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Lack of compensatory pAKT activation and eIF4E phosphorylation of lymphoma cells towards mTOR inhibitor, RAD001

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ABSTRACT

mTOR (mammalian target of rapamycin) inhibitors were recently found to be effective in the treatment of various human non-Hodgkin's lymphomas (NHLs). We recently reported that RAD001, an mTOR inhibitor, suppressed the growth of lymphoma cells at concentrations much lower than those required for carcinomas. However, the basis for the enhanced sensitivity to RAD001 is unknown. Seven aggressive NHL cell lines and seven carcinoma cell lines were used in this study. Cell cycle was analysed by flow cytometry. pAKT (phosphorylated AKT) (Ser^{473} and Thr^{308}), p-p70S6K, p-4E-BP1, p-mTOR, p-eIF4E (phosphorylated eIF4E), cyclin A, cyclin E, cyclin D3, c-Myc and insulin receptor substrate-1 (IRS-1) protein expression were assessed by immunoblotting. The PI3K/AKT/mTOR (phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin) signalling pathway was constitutively expressed in all seven lymphoma cell lines. RAD001 down-regulated p-mTOR, p-p70S6K, p-4E-BP1, cyclin A, cyclin E, cyclin D3, and c-Myc, but did not affect IRS-1. In parallel with RAD001-induced inhibition of cell viability, a dose- and schedule- dependent down-regulation of pAKT and p-eIF4E expressions was demonstrated. In contrast, a compensatory activation of pAKT and p-eIF4E, was observed in seven carcinoma cells. These findings indicate that the basis for enhanced activity of mTOR inhibitors in NHL may be the lack of compensatory activation of AKT and eIF4E phosphorylation in lymphoma cells.

1. Introduction

Although improvements in chemotherapy and immunotherapy regimens for the treatment of aggressive non-Hodgkin's lymphoma (NHL) have increased the occurrence of complete remission and have improved patient survival, a substantial portion of patients with NHL subsequently develop drug resistance and eventually die from progressive disease.[1,2]

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Therefore, novel salvage therapies with a favourable therapeutic index are urgently needed for patients who have already failed several lines of chemotherapy and immunotherapy regimens, or for patients who have recurrent NHL.

The phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT) and mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR) signalling pathway are reported to play a crucial role in the pathogenesis of a variety of NHLs, including mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), and anaplastic large cell lymphoma (ALCL).[3–5] In mammalian cells, the best characterised downstream effectors of mTOR are two key eukaryotic translation regulators, eukaryotic translation initiation factor 4E binding protein-1 (4E-BP1) and 40S ribosomal protein p70S6 kinase (p70S6K).[6,7] mTOR phosphorylation of 4E-BP1 inhibits its repression of eukaryotic initiation factor 4E (eIF4E) and ultimately contributes to enhance translation from a subset of genes that are required for cell cycle progression, cell growth, and apoptosis resistance.[8,9] Although the role of eIF4E in the oncogenesis of aggressive NHL remains unclear, it was recently reported that eIF4E itself has anti-apoptotic activity, and that eIF4E enhances c-Myc transcription in lymphoma cells and that c-Myc up-regulates eIF4E transcriptional activity.[10] Interestingly, recent studies have found that eIF4E can recapitulate AKT activity during oncogenesis and drug resistance in lymphoma cells *in vivo*.[11,12] p70S6K, 4E-BP1 and downstream eIF4E are good targets for inhibition by rapamycin, an mTOR inhibitor. Rapamycin and its analogues are thought to elicit anti-proliferative effects in NHL and may be useful adjunct treatments for a variety of aggressive NHL.

Indeed, mTOR inhibitors have been tested in haematologic malignancies and are found to be effective in the treatment of various human NHL.[13–15] For example, CCI-779 (temsirolimus), an analogue of rapamycin, has demonstrated substantial single-agent activity in relapsed MCL patients.[14] Recent studies also indicate that everolimus [RAD001,40-O-(2-hydroxyethyl)-rapamycin], the only orally active rapamycin derivative, is not only effective in a variety of human cancers, but is also active against MCL and DLBCL.[15–17] We have recently demonstrated that RAD001 suppresses the growth of lymphoma cells at concentrations much lower than those required for carcinomas.[18] However, the basis for enhanced RAD001 activity against lymphoma cells is unknown.

In this study, we examined the *in vitro* effects of RAD001 in seven NHL cell lines and in seven carcinoma cell lines. Results indicate that RAD001 50% inhibitory concentrations (IC50s) were generally lower for the lymphoma cell lines than for the seven carcinoma cell lines, confirming the generally good activity of mTOR inhibitors in the treatment of NHL. Furthermore, we showed that RAD001 induced G0/G1 cell cycle arrest and down-regulated phospho-p70S6K (p-p70S6K), phospho-4E-BP1 (p-4E-BP1), cyclin A, cyclin D3, and c-Myc. In parallel with RAD001-induced growth inhibition, phosphorylated AKT (pAKT), and phosphorylated eIF4E (p-eIF4E) were also down-regulated in lymphoma cells by RAD001 in a dose- and dosing schedule-dependent fashion. Conversely, a compensatory increase in pAKT and p-eIF4E levels was observed in the carcinoma cells. These findings indicate that the effectiveness of mTOR inhibitors in the treatment of NHL may involve the lack of compensatory pAKT and eIF4E phosphorylation in lymphoma cells.

2. Materials and methods

2.1. Cell culture and chemicals

Experiments used Pfeiffer (DLBCL), Ramos (EBV-negative Burkitt's lymphoma), Raji (EBV-positive Burkitt's lymphoma), and MC116 (EBV-negative undifferentiated lymphoma) aggressive B-cell lymphoma cell lines; SR786 [CD30 (Ki+) ALCL], H9 (HTLV-I negative T cell lymphoma), and Jurkat (acute T-cell leukaemia/lymphoma) aggressive T-cell lymphoma cell lines; Hep3B, HepG2, PLC5, and Huh7 hepatocellular carcinoma cell lines; TW01 and HONE1 nasopharyngeal cancer cell lines; and the gastric cell line NCI-N87. Pfeiffer, Ramos, Raji, MC116, H9, Jurkat, Hep3B, HepG2, PLC5, Huh7, and NCI-N87 cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). SR786 was obtained from DSMZ (Braunschweig, Germany). TW01 and HONE1 were obtained as previously described.[19] Lymphoma cell lines were maintained in RPMI-1640 medium and carcinoma cells were maintained in Dulbecco's modified Eagles' medium. All cells were incubated at 37 °C in a 5% CO_2 atmosphere. RAD001 was supplied by Novartis. BAFF was from Chemicon International (Temecula, CA), and LY294002 was from Sigma–Aldrich (St Louis, MO).

2.2. Measurement of growth inhibitory effects by MTT assay

Drug-induced *in vitro* growth inhibitory effects were determined using the MTT assay as previously described,[19] with slight modification. Briefly, cells were plated in 96-well plates at 5×10^3 cells/well. After overnight incubation, various concentrations of drugs were added to each culture in sextuplicate samples. Cells were exposed to drugs continuously. After 72 hours of culture, when cells in the drug-free control wells had reached 90% confluency, 20 μL of 5 mg/mL MTT (Sigma–Aldrich) in PBS was added to each well and incubated for 4 h at 37 °C. The formazan crystals that developed were dissolved in DMSO (carcinoma cells) or SDS-HCl and absorbance was determined with an ELISA reader at 540 nm. Absorbance values were presented as percentage of viability in control cells treated with solvent alone (cells not exposed to drug); and all values represent the mean ± standard deviation of three independent experiments ($P < 0.05$, at three independent experiments). Each experimental condition was performed in sextuplicate.

