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Rapalink-1 and Hydroxychloroquine Exhibit an Additive Effect in Undifferentiated Pleomorphic Sarcoma by Inducing Apoptosis

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Abstract. *Background/Aim: Advanced undifferentiated pleomorphic sarcoma (UPS) has a poor prognosis and there are few treatments that can improve overall survival. Recently, Rapalink-1, a third-generation mammalian target of rapamycin (mTOR) kinase inhibitor, has been developed and shown to be effective against other tumours. However, mTOR inhibitors have been shown to induce autophagy and resistance to anti-cancer drugs. This study aimed to investigate the antitumor effects of Rapalink-1 with an autophagy inhibitor. Materials and Methods: The antitumor effect of Rapalink-1 and/or hydroxychloroquine in three UPS cell lines was examined via cell viability analysis, western blotting, flow cytometry and immunofluorescence. Results: Rapalink-1 decreased cell proliferation and inhibited the PI3K/mTOR pathway. Combined treatment with Rapalink-1 and hydroxychloroquine enhanced the antitumor effect compared to treatment with Rapalink-1 alone by blocking the autophagy-inducing effect of mTOR inhibitors. Conclusion: Combined treatment with Rapalink-1 and hydroxychloroquine may be used as a potential therapeutic agent against UPS.*

Undifferentiated pleomorphic sarcoma (UPS) is the most common and most aggressive malignant soft tissue sarcoma (1). Surgical treatment and adjuvant chemotherapy are the first choices for localized disease. Chemotherapy and radiation therapy are the main treatments for metastatic disease, but their outcomes are generally poor (2). Various anti-cancer agents have been studied for soft tissue tumours and anthracyclines are most commonly used for the treatment of metastatic UPS (3). In addition, pazopanib, trabectedin, and ifosfamide with doxorubicin can be used for treatment. Despite the use of various anti-cancer agents, the progression-free survival for metastatic disease remains poor (4).

In recent years, molecularly targeted drugs have been developed for the treatment of malignant tumours. These drugs inhibit specific molecules related to tumour growth, such as the mammalian target of rapamycin (mTOR), which interacts with phosphatidylinositol 3-kinase (PI3K) to regulate a variety of cellular responses (5). PI3K/mTOR pathway is important for the regulation of hallmarks of cancer, and is also involved in tumour-promoting processes (6). Its activation is associated with poor prognosis in patients with soft tissue sarcoma (7). mTOR is an intracellular serine/threonine kinase that regulates cell growth, metabolism, and proliferation. It is composed of two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (8). mTORC1 is involved in mRNA translation, cell proliferation and autophagy, and is activated by the phosphorylation of ribosomal p70S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (9, 10). In contrast, mTORC2 is regulated by the phosphorylation of AKT and is related to cellular metabolism (11). First generation mTOR inhibitors, such as rapamycin, bind to the intracellular receptor FKBP12 and then to the FRB domain of mTOR, forming a stable complex consisting of rapamycin, FKBP12, and mTOR (12). The antitumor effect of first generation mTOR inhibitors on soft tissue sarcoma has been studied (13, 14), however, they are not effective enough to be the first choice of treatment. This is because, although the first generation mTOR inhibitors sufficiently inhibit mTORC1, they are associated with inadequate mTORC2 inhibition (15). Inability to inhibit mTORC2 leads to the reactivation of mTORC1 through AKT, which may lead to insufficient antitumor effects (16).

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Although second generation mTOR inhibitors such as MLN0128 inhibit both mTORC1 and 2, the effect only lasts for a short duration. In glioblastoma cells, MLN0128 inhibited both mTORC1 and mTORC2; however, the effect only lasted for a few h. In addition, the activity of MLN0128 was lower than that of rapamycin *in vivo* (17). Many studies are still being conducted; however, early reports did not clarify any clinical benefit of second generation mTOR inhibitor monotherapy in unselected populations (18). Thus, a third-generation mTOR inhibitor, Rapalink-1, was developed to overcome the shortcomings of each drug. Rapalink-1 is an mTOR inhibitor that binds rapamycin and a second generation mTOR inhibitor by a linker (19). It has a durable, long-lasting effect like rapamycin by binding to FKBP12 and an inhibitory effect on both mTORC1 and mTORC2 similar to that of a second generation mTOR inhibitor. Despite the reports on the anti-cancer efficacy of Rapalink-1 on glioblastoma and renal cell carcinoma (5, 18), its efficacy on UPS has not been reported. Inhibition of mTOR induces autophagy, a lysosome-dependent cellular survival mechanism. Autophagy provides recycled nutrients by breaking down unused cellular components (20). Recent studies have shown that autophagy plays an important role in cancer proliferation and resistance to chemotherapy (21), thus inhibition can improve the cytotoxicity of many chemotherapeutic agents (22-24). However, the ability of Rapalink-1 to induce autophagy is still unknown. Additionally, the therapeutic effect of the combined use of autophagy inhibitors remains unclear.

