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Inhibition of Retinoblastoma Cell Growth by CEP1347 Through Activation of the P53 Pathway

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Abstract. *Background/Aim: Despite advances in treatment modalities, the visual prognosis of retinoblastoma still remains unsatisfactory, underscoring the need to develop novel therapeutic approaches. Materials and Methods: The effect on the growth of six human retinoblastoma cell lines and a normal human fibroblast cell line of CEP1347, a small-molecule kinase inhibitor originally developed for the treatment of Parkinson's disease and therefore with a known safety profile in humans, was examined. The role of the P53 pathway in CEP1347-induced growth inhibition was also investigated. Results: CEP1347 selectively inhibited the growth of retinoblastoma cell lines expressing murine double minute 4 (MDM4), a P53 inhibitor. Furthermore, CEP1347 reduced the expression of MDM4 and activated the P53 pathway in MDM4-expressing retinoblastoma cells, which was required for the inhibition of their growth by CEP1347. Conclusion: We propose CEP1347 as a promising candidate for the treatment of retinoblastomas, where functional inactivation of P53 as a result of MDM4 activation is reportedly common.*

Although retinoblastoma is a rare form of cancer, it is one of the most common malignant intraocular tumors in children and affects one in 15,000-20,000 individuals worldwide (1, 2). Although it can be life-threatening if left untreated, the survival rate in developed countries is over 95% due to the availability of numerous treatment strategies that are effective for specific disease stages. These treatments include local treatments, such as laser therapy, cryotherapy, and radiation therapy, as well as systemic chemotherapy and enucleation performed to remove the entire tumor (3, 4). Ocular salvage is important in order to maintain the quality of life of pediatric patients considering their future. However, secondary enucleation is required in certain cases in which tumor control cannot be achieved by local treatment or systemic chemotherapy alone. Therefore, the ocular salvage rate remains below 50% (4, 5). Although chemotherapy with intra-arterial or intravitreal melphalan has improved the ocular salvage rate to approximately 70%, there is a risk of developing secondary cancer as it is an alkylating agent (6-9). Furthermore, intravitreal injection is highly invasive and may lead to severe side-effects such as retinal damage (10). Therefore, there is a need for novel treatment strategies for retinoblastoma. However, since the development of local intraocular chemotherapy with melphalan, there have been no new treatments that can be used widely in the clinical setting (10). As retinoblastoma is a rare disease, it can be challenging to perform clinical trials for novel therapeutics. As such, drug repositioning, which is a strategy to identify new uses for approved drugs or investigational drugs with a known safety profile for a different disease, may be an effective strategy to identify novel therapeutics for retinoblastoma. In the present study, we used several retinoblastoma cell lines to examine the efficacy against retinoblastoma of the existing small-molecule compound c-Jun N-terminal kinase (JNK)-pathway inhibitor CEP1347, which has a known safety profile in humans.

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Key Words: Drug repositioning, repurposing, MDM2, MDMX, CDKN1A, $P21^{WAF1/CIP1}$.

Materials and Methods

Reagents and antibodies. Antibodies against cyclin dependent kinase inhibitor 1A (CDKN1A, $p21^{Waf1/Cip1}$), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody against murine double minute 4 (MDM4) was purchased from Abcam (Cambridge, U.K.). Antibody against MDM2 was purchased from Merck Millipore (Darmstadt, Germany). Antibody against P53 was

purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). CEP1347 (product code FE29092) was synthesized at Tokyo Chemical Industrial Co., Ltd. (Tokyo Japan). *P53*-transcription inhibitor pifithrin-α was purchased from Merck Millipore. CEP1347 and pifithrin-α were dissolved in dimethyl sulfoxide to prepare 1 mM and 10 mM stock solutions, respectively.