2.3. Immunoblotting

After various treatments, cells were subjected to cell fractionation as previously described.[20] Total lysates from Pfeiffer, Ramos, Raji, MC116, H9, SR786, Jurkat, Hep3B, HepG2, PLC5, Huh7, NCI-N87, TW01, and HONE1 were prepared, and protein concentrations were determined using the Bio-Rad determination kit. Aliquots (15 μg) of lysates were subjected to immunoblotting. For reprobing, membranes were washed with a stripping buffer (2% sodium dodecyl sulphate, 7 μl of

β-mercaptoethanol, and 0.2% Tween 20 in 100 ml of Tris-buffered saline) for 30 min at room temperature to remove bound antibodies, and then subjected to immunoblotting. Antibodies for pAKT (Ser^{473}; sc-101629; Santa Cruz Biotechnology), pAKT (Thr^{308}; sc-16646-R; Santa Cruz Biotechnology), p70S6K (#2902; Cell signalling Technology), p-p70S6K (Thr^{389}; #2905; Cell signalling Technology), 4E-BP1 (#9452; Cell signalling Technology), p-4E-BP1 ($Thr^{37/46}$; #9451; Cell signalling Technology), mTOR #2972; Cell signalling Technology), phospho-mTOR (p-mTOR, Ser^{2448}; #2976; 49F9; Cell signalling Technology), eIF4E (#9742; Cell signalling Technology), p-eIF4E (Ser^{209}; #9741; Cell signalling Technology), cyclin D3 (112307; Novacastra), cyclin A (sc-239; Santa Cruz Biotechnology), cyclin E (sc-198; Santa Cruz Biotechnology), cyclin D1 (sc-753; Santa Cruz Biotechnology), p27 (sc-53906; Santa Cruz Biotechnology), insulin receptor substrate-1 (IRS-1; 17153; Sigma–Aldrich), and c-Myc (sc-40; Santa Cruz Biotechnology) were used in the present study. All experiments were repeated at least three times.

2.4. *Immunohistochemistry*

Cytospin preparations of Ramos cells were fixed in a 1:1 mixture of acetone–methanol. Immunostaining was performed using the avidinstreptavidin–peroxidase technique on acetone–methanol-fixed cytospin preparations according to the manufacturer's instructions (indirect immunoperoxidase methods).[20] Antibodies for pAKT (Ser^{473}; sc-101629; Santa Cruz Biotechnology), pAKT (Thr^{308}; sc-16646-R; Santa Cruz Biotechnology), p-p70S6K (Thr^{389}; sc-11759; Santa Cruz Biotechnology), p-4E-BP1 ($Thr^{37/46}$; #2855; 236B4, Cell signalling Technology), p-mTOR (Ser^{2448}; #2976; 49F9; Cell signalling Technology), and p-eIF4E (Ser^{209}; sc-12885; Santa Cruz Biotechnology) were used as primary antibodies in this indirect immunoperoxidase method of immunohistochemistry. Cell blocks of a human Karpas 299 ALCL (known to express pAKT, p-p70S6K, p-4E-BP1, p-mTOR, and p-eIF4E) were used as positive controls for pAKT, p-p70S6K, p-4E-BP1, p-mTOR and p-eIF4E. Antibodies for pAKT(Ser^{473}), pAKT (Thr^{308}), p-

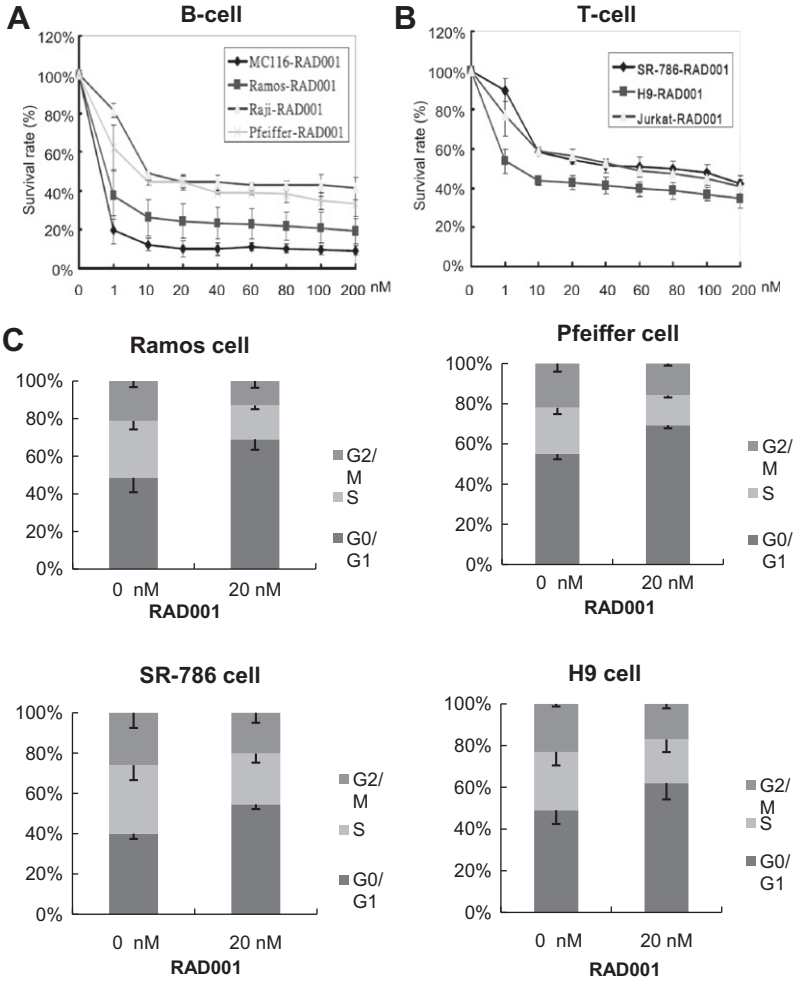

Fig. 1 – The inhibition of cell viability of RAD001 on cell growth and cell cycle arrest in aggressive B- and T-cell lymphoma cells. (A) B-cell NHLs, MC116 cells, Ramos cells, Raji cells and Pfeiffer cells (B) T-cell NHLs, SR786 cells, H9 cells and Jurkat cells were incubated with 0to 200 nM RAD001 for 72 hours. Cell numbers were measured by MTT assay and plotted as percentage of viability in control cells treated with solvent alone (cells not exposed to drug). All values represent the mean ± standard deviation of three independent experiments ($P < 0.05$, at three independent experiments). (C) Cell cycle arrest in RAD001-treated aggressive lymphoma cell lines (Ramos, Pfeiffer, SR786, and H9). Cell lines were incubated with 0 or 20 nM RAD001 for 24 hours, respectively, and thereby labelled with propidium iodine, followed by analysis with flow cytometry.

p70S6K, p-4E-BP1, p-mTOR and p-eIF4E have been used for the immunohistochemical detection of pAKT(Ser^{473}), pAKT (Thr^{308}), p-p70S6K, p-4E-BP1, p-mTOR, and p-eIF4E in ALCL tumour cells and breast cancer.[5,21]

2.5. Flow cytometric analysis

Cells were seeded at 2.0×10^5 per 60 mm dish and incubated overnight. Cells were then treated with 0 and 20 nM RAD001 for 24 h, and then both floating and attached cells were collected. The cells were fixed with −20 °C methanol for at least 30 min and then stained with 10 mg/ml propidium iodide and 100 mg/ml RNase A for 30 min in the dark. Cell cycle distribution was determined by flow cytometric analysis (Becton Dickinson FACScan). All cell cycle distributions represent the mean ± standard deviation of three independent experiments of flow cytometric analysis.

3. Results

3.1. RAD001 inhibits cell viability of NHL cells and carcinoma cells

To evaluate the RAD001 efficacy against a panel of cells representative of aggressive NHL cell lines, cells in logarithmic growth were incubated with RAD001 for 72 hours and cell survival was evaluated using a standard MTT colorimetric assay. As shown in Fig. 1A and B, RAD001 induced dose-dependent inhibition of cell viability in all 7 lymphoma cell lines, with 50% inhibitory concentrations (IC50s) ranging from 5 to 50 nM. In addition, there was no difference between (1) EBV-positive and EBV-negative (2) between the B-cells and T-cells.

The effects of RAD001 were also examined in a panel of hepatocellular carcinoma, gastric carcinoma and nasopharyngeal carcinoma cell lines. The inhibition of cell viability of RAD001 in carcinoma cells was strikingly different from those observed in aggressive lymphomas. In contrast to the 7 lymphoma cell lines, RAD001 induced a maximum of inhibition of cell viability of 30–50%, even at μM concentrations, in all carcinoma cells tested (Table 1).

