mTOR Inhibitors Induce Cell-Cycle Arrest and Inhibit Tumor Growth in Epstein–Barr Virus–Associated T and Natural Killer Cell Lymphoma Cells

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Abstract

Purpose: Epstein–Barr virus (EBV) infects B cells, as well as T cells and natural killer (NK) cells, and is associated with T cell or NK cell lymphoid malignancies. In various tumor cells, mTOR performs an essential function together with Akt with regard to cell growth. We investigated the effects of mTOR inhibitors on EBV-associated T- and NK-cell lymphomas.

Experimental Design: We investigated the Akt/mTOR activation pathway in EBV-positive and -negative T- and NK-cell lines (SNT13, SNT16, Jurkat, SNK6, KA3, and KHYG1). We evaluated the antitumor effects of mTOR inhibitors (rapamycin and its analogue, CCI-779) against these cell lines in culture and in a murine xenograft model that was established by subcutaneous injection of SNK6 cells into NOG mice.

Results: All EBV-positive and -negative T- and NK-cell lines tested displayed activation of the Akt/mTOR pathway, and treatment with mTOR inhibitors suppressed mTOR activation. The inhibitors induced G1 cell-cycle arrest and inhibited cell proliferation in T- and NK-cell lines. Overall, T cell lines were more sensitive to rapamycin, but there were no significant differences between EBV-positive and -negative cell lines. Treatment with rapamycin did not affect lytic or latent EBV gene expression. Intraperitoneal treatment with CCI-779 significantly inhibited the growth of established tumors in NOG mice and reduced the EBV load in peripheral blood.

Conclusion: These results suggest that inhibition of mTOR signaling is a promising new strategy for improving treatment of EBV-associated T- and NK-cell lymphoma.
proteins, have profound effects on cell growth regulation and are required for EBV latent infection and B-cell transformation (13). Latent membrane protein 1 (LMP1) is considered to be a major oncoprotein of EBV because activation of NF-κB is required for EBV-induced B-cell transformation and inhibition of it rapidly results in cell death (14, 15). On the other hand, Shair and colleagues have shown that LMP1 also activates PI3K/Akt signaling and this pathway could be an effective target for the treatment of EBV-associated B-cell lymphoma (16). It has previously been shown that the PI3K/Akt/mTOR pathway is activated in EBV-associated B-cell lymphomas and that rapamycin modulates the cell cycle and inhibits cell growth (17–20). Furthermore, constitutive activation of the mTOR signaling pathway has been demonstrated in tissue samples from EBV-positive posttransplant lymphoproliferative disorders (21). LMP1 is expressed in EBV-associated T- and NK-cell lymphoma, but PI3K/Akt/mTOR pathway activation in these cells has not been confirmed. Loong and colleagues examined the effects of rapamycin in an EBV-positive NK cell lymphoma xenograft model (22). In that study, rapamycin did not show any effects on the tumor growth, but inhibition of the mTOR pathway was not evaluated.

In the present study, we evaluated the antitumor effects of mTOR inhibitors on EBV-associated T- and NK-cell lines in culture and in a murine xenograft model and found that mTOR inhibitors induce G1 cell-cycle arrest and inhibit cell proliferation. These findings suggest that inhibition of mTOR signaling is a promising new strategy to improve treatment of EBV-associated T- and NK-cell lymphoma.

Materials and Methods

Cell lines and reagents

Of the cell lines used in the present study, SNT13 and SNT16 are EBV-positive T-cell lines, SNK6 and KAI3 are EBV-positive NK cell lines, and Jurkat and KHYG1 are EBV-negative T- and NK-cell lines, respectively (23–25). SNT13, SNT16, SNK6, and KAI3 were derived from patients with chronic active EBV disease or nasal NK/T cell lymphoma. MT2 cell line was established from cord mononuclear cells by coculture with adult T-cell leukemia cells and harbors human T-cell-leukemia virus type I. The MT2/rEBV/9-7 and MT2/rEBV/9-9 cell lines were established following infection of MT2 cells with the hygromycin-resistant EBV B95.8 strain (26). The MT2/hyg cell line was transfected with a hygromycin resistance gene. The NKl cell line was derived from a patient with NK cell leukemia, and the TL1 cell line was established from NKl cells infected with an Akata-transfected recombinant EBV strain carrying a neomycin resistance gene (27). Jurkat cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin (complete media). Complete media supplemented with 100 U/mL human IL2 were used for SNT13, SNT16, SNK6, KAI3, KHYG1, NKl, and TL1. Complete media supplemented with 0.2 mg/mL hygromycin were used for MT2/hyg, MT2/rEBV/9-7, and MT2/rEBV/9-9. TL1 underwent periodic selection with G418.

