001 for 9 days and 14 days, respectively (Figure 22a). RAD001-induced apoptosis was also associated with cleavage of PARP (113 kDa) into its death associated fragments (89 and 24 kDa) on day 9 for the HTLV-I positive cells, and on days 14 and 21 for CEM and Jurkat cells, respectively (Figure 22b). Collectively, these results show that long-term exposure to the mTOR inhibitor induces apoptosis in senescent HTLV-I positive and in growth arrested HTLV-I negative malignan T-cells.

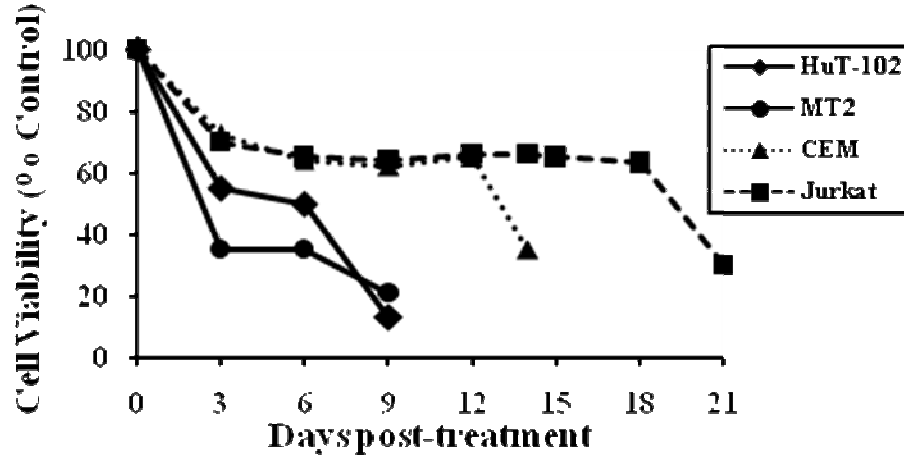

Fig. 20. Long-term treatment with RAD001 reduces the viability of ATL and malignant T-cells. HuT-102, MT2, CEM, and Jurkat cells were treated with 20 nM RAD001. Viability with respect to control (0.1% DMSO) cells was assessed at least every three days using trypan blue exclusion assay, followed by replenishment with fresh RPMI media and RAD001 or 0.1% DMSO. This was continuously repeated until the viability of cells decreased. Results are expressed as percentage of control, and are representative of at least three independent experiments.