This study aimed to investigate the anti-cancer effect and autophagy induction of Rapalink-1 on UPS cells. In addition, combination therapy of Rapalink-1 and hydroxychloroquine, an autophagy inhibitor, is demonstrated.

Materials and Methods

UPS cell lines and culture. The UPS cell lines Nara-H, Nara-F and GBS-1 were used. Nara-H and Nara-F were purchased from ScienStuff Co. (Nara, Japan) and GBS-1 was provided by the Cancer Institute of the Japanese Foundation for Cancer Research (Tokyo, Japan). Propagation was performed in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% foetal bovine serum and 100 U/ml penicillin at 37˚C in a humidified incubator with 5% CO_2.

Cell proliferation assay. Rapalink-1 was used as an mTOR inhibitor and hydroxychloroquine as an autophagy inhibitor. The cells were cultured in 96-well plates at a density of 1×10^4 cells/well for 48 h in DMEM. The medium was then removed and cells were cultured in DMEM containing each drug. To investigate the independent effect of Rapalink-1, different concentrations of Rapalink-1 (0.032, 0.16, 0.8, and 4 nM) were added to the cells for 24 or 48 h. To examine the combined effect of Rapalink-1 and hydroxychloroquine, 25 μM hydroxychloroquine was added for 12 h, washed with phosphate-buffered saline, and cells were treated with or without 0.8 nM Rapalink-1 for another 24 h. The cells were then cultured in DMEM containing 10 μL WST-1 (Sigma-Aldrich) for two h. Optical density at 420 nm was calculated using Maltiskan FC (Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting analysis. The cells were seeded in six-well plates at a density of 6×10^5 cells/well for 48 h. Next, the cells were treated with different concentrations of Rapalink-1 for 24 h. To examine the combinatory effect of Rapalink-1 and hydroxychloroquine, the cells were treated in the same way as in the cell proliferation assay. Following treatment, the cells were lysed with lysis buffer (Cell Signaling Technology, Beverly, MA, USA) for 20 min on ice. The cell lysate was centrifuged at 15,000 × *g* using a Tabletop Micro Refrigerated Centrifuge 3500 (Kubota Shoji Co. Ltd., Tokyo, Japan) for 30 min at 4˚C. The supernatant was retrieved and adjusted to the same amount of protein. These proteins were separated on NuPAGE 4%-12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and electrophoretically transferred to nitrocellulose membranes. Immunoblotting was performed with primary antibodies and anti-rabbit secondary antibodies in the iBind solution (iBind Western System, Life Technologies, Carlsbad, CA, USA) for three h. The antibodies used were 4EBP1, phospho-4EBP1, S6 ribosomal protein, phospho-S6 ribosomal protein, cleaved PARP, GAPDH (Cell Signaling Technology, Beverly, MA, USA), AKT, phospho-AKT (Abcam, Cambridge, UK), LC-3, and p62/SQSTM1 (MBL CO., Nagoya, Japan). LC3 has two molecular forms, LC3-I and LC3-II, and LC3-I is localized in the cytoplasm. LC3-1 binds to phosphatidylethanolamine and is converted to LC3-II, which binds to the membrane of autophagosomes. Therefore, LC3-II is used as an autophagic marker. p62/SQSTM1 is degraded by the autophagy process and its intracellular level is considered to be an autophagic marker. p62/SQSTM1 protein is present in inclusion bodies containing aggregates of polyubiquitinated proteins that are broken by autophagy (25). Finally, protein signals were identified using a Novex AP Chemiluminescent Detection Kit (Life Technologies) and a LAS-4000 image analyser (Fujifilm Co., Tokyo, Japan).

Flow cytometry. Apoptosis was detected by staining the cells with Annexin V-FITC kit (Beckman Coulter, Inc., Brea, CA, USA). The cells were cultured in six-well plates at a density of 6×10^5 cells/well for 48 h. To examine the combinatory effect of Rapalink-1 and hydroxychloroquine, the cells were treated in the same way as in the cell proliferation assay. The cell lysate was centrifuged at 500 × *g* at 4˚C for five min and the supernatant was removed. The cells were mixed in 1× binding buffer at a concentration of 1×10^6 cells /ml. Annexin V-FITC and propidium iodide (PI) were added to 100 μl of cell lysate and incubated on ice for 15 min in the dark. Then, 400 μl of 1× binding buffer was added, and apoptosis was detected using a CytoFLEX S flow cytometer (Beckman Coulter).