3.2. RAD001 blocks cell cycle progression in NHL cells

Flow cytometry analysis was performed after 24 hours of drug exposure to determine whether RAD001 blocked cell cycle progression in the G0/G1 phase. Compared with the untreated control groups, aggressive lymphoma cell lines treated with RAD001 alone for 24 hours exhibit G1 cell cycle arrest. As shown in Fig. 1C, cell cycle distribution showed that there was an increase in the percentage of cells in the G0/G1 and a concomitant decrease in the percentage of cells in the G2/M, indicating that RAD001 blocked cell cycle progression in the G0/G1 phase. The percentage of G0/G1 phase cells was increased 42.8% ($P = 0.019$), 26.7% ($P = 0.001$), 35.0% ($P = 0.002$), and 24.5% ($P = 0.001$), respectively, for Ramos cell, Pfeiffer cell, SR786 cell and H9 cell, respectively, after 24 hours of 20 nM RAD001 treatment (Fig. 1C). These results indicated that apoptosis was not the major mechanism of RAD001-induced inhibition of cell viability of aggressive NHL cells.

3.3. RAD001 down-regulates the function of critical cell cycle regulatory proteins

Since RAD001 blocked cell cycle progression in the G0/G1 phase in all seven lymphoma cell lines, we assessed whether critical cell cycle regulatory proteins were affected by RAD001. We found that RAD001 strongly reduced cyclin D3 expression and c-Myc expression in 6 lymphoma cell lines, including Ramos, SR786, MC116, Pfeiffer, H9 and Jurkat cells (Fig. 2A, B). In parallel, a sustained decrease in cyclin A and cyclin E were observed in Ramos and SR786 cell lines during a dose-dependent course of RAD001 treatment, while cyclin D1 expression was not significantly changed in Ramos and SR786 cell lines (Fig. 2A). However, the p27 expression was not affected by RAD001 in Ramos, SR786, MC116, Pfeiffer, H9 and Jurkat cells (Fig. 2A, B). These data suggest that RAD001 is a promising therapeutic cytostatic agent in aggressive

Table 1 – Anti-proliferative activity of RAD001 in a panel of cancer cell lines, evaluated in *in vitro* culture cells.

Type of cancer	Cell-line	IC_{50} (nM)	Maximum inhibitory effect: (% of control at highest RAD001 concentration tested)
EBV-positive Burkitt's lymphoma	Raji	3.7	
Diffuse large B-cell lymphoma	Pfeiffer	1.8	
EBV-negative Burkitt's lymphoma	Ramos	4.0	
EBV-negative undifferentiated lymphoma	MC116	<1.0	
CD30 (Ki+) anaplastic large cell lymphoma	SR786	44	
Human T-cell lymphotropic virus type 1-negative T cell lymphoma	H9	12	
Acute T-cell leukaemia/lymphoma	Jurkat	19	
Hepatocellular carcinoma	Hep3B	>100	~30% (at 100 nM)
Hepatocellular carcinoma	HepG2	>100	~20% (at 100 nM)
Hepatocellular carcinoma	PLC5	>100	~30% (at 100 nM)
Hepatocellular carcinoma	Huh7	>100	~20% (at 100 nM)
Nasopharyngeal cancer	TW01	>100	~30% (at 10 μM)
Nasopharyngeal cancer	HONE1	>100	~30% (at 10 μM)
Gastric cancer	NCI-N87	>100	~50% (at 10 μM)

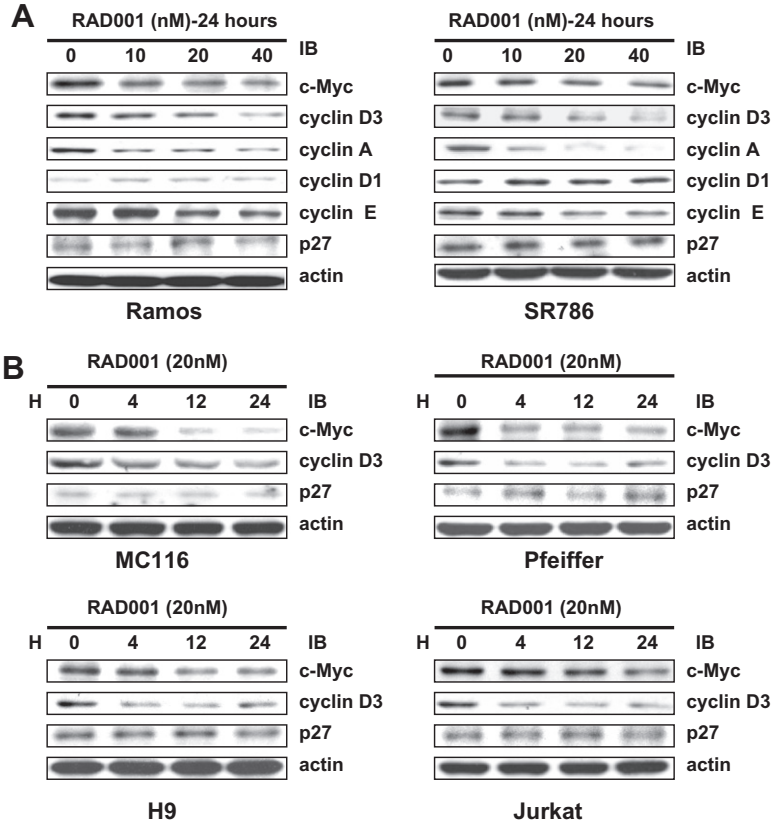

Fig. 2 – RAD001 down-regulates the functioning of critical cell cycle regulatory proteins. (A) RAD001 down-regulates c-Myc, cyclin D3, cyclin A, cyclin E, but not cyclin D1 and p27. Ramos cells and SR786 cells were treated with RAD001, 0, 10, 20, 40 nM. The whole cell lysates were subjected to immunoblotting with antibody for cyclin D1, cyclin D3, cyclin A, cyclin E, p27 and c-Myc. The membrane was reprobed with anti-actin antibody as a loading control. (B) RAD001 down-regulates c-Myc and cyclin D3, but not p27 in other B-cell NHLs and T-cell NHLs. Following RAD001 (20nM) treatment, the expression of c-Myc, cyclin D3 and p27 in MC116 cells, Pfeiffer cells, H9 cells and Jurkat cells was analysed by immunoblotting with specific antibodies after the indicated time. The membrane was reprobed with anti-actin antibody as a loading control.

lymphoma cells that functions through blocking cell cycle progression in the G0/G1 phase.

3.4. *RAD001 down-regulates mTOR signalling pathway in NHL cells*

Recent studies have demonstrated that the PI3K/AKT/mTOR signalling pathway plays an important role in the lymphomagesis of a variety of NHLs.[3–5] In the present study, we tested whether inhibition of mTOR down-regulated the PI3/AKT/mTOR signalling pathway in these lymphoma cell lines. We cultured Pfeiffer, Ramos, Raji and MC116 aggressive B-cell lymphoma cell lines; SR786, H9 and Jurkat aggressive T-cell lymphoma cell lines, and then these cells were incubated with RAD001. We found that RAD001 treatment caused a marked dose-dependent and schedule-dependent (data not shown) decrease in the phosphorylation levels of p70S6K and 4E-BP1 of these 7 lymphoma cell lines (Fig. 3). In addition, RAD001 incubation caused a dose-dependent and schedule-dependent (data not shown) decrease in p-mTOR activation on Ser^{2448} in these 7 lymphoma cell lines (Fig. 3). To further confirm the biological significance of our observations, we examined the expression pattern of p-p70S6K, p-4E-BP1 and p-mTOR in Ramos cells using immunohistochemistry. In Ramos cells, treatment with RAD001 caused a dose-dependent decrease in the expression of p-p70S6K, p-4E-BP1 and p-mTOR (Fig. 4).