Rapamycin (Cell Signaling Technology) and CCI-779 (Sigma) were dissolved in DMSO and ethanol, respectively. The autophagy inhibitor 3-methyladenine (3-MA; Sigma) was dissolved in DMSO.

Immunoblotting

Cells were lysed directly in sample buffer. Equal amounts of protein were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with antibody. Antibody against phospho-Akt (Ser473), Akt, phospho-4E-BP1 (Ser65), 4E-BP1, phospho-Atg12, and caspase-3 (Cell Signaling Technology); β-actin and PARP (Sigma); and retinoblastoma protein (Rb) and CDK2 (BD Pharmingen Biosciences) were used for immunoblots. To compare the amount of each protein, densitometric analysis was performed using ImageJ software version 1.46r (NIH, Bethesda, MD).

Cell numbers and viability

Cells were cultured in 24-well plates at 2 × 10⁵/mL, and cell numbers and viability were assayed by trypan blue exclusion using a Countess automated cell counter (Invitrogen). Experiments were performed at least in triplicate.

Cell proliferation assay

Cell proliferation was measured by MTS assay using CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent (Promega). Briefly, 100 μL of cell suspension and 20 μL of MTS reagent were incubated in a 96-well plate for 1 hour at 37°C, and formazan absorbance was measured at a wavelength of 490 nm. Experiments were performed in triplicate.

Annexin V analysis of apoptosis

Apoptosis was measured using an Annexin V-PE/7-AAD apoptosis assay kit (BD Pharmingen Biosciences) in
accordance with the manufacturer’s instructions. Cells were analyzed by flow cytometry, and viable cells were defined as negative for Annexin V-phycocerythrin (PE) and 7-aminoactinomycin D (7-AAD) staining, whereas apoptotic cells were defined as positive for Annexin V-PE and negative for 7-AAD staining.

Cell-cycle assay

Cells were treated with various concentrations of rapamycin or CCI-779 for 24 hours, fixed with 70% ethanol, and then washed with ice-cold PBS. Fixed cells were treated with 10 µg/mL DNase-free RNase and stained with 5 µg/mL propidium iodide (Sigma). Experiments were performed in triplicate.

Real-time RT-PCR

Viral mRNA expression was quantified by RT-PCR, as described previously, using β2-microglobulin as an endogenous control and reference gene for relative quantification (28, 29). Each experiment was performed in triplicate.

Xenograft model

Mice of the NOD/Shi-scid/IL-2Rγnull (NOG) strain were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained under specific pathogen-free conditions in the animal facility of Nagoya University (Nagoya, Japan). The Ethics Review Committee of the Institute approved the experimental protocol. The Ethics Review Committee of the Institute approved the experimental protocol. The Ethics Review Committee of the Institute approved the experimental protocol.

Inhibition of mTOR signaling by rapamycin suppresses growth of EBV-positive and -negative T- and NK-cell lines

To confirm activation of the mTOR signaling pathway, we examined the status of phospho-p70S6K (Thr389) and phospho-4E-BP1 (Ser65) in an EBV-negative T cell line (Jurkat), EBV-positive T cell lines (SNT13 and SNT16), an EBV-negative NK cell line (KHYG1), and EBV-positive NK cell lines (KAI3 and SNK6). As shown in Fig. 1A, the phosphorylated form of 4E-BP1 was detected in all cell lines tested, regardless of EBV status. Phosphorylated p70S6K was not detected in KIA3 but was detected in the other cell lines tested. Treatment of cell lines with rapamycin induced almost complete inhibition of p70S6K phosphorylation at T389 and partial (20%–80%) inhibition of 4E-BP1 phosphorylation at S65, confirming the role of mTOR as their kinase and indicating the sensitivity of these cells to mTOR inhibitors (Fig. 1A and Supplementary Fig. S1A). Treatment of the cell lines with higher concentrations of rapamycin (100 nmol/L) did not show complete inhibition of 4E-BP1 phosphorylation (data not shown). As it has been well documented that mTOR functions downstream of the PI3/Akt pathway (5), we examined the activation of Akt. As shown in Fig. 1A, phosphorylation at S473 was detected in all T cell lines indicating strong activation of Akt. However, phospho-Akt was not detected in NK cell lines. After treatment with rapamycin, a compensatory increase in Akt phosphorylation was observed in 4 of the 6 tested cell lines (SNT16, SNT13, KHYG1, and SNK6).