Fluorescence microscopy images of immunocytochemical staining for LC3. The cells were cultured in six-well plates on 25-mm circular coverslips at a concentration of 1×10^6 cells/well for 48 h. To examine the combinatory effect of Rapalink-1 and hydroxychloroquine, the cells were treated in the same way as in the cell proliferation assay. Following treatment, the cells were fixed with 4% paraformaldehyde in phosphate buffer for 10 min. Anti-LC3 antibody was added to the cells for one hour at room temperature to examine autophagy. Then, anti-IgG secondary antibody (Alexa Fluor 488, code no. A11008; Invitrogen) was

added, and the cells were incubated for 30 min. The cells were observed under an epifluorescence microscope (FSX100, Olympus Optical Co., Ltd., Tokyo, Japan) with a 50× objective lens.

Fluorescence microscopy images of Annexin V-FITC stained cells. The cells were cultured and treated with Rapalink-1 and hydroxychloroquine, as described above. The cells were then incubated with Annexin V-FITC, PI, and Hoechst 33342 in the dark for 15 min using a Promokine Apoptotic/Necrotic/Healthy Cell detection kit (PromoCell GmbH, Heidelberg, Germany). The cells were observed under an epifluorescence microscope (FSX100, Olympus Optical Co., Ltd., Tokyo, Japan) with a 50× objective lens.

Statistical analysis. All graphs and analyses were performed using GraphPad Prism 9 software (GraphPad, San Diego, CA, USA). One-way analysis of variance and Bonferroni post-hoc tests were used to examine the statistical differences among the groups. $p<0.05$ was considered statistically significant in all analyses.

Results

Cell proliferation assay. Initially, the effect of Rapalink-1 on UPS cell lines was examined. Rapalink-1 was shown to reduce the viability of UPS cell lines in a concentration- and time-dependent manner. Rapalink-1 significantly inhibited cell proliferation in all treated cells treated at a concentration of 0.8 nM or higher compared to the control at 24 and 48 h ($p<0.05$, Figure 1A). Additionally, the effects of combining Rapalink-1 and hydroxychloroquine were tested. Compared to Rapalink-1 alone, combination of Rapalink-1 and hydroxychloroquine significantly inhibited cell proliferation from 76.4% to 46.5% for Nara-H, from 55.7% to 29.2% for Nara-F, and from 61.2% to 22.3% for GBS-1 ($p<0.05$, Figure 1B).

Western blot analysis. First, the effect of Rapalink-1 on the PI3K/mTOR pathway was examined. In Nara-H, Nara-F, and GBS-1 cells, Rapalink-1 inhibited the phosphorylation of 4EBP1, RPS6, and AKT, which are downstream proteins of the PI3K/mTOR pathway. The effect of Rapalink-1 on autophagy was also determined. Rapalink-1 increased LC3-II levels and decreased p62/SQSTM1 levels in a concentration-dependent manner (Figure 2A), which suggests that Rapalink-1 induces autophagy. The combination of Rapalink-1 and hydroxychloroquine increased LC3-II and p62/SQSTM1 levels compared to Rapalink-1 or hydroxychloroquine alone. In addition, the expression of cleaved PARP, which was used as a marker of apoptosis, was enhanced by the combination treatment (Figure 2B).

Flow cytometry. To examine the effect of Rapalink-1 and the combination treatment of Rapalink-1 and hydroxychloroquine on cell apoptosis, flow cytometry was performed to determine the percentage of apoptotic cells. The Annexin V-FITC-positive and PI-negative cells were considered as early apoptotic cells. Rapalink-1 significantly increased the percentage of apoptotic cells in Nara-H to 9.85%, compared to 2.65% in controls. Similarly, after Rapalink-1 treatment, the percentage of apoptotic cells increased significantly from 2.47% to 12.22% in Nara-F and from 0.36% to 17.45% in GBS-1 compared to controls. In addition, the combination of Rapalink-1 and hydroxychloroquine significantly increased the percentage of apoptotic cells to 15.1% in Nara-H, to 22.46% in Nara-F and to 53.12% in GBS-1 compared to Rapalink-1 alone (Figure 3A and B).

Fluorescence microscopy images. The expression of LC3 in UPS cells increased after treatment of Rapalink-1. Moreover, it was also significantly increased following the combination treatment (Figure 4A). Apoptotic cells were also detected using the Promokine Apoptotic/Necrotic/Healthy Cell detection kit. Early apoptotic cells that were stained with Annexin V-FITC, but not PI, were increased following the combination treatment compared to controls, Rapalink-1 or hydroxychloroquine alone (Figure 4B).

