A rapamycin derivative (everolimus) controls proliferation through down-regulation of truncated CCAAT enhancer binding protein β and NF-κB activity in Hodgkin and anaplastic large cell lymphomas

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Abstract
The immunosuppressive macrolide rapamycin and its derivate SDZ RAD (RAD, everolimus) inhibit the mammalian target of rapamycin (mTOR) signaling pathway. In this study, we provide evidence that RAD has profound anti-proliferative activity in vitro and in NOD/SCID mice in vivo against Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) cells. Moreover, we identified two molecular mechanisms that showed how RAD exerts anti-proliferative effects in HL and ALCL cells. RAD down-regulated the truncated isoform of the transcription factor CCAAT enhancer binding protein (C/EBP)β, that is known to disrupt terminal differentiation and induce a transformed phenotype. Furthermore, RAD inhibited constitutive NF-κB activity, that is a critical survival factor of HL cells. Pharmacological inhibition of the mTOR pathway by RAD therefore interferes with essential proliferation and survival pathways in HL and ALCL cells and might serve as a novel treatment option.

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Introduction

Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) share morphologic and immunophenotypic markers in a subgroup of cases although they are biologically distinct entities.\(^1\) Therefore, pathologic diagnosis is sometimes difficult to achieve and these cases are classified as “grey zone lymphomas”.\(^2\) Moreover, in both entities novel therapeutic options are needed, as curative therapy of HL is compromised by a high risk of long-term complications and anaplastic lymphoma kinase (ALK) negative ALCL still have very unfavorable prognosis with current treatment strategies.\(^3-5\)

The macrocyclic lactone SDZ RAD (RAD, INN: everolimus) is a rapamycin derivative with potent immunosuppressive and anti-proliferative properties.\(^6-10\) It is further known to inhibit growth factor-driven cell proliferation of hematopoietic and nonhematopoietic cells.\(^6,10\) In addition, RAD is a potent inhibitor of human Epstein-Barr virus (EBV)-transformed B lymphocytes \textit{in vitro} and \textit{in vivo}, arresting cell-cycle progression and increasing apoptotic rate of EBV+ B cells.\(^10\) Therefore, it has been suggested that RAD might be effective in prevention and treatment of human posttransplant lymphoproliferative disorders.\(^10\)

Here, we investigated whether RAD inhibits tumor cell proliferation of HL and ALCL. We show that RAD significantly inhibits proliferation of HL and ALCL cells \textit{in vitro} and arrests cell-cycle progression in G0/G1. Furthermore, we demonstrate that \textit{in vivo}, RAD markedly suppresses tumor cell proliferation of HL and ALCL cells, xenotransplanted into NOD/SCID mice. Our data suggest that RAD might be used in combination chemotherapy for the treatment of HL and ALCL. Moreover, we studied the mechanisms of proliferation arrest mediated by the mTOR inhibitor RAD to identify the molecular targets in HL and ALCL. The TOR pathway controls the translation initiation machinery in response to nutrients and growth factors thereby coordinating cell growth with cell division.\(^11\) A transcription factor that is a critical target of mTOR is the CCAAT/enhancer binding protein (C/EBP)\(\beta\).\(^11-13\) C/EBP\(\beta\) has previously been identified as an essential downstream target in tumors expressing activated cyclin D1.\(^14\) Our data demonstrate
that ALCL and some HL cells express abundant amounts of C/EBPβ, in particular of its rapamycin sensitive, truncated (Tr) isoform LIP that emerges as a results of alternative translation initiation of the C/EBPβ mRNA. We further show that RAD treatment inhibits proliferation of HL and ALCL cells and down-regulates expression of the LIP C/EBPβ isoform. Ectopic expression of LIP in the ALCL cell line Karpas 299 abrogated the anti-proliferative effect of RAD, suggesting that translationally deregulated expression of C/EBPβ plays an important role in ALCL.