Inhibition of mTOR signaling by rapamycin suppresses growth of EBV-positive and -negative T- and NK-cell lines

To determine whether T- and NK-cell lines were sensitive to mTOR inhibitors, they were exposed to 10 to 50 nmol/L of rapamycin, and cell counts were determined after 48 and 72 hours. Neither fresh medium nor additional drugs were added during the observation period. As shown in Fig. 1B and Supplementary Fig. S2A, we found that growth in T- and NK-cell lines was inhibited by rapamycin in a dose-dependent manner. In all T- and NK-cell lines, inhibition of cell growth by rapamycin was statistically significant at 72 hours at all concentrations. Overall, T cell lines were more sensitive to rapamycin than NK cell lines. In comparison, at the highest concentration (50 nmol/L) of rapamycin, cell growth inhibition of SNT16 cells was significantly higher than in Jurkat cells, whereas there were no significant differences between Jurkat and SNT13 cells. On the other hand, no significant differences were observed among EBV-positive and -negative NK cell lines at concentration of 50 nmol/L (Fig. 1C). The results of cell proliferation assay using MTS are shown in Supplementary Fig. S2B. Treatment with 10 to 50 nmol/L rapamycin for 72 hours inhibited proliferation of all T- and NK-cell lines in a dose-dependent
manner. When compared with untreated cells, inhibition of cell proliferation by rapamycin was statistically significant with 25 and 50 nmol/L in all T- and NK-cell lines. No significant differences were observed among EBV-positive and -negative cell lines. All T- and NK-cell lines tested showed little loss in viability, even at 50 nmol/L rapamycin (data not shown). Treatment of cells with rapamycin over longer periods of time resulted in little or no decrease in viability when compared with untreated cells (Supplementary Fig. S2C).

Rapamycin induces G1 cell-cycle arrest in T- and NK-cell lines

Induction of cell-cycle arrest by mTOR inhibitors has been reported to inhibit cancer cell growth (10, 11). We examined whether growth inhibition of T and NK cells by rapamycin was a result of cell-cycle arrest. T and NK cells were treated with various concentrations of rapamycin for 24 hours, stained with propidium iodide, and then analyzed using flow cytometry. Significant increases in cells in G1 phase were observed after treatment with rapamycin in Jurkat, SNT16, and SNT13, and KHYG1 cells (Fig. 2A). A similar trend was observed in SNK6 and KAI3 cells, but the increase in cells in the G1 phase was not significant (Fig. 2A). Consistent with G1 cell-cycle arrest, reduced levels of corresponding to cell-cycle markers CDK2 and Rb were also observed in all cell lines tested together with increased levels of p27 Kip1 in Jurkat, SNT16, and KHYG1 following treatment with rapamycin for 24 hours (Fig. 2B). Together, these results suggest that rapamycin induced G1 cell-cycle arrest in T- and NK-cell lines.

CCI-779 induces inhibition of cell proliferation by G1 cell-cycle arrest in T- and NK-cell lines

We evaluated the effects of CCI-779 on T- and NK-cell lines. CCI-779 is a soluble ester analogue of rapamycin and
is more suitable as an intravenous agent than rapamycin (11). Treatment with various concentrations of CCI-779 for 72 hours inhibited proliferation of SNT16 and SNK6 cells in a dose-dependent manner (Supplementary Fig. S3A). As shown in Supplementary Fig. S3B, phosphorylation of both 4E-BP1 and p70S6K was reduced by treatment with CCI-779, suggesting that mTOR activation was inhibited in these cell lines. Cell-cycle analysis showed an increase in G1 phase cells following treatment with CCI-779, but no decrease in S-phase was observed in SNT16 cells (Supplementary Fig. S3C). Immunoblotting of cycle markers showed reduced levels of Rb in both cell lines and increased levels of p27 Kip1 in SNT16 cells, but no decreases in CDK2 were observed (Supplementary Fig. S3B). These results suggest that CCI-779 induces inhibition of cell proliferation by G1 cell-cycle arrest, as seen in T- and NK-cell lines treated with rapamycin.