Discussion

UPS is the most frequent malignant soft tissue sarcoma with poor prognosis due to its aggressiveness (4). Various anti-cancer drugs, including mTOR inhibitors, have been studied, but no drug has significantly improved survival rates yet (2, 3). This study investigated the anti-cancer effect of a third-generation mTOR inhibitor, Rapalink-1, on UPS cells. Additionally, the induction of autophagy in UPS cells was investigated using Rapalink-1, and the anti-cancer effect of combination therapy with hydroxychloroquine, an autophagy inhibitor. The experiments showed that Rapalink-1 inhibited the proliferation of UPS cells by inhibiting both mTORC1 and mTORC2. It was also found that combination treatment with Rapalink-1 and hydroxychloroquine was more effective than Rapalink-1 alone, because of the induction of autophagy by Rapalink-1.

The PI3K/mTOR pathway plays a crucial role in cell growth, structural cytoskeleton remodelling, and cell metabolism reprogramming in many types of cancer. In soft tissue sarcomas, activation of PI3K/mTOR pathway has worse prognosis (7). Thus, the inhibition of this pathway is expected to improve treatment outcomes. mTOR is composed of two complexes, and inhibition of only one of them causes the other to be activated *via* feedback loops (16). It has been proposed that blocking the entire pathway is necessary for sufficient anti-cancer effects (26). A previous study showed that rapamycin suppressed the phosphorylation of p70S6K, but not 4EBP1, in mTORC1. After rapamycin treatment, 4EBP1 is dephosphorylated within a few h and induces rapamycin resistance (17). In contrast, mTOR kinase inhibitor (TORKi), a second generation mTOR inhibitor, can inhibit both complexes (27, 28). However, TORKi as