Our further analysis of the molecular mechanisms of RAD effects also identified the NF-κB signaling pathway as a novel target. We had previously shown that NF-κB is constitutively activated and serves as a survival factor of HL cells. Here we show that RAD down-regulates constitutive NF-κB DNA binding activity in HL L540cy cells and ALCL Karpas 299 cells. Overexpression of NF-κB-p65 rescues L540cy cells from RAD-mediated proliferation arrest, indicating that RAD exerts its anti-proliferative effects at least in part through inhibition of constitutive NF-κB. We conclude that pharmacologic inhibition of mTOR signaling by RAD affects at least two important proliferation and survival pathways in HL and ALCL and that RAD treatment might therefore serve as an alternative therapeutic approach.
Methods

Cell Culture

Human cell lines analyzed in this study were as follows: the HL cell lines, L428, KM-H2, L1236, HD-LM2 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany) and L540cy, a subclone of the HL cell line L540 that grows in SCID mice (kindly provided by A. Engert, University Hospital Cologne, Germany); the ALCL cell lines Karpas 299, JB6 and SU-DHL1 carrying the t(2;5) chromosomal translocation and expressing NPM-ALK that involves the fusion of the nucleophosmin (NPM) gene to an anaplastic lymphoma kinase ALK (DSMZ, Braunschweig, Germany) and the NPM-ALK negative ALCL cell line FE-PD (kindly provided by Dr. Michael Hummel, Berlin, Germany). Cell lines were maintained in RPMI 1640 (Biochrom, Berlin, Germany), 10% heat-inactivated FCS, 100 units/ml penicillin and 100 µg/ml streptomycin, 1 mmol/liter sodium pyruvate, 2 mmol/liter glutamine (GIBCO, Karlsruhe, Germany). Cell lines were treated with RAD (Novartis Pharma, Basel, Switzerland) in various concentrations (0 to 50 nM) for 24, 40, 48 and 96 h as indicated. Electroporation of L540cy cells was performed using a Gene-Pulser II (Bio-Rad, München, Germany) with 960 microfarads (µF) and 0.18 kV. Transfection-associated cell death was negligible. Cells were transfected with 6 µg 6NF-κBtkluc, 40 µg p65-GFP expression plasmid or 36 µg pcDNA3 along with 4 µg pEGFP-N3 plasmid. Luciferase assays were performed as previously described.

DNA constructs

The following expression plasmids were used: p65-pEGFP (kindly provided by J. Schmid, Vienna, Austria), pcDNA3 (Invitrogen, Karlsruhe, Germany), and pEGFP-N3 (Clontech Laboratories, Heidelberg, Germany). C/EBPβ constructs: rC/EBPβ-Tr construct was cloned as previously described and was C-terminally tagged with a FLAG-epitope and cloned in
pMSCVpuro vector (Clontech). Fusion protein NPM-ALK was cloned in pcDNA3 (kindly provided by SW. Morris, Tennessee, USA).

**Retroviral methods**

The amphotropic-packaging cell line Phoenix A was transiently transfected with the calcium phosphate-DNA precipitation method, and infectious virus was harvested after 48 h. Karpas 299 target cells (5 x10^5) were infected as described^9^ and selected for puromycin (1,5 µg/ml) resistance.

**Proliferation assay and cell-cycle analysis**

Cell lines were cultured in triplicate at 1 x 10^5 cells per six well for 96 h in the presence of various concentrations of RAD and counted in Neubauer chambers. Cell counts were determined as cells/ml. For cell-cycle analysis, cell lines were cultured in 0-10 nM of RAD for 48 and 96 h. The cells were washed with PBS and fixed in 70% ice cold ethanol (-20°C). After at least o/n at -20°C, cells were washed and stained with 4 mg/ml propidium iodide (Sigma, Deisenhofen, Germany) and incubated with 10 mg/ml RNase A (Roche, Basel, Switzerland) for 10 min at RT (dark). DNA content was then measured by flow cytometry analysis. Statistical analysis was performed via ModFit LT program.