Cell culture. The Y79, WERI-Rb-1, NCC-RbC-54, NCC-RbC-60, and NCC-RbC-83 human retinoblastoma cell lines were obtained from the Riken BioResource Center (Tsukuba, Japan). The NCC-RbC-51 human retinoblastoma cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). NCC-RbC-54, NCC-RbC-60, and NCC-RbC-83 were maintained in RPMI1640 medium supplemented with 20% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 50 μM 2-mercaptethanol (Nacalai Tesque, Inc., Kyoto, Japan). The other retinoblastoma cell lines were maintained in RPMI1640 medium supplemented with 10% (Y79 and WERI-Rb-1) or 20% (NCC-RbC-51) FBS. IMR90 normal human fetal lung fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Furthermore, the culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. All IMR90 experiments were performed using cells with a low passage number (<8).

Cytotoxicity analysis. Cell viability and growth inhibition were determined by tetrazolium salt reduction method using WST-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) as described previously (11, 12). Cells in 96-well plates were treated with or without 100-1000 nM CEP1347 for 6 days. WST-8 reagent was then added and the cells were incubated for 1-3 h at 37˚C. Absorbance (optical density, OD) at 450 nm was measured using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA). Relative cell viability was calculated as the absorbance of treated samples as percentage relative to that of the controls. To determine the growth inhibition rate, we used the following formula as previously reported (13):

Growth inhibition (%)=(1−OD treatment/OD control) × 100 (%)

Cell growth-curve analysis was performed by trypan blue exclusion. Cells (1×10^5/well) plated in 6-well plates were treated with or without 500 nM CEP1347, stained with 0.2% trypan blue for 1 min at room temperature, and the number of viable cells was determined using a hemocytometer.

Immunoblot analysis. Immunoblot analysis was conducted as previously described (11, 12, 14). Cells were washed with ice-cold PBS and lysed in RIPA buffer [10 mM Tris-HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1.5 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, and 1% protease inhibitor cocktail set III (Sigma)]. The same volume of 2× Laemmli buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, and 10% 2-mercaptoethanol] was immediately added and boiled at 95˚C for 10 min. The protein concentrations of the cell lysates were measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham MA, USA). Cell lysates containing equal amounts of protein were separated by SDS- polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was probed with primary antibody against CDKN1A, GAPDH, MDM4, MDM2 and P53 and then with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody according to the protocol recommended by the manufacturer of each antibody. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and detected by a ChemiDoc Touch (Bio-Rad).

Statistical analysis. All data are shown as means±standard deviation (SD). Differences were compared using two-tailed Student's *t*-test. Differences with a *p*-value of less than 0.05 were considered statistically significant.

Results

CEP1347 suppresses the proliferation of retinoblastoma cells. In order to identify drug candidates for retinoblastoma, the drug repositioning strategy was used to assess the efficacy of existing small-molecule compounds with a known safety profile in humans against six different retinoblastoma cells lines. Specifically, the water-soluble tetrazolium salt (WST-8) assay was performed to measure the metabolic activity of cells to reduce WST-8 to a soluble formazan product as an indicator of the ability of each drug to suppress cell proliferation. As shown in Figure 1, the JNK-pathway inhibitor CEP1347, which was developed for Parkinson's disease as an inhibitor of nerve cell apoptosis (15, 16), was effective against five out of the six retinoblastoma cell lines. It suppressed the proliferation of retinoblastoma cells in a dose-dependent manner within the range of concentrations that was not toxic to normal IMR90 cells. Of note, the effects of CEP1347 were not noted in NCC-RbC-51 cells, suggesting that they are more resistant to CEP1347 than the other cell lines (Figure 1).

In order to confirm the results from the WST-8 assay, a dye-exclusion test was performed to examine the effects of CEP1347 on the proliferation of retinoblastoma cells at a concentration (500 nM) non-toxic to IMR90 cells. In good accordance with the results shown in Figure 1, the growth of Y79 and NCC-RbC-54 was substantially suppressed by CEP1347, whereas that of NCC-RbC-51 was only modestly inhibited (Figure 2). Taken together, CEP1347 had anti-proliferative effects against most of these retinoblastoma cell lines.