**Rapamycin induces little or no apoptosis in T- and NK-cell lines**

To evaluate whether rapamycin induces apoptosis, T- and NK-cell lines were treated with 50 nmol/L of rapamycin for 24 and 48 hours, and the cleavage of caspase-3 and PARP was analyzed by immunoblotting. As shown in Fig. 3A and Supplementary Fig. S1B, slightly increased levels of cleaved caspase-3 were detected in SNK6 cells, and slightly increased levels of cleaved PARP were detected in Jurkat, SNK6, and KAI3 cells after 24-hour treatment with rapamycin. Decreased levels of PARP or caspase-3 were not clearly identified. Similar results were seen in T- and NK-cell lines treated with rapamycin for 48 hours (data not shown). Furthermore, apoptosis was analyzed by flow cytometry after Annexin V staining. SNT16 cells treated with rapamycin showed modest increases in apoptotic cells (Annexin V–positive and 7-AAD–negative) when compared with untreated cells (Fig. 3B). Similar results were seen in SNT13 (data not shown). On the other hand, no increase in the number of apoptotic cells was confirmed in SNK6, KAI3, KHYG1, or Jurkat cells (Fig. 3B and data not shown). Taken together, these results suggest that rapamycin induces little or no apoptosis in T- and NK-cell lines.

**Effects of rapamycin on autophagy of T- and NK-cell lines**

Previous studies have shown that mTOR inhibitors induce autophagy in various cancer cells because the mTOR pathway plays a crucial initiating role in...
autophagy (33, 34). To evaluate whether rapamycin induces autophagy in T- and NK-cell lines, protein expression in LC3-I and LC3-II was examined by immunoblotting. Conversion of LC3-I to the lower migrating form LC3-II has previously been used as an indicator of autophagy. The 3-MA inhibition of LC3-I reduction seems to be true for Jurkat and SNT16 but not for KHYG1, SNT13, or SNK6 when comparing LC3II/LC3I levels from rapamycin and rapamycin + 3-MA samples (Fig. 3C and Supplementary Fig. S1C). On the other hand, treatment with rapamycin did not increase the expression of proautophagic protein Atg5-Atg12 (Fig. 3C). Furthermore, pretreatment with 100 or 250 nmol/L 3-MA did not prevent the rapamycin-induced cell growth inhibition in Jurkat and SNT16 cells (Fig. 3D). Treatment with combination of 3-MA and rapamycin did not show significant differences in the number of cells when compared with rapamycin alone. These results suggest that autophagy may not be a crucial mechanism of rapamycin-induced cell growth inhibition in these cell lines.

Presence of EBV in an NK cell line increases susceptibility to rapamycin

To directly compare the effects of rapamycin in EBV-positive and -negative cell lines, we administered rapamycin to MT-2/hyg, MT2/rEBV/9-7, MT2/rEBV/9-9, NKL, and TL1. As shown in Fig. 4A, the EBV-positive NK cell line (TL1) was more sensitive to rapamycin than its parent cell line (NKL). On the other hand, rapamycin showed almost equal effects on EBV-positive T cell lines (MT2/rEBV/9-7 and MT2/rEBV/9-9) and a control cell line (MT-2/hyg; Fig. 4A). Cell-cycle analysis showed rapamycin-induced G1 cell-cycle arrest in NKL and TL1 cells, but the effects of rapamycin on the 2 cell lines were similar (Fig. 4B). Significant increases in cells in S-phase were observed in MT2/rEBV/9-7 when compared with MT2/hyg. The difference was not significant between TL1 and NKL cells or between the EBV-positive and -negative T or NK cells. We evaluated the effects of rapamycin on Akt/mTOR pathway activation in these cell lines. Compared with NKL cells, expression of phospho-Akt was upregulated in TL1 cells. On the other hand, phospho-Akt was expressed...
in MT-2/hyg and MT2/rEBV/9-7 cells but there were no differences in expression levels (Fig. 4C). Treatment with rapamycin decreased phospho-Akt expression levels in TL1 cells, whereas a compensatory increase in Akt phosphorylation was observed in MT2/hyg and MT2/rEBV/9-7 cells (Fig. 4C and D). Activation of mTOR was confirmed by expression of phospho-p70S6K in these cell lines, and it was suppressed by treatment with rapamycin (Fig. 4C).

