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Chloroquine Combined With Rapamycin Arrests Tumor Growth in a Patient-derived Orthotopic Xenograft (PDOX) Mouse Model of Dedifferentiated Liposarcoma

NORIYUKI MASAKI[1,2,3], YUSUKE AOKI[1,3], YUTARO KUBOTA[1,3], KOYA OBARA[1,3], JUN MIYAZAKI[4] and ROBERT M. HOFFMAN[1,3]

[1]*AntiCancer Inc, San Diego, CA, U.S.A.;*
[2]*Graduate School of Medicine, International University of Health and Welfare, Tokyo, Japan;*
[3]*Department of Surgery, University of California, San Diego, CA, U.S.A.;*
[4]*Department of Urology, School of Medicine, International University of Health and Welfare, Narita, Japan*

Abstract. *Background/Aim: Dedifferentiated liposarcoma (DDLS) is a type of soft-tissue sarcoma with a poor prognosis due to distant metastasis and resistance to chemotherapy. The antimalarial drug chloroquine (CQ) can induce apoptosis in cancer cells. CQ in combination with rapamycin (RAPA), an mTOR inhibitor, has shown efficacy on osteosarcoma and other types of cancer. In the present study the efficacy of RAPA combined with CQ on the treatment of a DDLS patient-derived orthotopic xenograft (PDOX) model was investigated. Materials and Methods: A patient-derived DDLS was transplanted into the left retroperitoneum of nude mice to establish a DDLS PDOX nude-mouse model. The mice were randomly divided as follows: untreated control group; CQ group; RAPA group; combined CQ and RAPA group (n=7 for all groups). During the treatment period, tumor volume was measured every 3-4 days with calipers. After 2 weeks treatment, the mice were sacrificed, and H&E staining was performed for histological evaluation. The TUNEL assay was performed to detect apoptosis. Results: The combination of CQ and RAPA arrested tumor growth in the DDLS PDOX compared to the untreated control (p=0.009) and was significantly more effective than RAPA alone (p=0.009). RAPA alone slowed tumor growth, but the difference was not statistically significant (p>0.05). CQ was not active alone (p>0.05). The number of apoptotic TUNEL-positive cells was significantly higher in the CQ plus RAPA group than in the other groups (p=0.02). Conclusion: Combination therapy with CQ and RAPA arrested tumor growth in a DDLS PDOX model by inducing apoptosis.*

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer Inc, 7917 Ostrow St, San Diego, CA, 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com

Key Words: Dedifferentiated liposarcoma, PDOX, patient-derived orthotopic xenograft, combination therapy, mTOR inhibitors, rapamycin, chloroquine, apoptosis.

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Dedifferentiated liposarcoma (DDLS) has a poor prognosis due to distant metastasis and resistance to chemotherapy (1, 2). For patients with recurrent or metastatic dedifferentiated liposarcoma, ifosfamide and doxorubicin are first-line treatment (3-7), but with limited efficacy (1).

Our laboratory established the technique of surgical orthotopic implantation in 1988, pioneering the patient-derived orthotopic xenograft (PDOX) mouse model (8, 9). We have shown that the PDOX mouse model, unlike the subcutaneous PDX mouse model, retains the metastatic potential of the original tumor after transplantation in nude mice (9, 10).

Rapamycin (RAPA), a mammalian target of rapamycin (mTOR) inhibitor, is used in transplant medicine as an immunosuppressive agent (11), as well as for treatment of renal cell carcinoma (12). mTOR plays a central role in the regulation of various cellular functions such as survival and proliferation (13). We have previously demonstrated the anticancer effects of mTOR inhibitors in combination with multiple agents in PDOX mouse models of osteosarcoma (14, 15).

Chloroquine (CQ) is an antimalarial drug developed in Germany in 1934 (16). CQ is an autophagy inhibitor and can induce apoptosis in cancer cells (17). Previous studies have demonstrated the efficacy of CQ in combination with cancer-chemotherapy drugs in metastatic prostate cancer cell lines and hepatocarcinoma cell lines *via* increased apoptosis (18, 19). The combination of CQ and the mTOR inhibitor temsirolimus has shown efficacy in solid tumors and melanoma, in a Phase I clinical trial (20).