CEP1347 activates the P53 pathway in CEP1347-sensitive cell lines. The expression of functional proteins with known antiproliferative properties was examined in order to understand the mechanisms underlying the effects of CEP1347 on the proliferation of retinoblastoma cells. Our initial findings suggested that CEP1347 selectively induced the expression of P53 in CEP1347-sensitive cell lines. As *P53* mutation is not common in retinoblastoma (17, 18), the CEP1347-induced increase in P53 protein likely led to the activation of P53. Thus, the expression of CDKN1A (P21), which is a cyclin-

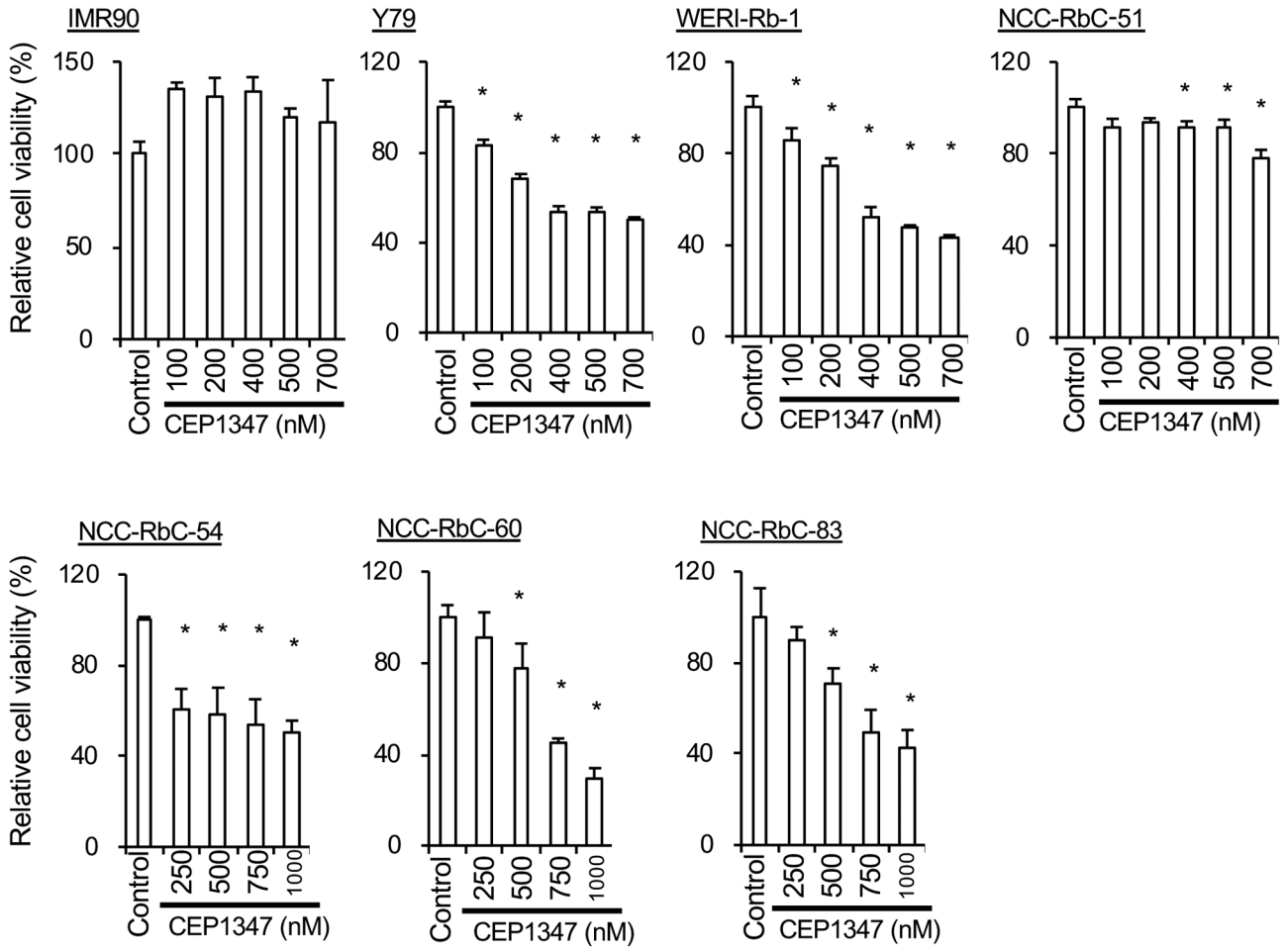

Figure 1. *Growth-inhibitory effect of c-Jun N-terminal kinase-pathway inhibitor CEP1347. Human retinoblastoma cell lines (Y79, WERI-Rb-1, NCC-RbC-51, NCC-RbC-54, NCC-RbC-60, and NCC-RbC-83) and a human normal lung fibroblast cell line (IMR90) were treated without (Control) or with the indicated concentrations of CEP1347 for 6 days. The cell viability relative to the control was then determined using WST-8. Data are represented as means+standard deviation from triplicate samples of a representative experiment repeated with similar results. *Significantly different at p<0.05 vs. control by Student's t-test.*

dependent kinase inhibitor with potent antiproliferative effects and target gene of P53, was further examined in order to monitor the activity of P53. As a result, the increase in the level of P53 correlated with the level of CDKN1A in CEP1347-sensitive Y79 and NCC-RbC-54 cells, whereas their levels did not increase in CEP1347-insensitive NCC-RbC-51 cells (Figure 3). Therefore, CEP1347 may suppress the proliferation of retinoblastoma cells by activating P53.

The antiproliferative effects of CEP1347 on retinoblastoma cells are P53-dependent. In order to clarify whether the antiproliferative effects of CEP1347 against retinoblastoma cells are regulated by activation of the P53 pathway, we examined whether the suppression of P53 affected the antiproliferative effects of CEP1347 on retinoblastoma cells. As gene transfer efficiency of the retinoblastoma cell lines used in the study was poor, a pharmacological approach was used instead of a genetic approach, such as gene knockdown; specifically, an inhibitor of *P53* transcription, pifithrin-α, was used to suppress the function of P53 (19, 20). Although CEP1347 alone induced the expression of P53 and CDKN1A in retinoblastoma cells, the addition of pifithrin-α suppressed the expression of CDKN1A, but not P53, in a dose-dependent manner (Figure 4A). This revealed that pifithrin-α suppressed the transcriptional activity of P53. Next, the effects of pifithrin-α on the antiproliferative effects of CEP1347 were examined. As shown in Figure 4B, pifithrin-α reduced the antiproliferative effects of CEP1347 on retinoblastoma cells in a dose-dependent manner. Collectively, these findings suggest that the antiproliferative effects of CEP1347 on retinoblastoma cells are at least partially attributed to the function of P53.