**NOD/SCID mice experiments**

Immunodeficient 7-8 week-old NOD/SCID mice were kept under pathogen-free conditions in a laminar flow unit, and were supplied with sterile food and water. 1 x 10^7 L540cy and 1 x 10^7 Karpas 299 cells were inoculated into mice subcutaneously in 100 µl PBS together with 100 µl matrigel (Becton Dickinson, Heidelberg, Germany), respectively. Palpable subcutaneous tumors developed one to two weeks after cell injection. RAD treatment (5 mg/kg/day) was initiated after palpable tumor growth and was given once a day by gavage. Tumor volume (V) was calculated
in all experiments according to \( V = \frac{ab^2}{2} \), where \( a \) and \( b \) designate long and short diameters of the tumor, respectively. The transplanted mice were monitored for tumor growth for a period of up to 3-4 weeks. Mice experiments were performed by EPO GmbH (Berlin, Germany) according to the German Animal Protection Law with permit of the responsible authorities. At the end of the study a complete autopsy was performed, including the tumors, all internal organs (liver, kidneys, heart, spleen, lung, small and large bowel) and lymph nodes. In the second experiment, \( 1 \times 10^7 \) vector and C/EBP\(\beta\)-Tr transfected Karpas 299 cells were inoculated into mice. RAD treatment (5mg/kg/day) was initiated one day after tumor cell inoculation and was given once a day by gavage. Statistical significance was determined by U-Test of Mann and Whitney; * \( p<0.05 \).

**Immunoblotting**

Cell extracts were prepared and quantitated as described.\(^{22}\) Proteins (30 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Protein load was normalized by Ponceau Red staining. Membranes were incubated with rabbit polyclonal anti-C/EBP\(\beta\), anti-p50, anti-p65, anti-I\(\kappa\)B\(\alpha\) antibodies (Santa Cruz) and with rabbit polyclonal anti-phospho S6 ribosomal protein (Ser235/236; New England Biolabs, Frankfurt, Germany), followed by goat anti-rabbit HRP-conjugated antibodies (Dianova, Hamburg, Germany) and anti-Flag tag (Eastman Kodak, Rochester, USA) followed by goat anti-mouse HRP-conjugated antibodies (Pharmingen), and detected by enhanced chemiluminescence (Amersham Pharmacia, Freiburg, Germany).

**Electrophoretic mobility shift assay (EMSA)**

Whole cell extracts were prepared essentially as described previously.\(^{23}\) After washing the cells with PBS, lysis buffer (20 mM HEPES [pH 7.9], 350 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM MgCl\(_2\), 10% Glycerol, 1% Nonident P-40, 1 mg/ml Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin
A, 10 mM NaF, 8 mM β-glycerophosphate and 1 mM DTT) was added and after 10 min incubation at 4 °C the lysate was centrifuged for 10 min at 14 000 rpm, 4 °C. EMSA was performed as described previously.15

**Northern blot analysis**

Total RNA was prepared using the RNeasy kit (QIAGEN, Hilden, Germany). 10 µg total RNA was subjected to gel electrophoresis on a 1.1 % formaldehyde–1.2 % agarose gel and transferred to a nylon membrane (Appligene, Heidelberg, Germany). After UV cross-linking, the membrane was prehybridized (ExpressHyb solution, Clontech) and thereafter hybridized with [α-32P]deoxycytidine triphosphate ([α-32P]dCTP)–labeled random prime-labeled DNA probes (IκBα) overnight at 68 °C. Membranes were washed at room temperature in 2 x SSC and 0.05% SDS and then in 0.1 x SSC and 0.1% SDS.
Results