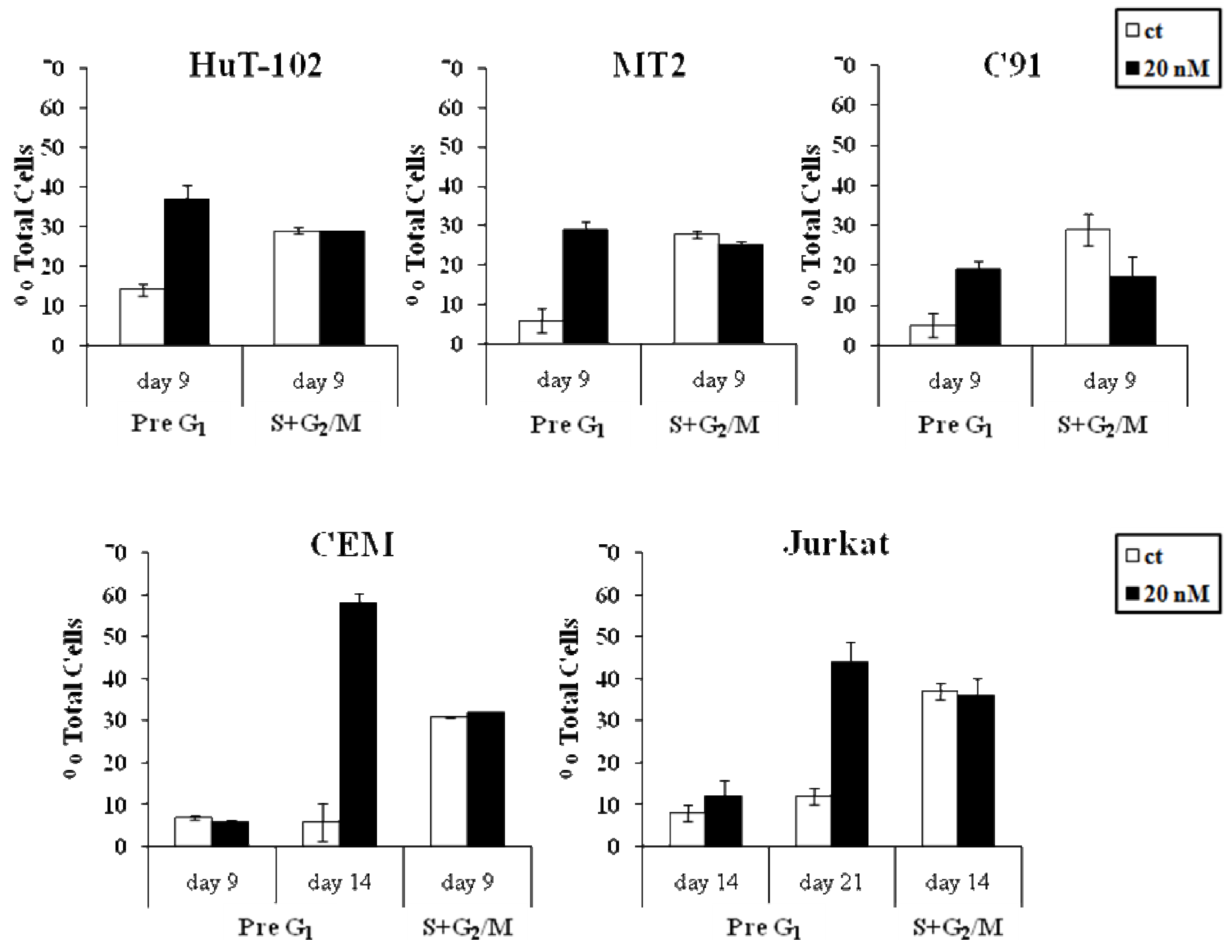

Fig. 21. Long-term treatment with RAD001 induces an increase in Pre-G_1 in HTLV-I positive and HTLV-I negative malignant T-cells. RAD001-treated cells were stained with PI (50 μg/ml) and the cell cycle analysis was performed using a FACScan flow cytometer. The pre-G_1 percentage represents apoptotic cells. Cycling cells, the sum of (S+G_2/M) phases, are a percentage of nonapoptotic cells. The results are an average of two independent experiments and are expressed as the mean values ± SD.