3.5. *Lack of compensatory pAKT activation and eIF4E phosphorylation in RAD001-treated NHL cells*

We found that RAD001 caused a dose-dependent and schedule-dependent (data not shown) decrease in AKT (Ser^{473} and Thr^{308}) phosphorylation and p-eIF4E activation on Ser^{209} in all 7 lymphoma cell lines, including Pfeiffer, Ramos, Raji and MC116 aggressive B-cell lymphoma cell lines; SR786, H9 and Jurkat aggressive T-cell lymphoma cell lines (Fig. 5). To further confirm the biological significance of these observations, we examined the expression pattern of pAKT and p-eIF4E in Ramos cells by immunohistochemical staining. Notably, in RAD001-treated Ramos cells, the expression of pAKT (Ser^{473}), pAKT (Thr^{308}) and p-eIF4E decreased in a dose-dependent manner as compared with control cells (Fig. 4). In contrast, a dose-dependent and schedule-dependent (data not shown) increase in phosphorylation of AKT (Ser^{473}) and eIF4E (Ser^{209}) was found in all 7 carcinoma cell

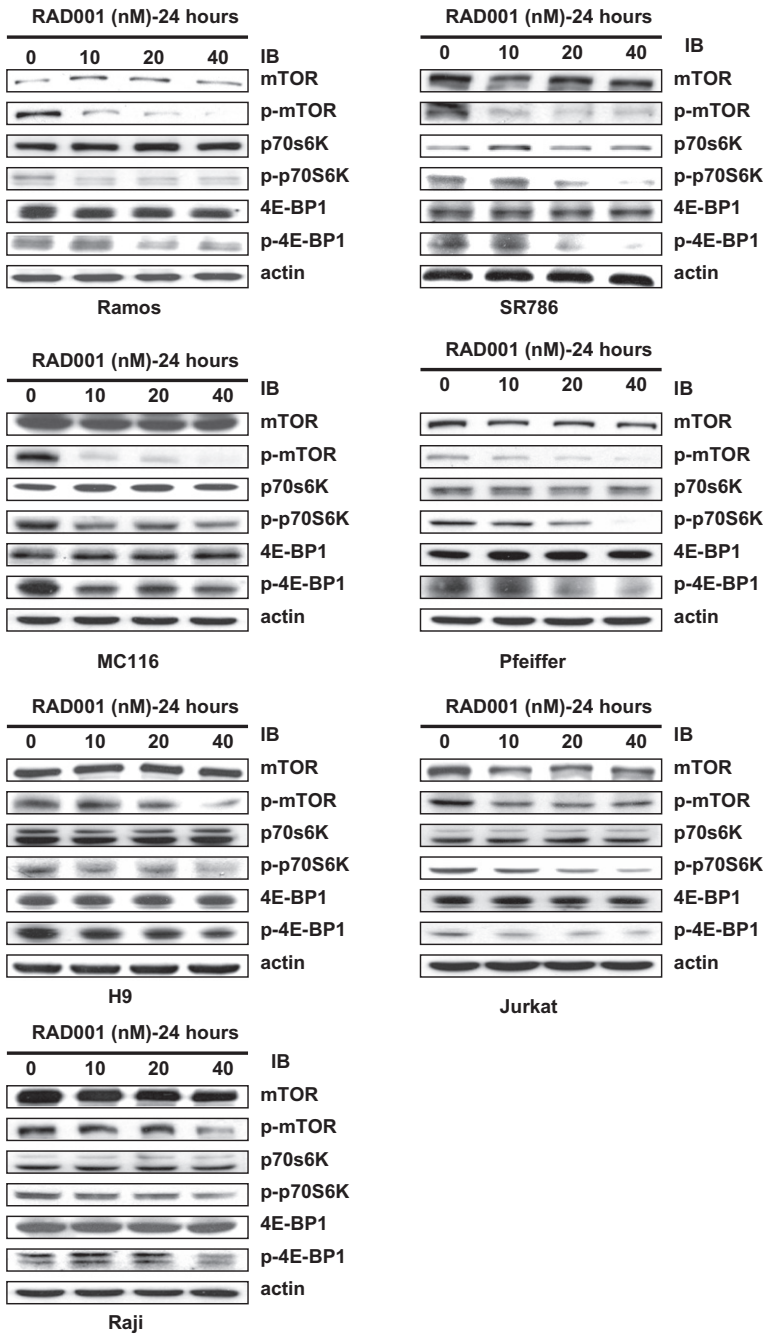

Fig. 3 – RAD001 down-regulates mTOR signalling pathway, including p-mTOR, p-p70S6K and p-4E-BP1 in aggressive lymphoma cells. The protein was isolated from whole cell lysates prepared from Ramos cells, SR786 cells, MC116 cells, Pfeiffer cells, H9 cells, Jurkat cells, and Raji cells with the mTOR inhibitor, RAD001, at a concentration of 0, 10, 20, or 40 nM. Immunoblotting (IB) of level of mTOR, p-mTOR (Ser^{2448}), p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1 in this protein was performed. The membrane was reprobed with anti-actin antibody as a loading control.

lines, including Hep3B, HepG2, PLC5 and Huh7 (hepatocellular carcinoma cell lines); TW01 and HONE1 (nasopharyngeal cancer cell lines); and the gastric cell line NCI-N87 (data not shown), after RAD001 treatment (Fig. 6).

Recent studies suggest that mTOR inhibition may result in AKT activation in cancer cell lines through enhanced insulin receptor substrate (IRS-1) expression and abrogation of negative feedback-induced down-regulation of receptor tyrosine kinase signalling.[21,22] In the present study, we demonstrated that RAD001 caused an increase in the expression of IRS-1 in these 7 carcinoma cells (Fig. 6; NCI-N87 cell, data not shown). Further, to determine whether the lack of induction of AKT activity in these seven lymphoma cells lines resulted from a lack of insulin-like growth factor-1 (IGF-1) responsiveness, we examined the expression of IRS-1 in RAD001-treated lymphoma cells lines. As shown in Fig. 7A, IRS-1 protein levels were not altered after increasing concentrations of and prolonged exposure to RAD001.