RAD inhibits tumor cell proliferation of HL and ALCL in vitro

The indicated HL and ALCL cell lines (as shown in figure 1) were grown for 96 hours in 0-50 nM RAD to determine its effect on cell proliferation. The HL cell lines L1236 and L540cy (a subclone of the HL cell line L540 that forms tumors in SCID mice) and the ALCL cell lines Karpas 299 (ALK+) and FE-PD (ALK-) were highly sensitive to RAD-mediated inhibition of proliferation (Figure 1a, b). In L540cy, L1236 and FE-PD cells treatment with 10 nM RAD resulted in 80% inhibition of proliferation. Proliferation of Karpas 299 cells was already inhibited to 90% by 1 nM RAD with no further increase after dose escalation. These results were comparable to the inhibition of EBV+ B cell lines derived from patients with posttransplant lymphoproliferative disorders.10 The HL cell lines KM-H2, L428 and HD-LM2 and the ALCL cell lines JB6 and SU-DHL-1 were less sensitive towards RAD treatment excluding generalized toxicity of RAD. Even the maximally inhibited cultures (10-20 % of control) such as Karpas 299, FE-PD, L540cy or L1236 had either increased or maintained cell numbers per well after RAD treatment compared to starting cultures. The degree of response was confirmed by determination of [3H] thymidine incorporation (data not shown).

Figure 1

A

B
**RAD blocks cell-cycle progression in HL and ALCL**

Flow cytometry analysis was performed after 48 h (Figure 2, Table 1) and 96 h (data not shown) to analyze whether RAD blocks cell-cycle progression in G0/G1 phase as previously reported. Increase of G0/G1 and concomitant decrease in G2/M cells was observed in all RAD-treated HL and ALCL cell lines. There was no increase in cells in sub-G1 phase and no enhanced expression of Annexin V (data not shown), indicating that RAD did not induce apoptosis leading to loss of DNA content. These data suggest that failure to expand in cell numbers is due to cell-cycle arrest in G0/G1 rather than apoptosis or necrosis.

**Figure 2**

![Flow cytometry analysis results](image)

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Table 1. Cell Cycle profiles

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<td>76%</td>
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RAD treatment of HL and ALCL cells in xenotransplant models

A xenotransplant model of the HL cell line L540cy and the ALCL cell line Karpas 299 in NOD/SCID mice was established to determine the effect of RAD in vivo (Figure 3). Mice were injected subcutaneously with 1 x 10^7 L540cy cells or Karpas 299 cells. Treatment with RAD started when tumors were palpable (day 8 or day 6). In the untreated L540cy control group, tumors rapidly grew and reached a mean tumor volume of 10,5 mm^3 within 23 days. In the RAD-treated group, tumor growth was delayed and by day 23 tumors had a mean volume of about 2,4 mm^3 (Figure 3a). Subcutaneously injected Karpas cells grew to a mean tumor volume of 32,7 mm^3 after 16 days (Figure 3b). RAD treatment resulted in a delay of tumor development and a reduction of tumor size (mean volume: 13,7 mm^3). Furthermore, in RAD-treated mice no lymph node metastasis of Karpas 299 cells were detected whereas untreated animals showed invasion of axillary and inguinal lymph nodes. Untreated animals were sacrificed at day 16 because of massive tumor size. Our data indicate that treatment of HL and ALCL cells with RAD significantly inhibits tumor cell proliferation and metastasis in xenotransplant models.
Figure 3

A

L540cy

Mean Tumor Volume (mm$^3$)

Solvent

RAD

0 5 10 15 20 25

days

B

Karpas 299

Mean Tumor Volume (mm$^3$)

Solvent

RAD

0 5 10 15

days
RAD controls tumor cell proliferation through modulation of the CCAAT/enhancer binding protein (C/EBP)β protein isoform ratio in HL and ALCL cells