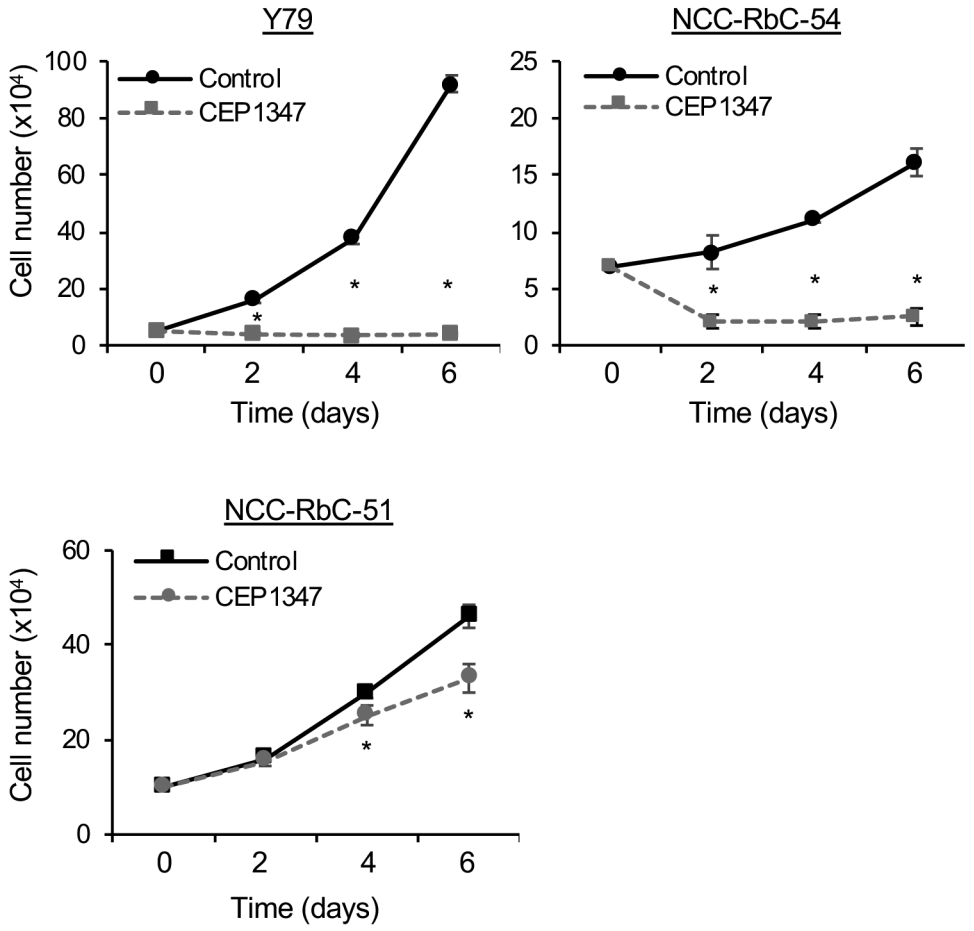

Figure 2. *Differential sensitivities to CEP1347 among retinoblastoma cell lines. Cell-growth curves were determined for the indicated cell lines treated without (Control) or with 500 nM CEP1347. Data are presented as means±standard deviation from triplicate samples of a representative experiment repeated with similar results. *Significantly different at p<0.05 vs. control by Student's t-test.*

CEP1347 reduces the expression of MDM4, a negative regulator of P53. Further analyses were performed to examine the mechanisms underlying the activation of P53 by CEP1347. *P53* is not mutated in retinoblastoma; however, amplification and polymorphism of *MDM4*, which negatively regulates P53, are commonly noted in patients with retinoblastoma. This mechanism is thought to play a role in the deactivation of the P53 pathway (21-23). Thus, the expression of MDM4 was examined in retinoblastoma cell lines. As shown in Figure 5, MDM4 was expressed in CEP1347-sensitive cell lines, but not in CEP1347-insensitive NCC-RbC-51 cells. Next, the impact of CEP1347 on the expression of MDM4 in retinoblastoma cells was further examined. The expression of MDM4 in CEP1347-sensitive retinoblastoma cells was down-regulated by CEP1347 in a dose-dependent manner, whereas that of P53 was up-regulated by CEP1347. On the other hand, there was no significant trend in the expression of MDM2 regarding

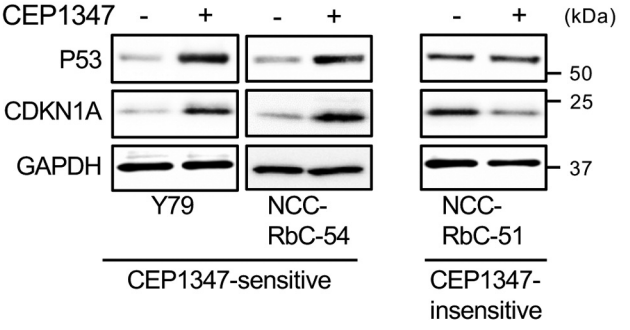

Figure 3. *CEP1347 activates the P53 pathway in sensitive retinoblastoma cell lines. CEP1347-sensitive and -insensitive retinoblastoma cell lines were treated with or without 500 nM CEP1347 for 6 days then subjected to immunoblot analysis of P53 and cyclin-dependent kinase inhibitor 1A (CDKN1A) expression. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.*