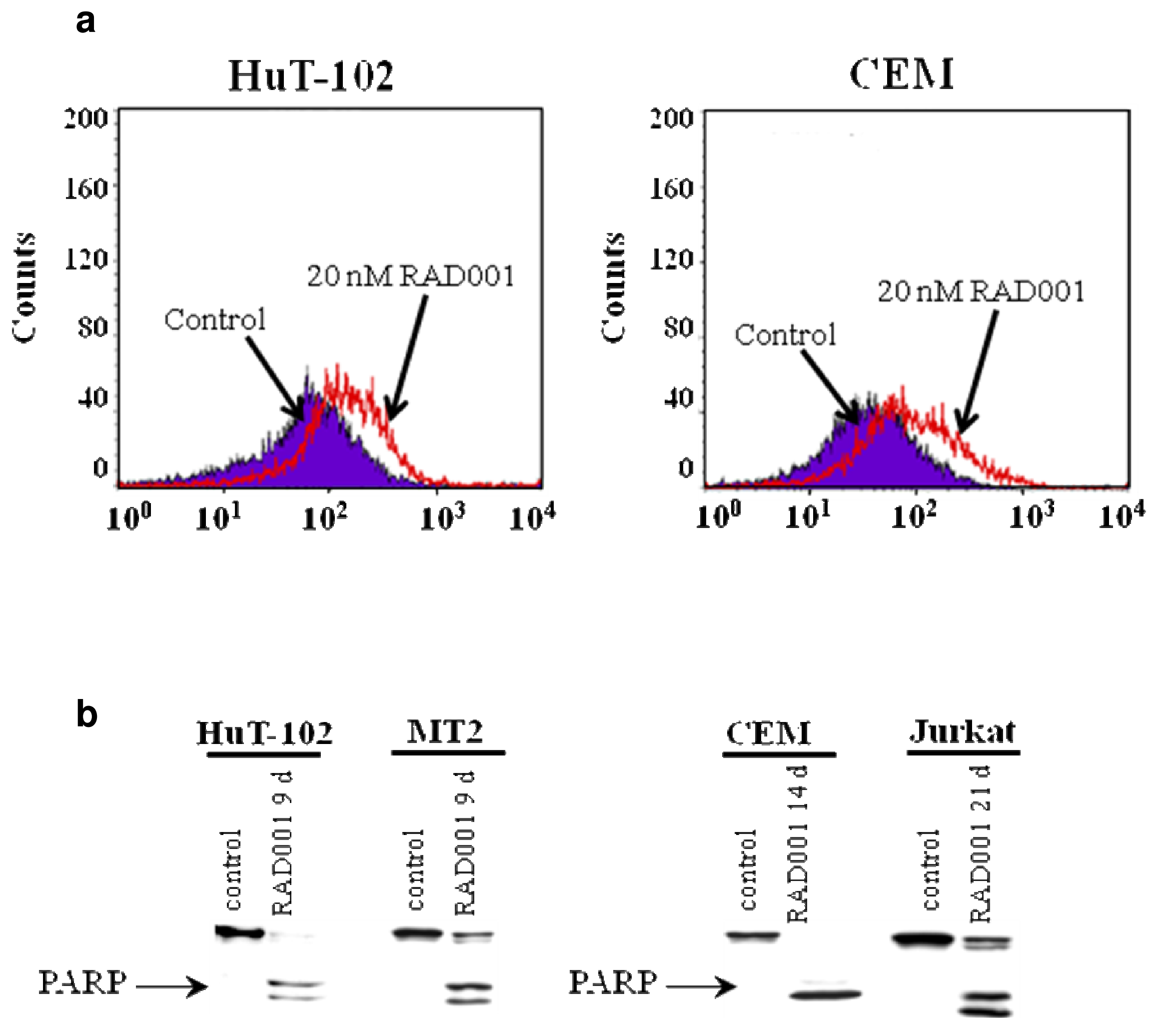

Fig. 22. RAD001 causes TUNEL positivity and PARP cleavage in HTLV-I positive and negative malignant T-cells. (a) TUNEL analysis of CEM and HuT-102 cells treated with 20 nM RAD001 for 9 days and 14 days, respectively. Panels represent overlay of TUNEL positivity of treated cells (open graphs) over control cells (solid graphs). The results are representative of two independent experiments. (b) RAD001 causes PARP cleavage (arrow) in HTLV-I-positive cells (HuT-102, MT2) and HTLV-I-negative cells (CEM, Jurkat) cells treated with 20 nM RAD001 for at the indicated time points. Total SDS protein lysates (30 μg/lane) were prepared and immunoblotted against PARP antibody.

2. Effect of Long-term RAD001 Treatment on Bcl-2 Family Members

It has been previously established that aberrant expression of Bcl-x_L may increase the survival of virus-infected T-cells as well as their resistance to apoptotic signals, thereby contributing to HTLV-I–induced leukemogenesis[147]. Consequently, we investigated the involvement of the Bcl-2 family members in RAD001-induced apoptosis. A significant decrease in the antiapoptotic Bcl-x_L proteins in HuT-102, MT2, CEM, and Jurkat cells, and an increase in pro-apoptotic Bax proteins, were observed after prolonged RAD001 treatment (Figure 23). We were unable to detect the expression of Bax in Jurkat cells, which has been previously attributed to frameshift mutations that result in premature termination of translation of Bax RNA[148]. Bcl-2 proteins were not altered in any of the tested cell lines; however, Bax to Bcl-x_L ratio increased, thus supporting a potential role for these apoptotic regulators in RAD001-induced cell death of malignant T-cells.