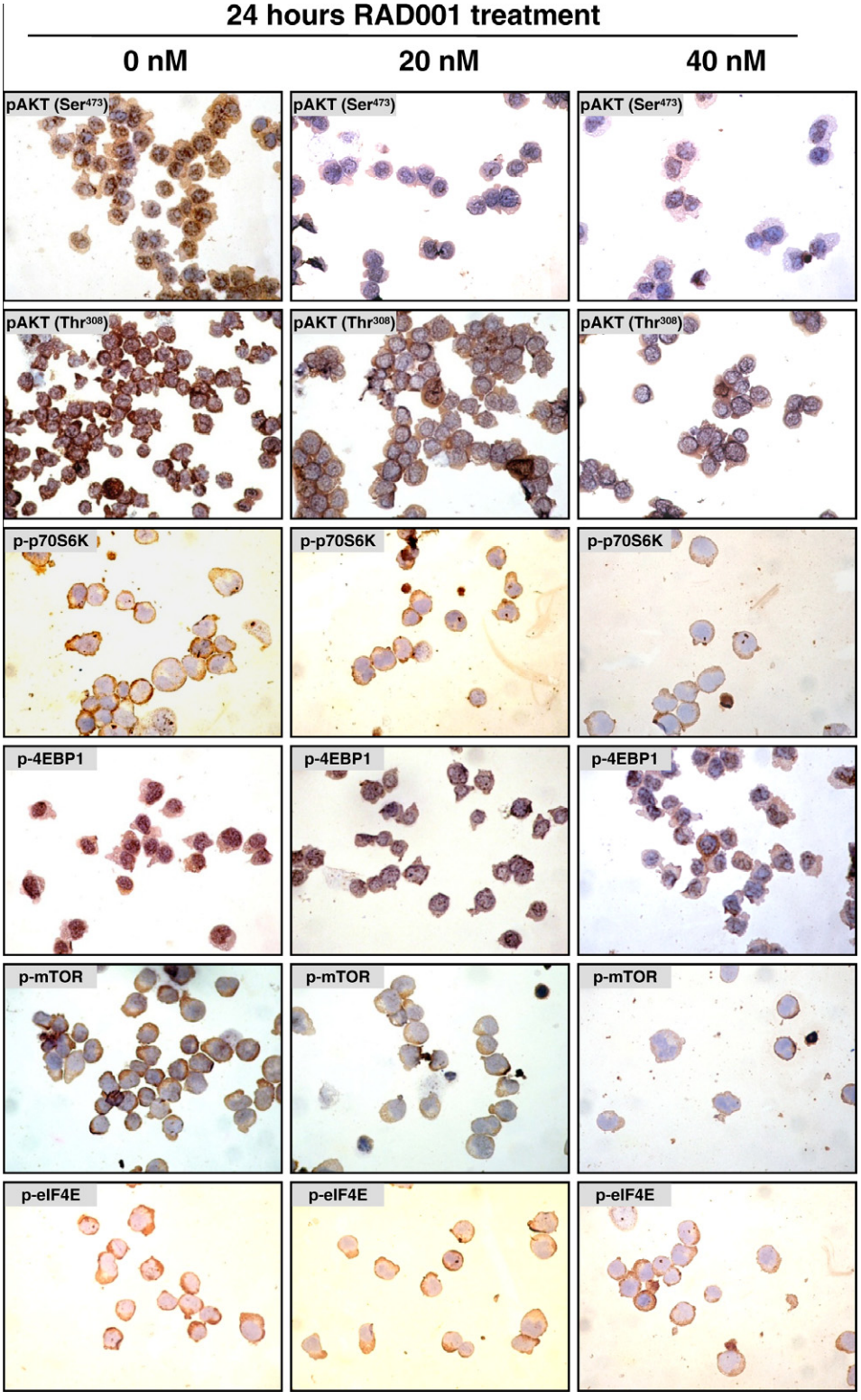

Fig. 4 – RAD001 down-regulates PI3K/AKT/mTOR signalling pathway in aggressive lymphoma cells. After 24 hours of 0, 20 and 40 nM RAD001 treatment, the dose-dependent decreased expression of pAKT (Ser^{473}), pAKT (Thr^{308}), p-p70S6K, p-4E-BP1, p-mTOR (Ser^{2448}), and p-eIF4E (Ser^{209}) is found in tumour cells of post-RAD001-treated Ramos cell lines by immunohistochemical staining. Left top panel, after 24 hours 0 nM RAD001; Mid top panel, after 24 hours 20 nM RAD001; Right top panel, after 24 hours 40 nM RAD001.

Based on these findings, we hypothesised that the compensatory pathway that exists in carcinoma cells may not be present in these aggressive lymphoma cell lines. To further confirm this hypothesis, we attempted to elucidate whether or not the combination of a PI3K inhibitor (LY294002) with RAD001 enhanced the down-regulation of PI3K/AKT/mTOR

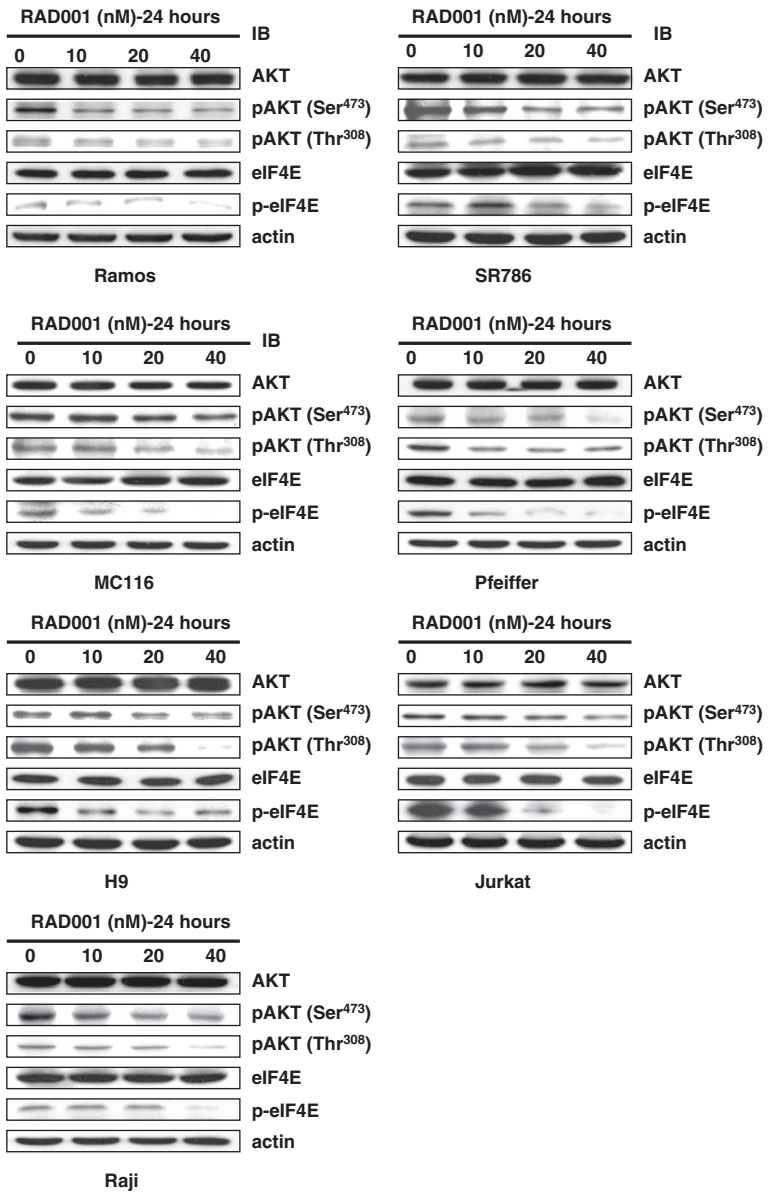

Fig. 5 – Inhibition of mTOR in aggressive lymphoma cells is associated with the lack of compensatory activation of AKT and eIF4E phosphorylation. RAD001 down-regulates pAKT (Ser^{473}), pAKT (Thr^{308}) and p-eIF4E. The whole cell lysates from RAD001-treated Ramos cells, SR786 cells, MC116 cells, Pfeiffer cells, H9 cells, Jurkat cells, and Raji cells were individually subjected to immunoblotting with anti-AKT anitbody, anti-pAKT (Ser^{473}) antibody, pAKT (Thr^{308}) antibody, anti-eIF4E antibody and anti-p-eIF4E (Ser^{209}) antibody. The membrane was reprobed with anti-actin antibody as a loading control.

signalling. We selected 2 aggressive B-cell lymphoma cell lines (Pfeiffer, and Raji) and 2 aggressive T-cell lymphoma cell lines (H9, and Jurkat), and found that treatment with RAD001 alone does not result in the compensatory activation of IRS-1, pAKT (Ser^{473}), and pAKT (Thr^{308}), whereas treatment with PI3K inhibitor (LY294002) alone down-regulated AKT phosphorylation (Ser^{473} and/or Thr^{308}) but not IRS-1 in these lymphoma cells (Fig. 7B). The combination of LY294002 and RAD001 did not enhance the down-regulation of IRS-1, pAKT (Ser^{473}) and pAKT (Thr^{308}) in these lymphoma cell lines (Fig. 7B). In another two lymphoma cell lines (Ramos cell, and SR786 cell), we found that the addition of LY294002 to these lymphoma cell lines did not enhance the down-regulation of IRS-1, pAKT (Ser^{473}), and pAKT (Thr^{308}) (Fig. 7C). Furthermore, in Pfeiffer cell and SR786 cell, we found that the cell viabilities of these lymphoma cell lines were not synergistically inhibited but were additively inhibited by the combination of LY294002 and RAD001. Since compensatory activation of AKT phosphorylation was found in carcinoma cells during RAD001 treatment, we select two carcinoma cells (HepG2 cell, and PLC5 cell) to examine whether the addition of LY294002 can increase the inhibition of cell viability of RAD001 in these carcinoma cells. We found that, in these carcinoma cells, the combination LY294002 and RAD001 had more additive effects of cell viability inhibition than lymphoma cells (Supplementary Fig. 1).