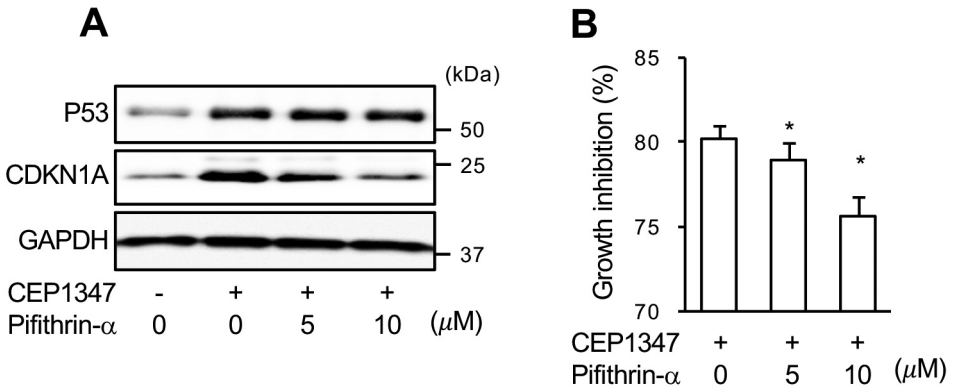

Figure 4. *P53 is critically involved in the growth-inhibitory effect of CEP1347 on retinoblastoma cell lines. Y79 cells were treated with or without CEP1347 (500 nM) in the absence or presence of P53-transcription inhibitor pifithrin-α for 6 days and then subjected to immunoblot analysis of P53 and cyclin-dependent kinase inhibitor 1A (CDKN1A) expression (A) or the determination of cell viability using WST-8 to assess the degree of growth inhibition caused by CEP1347 treatment (B). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Data are presented as means+standard deviation from triplicate samples of a representative experiment repeated with similar results. *Significantly different at p<0.05 vs. 0 μM pifithrin-α by Student's t-test.*

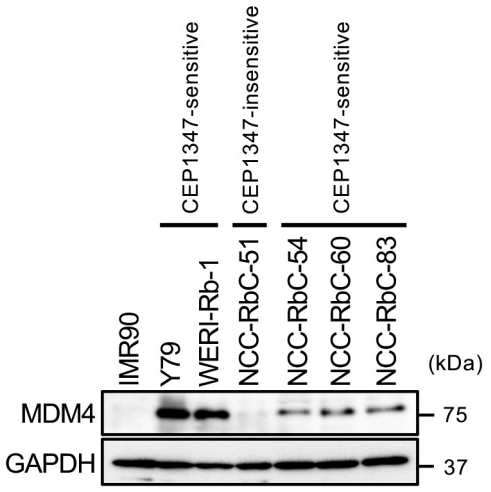

Figure 5. *CEP1347-sensitive retinoblastoma cell lines express murine double minute 4 (MDM4). The expression levels of MDM4 in the studied cell lines were determined by immunoblot analysis. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.*

exposure to CEP1347 (Figure 6A). In CEP1347-insensitive NCC-RbC-51 cells, the level of MDM4 remained below the detection limit when the cells were treated with CEP1347 in the range of concentrations examined. Next, the time course of the expression of MDM4 and P53 was examined to assess whether the down-regulation of MDM4 by CEP1347 was associated with the increased level of P53. As shown in Figure 6B, the expression of MDM4 was negatively correlated with P53 at all time points. Collectively, these findings suggest that CEP1347 suppresses the proliferation of retinoblastoma cells by inducing and activating the expression of P53 *via MDM4*, which negatively regulates the expression of *P53*.