As shown in Figure 4a (lower panels), C/EBPβ proteins were highly expressed in all HL and ALCL cell lines examined. Of particular interest was the observation that the truncated LIP-isoform of C/EBPβ that is highly expressed in breast cancer cells24,25, was also highly expressed in Hodgkin L540cy and in ALCL Karpas 299, FE-PD, JB6 and SU-DHL-1 cells (Figure 4a) and moderately expressed in Hodgkin L1236 and L428 cells. We and others12,24,25 have recently shown that the truncated isoform promotes proliferation and may induce transformation and that rapamycin blocks expression of the truncated isoform through partial inhibition of the translation initiation factor eIF4E.12 As shown in Figure 4a, treatment with 10 nM RAD for 96 h down-regulated the activity of the mTOR signalling pathway as evidenced by strongly diminishing phosphorylation of the ribosomal S6 protein (Figure 4a, upper panels) and 4E-BP1 (data not shown). Concomitantly, the ratio of C/EBPβ isoform expression was modulated such that the truncated C/EBPβ isoform was specifically decreased in all cell lines examined (Figure 4a).

To determine whether abrogation of expression of the truncated LIP-isoform of C/EBPβ contributes to the inhibition of proliferation of RAD-treated Karpas 299 cells, we stably introduced the truncated C/EBPβ isoform (fused to a FLAG immuno-epitope) by retroviral gene transfer (Tr-Flag, Figure 4b). Ectopic expression of the FLAG-tagged truncated C/EBPβ isoform alleviated RAD-induced proliferation arrest as shown in Figure 4c (RAD treatment for 48 h). Thus, translational up-regulation of the truncated C/EBPβ isoform sustains proliferation and might thereby contribute to transformed phenotype in Karpas 299 cells. This suggests translational control of C/EBPβ isoform expression as a target of proliferation control and therapeutic intervention.
**RAD down-regulates constitutive NF-κB activity in L540cy and Karpas 299 cells**

Activated NF-κB p50/p65 has been shown to be essentially required for HL tumor cell proliferation and survival. We therefore asked whether RAD also affects the function of NF-κB in HL cells. As shown in Figure 5b 10 nM RAD down-regulated NF-κB DNA binding activity and inhibited proliferation of L540cy cells in a time-dependent fashion (Figure 5a). Similarly, RAD inhibited constitutive NF-κB activity in the ALCL cell line Karpas 299 whereas NF-κB activity did not change in the other RAD-treated HL and ALCL cell lines (Figure 5b and data not shown). To further determine RAD effects on transcriptional activity of NF-κB we performed luciferase assays with a NF-κB dependent reporter construct (Figure 5c). Our data demonstrate that RAD treatment down-regulated transcriptional activity of NF-κB in L540cy cells.
To investigate whether reduced NF-κB p50, p65 protein levels may account for the down-regulated NF-κB DNA binding activity in L540cy cells, we performed Western blot analysis. NF-κB p50 and p65 levels remained unaltered over time (Figure 5d), indicating that the loss of NF-κB activity could not be explained by down-regulated expression levels. We next analyzed inhibitor kappa B (IκB)α protein and mRNA expression in response to RAD (Figure 5e), because IκBα is the main inhibitor and an important target gene of NF-κB. As expected, IκBα mRNA was significantly reduced since NF-κB activity was down-regulated (Figure 5e; 24, 48, 96 h). However, expression of IκBα protein that has a very short half life, remained unaltered in L540cy cells over 96 h in contrast to its mRNA expression, indicating a posttranslational stabilization of the protein (Figure 5e). We conclude that the loss of NF-κB activity is caused by the posttranslational stabilization of IκBα and results in down-regulation of mRNA expression of IκBα as its main target. Our results clearly show that RAD treatment can functionally interfere with the NF-κB signaling pathway.
Figure 5

A

B

C

D

E

Figure 5

A

B

C

D

E

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NF-κB-p65 overexpression rescues L540cy cells from RAD-mediated inhibition of proliferation