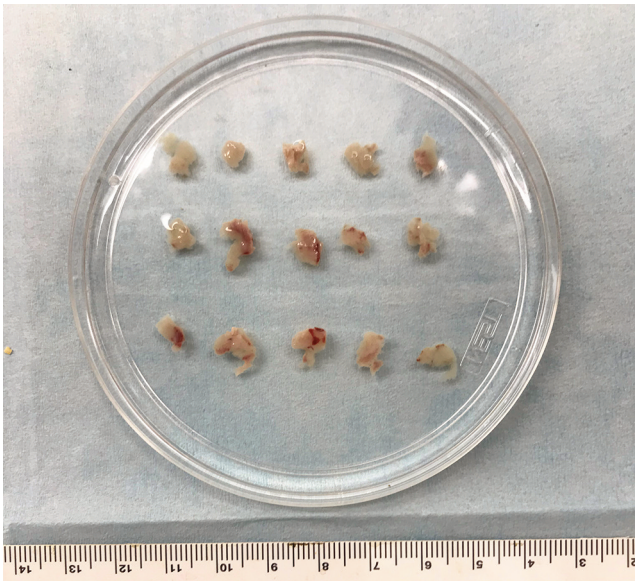

Figure 1. *Minced fragments of dedifferentiated liposarcoma tumor tissue and surrounding normal tissue prepared for orthotopic implantation.*

In the present study, we established a PDOX mouse model of DDLS and investigated the efficacy of combination treatment with CQ and RAPA.

Materials and Methods

Animals. Athymic (nu/nu) nude male mice, 4-6 weeks old, (AntiCancer, Inc., San Diego, CA, USA) were used under an AntiCancer, Inc. Institutional Animal Care and Use Committee (IACUC) protocol. This was specifically approved for the present study, which followed the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals, Assurance No. A3873-1.

Patient-derived tumor. The DDLS surgical specimen was from a primary retroperitoneal DDLS in a male in his 80s. The patient previously underwent surgical resection. The patient's tumor was provided as a discarded pathology specimen at the Department of Surgery, University of California, Los Angeles, CA, USA (UCLA). All experiments were performed in accordance with the Declaration of Helsinki and regulations on human research. Informed consent was obtained from the patient as part of a UCLA Institutional Review board approval protocol to obtain the tumor specimen (IRB #10-001857).

Establishment of a DDLS PDOX nude-mouse model. The tumor was minced to approximately 40 mm^3 in size (Figure 1). All surgical procedures were performed under anesthesia induced by a ketamine mixture. After anesthesia, a 1.5 cm incision was made in the left retroperitoneum (Figure 2A), and a 40 mm^3 tumor was implanted on the retroperitoneum in the back of the kidney (Figure 2B). The wound was closed with 5-0 nylon sutures (Figure 2C). The tumor grew to 50-100 mm^3 in size within 10 days of implantation and could be measured through the skin (Figure 2D).

Reagents. Rapamycin (HY-10219) (MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in phosphate-buffered saline (PBS) with 4.8% Tween 80, 4.8% polyethylene glycol 400 and 4% ethanol. Chloroquine diphosphate (C6628) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS.

Treatment schedule. Ten days after orthotopic transplantation, treatment was initiated when tumor volume reached 50-100 mm^3 and continued for 15 days. The DDLS-PDOX mouse model was randomly assigned to four groups of 7 mice per group as follows: untreated control; CQ [100.0 mg/kg/day, intraperitoneal (*i.p.*) injection]; RAPA (1.0 mg/kg/day, *i.p.* injection); combination of CQ (100.0 mg/kg/day, *i.p.* injection) and RAPA (1.0 mg/kg/day, *i.p.* injection) (Figure 3).