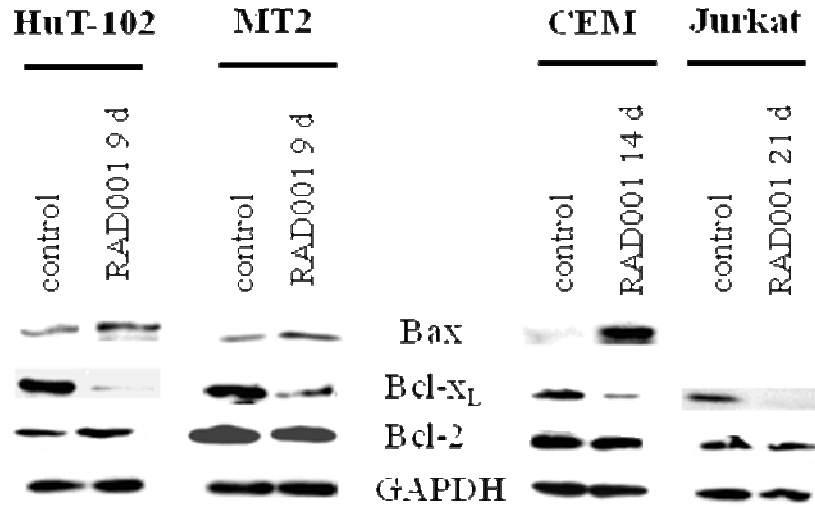

Fig. 23. RAD001 upregulates Bax and reduces Bcl-x_L proteins in malignant T-cells. HTLV-I positive cells (HuT-102, MT2) and HTLV-I negative cells (CEM, Jurkat) were treated with 20 nM RAD001 at the indicated time points. Total SDS protein lysates (30 μg/lane) were prepared and immunoblotted against Bax and Bcl-$x_{L/s}$ antibodies. Blots were re-probed with GAPDH to ensure equal protein loading. Results are representative of two independent experiments.

CHAPTER IV

DISCUSSION

ATL remains of poor prognosis with a median survival of six months in the acute form[95]. Despite recent progress in ATL therapy, the search for new effective drugs is absolutely warranted to reduce or eliminate the emergence of resistant clones, the principal cause of the poor outcome of ATL patients. On the other hand, HTLV-I-negative peripheral T-cell lymphomas (PTCL) also carry a poor prognosis and a higher rate of relapse as compared to B-cell lymphomas[149].

Our results show that clinically achievable concentrations of the mTOR inhibitor RAD001 induced cytostatic growth inhibition and G_1 cell cycle arrest in HTLV-I-positive and -negative malignant T-cells upon short-term treatment, senescence in HTLV-I-positive cells only, and apoptosis in all malignant T-cells upon long-term treatment. Most importantly, RAD001 suppressed the growth of primary ATL cells from two patients while no effect was observed on normal T-lymphocytes with hundred fold higher RAD001 concentrations. Interestingly, no dose-dependent growth inhibition was observed as 2 to 2000 nM of RAD001 gave almost similar maximal growth inhibition in malignant T-cells. Similar observations were described in RAD001-treated lung cancer cells [114] and in mantle cell lymphoma[74]. The observed growth inhibition, senescence, and apoptosis induction correlate with changes in major modulators of these pathways as summarized in Table 1. Overall, HTLV-I-positive cells treated with RAD001 show an early increase in the levels of p21 proteins and undergo Tax protein degradation, likely contributing to the senescent