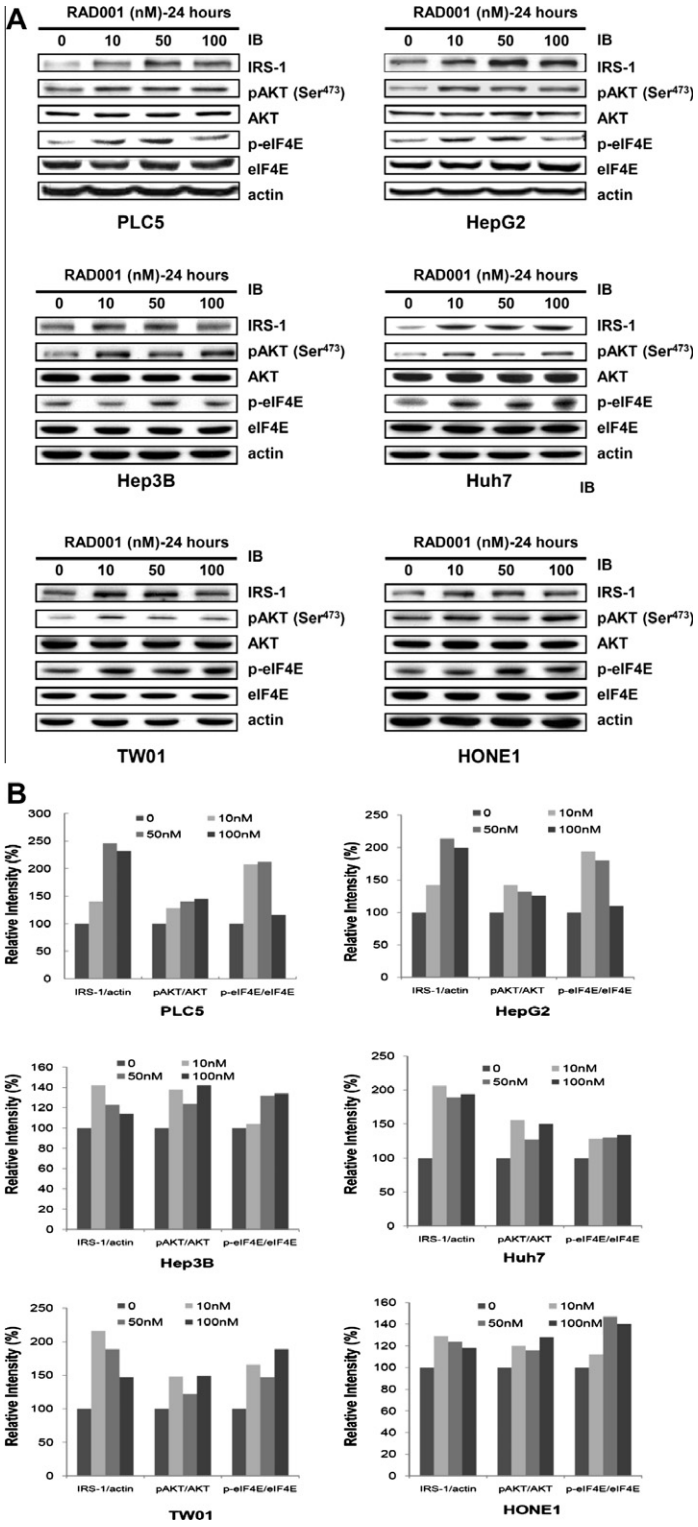

Fig. 6 – Inhibition of mTOR in carcinoma cells is associated with the up-regulation of the feedback IRS-1/PI3K/AKT signalling. (A) RAD001 up-regulates IRS-1 expression and results in a compensatory activation of AKT and eIF4E phosphorylation in carcinoma cells. The protein was isolated from whole cell lysates prepared from PLC5 cells, HepG2 cells, Hep3B cells, Huh7 cells, TW01 cells and HONE1 cells with the mTOR inhibitor, RAD001, at a concentration of 0, 10, 50, or 100 nM of 24 hours DAR001. Immunoblotting (IB) of level of IRS-1, AKT, pAKT (Ser^{473}), eIF4E and anti-p-eIF4E (Ser^{209}) in this protein was performed. The membrane was reprobed with anti-actin as a loading control. (B) Densitometries of IRS-1 protein expression relative to beta-actin expression, pAKT protein expression relative to total AKT protein expression and p-eIF4E protein expression relative to total eIF4E expression normalised to control (0 nM of 24 hours RAD001) are found in PLC5 cells, HepG2 cells, Hep3B cells, Huh7 cells, TW01 cells and HONE1 cells after incubation with RAD001 (10, 50, or 100 nM) for 24 hours.

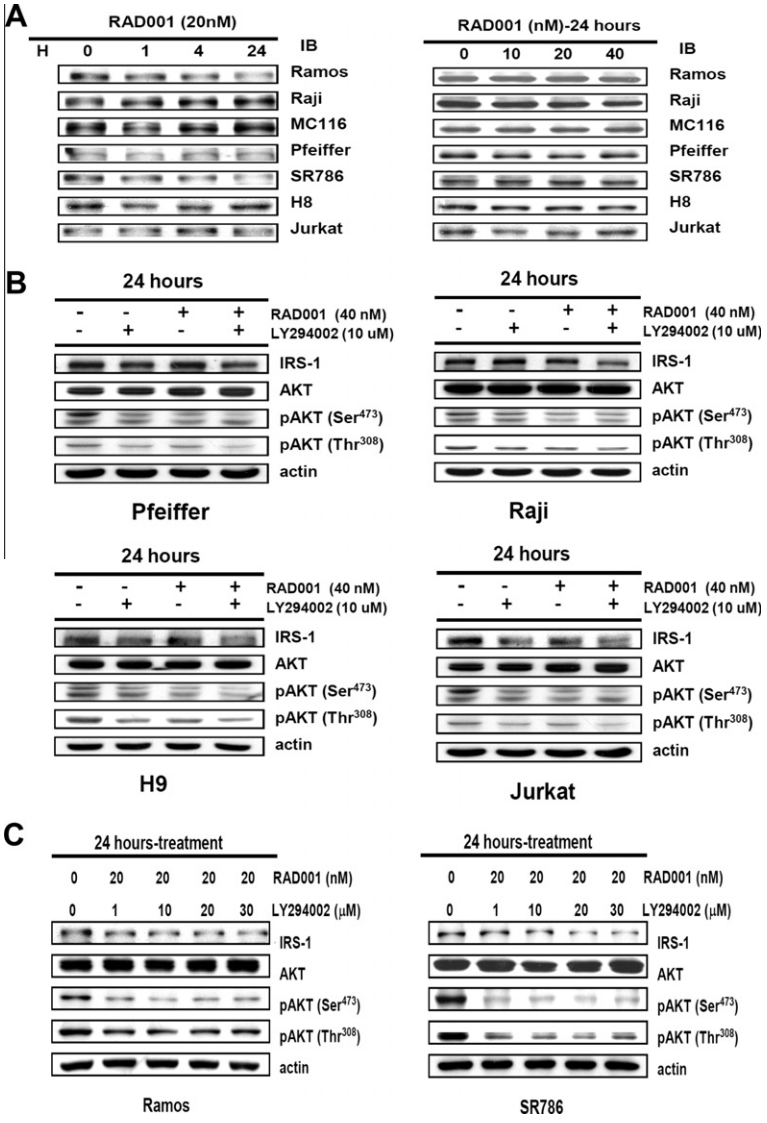

Fig. 7 – Inhibition of mTOR in aggressive lymphoma cells is associated with the lack of compensatory activation of AKT and eIF4E phosphorylation but not associated with the up-regulation of the feedback IRS-1/PI3K/AKT signalling. (A) Following RAD001 (20nM) treatment, the protein level of IRS-1 determined by immunoblotting (IB) shows no time-dependent decrease in the seven lymphoma cell lines. Similarly, a change of IRS-1 protein levels is also not found in a dose-dependent of RAD001 treatment. (B) Treatment with RAD001 alone does not result in the compensatory activation of IRS-1 and AKT, and with PI3K inhibitor (LY294002) alone down-regulates AKT phosphorylation in aggressive lymphoma cells. However, the combination of LY294002 and RAD001 does not enhance the down-regulation of IRS-1 and pAKT. Pfeiffer cells, Raji cells, H9 cells and Jurkat cells were treated with RAD001 (40 nM) or LY294002 (10 μM) alone or the combination. (C) The addition of LY294002 to RAD001-treated lymphoma cells does not enhance the down-regulation of IRS-1, pAKT (Ser^{473}) and pAKT (Thr^{308}). Ramos cells and SR786 cells were treated with 20nM RAD001 for 24 hr with or without 0–30 μM LY294002 24hr-treatment as indicated. The whole cell lysates were subjected to immunoblotting with antibody for IRS-1, total AKT, pAKT (Ser^{473}), and pAKT (Thr^{308}). The membrane was reprobed with anti-actin and anti-total AKT antibody as a loading control.