To examine a potential causal relationship between down-regulation of constitutive NF-κB and RAD-mediated inhibition of proliferation in L540cy cells, we transiently transfected L540cy cells with a NF-κB-p65-GFP expression plasmid (Figure 6a) and determined rescue from RAD-mediated proliferation arrest (Figure 6b). As shown in Figure 6b, treatment with 10 nM RAD of control-transfected L540cy cells for 24 h resulted in approximately 40% inhibition of proliferation (cell count, see also Figure 5a). p65-GFP-transfected L540cy cells, however, became resistant to RAD treatment, indicating that overexpression of NF-κB-p65 rescues L540cy cells from RAD-mediated proliferation arrest. The p65-GFP-mediated activation of NF-κB was controlled by reporter assay and EMSA analysis.23 Furthermore, the cotransfection of IκBα reduced NF-κB activity of the construct suggesting proper regulation of the fusion protein.23 Thus, our data show that in addition to regulation of C/EBPβ isoform expression, the NF-κB system is also involved and a target in RAD-mediated inhibition of proliferation in L540cy cells. We suggest that in L540cy cells down-regulation of both the truncated LIP isoform and NF-κB activity contribute to the RAD-mediated effect but neither change is sufficient to the effect.
Figure 6

A

L540cy control  L540cy p65-GFP

p65-GFP  p65

B

0 nM RAD  10 nM RAD

cell count in %

L540cy control  L540cy p65-GFP
Discussion

Data presented here show that the macrolide fungicide RAD, a known immunosuppressant and anti-tumor agent for human posttransplant lymphoproliferative disorders\textsuperscript{10}, inhibits tumor cell proliferation of HL and ALCL (Figure 1). We show that RAD potently suppresses cell-cycle progression in G0/G1 phase of HL and ALCL cells (Figure 2, Table 1) and significantly delays tumor development in xenotransplanted NOD/SCID mice (Figure 3). Moreover, we identify two molecular targets that account for the RAD-mediated substantial anti-proliferative effects, through down-regulation of both, the truncated isoform of the transcription factor CCAAT enhancer binding protein (C/EBP)\textgreek{b} and NF-\kappaB activity.

Tumor cell lines used in this study are derived from patients with advanced stages of HL and ALCL that were heavily pre-treated. It is therefore conceivable that less malignant forms of HL and ALCL cases that would have to be obtained earlier in treatment are even more sensitive to RAD, when tested in future studies. In contrast to the apoptotic effect of RAD or rapamycin on EBV+ B cells and B-percursor leukemia cells\textsuperscript{10,26}, programmed cell death did not appear to play a major role in its anti-tumor activity towards HL and ALCL cells (data not shown). Majewski et al. demonstrated that RAD induced apoptosis in EBV+ B cell lines in contrast to HTLV-I+ malignant T cell lines and Brown et al. used a B-precursor acute lymphoblastic leukemia cell line to determine rapamycin induced apoptosis. The ALCL cell lines and two of the HL cell lines (L540cy and HD-LM2) were of T cell origin and might be relatively resistant to induction of apoptosis by RAD in contrast to the B cell lines used in former studies. In addition HL cell lines are known to be resistant towards induction of apoptosis by various drugs.\textsuperscript{16}

Our data also suggest that RAD might not be used as a single therapeutic drug since it did not eradicate xenotransplanted tumor cells but only delayed tumor outgrowth. However, from two studies it is already known that rapamycin can not only enhance apoptosis but also potentiate dexamethasone-induced apoptosis and increase sensitivity to cisplatin in lymphoblastoid
cells. Therefore, in clinical settings of HL and ALCL RAD might be used in combination therapy with conventional chemotherapeutic drugs.