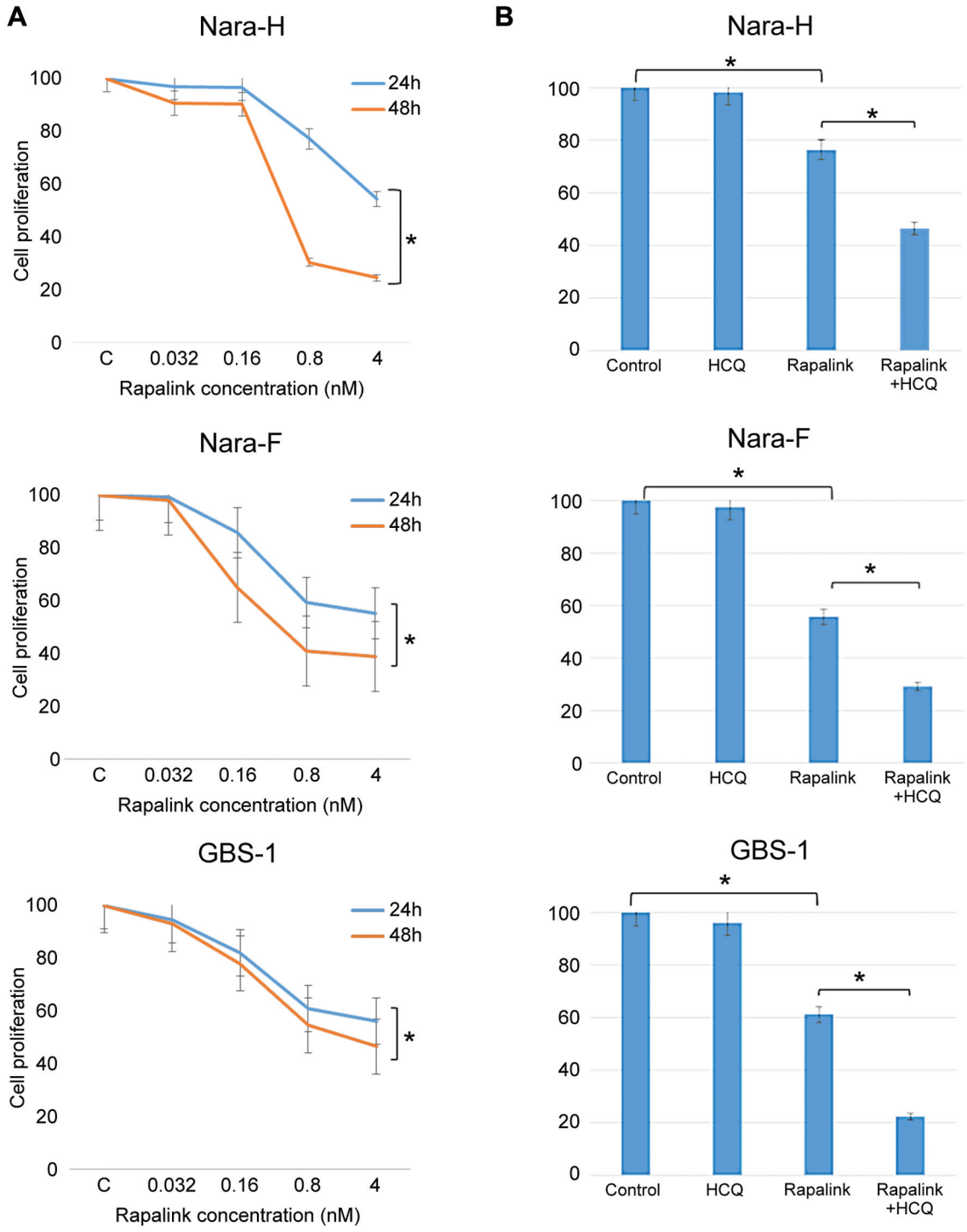

Figure 1. *Results of WST-1 cell proliferation assay in UPS cell lines. (A) Nara-H, Nara-F, and GBS-1 cells were treated with various doses (0.032-4 nM) of Rapalink-1 for 24 and 48 h. Rapalink-1 significantly inhibited proliferation at a concentration of 0.8 nM compared to the control at 24 and 48 h in all cells (*p<0.05). There was also a significant difference between 0.16 nM and 4 nM concentrations, and a significant difference between 24 and 48 h at concentrations of 0.8 nM and 4 nM (*p<0.05). One-way analysis of variance and Bonferroni post-hoc tests were used to examine for statistical differences between the groups. (B) Nara-H, Nara-F, and GBS-1 cells were treated with 25 μM hydroxychloroquine for 12 h, and then cells were treated with or without 0.8 nM Rapalink-1 for another 24 h. Rapalink-1 significantly inhibited UPS cell proliferation compared to the control (*p<0.05). Combination treatment with Rapalink-1 and hydroxychloroquine significantly inhibited UPS cell proliferation compared to Rapalink-1 alone (*p<0.05). UPS, Undifferentiated pleomorphic sarcoma; HCQ, hydroxychloroquine.*