3.6. *Up-regulation of AKT/mTOR signalling pathway by BAFF lessens the inhibition of cell viability of RAD001 in B-cell NHL cells*

In Pfeiffer cells and Ramos cells, we recently demonstrated that B cell–activating factor belonging to the TNF family (BAFF) activates AKT and NF-κB.[20] A recent study of multiple signalling pathway promoting the growth and survival of B-lymphocyte, the authors suggest that BAFF stimulation activate possible survival pathways utilising the survival kinases AKT.[23] In addition, AKT dependent protection operates through mTOR, and enhances the efficiency of translation by inactivating the translation inhibitor 4E-BP1 as well as up-regulating the phosphorylation of eIF4E.[24] Therefore, we examined whether BAFF can activate the AKT/mTOR pathway (activation of AKT phosphorylation, and up-regulation of mTOR downstream effectors, such as p70S6K, p-4E-BP1, and eIF4E) in aggressive B-lymphoma cells. In Pfeiffer cells, we

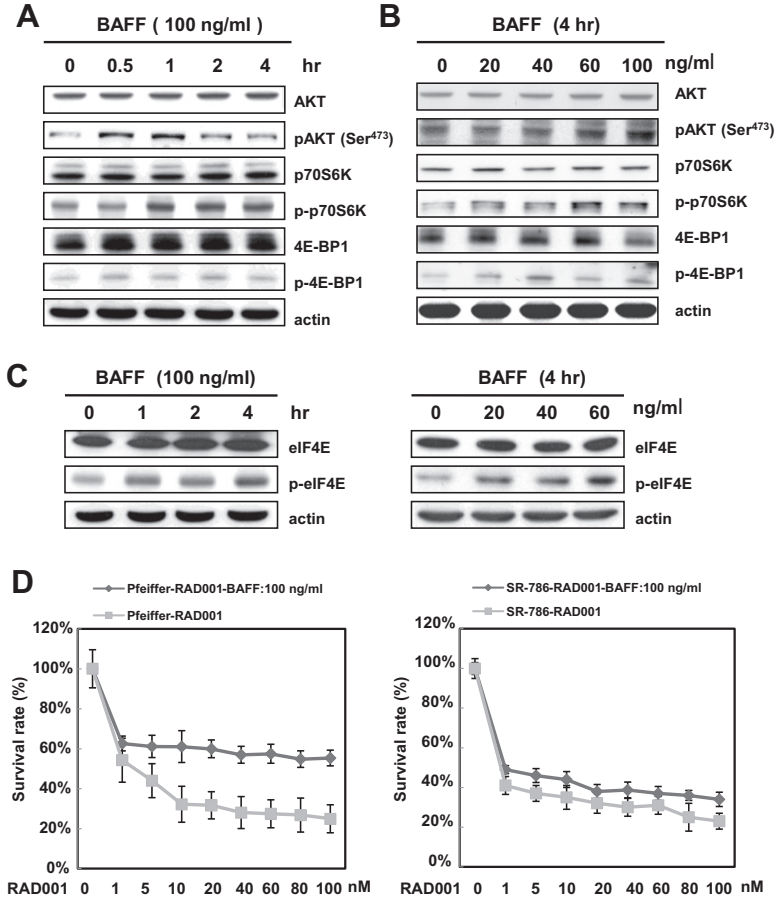

Fig. 8 – BAFF activates the PI3K/AKT/mTOR signalling transduction pathway. (A) BAFF induces a schedule-dependent activation of pAKT (Ser^{473}), p-p70S6K and p-4E-BP1. (B). BAFF treatment increases a dose-dependent level of pAKT (Ser^{473}), p-p70S6K and p-4E-BP1. (C) BAFF induces a schedule-dependent and dose-dependent increase in phosphorylation of eIF4E (Ser^{209}). The whole cell lysates were subjected to immunoblotting with anti-body for total AKT, pAKT (Ser^{473}), p70S6K, p-p70S6K, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E (Ser^{209}). The membrane was reprobed with anti-actin as a loading control. (D) Pretreatment with BAFF decreases the inhibition of cell viability of Pfeiffer cells and SR786 cells to RAD001. Cell numbers were measured by MTT assay and plotted as a percentage of viability in control cells treated with solvent alone (cells not exposed to drug). All values represent the mean ± standard deviation of three independent experiments ($P < 0.05$, at three independent experiments).

found that a marked schedule-dependent increase in the level of pAKT (Ser^{473}), p-p70S6K, p-4E-BP1 and p-eIF4E during the course of BAFF treatment (Fig. 8A and C). Similarly, in BAFF-treated Pfeiffer cells, the expression of pAKT (Ser^{473}), p-p70S6K, p-4E-BP1 and p-eIF4E increased in a dose-dependent manner as compared with control cells (Fig. 8B and C). Next step, we want to evaluate whether up-regulation of AKT/mTOR signalling pathway can decrease the inhibition effect of cell viability of Pfeiffer cell and SR-786 from RAD001. As shown in Fig. 8D, pretreatment with BAFF decreased the inhibition of cell viability of Pfeiffer cells and SR-786 cells to RAD001, indicating the up-regulation of AKT/mTOR signalling pathway can reverse the inhibition of cell viability of RAD001 in aggressive lymphoma cells.

4. Discussion

In this study, we demonstrated that RAD001-induced mTOR inhibition suppresses NHL cell growth by blocking cell cycle progression in the G0/G1 phase and down-regulating the activation of pAKT and p-eIF4E. Our data also showed that RAD001 IC50s in these lymphoma cell lines appear to be much lower than those obtained from the carcinoma cell lines, in which compensatory pAKT activation and eIF4E phosphorylation were observed following RAD001 exposure. These data suggest that the effectiveness of mTOR inhibitors against human NHL is due to a lack of compensatory pAKT and eIF4E activation in the lymphoma cell lines.

Recent studies have demonstrated that PI3K/AKT/mTOR signalling contributes to cancer cell survival, promotes chemotherapy resistance through disruption of apoptosis and initiates cap-dependent translation of mRNAs essential for cell cycle progression, differentiation and growth.[25–27] Based on these findings, it is reasonable to speculate that therapeutic targeting of mTOR using RAD001 can effectively treat tumours with PI3K/AKT/mTOR signalling. However, in the present study, we found that the pAKT was activated in gastric, nasopharyngeal and hepatocellular carcinoma cells

following RAD001 treatment. These findings are in line with recent observations that rapamycin-induced inhibition of mTOR results in a compensatory activation of pAKT (Ser^{473}) in lung cancer, breast cancer and brain tumour cells.[21,28,29] In their analyses, the possible mechanism of compensatory pAKT activation is dependent on PI3K activity. Another possible mechanism is that the mTOR inhibitor may indirectly activate the mTOR-rictor (mTORC2) to phosphorylate AKT.

Indeed, mTOR-rictor, one of two distinct mTOR complexes, can directly phosphorylate AKT on Ser^{433}, and thereby make easy phosphorylation on Thr^{308} through PDK1 activation.[30] Upon activation of AKT, another complex, mTOR-raptor (mTORC1), phosphorylates its downstream target p70S6K and subsequently rpS6, thereby increasing tumour cell proliferation and protein translation.[31] Activated p70S6K phosphorylates mTORC1 at Ser^{2448},[32] completing a positive signalling loop involving p70S6K, mTORC1 and AKT (Thr^{308}). In the present study, RAD001 inhibition of mTOR resulted in decreased p70S6K and 4E-BP1 phosphorylation in lymphoma cells. In addition, we showed that RAD001 down-regulated p-mTOR activation on Ser^{2448} and pAKT activation on Ser^{473}. These findings are in line with a previous study reporting that the inhibition of mTOR with rapamycin results in reduced mTOR (Ser^{2448}) and AKT (Ser^{473}) phosphorylation in ALCL cells.[5] Interestingly, we also found that RAD001 down-regulated AKT activation on Thr^{308}, suggesting that AKT (Thr^{308}) phosphorylation is dependent on the AKT (Ser^{473}) activation status. Taken together, these findings suggest that inhibition of mTOR with RAD001 in lymphoma cell lines not only down-regulates its downstream effectors but also inhibits the feedback signalling from mTOR to AKT (Ser^{473}), from AKT (Ser^{473}) to AKT (Thr^{308}), and from p70S6K to mTOR.