Discussion

In the present study, we identified CEP1347, which was originally developed as a treatment for Parkinson's disease, as being effective against retinoblastoma. We demonstrated that activation of the P53 pathway is involved in the antiproliferative effects of CEP1347 on retinoblastoma cells. Furthermore, CEP1347 suppressed the expression of MDM4 in retinoblastoma cells and consequently activated the P53 pathway.

It is well known that MDM2, which is overexpressed in some cancer cells, regulates the expression of the tumor suppressor P53 *via* degradation by poly-ubiquitination. MDM4 is known to maintain the expression of MDM2 and thereby suppress the expression of P53 by binding to the active domain (RING finger domain) of MDM2 and subsequently interfering with its self-ubiquitination and auto-degradation (24). Significantly, while amplification and polymorphism of the *MDM4* gene have been reported, the *P53* gene is wild-type in retinoblastoma (17, 18). Therefore, previous studies focused on the suppression of MDM4, which is an antagonist of P53, in order to activate P53 and suppress the proliferation of retinoblastoma cells (22, 23, 25). In the present study, we demonstrated that CEP1347 was highly effective at suppressing the proliferation of retinoblastoma cells with high levels of MDM4 expression, and that CEP1347 reduced the expression of MDM4 and increased that of P53. This suggests that CEP1347 induces the activation of P53 by suppressing the expression of MDM4. However, we noted no significant trend in terms of the effects of CEP1347 on the expression of

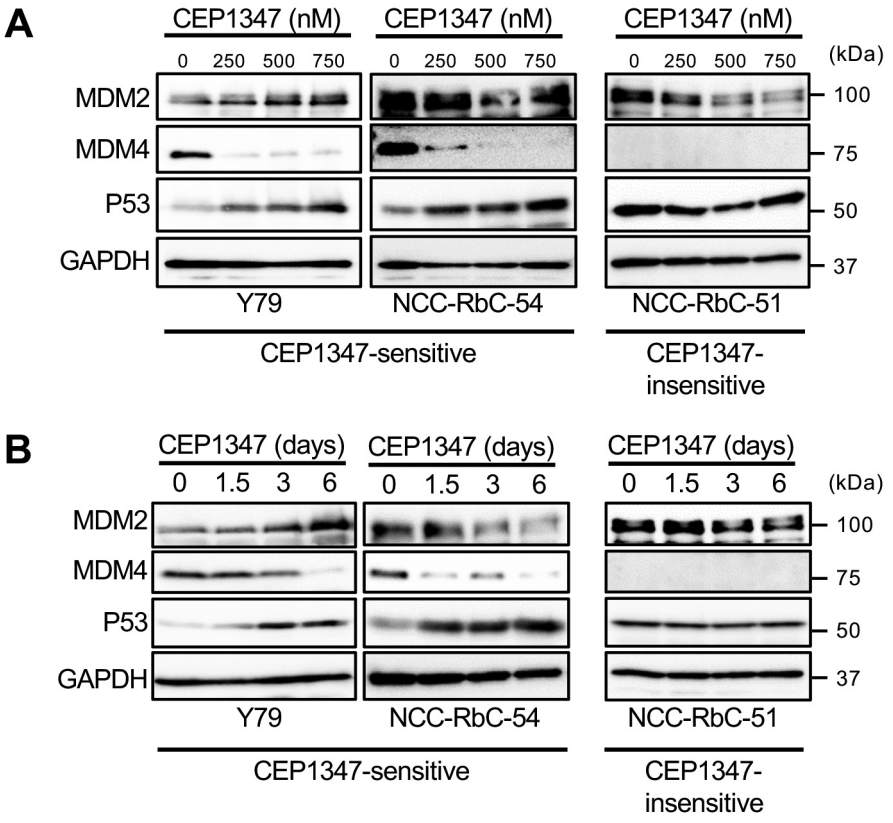

Figure 6. *Reciprocal changes in murine double minute 4 (MDM4) and P53 levels induced by CEP1347 in sensitive retinoblastoma cell lines. CEP1347-sensitive and insensitive retinoblastoma cell lines were treated with the indicated concentrations of CEP1347 for 6 days (A) or treated with 500 nM CEP1347 for the indicated times (B) and then subjected to immunoblot analysis to determine the expression levels of murine double minute 2 (MDM2), MDM4, and P53. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.*