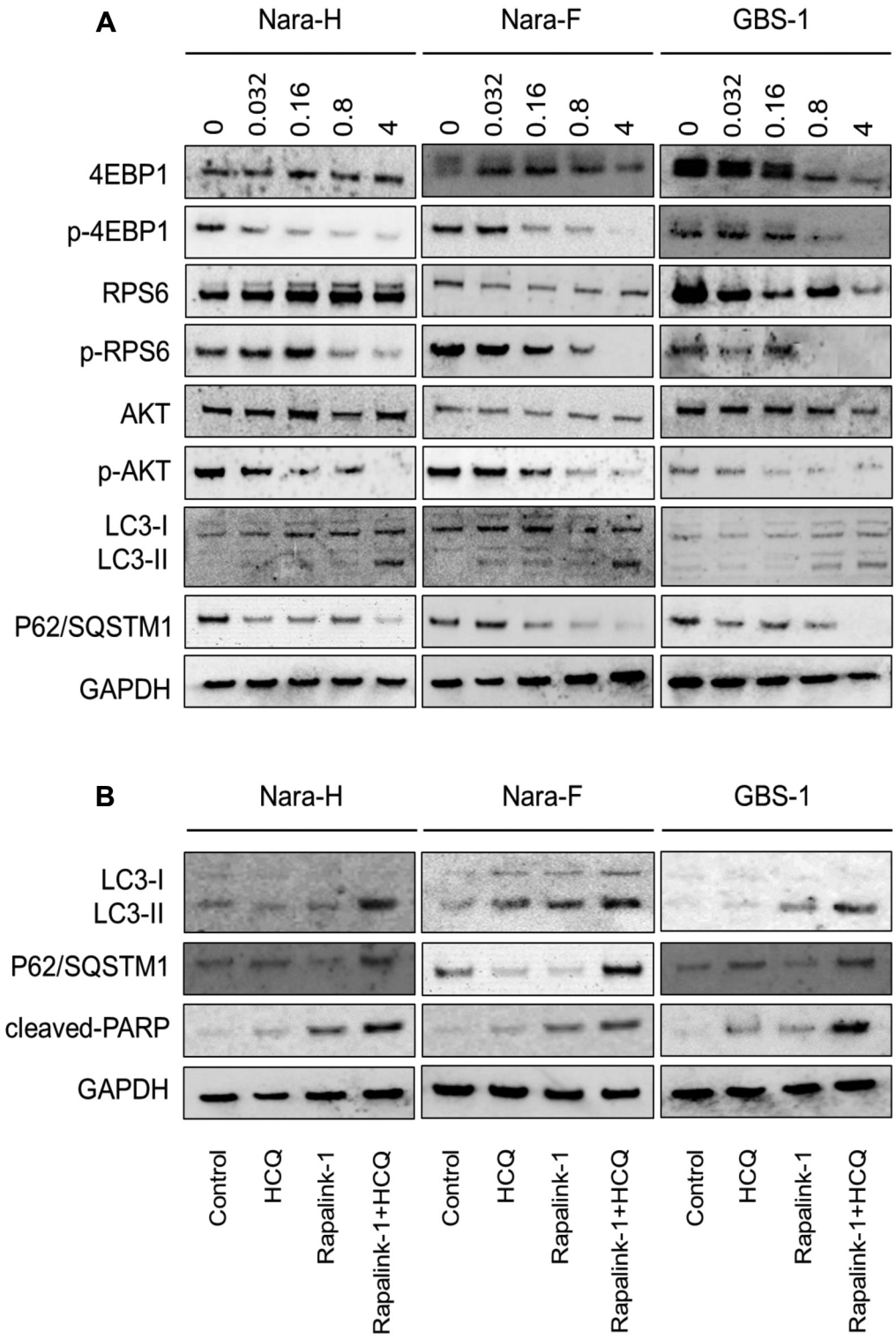

Figure 2. *Western blot analysis of the PI3K/mTOR pathway, autophagy, and apoptosis in UPS cells during hydroxychloroquine and Rapalink-1 treatment. (A) UPS cell lines were treated with various doses (0.032-4 μM) of Rapalink-1. Rapalink-1 inhibited PI3K/mTOR signalling protein marker expression and increased autophagy-related protein expression. (B) Combination treatment with Rapalink-1 and hydroxychloroquine inhibited autophagy and induced apoptotic markers compared to treatment with Rapalink-1 alone. UPS, Undifferentiated pleomorphic sarcoma; HCQ, hydroxychloroquine.*