Recent phase I/II studies using mTOR inhibitors, including CCI-779 (temsirolimus) and RAD001 (everolimus), in a variety of haematologic and non-haematologic malignancies showed that the effects of RAD001 were unexpectedly modest.[33,34] The suboptimal anti-tumour effect of mTOR inhibitors in these tumours is reported to be associated with activation of a compensatory signalling pathway such as the IGF-1/IRS-1/PI3K/AKT pathway.[21,35,36] For example, recent studies of mTOR inhibitors in multiple myeloma cell lines and breast cancer cell lines have demonstrated that rapamycin or RAD001 can paradoxically increase AKT phosphorylation through enhancement of IGF-1/IRS-1 receptor interaction following the prevention of p70S6K-mediated phosphorylation of IRS-1 on Ser^{312} and/or $Ser^{636/639}$ and further IRS-1 degradation.[21,35] Therefore, it would be interesting to elucidate whether or not this alternative compensatory pathway does exist in aggressive NHL cell lines. We found that in these aggressive NHL cells lines, RAD001 did not influence IRS-1 expression and that inhibition of PI3K/AKT with LY294002 did not intensify the therapeutic effects of RAD001. These findings suggest that the feedback up-regulation of IGF-1/IRS-1/PI3K/AKT signalling is not a frequent event in aggressive NHL cells.

Although it has been demonstrated that AKT activation plays a crucial role in NHL survival signalling pathways, recent studies have clearly shown that eIF4E has oncogenic effects in tumour genesis, eIF4E is highly expressed in a variety of human cancers, including NHL;[9,37,38] and increased expression of eIF4E is associated with the poor prognosis of these tumour.[39,40] Theoretically, mTOR inhibitors should down-regulate the cap-dependent translation function of eIF4E via inhibition of 4E-BP1 phosphorylation.[41] However, recent studies of mTOR inhibitors in human non-small cell lung cancer (NSCLC), breast cancer and glioblastoma cell lines have demonstrated that rapamycin inhibits the activation of p70S6 kinase and 4E-BP1, but paradoxically increases the phosphorylation of both AKT and eIF4E.[28,29] In their analyses, the effect of rapamycin-induced eIF4E phosphorylation is dependent on PI3K activity but is independent of AKT, extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signalling pathways.[28,29] A further analysis has found that the combination of rapamycin and CGP57380 (MNK1 and MNK2 inhibitor) prevents eIF4E phosphorylation and has synergistic inhibitory effects in NSCLC cancer cell lines.[29]

In the present study, we found that treatment of seven NHL cell lines with RAD001 resulted in a marked dose-dependent and schedule-dependent decrease in eIF4E phosphorylation and pAKT activation. However, compensatory activation of eIF4E phosphorylation and AKT phosphorylation during RAD001 treatment was observed in gastric, nasopharyngeal and hepatocellular carcinoma cells. Furthermore, up-regulation of eIF4E phosphorylation through the activation of BAFF/AKT/mTOR signalling pathway affects the inhibition of cell viability of RAD001 in NHLs. Overall, these findings suggest that the basis for enhanced activity of mTOR inhibitors in NHL may be the lack of compensatory activation of AKT and eIF4E phosphorylation in lymphoma cells. In addition to the down-regulation of AKT and eIF4E phosphorylation, we found that RAD001 caused a dose- and schedule- dependent decrease in the expression of c-Myc, indicating that a positive loop exists between c-Myc and eIF4E. Our results are consistent with those reported in a recent study conducted in a murine model of lymphomagenesis, in which increased levels of eIF4E were associated with an enhancement of c-Myc mRNA translation activity and that the activation of eIF4E was linked to an increase in c-Myc activity.[42] We also showed that RAD001 caused cell cycle arrest in the G1 phase which was associated with modulation of critical cell cycle regulatory proteins like cyclin D3, cyclin A and cyclin E, but not cyclin D1 and p27. This observation is in agreement with recent observations that mTOR inhibitors can inhibit cell cycle progression in DLBCLs (cyclin D3, cyclin A, and cyclin E were down-regulated) and MCLs (cyclin D3, cyclin A, and cyclin E were down-regulated; cyclin D1 and p27 were unchanged) through G1 arrest.[15,43] A portion of RAD001 anti-tumour effects in aggressive NHL cells stems from the inhibition of activation of pAKT, p-eIF4E, c-Myc and critical cell cycle regulators in the Go/G1 phase.

In summary, our results indicate that RAD001 suppresses NHL cell growth at concentrations that are much lower than those required for inhibition of carcinomas. Although the precise mechanisms responsible for the differential effects of RAD001 on carcinoma cells and lymphoma cells remains unclear, the associated mechanism of action involves down-regulation of phosphorylation-dependent AKT, mTOR, and eIF4E signalling, inhibition of c-Myc expression, and the prevention of feedback up-regulation of IGF-1/IRS-1/PI3K/AKT signalling

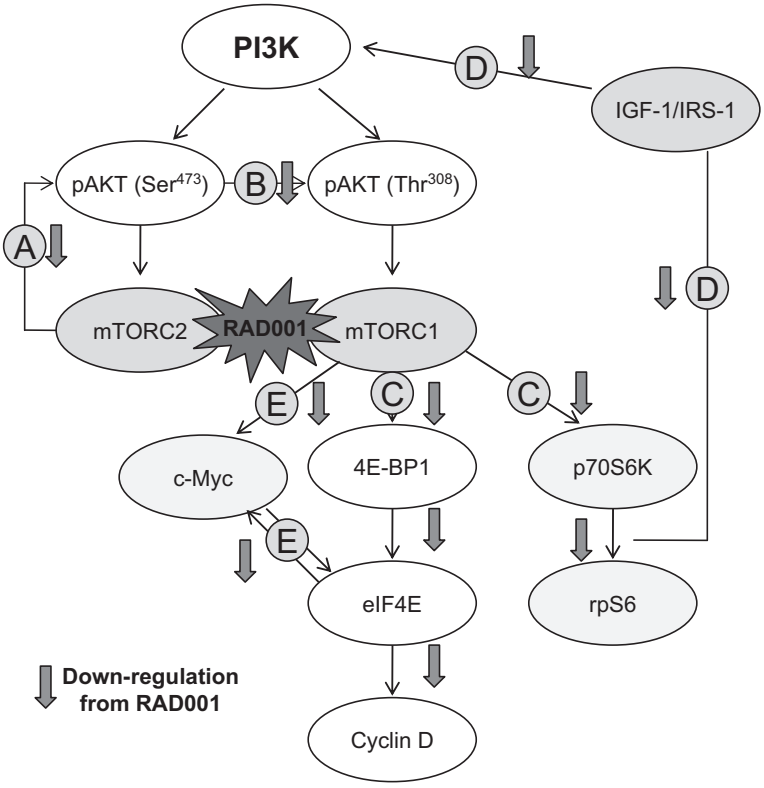

Fig. 9 – Lack of compensatory pAKT activation and eIF4E phosphorylaiton of lymphoma cells towards mTOR inhibitor, RAD001. (A) RAD001 down-regulates AKT phosphoryation (Ser^{473}) in lymphoma cells through the inhibition of mTORC2 (B) The inactivation of AKT(Ser^{473}) further impedes AKT phosphoryation on Thr^{308} (C) RAD001 down-regulates p70S6K phosphorylation, as well as of mTOR activation on Ser^{2448} through the inhibition of mTORC1. (D) Lack of alternative compensatory activation of IGF-1/IRS-1/PI3K in RAD001-treated lymphoma cells. (E) RAD001 inhibits activation of c-Myc, as well as p-eIF4E, suggesting a positive loop between c-Myc and eIF4E exists in lymphoma cells. These findings suggest that the mechanism of good activity of RAD001 in lymphoma cells is at least partly due to down-regulation of AKT/mTOR/4E-BP1/eIF4E signalling and the prevention of feedback up-regulation of IGF-1/IRS-1/PI3K/AKT signalling.

(Fig. 9). Alternatively, RAD001 effectively inhibits the mTORC2 and, therefore, AKT (Ser^{473}) phosphorylation may explain the possibility of the greater antitumour effect of RAD001 in these lymphomas (Fig. 9). [44,45] Since activation of AKT, eIF4E, c-Myc and mTOR signalling pathways is also closely associated with cell proliferation, cell cycle progression, tumour progression and chemoresistance in human NHLs, RAD001 may be a useful adjunct in the treatment of this group of tumours. Additional investigation of RAD001 molecular mechanisms and the biologic significance of differences between carcinoma cells and lymphoma cells are needed.

Conflict of interest statement

None declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.01.003.

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