Effects of rapamycin on lytic and latent EBV gene expression in T- and NK-cell lines

Expression of the following 8 viral genes was analyzed using real-time RT-PCR: lytic genes encoding BZLF1 and gp350/220; latent genes encoding EBV nuclear antigen (EBNA) 1, EBNA2, latent membrane protein (LMP) 1, LMP2, EBER1, and BamHI-A rightward transcript (BART). Although EBV-positive T- and NK-cell lines are considered to be latency type II, BZLF1 was detected in all tested cell lines except TL1, and gp350/220 was detected in 2 T cell lines (SNT13 and MT2; Fig. 5). The expression of these lytic genes may come from small fractions of EBV-positive T and NK cells that spontaneously undergo lytic replication. Expression of BZLF1 decreased in rapamycin-treated SNT16 and KAI3 cells, but this effect was not observed in other cell lines (Fig. 5). Expression of 6 latent genes did not differ significantly between rapamycin-treated cells and controls. Representative results for 2 latent genes (those encoding LMP1 and EBER1) are shown in Fig. 5.

CCI-779 inhibited growth of established tumor in NOG mice

We further extended our studies to an in vivo xenograft model to validate the significance of our in vitro findings. Because of the poor solubility of rapamycin, we used a...
A water-soluble ester derivative of rapamycin, CCI-779, was used for treatment of the xenograft model. Subcutaneous inoculation of SNK6 cells into NOG mice resulted in tumor formation at the site of injection in all mice. Four days after the inoculation of SNK6 cells, mice were treated with CCI-779 (10 mg/kg, i.p.) for 3 weeks. Mice generally tolerated CCI-779 with no apparent toxicity throughout the experiment. Figure 6A demonstrates a significant antitumor effect of CCI-779 on tumor growth that was evident 10 days after the start of treatment. Progressive tumor growth was prevented during treatment with CCI-779, and tumor growth after treatment was suppressed for approximately 2 weeks. Subsequently, progressive tumor growth was renewed, but the tumor volume in CCI-779–treated mice was significantly smaller than in control mice at the end of the study (2,080 \( \pm \) 410 mm\(^3\) for CCI-779 group and 3,700 \( \pm \) 300 mm\(^3\) for control group; \( P < 0.01 \)). We repeated the NOG mouse experiments to confirm the findings, and the results are shown in Supplementary Fig. S4. Tumor volume in CCI-779–treated mice was significantly smaller than in control mice from 10 days after the start of treatment until the end of the observation. Peripheral blood was obtained every 2 weeks and EBV load, which may reflect tumor progression, was measured by real-time PCR. EBV load in CCI-779–treated mice was significantly lower than in the control group but increased rapidly after treatment (Fig. 6B). Histologic analyses of tumor explants showed more vacuolar changes in the CCI-779 group (Fig. 6C). To determine whether the observed tumor growth suppression was caused by inhibition of cell proliferation, we performed immunohistochemistry for Ki-67 expression (Fig. 6C). As shown in Fig. 6D, the average proliferation index in 5 randomly selected microscopic fields of CCI-779–treated mice was significantly decreased after 3 weeks of treatment (approximately 30% reduction, \( P < 0.01 \)).

**Discussion**

The mTOR is a highly conserved serine/threonine kinase and is a central regulator of cell growth, metabolism, and aging (35). Constitutive PI3K/Akt/mTOR activation is critically involved in a variety of cancers and hematologic malignancies (5, 6, 35). Previous studies have shown that the PI3K/Akt/mTOR pathway is activated in EBV-associated B-cell lymphoma, but it is unclear whether this pathway is activated in EBV-associated T- and NK-cell lymphomas (17, 18). This study demonstrated that mTOR is activated in EBV-associated T- and NK-cell lymphoma cells and that mTOR inhibitors induce G1 cell-cycle arrest and inhibit cell proliferation both in vitro and in vivo.