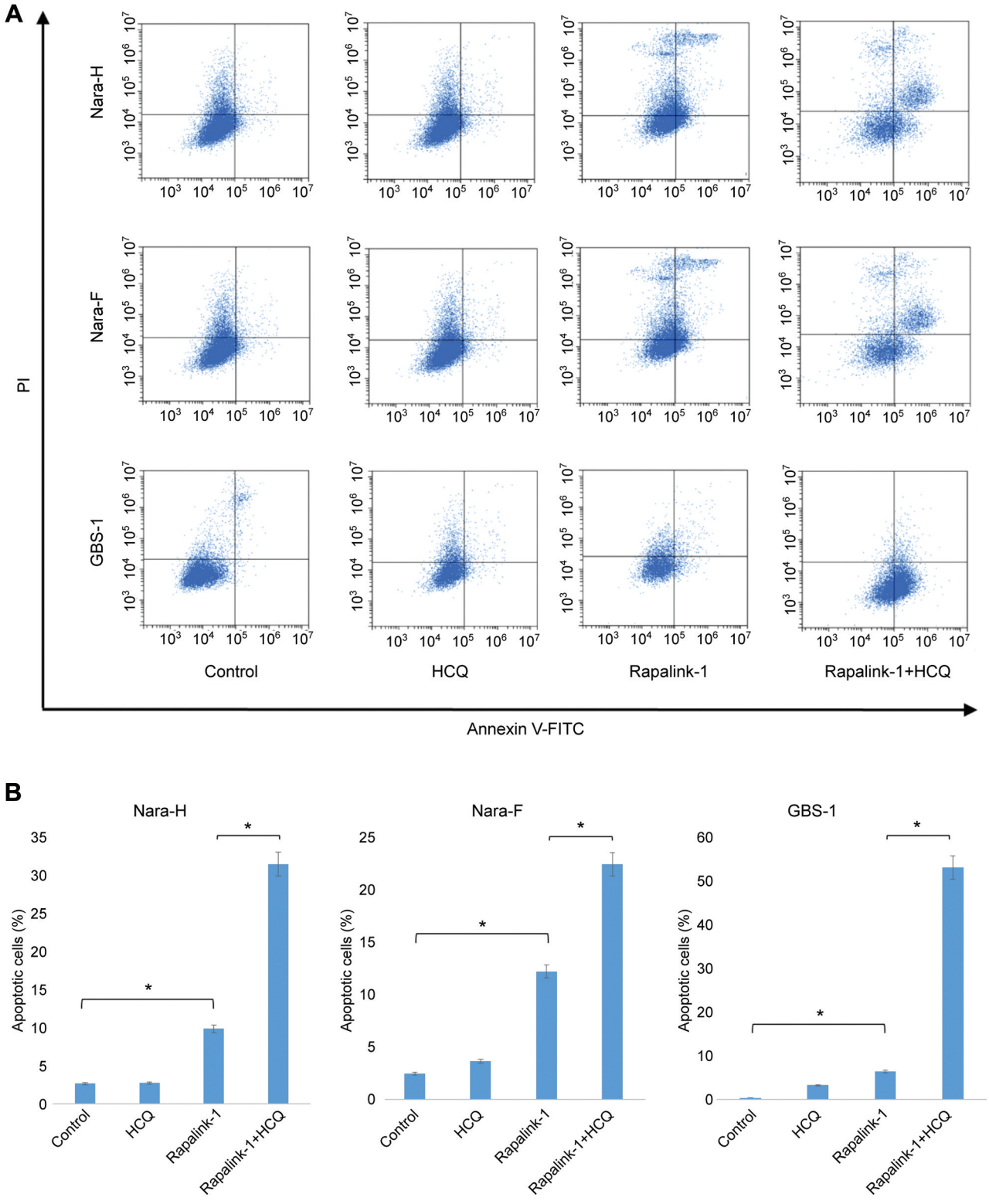

Figure 3. *Flow cytometry analysis of apoptosis during hydroxychloroquine and Rapalink-1 treatment. (A) Apoptosis detection by Annexin V-FITC/PI double staining using flow cytometry. (B) Rapalink-1 increased apoptotic cells significantly in all cell lines compared to controls (*p<0.05). Combination treatment with Rapalink-1 and hydroxychloroquine increased apoptotic cells significantly in all cell lines compared to treatment with Rapalink-1 alone (*p<0.05). HCQ, Hydroxychloroquine.*