The short and long axes of the tumors were measured using calipers, and the mice were weighed once a week. Tumor volume was determined as (short-axis diameter)^2×long-axis diameter×0.5. All mice were sacrificed on day 15 after administration. Tumors were then removed for histological evaluation.

Hematoxylin and eosin staining. Specimens were prepared and stained according to the standard hematoxylin-eosin protocol for histo-pathological evaluation (21).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Apoptosis was assessed using the One-step TUNEL In Situ Apoptosis Kit (Green, FITC) (E-CK-A320) (Elabscience, Houston, TX, USA). Fluorescence images were obtained using a IX71 microscope (Olympus Corporation, Tokyo, Japan). Six views were randomly selected from two tumor sections per group. The cells were observed under the fluorescence microscope at 200× magnification. Positive cell counts were expressed as mean±standard error of the mean (SEM).

Statistical analysis. EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), was used to perform all statistical analyses (22). The parametric test for between-group comparisons was the Tukey-Kramer HSD. Nonparametric tests were conducted using the Kruskal-Wallis test, and comparisons between groups were evaluated using the Steel-Dwass technique. Graphs show means±standard deviation (SD) or SEM. A p-value ≤0.05 was defined statistically significant.

Results

Treatment efficacy on DDLS-PDOX. The combination of CQ and RAPA arrested tumor growth in the DDLS PDOX compared to the untreated control (p=0.009) and was significantly more effective than RAPA alone (p=0.009). RAPA alone slowed tumor growth, but the difference was not statistically significant compared to the untreated control (p=0.2). CQ was not active alone (Figure 4). There was no significant difference in mouse body weight among the four groups (Figure 5).

Histology of DDLS-PDOX. PDOX tissue in the untreated control and CQ alone consisted of dysplastic cells (Figure 6A and B). Treatment with RAPA alone as well as with the