phenotype. In fact, induction of p21 is closely linked with tumor senescence caused by cancer therapeutic agents[150]. In addition, Tax was shown to activate AP-1 independently of NF-κB signaling, and to promote cell proliferation and survival through the PI3K/AKT pathway[113]. Although Tax is undetectable in circulating ATL cells at the protein level, our previous demonstration that Tax is continuously degraded by the proteasome[151, 152] may account for the subsequent presentation of Tax peptides on MHC class I molecules[153] and for the high frequency of circulating Tax-specific cytotoxic T-lymphocytes found in most HTLV-I-infected individuals[154]. Moreover, Tax transgenics develop murine ATL with quite similar features to human ATL, formally demonstrating that Tax is the driving oncogene in ATL[155]. However, the dramatic toxicity of proteasome inhibitors on ATL cells[113] prevented us from directly demonstrating that inhibition of Tax degradation protects ATL cells from RAD001-induced growth inhibition and senescence.

In contrast, a different mechanism of growth inhibition was observed in RAD001-treated HTLV-I-negative malignant T-cells. Indeed, the G_0/G_1 cell cycle arrest observed in RAD001-treated malignant T-cells is unlikely a result of p21 induction, particularly that HTLV-I-negative cells show a higher percentage of arrested cells than HTLV-I-positive cells, with no detectable p21 protein expression. In these HTLV-I-negative cells, RAD001 induced an early increase in phosphorylated p53 proteins leading to cell cycle arrest. Interestingly, primary ATL cells from the newly diagnosed acute ATL patient were killed by RAD001 whereas leukemic cells from the other relapsed ATL patient displayed a cytostatic growth inhibitory effect similar to the ATL cell lines. This observation suggests that perhaps senescence induction is an alternative tumor inhibitory mechanism when cancer cells develop resistance and fail to undergo cell death. Recently, treatment with

Rapamycin for 2 days was shown to inhibit the growth of HTLV-I-positive cell lines in a dose-independent manner and no apoptosis induction, while HTLV-I-negative cells and normal lymphocytes were not affected by the treatment[156]. Other reports have shown autophagy induction by mTOR inhibition[143, 157], but to our knowledge this is the first report showing senescence induction by single RAD001 treatment. It would be interesting to decipher the role of HTLV-I infection in senescence induction. Interestingly, combined Rapamycin and fluorouracil treatment of hepatocellular carcinoma cells that have high rate of HPV infection, resulted in synergistic growth inhibition and induced apoptosis and senescence[62].

RAD001-treated malignant T-cells displayed elevated levels of the DNA damage protein γH2AX and of PUMA- α, the latter of which has been shown to uncouple p53 and Bcl-x_L in the cytoplasm and induce apoptosis[158]. Although γH2AX induction classically occurs in cells undergoing DNA damage due to apoptosis[159], interestingly in RAD001-treated ATL and malignant T-cells, changes in γH2AX and PUMA-α protein levels, as well as phosphorylation of p53, are all early events that appear when apoptosis is still undetectable. Therefore, DNA damage appears to be a cause rather than a consequence of apoptotic cell death.

It is not surprising that apoptosis detection was earlier in RAD001-treated senescent HTLV-I-positive than HTLV-I-negative malignant T-cells, as senescence presents a powerful barrier to tumorigenesis and may promote an earlier cell death signaling pathway in senescent *versus* growth arrested cells. Indeed, prolonged RAD001 treatment resulted in increased levels of Bax and decreased levels of Bcl-x_L proteins resulting in apoptosis induction most likely through caspase activation. While increased

apoptotic Bax to Bcl-x_L ratio could be an event associated with apoptosis induction, it is more likely that it has sensitized cells later to undergo apoptosis and, thus, highlights the major role of this family in RAD001-mediated cell death of malignant T-cells. In fact, increased bcl-2 protein levels were recently shown to modulate RAD001 sensitivity in small cell lung cancer cells with a constitutively active AKT/mTOR signaling[160].