Both p70S6K and 4E-BP1 are considered as key mTOR target proteins, and treatment of cell lines with rapamycin or CCI-779 resulted in complete inhibition of p70S6K phosphorylation, whereas inhibition of 4E-BP1 phosphorylation was partial. In some other studies using EBV-positive B cell lines (21) or other cell lines (36, 37), inhibition of 4E-BP1 phosphorylation by rapamycin was partial, whereas inhibition of p70S6K phosphorylation was complete. The reason is uncertain, but activation of survival signaling pathways such as Akt may prevent complete inhibition of 4E-BP1 phosphorylation (37). It has been shown that rapamycin induces Akt activation through an mTOR
complex, resulting in the attenuating growth-inhibitory effect of rapamycin (37). As shown in Supplementary Fig. S1A, the increased ratio of phospho-Akt/Akt was marked in KHYG1 and SNK6. On the other hand, small increases in the ratio of phospho-Akt/Akt was observed in SNT13 and SNT16 cells, but not in Jurkat cells. Associations between EBV presence and rapamycin-inducing Akt phosphorylation were inconclusive.

The presence of EBV may have some effect on sensitivity to mTOR inhibitors because LMP1 is known to activate the PI3/Akt pathway, as well as the NF-κB, c-JNK, and p38 MAPK signaling pathways (16, 38). The EBV-positive T- and NK-cell lines used in this study are classified as latency type II and express LMP1. However, there were no marked differences in sensitivity to rapamycin between EBV-positive and -negative cell lines. It is possible that LMP1 has only a small impact on Akt activation in these cell lines because expression of phospho-Akt was similar between EBV-positive and -negative cell lines (Fig. 1A).

T cell lines appeared to be more sensitive to rapamycin and expression of phospho-Akt in T cell lines was higher than in NK cell lines regardless of the presence of EBV. Previous studies have shown that cells with increased Akt activation are more sensitive to mTOR inhibitors (32, 39). The tumor cell origin, rather than the existence of EBV, may have a greater effect on Akt activation and sensitivity to mTOR inhibitors. Interestingly, an artificially EBV-infected NK cell line (TL1) was more sensitive to rapamycin than its parent line (NKL), but there were no differences between EBV-infected T cell lines (MT2/rEBV/9-7 and MT2/rEBV/9-9) and the parental line (MT2/hyg). These differences could be explained by Akt activation, as expression of phospho-Akt was increased in TL1 cells, but not in MT2/rEBV/9-7 cells when compared with the parental lines. Furthermore, LMP1 mRNA was detected in TL1 cells but...
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not in MT2/EBV/9-7 cells (Fig. 5). LMP1-induced PI3K/Akt activation might have some impact on dysregulation of mTOR and sensitivity to mTOR inhibitors but further studies are required.

We established a murine xenograft model of EBV-associated NK cell lymphoma and demonstrated tumor growth suppression by CCI-779 in vivo. The results of this study showed that T cell lines were more sensitive to rapamycin in vitro, but unfortunately, the xenograft model of T-cell lymphoma could not be established. When compared with SNK6 cells, SNT13 and SNT16 cells were more dependent on IL2 for their growth in vitro (data not shown). High dependency on IL2 for cell growth might explain the difficulty in establishing a xenograft model using SNT13 or SNT16 cells. Because rapamycin strongly suppresses IL2-stimulated T-cell proliferation, it has been used as an immunosuppressive agent in posttransplant patients (40). Furthermore, several studies have confirmed the antitumor activity of mTOR inhibitors on T-cell acute lymphoblastic leukemia or T-cell lymphoma (41, 42). Further investigations are required to determine whether mTOR inhibitors have more therapeutic potential with EBV-associated T-cell lymphoma than NK cell lymphoma.

In the present study, mTOR inhibitors elicited a cytostatic response in T and NK lymphoma cells resulting in G1 cell-cycle arrest. Consistent with G1 cell-cycle arrest, diminished CDK2 and Rb expression and increased p27 Kip1 expression but progressive tumor growth was subsequently renewed, was completely prevented during treatment with CCI-779, lymphomas. In the murine xenograft model, tumor growth mTOR inhibitors (33, 34, 41–43), although we found that apoptosis is also considered to be a crucial antitumor effect of (10, 11). On the other hand, induction of autophagy or mTOR inhibitors have been demonstrated in various types of cancers (40). Furthermore, several studies have confirmed the antitumor activities of bortezomib and the histone deacetylase inhibitor valproic acid on EBV-associated lymphoma cells (15, 48–50). Combinations of these agents and mTOR inhibitors are promising strategies to improve the treatment of EBV-associated T- and NK-cell lymphomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: J. Kawada, Y. Ito, H. Kimura
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