MDM2 (Figure 6). Thus, the effects of CEP1347 on activation of the P53 pathway are not due to the suppression of MDM2 expression *via* MDM4. Indeed, MDM4 is also known to inhibit P53 by binding directly to the transcriptional domain of P53 without the involvement of MDM2 (26). Our study did not exclude the possibility that there are mechanisms other than those that involve MDM4 that explain the effects of CEP1347 on P53. For example, CEP1347 is known not only as a pan-mixed lineage kinase (MLK) inhibitor, but also as an inhibitor of the oncogenic P21-activated kinase 1 (PAK1) pathway (27). One previous study demonstrated that pharmacological (FRAX-1036) and genetic suppression of PAK1 reduced the growth of ovarian cancer cells both *in vitro* and *in vivo*, and that these inhibitory effects were dependent on the activation of P53 and subsequent increase in P21 (28). More recently, MLK3 was reported to function in the tumorigenesis of breast cancer cells by activating PAK1 (29). These studies collectively demonstrated that CEP1347 can also regulate the MLK-PAK1 pathway independent of MDM4. Thus, it is possible that the antiproliferative effects of CEP1347 in retinoblastoma are due to the activation of P53 *via* the MLK3-PAK1 pathway. Regardless of the underlying mechanisms, we demonstrated novel pharmacological effects of CEP1347 on activation of the P53 pathway in cancer cells. Based on our study, CEP1347 may be a novel and safe strategy for controlling retinoblastoma and other human cancer types in which the *P53* gene is intact.

In terms of other strategies that target P53 in retinoblastoma, one study demonstrated that NUTLIN3A, an inhibitor of MDM2/MDM4–P53 interaction, suppresses the proliferation of retinoblastoma cells both *in vitro* and *in vivo* (21, 30). However, although overexpression of MDM4 is frequent in human retinoblastoma cells, it has been reported that the level of MDM2, which is the target of NUTLIN3A, is often below the limit of detection in human retinoblastoma cells (31). In addition, retinoblastoma is protected by the blood–retinal barrier and many drugs, including NUTLIN3A, do not penetrate this barrier effectively (21, 32, 33). On the other hand, CEP1347 was designed as a treatment for Parkinson's disease and its safety profile in humans has been well characterized. In addition, it can pass the blood–brain barrier and effectively target tumor cells implanted in the brain (15, 16, 34, 35). The blood–retinal barrier is similar to the blood–

brain barrier in terms of its structure and protein complex composition, in addition to its permeability and transport efficiency; drug permeability into the retina was even proposed as an indicator of drug permeability of the central nervous system (36, 37). Thus, CEP1347 may be able to pass through the blood–retinal barrier. Currently, none of the drugs that act on retinoblastoma by activating P53, including NUTLIN3A, have a strong clinical potential. However, CEP1347 may be a candidate for the treatment of retinoblastoma.

In summary, we demonstrated that CEP1347 activates P53 and exerts antiproliferative effects on retinoblastoma cells that overexpress MDM4. As its safety profile in humans has been well characterized and it effectively penetrates vascular barriers, such as the blood–brain barrier, CEP1347 may be a strong candidate drug for the treatment of retinoblastoma.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

MO and CK designed the research. KT and MO performed the experiments under the supervision of MO and CK. KT, S Suzuki, TS, S Seino, MY, HY, CK, and MO were involved in data interpretation. KT and MO drafted the article and prepared the figures with the help of CK. S Suzuki, TS, S Seino, MY, and HY reviewed the draft and, based on their inputs, KT and MO edited the article under the supervision of CK. All Authors read and approved the final version of the article.

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