Novel concepts for the treatment of HL and ALCL have to be based on insights of the molecular mechanisms that are responsible for deregulated proliferation and differentiation. Rapamycin inhibits the protein kinase activity of mTOR and thereby controls the translation of key mRNAs that encode proteins that are required for cell-cycle progression and differentiation. We recently demonstrated that inhibiting the mTOR signaling pathway by rapamycin controls the protein isoform expression of the transcription factor C/EBP through alteration of the activity of the eukaryotic translation initiation machinery. Different translationally initiated C/EBP proteins display isoform-specific biological activities that adjust cell proliferation versus differentiation. The full-length C/EBP isoform functions as a transcriptional activator that induces differentiation, whereas a truncated, downstream initiated isoform permits the cell cycle to proceed. Moreover, the truncated isoform can counteract the functions of full-length isoform. Therefore, the biological effects evoked by C/EBP proteins strongly depend on the ratio of C/EBP isoforms. We show that C/EBP and in particular its truncated, proliferation supporting isoform is abundantly expressed in ALCL and some HL cells (Figure 4a). Similarly to what has been shown in rapamycin treated adipocytes, RAD strongly reduced expression of the truncated C/EBP isoform, whereas the full-length isoform remained relatively constant. Karpas 299 cells that were stably transfected with truncated C/EBP were less sensitive to RAD-mediated inhibition of proliferation in vitro demonstrating a growth supporting function for C/EBP in ALCL (Figure 4b, c). Taken together, our data suggest that modulation of the translational control of the C/EBP isoform expression by RAD essentially contributes to inhibition of proliferation in HL and ALCL cells.

Increased NF-κB activity is an essential factor for malignant transformation in HL. Our previous data showed that constitutive NF-κB activity is a survival factor for HL cells.
Furthermore, pharmacologic inhibition of IKK/NF-κB activity by arsenic can also overcome drug resistance and therefore is a powerful treatment option for HL.\textsuperscript{23} Several reports suggested that rapamycin can prevent activation of NF-κB by stabilization of its major inhibitor IκBα.\textsuperscript{33-36} Here we demonstrate that RAD strongly inhibits NF-κB DNA-binding activity only in L540cy HL and Karpas 299 ALCL cells (Figure 5), whereas more conventional mTOR pathway targets such as ribosomal S6 protein were down-regulated in all cell lines (Figure 4a, upper panels). Thereby, in L540cy cells the loss of NF-κB activity is caused by the posttranslational stabilization of its main inhibitor IκBα that has been previously described in Jurkat T cells.\textsuperscript{31} We further show that over-expression of NF-κB-p65 rescues L540cy cells from RAD-mediated inhibition of proliferation, indicating that RAD also exerts its anti-proliferative effects and at least in part through inhibition of constitutive NF-κB (Figure 6).

In conclusion, our data provide evidence for the inhibitory activity of RAD against HL and ALCL cell proliferation in tissue culture and in the animal. These results suggest that important targets of the anti-proliferative activity of RAD include C/EBPβ isoform expression and constitutive NF-κB activity. Therefore, targeting mTOR dependent signal transduction may represent a novel and an alternative strategy for potent lymphoma combination therapy. This view is in agreement with conclusions drawn from classical tumor virology that suggested that inhibition of translation by rapamycin through mTOR and its downstream targets S6K and 4EBPs effectively blocks oncogenic transformation induced by a subgroup of pathways such as activated P3K or Akt but not by other oncoproteins.\textsuperscript{37} Our study might therefore provide a basis for testing RAD in new clinical trials to overcome classical drug resistance and achieve improved outcome in incurable cases of HL and ALCL. Further studies are needed to determine its efficacy in combination with standard therapeutic drugs.
Acknowledgments

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References


Figure Legends

Figure 1

RAD inhibits proliferation of HL and ALCL cells. (a) The HL cell lines (L540cy, L1236, KM-H2, L428, HD-LM2) and (b) the ALCL cell lines (Karpas 299, FE-PD, JB6, SU-DHL1) were incubated with 0-50 nM RAD for 96 h. Cell counts are given as the mean of three independent experiments. Counts of RAD-treated lymphoma cells are given relative to counts of untreated cells which were set arbitrarily at 100% for each cell line.
**Figure 2**

RAD mediates inhibition of cell-cycle progression in HL and ALCL cells. Cell lines were incubated with 0-10 nM RAD for 48 h, labeled with propidium iodine, and analyzed by flow cytometry. Cell-cycle profiles of L540cy and Karpas 299 cells are shown as representative examples.
Table 1