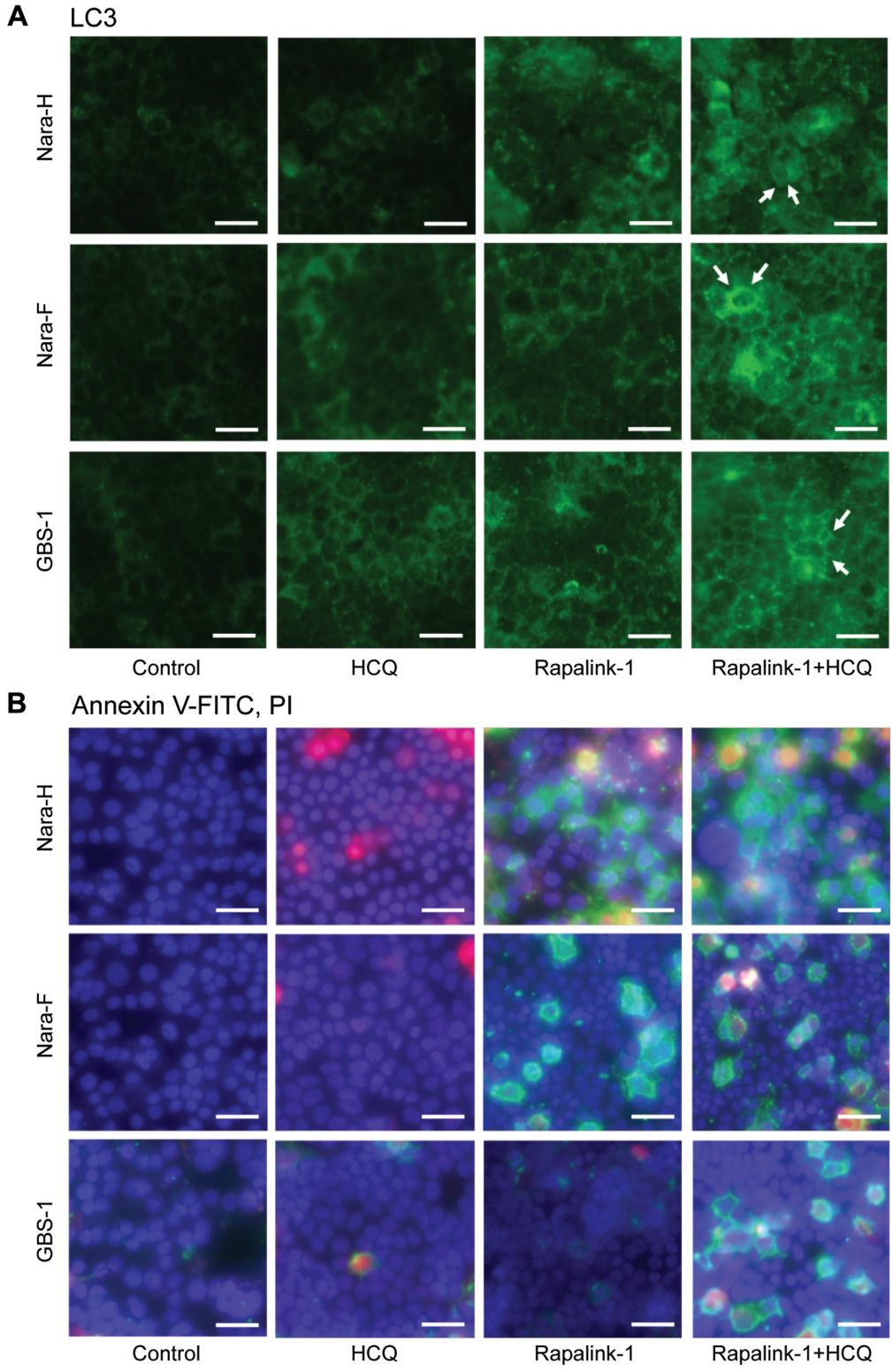

Figure 4. *Fluorescence microscopy images analysing autophagy and apoptosis in UPS cells during hydroxychloroquine and Rapalink-1 treatment. (A) LC3 was detected by immunolabeling after each treatment. LC3-positive puncta increased after combination treatment compared to the control and other treatment (arrows). (B) Apoptotic cells were detected by Annexin V-FITC/PI double staining. Living cells were labelled blue, while early and late apoptotic cells were identified as green and red. Early apoptotic cells increased after combination treatment compared to the other treatment (scale bars=20 μm). HCQ, Hydroxychloroquine.*

MLN0128 did not show sufficient efficacy in clinical trials in glioblastoma and metastatic prostate cancer due to its short duration of action (17, 29). Rapalink-1 combines the effects of rapamycin on mTORC1 and dual mTORC1 and mTORC2 inhibition effects of MLN0128. A strong anti-cancer effect of Rapalink-1 has been observed in glioblastoma, prostate cancer and renal cell carcinoma by blocking both mTORC1 and mTORC2 (5, 30, 31). In this study, it was found that Rapalink-1 inhibited the proliferation of UPS cell lines in a dose- and time-dependent manner, and decreased RPS6, 4E-BP1 and AKT phosphorylation in a dose-dependent manner. These results suggest that Rapalink-1 inhibits the mTOR pathway in UPS cells.

Autophagy maintains cellular homeostasis during metabolic stress and prevents carcinogenesis by protecting normal cells (32, 33). In advanced cancer, autophagy acts as a survival mechanism that is induced by various intra- and extracellular stresses in the cell (34). Targeting the PI3K/mTOR pathway may not be effective in malignant tumours, because one mechanism of resistance to the inhibition of this pathway is the induction of autophagy (35). Autophagy acts as a self-defence mechanism by allowing tumour cells to escape apoptosis, thereby promoting drug resistance (36-38). In sarcomas such as osteosarcoma and UPS, mTOR inhibitors such as rapamycin are known to act as potent inducers of autophagy (39). Previous studies have shown that inhibition of autophagy by hydroxychloroquine can enhance the cytotoxicity of chemotherapy (24). Hydroxychloroquine is involved in the inhibition of autophagy, because it affects lysosomal acidification and subsequently inhibits autophagosome-lysosome fusion (34). It was found that Rapalink-1 increased the level of the autophagy marker, LC-3II, and decreased the level of p62/SQSTM1 in UPS cell lines in a dose-dependent manner. In other words, Rapalink-1 induced autophagy as well as rapamycin. In contrast, the western blot results showed that the levels of LC-3II and p62/SQSTM1 in cells treated with Rapalink-1 and hydroxychloroquine were higher than those in cells treated with Rapalink-1 alone. Immunocytochemical analysis showed that the number of LC3 positive puncta increased following combination treatment, compared to Rapalink-1 alone. These results may be due to the fact that hydroxychloroquine inhibits the late phase of autophagy, which leads to increased accumulation of autophagosomes and increased levels of LC3-II and p62/SQSTM1. Next, it was examined whether hydroxychloroquine increased apoptosis because of impaired autophagy. In this study, cell proliferation was markedly inhibited after treatment with Rapalink-1 and hydroxychloroquine. Western blot analysis showed that the cleaved PARP levels increased in cells with combination treatment compared to Rapalink-1 alone. Additionally, flow cytometry analysis and fluorescence microscopy images showed that the number of apoptotic cells increased following combination treatment. These results show that Rapalink-1 alone mildly induced apoptosis in UPS cells, while the combination treatment induced significantly greater apoptosis.

In conclusion, this study showed that Rapalink-1 had a significant antitumor effect in UPS cells by inhibiting the PI3K/mTOR pathway. Moreover, it was found that hydroxychloroquine enhanced apoptotic cell death by inhibiting autophagy. A limitation of this study is that only three cell lines were examined *in vitro*. Further studies are needed to clarify whether Rapalink-1 has an antitumor effect in other UPS cells *in vivo*. Overall, combination treatment with Rapalink-1 and hydroxychloroquine may be an effective treatment for UPS.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

TN wrote this article. TN, YI, ON, TY conceived and designed the study, analysed and interpreted the data. YN, YK were involved in the data analysis. All Authors critically revised the report, commented on drafts of the manuscript and approved the final report.

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