It has been shown that mTOR inhibitors have the ability to promote Akt activity by a feedback mechanism in several tumor cells, therefore affecting the outcome of their survival[161]. A similar situation is likely present in ATL and malignant T-cells, as the combination treatment of RAD001 with Luteolin, a bioactive flavonoid recently shown to inhibit activation of Akt signaling in cancer cells[162], resulted in enhanced growth suppressive effects (data not shown). These observations indicate that RAD001 may be initially activating Akt in these cells. However, levels of activated Akt in numerous tumor cells do not necessarily correlate with the antiproliferative effects of RAD001, suggesting that the resistance to mTOR inhibitors is not entirely due to rapalog-induced Akt activation[163].

PI3K/AKT/mTOR pathway is activated in several cancers, namely leukemias, lymphomas, and in HTLV-I-transformed cells[60, 164], making it an ideal target for therapy[165-168]. Unfortunately, Rapamycin and the PI3K inhibitor LY294002 have toxic side effects and the *in vitro* effective concentrations may not be reached in the plasma of patients. This led to the development of more bioavailable and specific second generation compounds of these drugs[169], such as RAD001[170], or to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, and targeted therapies[171, 172]. Therefore, mTOR inhibitors as single agents or in combination therapy are showing promise in several

clinical trials of leukemias and lymphomas[173]. In fact, a variety of hematological disorders and tumors harbor mutations, silencing, or deletions in the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN), a negative regulator of the PI3K/Akt/mTOR pathway, resulting in a constitutive activation of mTOR signaling[174]. Other aberrations implicated in these malignancies include abnormalities of the Akt/PI3K/mTOR signaling pathway or the G_1 checkpoint, such as pRb, p27, and cyclin D1, all of which render these hematological disorders sensitive to mTOR inhibition[173, 175]. Consistent with these observations, the clinical anti-leukemic activity of RAD001 is currently being assessed in a phase II clinical trial in patients with refractory or advanced Non-Hodgkin's lymphoma[165, 176], in phase II trial for relapsed mantle cell lymphoma[173], and in phase II trials for recurrent/refractory indolent lymphoid malignancies including CLL[177]. Other mTOR inhibitors are also being investigated in phase III clinical trial for mantle-cell lymphoma[67], phase II for Non-Hodgkin's lymphoma[178], and other hematologic malignancies[88]. Based on pre-clinical data suggesting additive or synergistic effects in combination with paclitaxel, carboplatin, vinorelbine, doxorubicin, and campthotecin, the efficacy of mTOR inhibitors in combination with a broad range of chemotherapeutic agents is also being evaluated in ongoing clinical trials[175]. However, lymphopenia have been encountered in some patients treated with mTOR inhibitors, necessitating a more careful administration of these drugs[179].

Overall, we demonstrate that RAD001 targets key cellular pathways in malignant T-cells, supporting a potential therapeutic role for RAD001 and other mTOR inhibitors in ATL and HTLV-I-negative peripheral T-cell lymphomas.

Table 5. Effect of RAD001 on the growth and cell death of HTLV-I positive and negative malignant T-cells

	Short-term treatment *		Long-term treatment [†]	
	HTLV-I positive	HTLV-I negative	HTLV-I positive	HTLV-I negative
Growth inhibition	+ [‡]	+	+ + +	+ + +
Cell cycle arrest	+	+ +	— [§]	—
Autophagy	—	—	ND **	ND
Senescence	—	—	+ + +	—
Apoptosis	—	—	+ + +	+ + +
Tax	Decrease	—	Decrease	—
p21	Increase	—	No change	—
p-p53	No change	Increase	ND	ND
PUMA-α	Increase	No change	Increase	Increase
γH_2AX	Increase	Increase	ND	ND
Bax	No change	No change	Increase	Increase
Bcl-x_L	No change	No change	Decrease	Decrease

* up to 3 days
[†] up to 21 days
[‡] + <50%, + + 50% - 80%, + + + >80%
[§] not present
** ND not determined

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