Relative increases and decreases of RAD-treated cells in G₀/G₁ and G₂/M phases of the cell-cycle of HL and ALCL cells. Cell-cycles of HL and ALCL cells were analyzed by propidium iodide staining and statistical analysis was performed via ModFit LT program. Untreated cells in G₀/G₁ and G₂/M phase were set arbitrarily at 100 % for each cell line.
Figure 3

RAD-mediated inhibition of in vivo proliferation of HL and ALCL cells. NOD/SCID mice were inoculated s.c. with (a) L540cy and (b) Karpas 299 cells and daily treatment with RAD (5 mg/kg) was started when tumors were palpable. Tumor volume (mean in mm³) was compared between RAD-treated versus control animals. Statistical significance was determined by U-Test of Mann and Whitney; * p<0.05.
Constitutive ectopic expression of truncated C/EBPβ renders ALCL cells resistant to RAD-mediated inhibition of proliferation. (a) Down-regulation of the mTOR target gene, ribosomal S6 protein, was demonstrated after treatment of cell lines with 0-10 nM RAD for 96 h (upper panels). Endogenous expression of C/EBPβ protein isoforms was determined in HL and ALCL cells, that were treated with 0-10 nM RAD for 96 h (lower panels). (Fl) Full-length isoform; (Tr) truncated isoform. (b) FLAG-tagged truncated C/EBPβ was ectopically expressed in Karpas 299 cells by retroviral transduction. Ectopic and endogenous expression of C/EBPβ protein isoforms was analyzed by immunoblotting with antibodies against C/EBPβ after treatment with 0-10 nM RAD for 96 h (c) Control and C/EBPβ-Tr transfected Karpas 299 cells were incubated with 0-10 nM RAD for 48 h. RAD-treated and untreated cells were counted and counts of untreated cells were set arbitrarily at 100%. Cell counts are given as the mean of three independent experiments.
Figure 5

Down-regulation of NF-κB DNA binding activity by RAD in L540cy and Karpas 299 cells. (a) The HL cell line L540cy was incubated with 0-10 nM RAD for 24, 48, 96 h. Cell counts are given as the mean of three independent experiments. Counts of RAD-treated lymphoma cells are given relative to counts of untreated cells which were set arbitrarily at 100%. (b) L540cy and Karpas 299 cells were treated with 0-10 nM RAD for indicated times. Whole cell extracts were analyzed by EMSA for NF-κB DNA binding activity. Free DNA probe is not shown. n.s indicates nonspecific. (c) L540cy cells were transfected with a NF-κB dependent reporter construct (6NF-κBtkluc) and transcriptional activity of NF-κB was detected 24 and 48 h after treatment with 0-10 nM RAD. Luciferase counts are given relative to control treated L540cy cells, that are set arbitrarily at 1. Luciferase activity was determined for triplicate experiments. (d) Protein expression levels p50 and p65 in response to RAD analyzed by Western blot analysis in L540cy cells. (e) Protein and total mRNA was prepared after indicated times and analyzed for expression of IκBα by Western and Northern blotting in L540cy cells.
Figure 6

Overexpression of NF-κB-p65 protects L540cy cells from RAD-induced cell-cycle arrest in G₀/G₁. (a) L540cy cells were transfected with a p65-GFP expression construct or with control (pcDNA3) and EGFP expressing (pEGFP-N3) constructs. Twenty-four hours after transfection, whole cell extracts were analyzed by Western blotting for the expression of p65-GFP and endogenous p65 using a p65-specific antibody. (b) L540cy cells were transfected with a p65-GFP expression construct or with pcDNA3 and pEGFP-N3 constructs. Six hours after transfection, cells were incubated with 0 and 10 nM RAD for 24 h. RAD-treated and untreated cells were counted and counts of untreated cells were set arbitrarily at 100%. Cell counts are given as the mean of three independent experiments.
A rapamycin derivative (everolimus) controls proliferation through down-regulation of truncated CCAAT enhancer binding protein β and NF-κB activity in Hodgkin and anaplastic large cell lymphomas

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