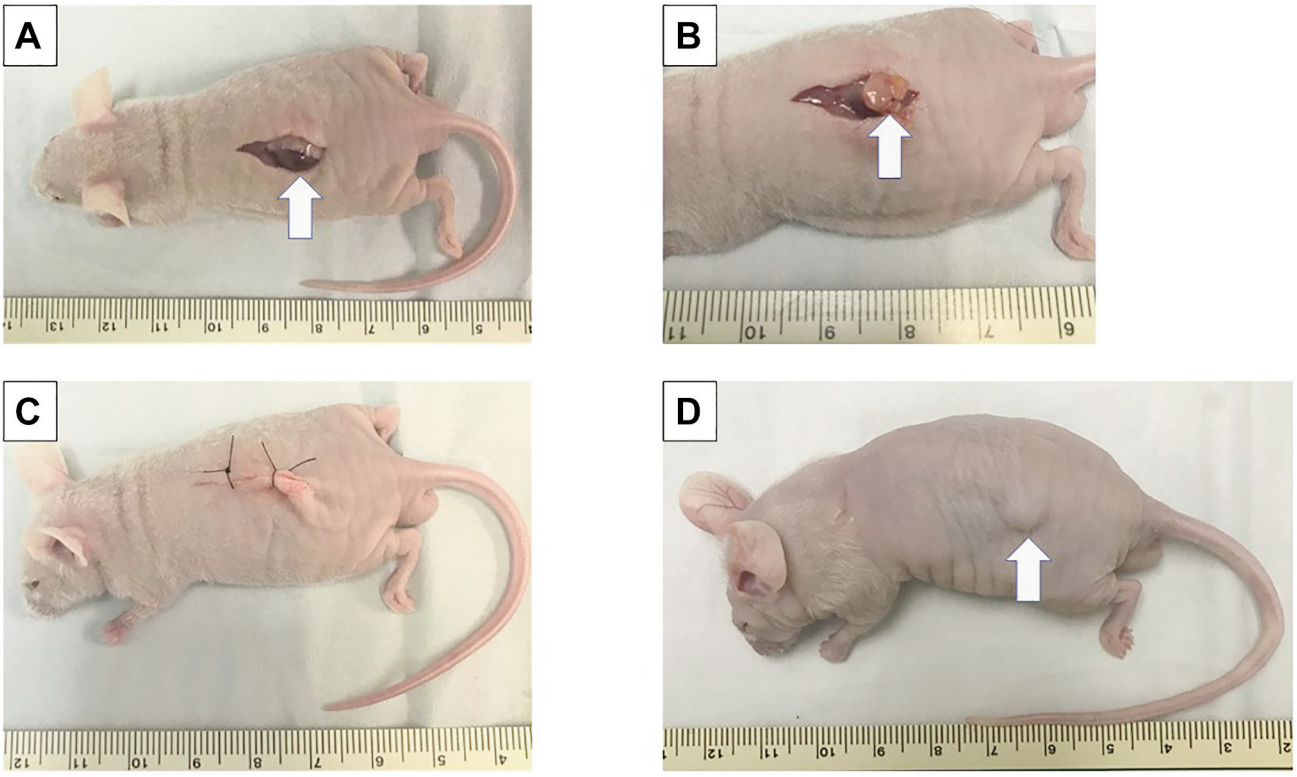

Figure 2. *Establishment of a dedifferentiated liposarcoma patient-derived orthotopic xenograft model. (A) Left retroperitoneal incision of approx. 1.5 cm. White arrow indicates left kidney. (B) The tumor was sutured to the fatty tissue of the retroperitoneum in the back of the kidney with an 8-0 nylon suture. White arrow indicates the implanted tumor fragment. (C) The wound was closed with 5-0 nylon sutures. (D) White arrow indicates tumor grown to approximately 70 mm^3, 10 days after transplantation.*

combination of CQ and RAPA showed fibrotic tissue with reduced cancer cell density (Figure 6C and D).

TUNEL assay of DDLS-PDOX. CQ combined with RAPA induced more apoptotic TUNEL-positive cells (24.33 cells) than the untreated control (3.33±1.60 cells); CQ alone (6.00±1.54 cells); and RAPA alone (7.00±1.93 cells) (p=0.02 for each comparison) (Figure 7 and Figure 8).

Discussion

Soft tissue sarcomas (STS) are rare mesenchymal malignant tumors that account for less than 1% of all adult malignancies. Liposarcoma is the most common histologic type of STS (23). In contrast to well-differentiated liposarcoma, which is less metastatic, the clinical behavior of DDLS is more aggressive, with a larger tendency for local recurrence and metastasis (2, 23). The five-year survival rate of well-differentiated is approximately 90%, whereas that of DDLS is 28% (1, 24). Because STS is a rare cancer with a small number of patients, the development of new drugs has lagged behind that for other, more frequent cancer types

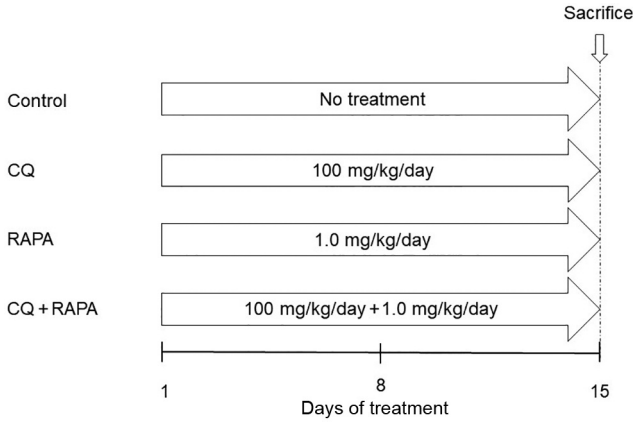

Figure 3. *Treatment scheme. CQ, Chloroquine; RAPA, rapamycin.*

(25). Although pazopanib, eribulin, and trabectedin have been used to treat STS since 2010 (26-28), antitumor drugs such as doxorubicin and isophosphamide, developed in the 1970s and 1980s, are still the standard first-line treatment for STS (3-7).

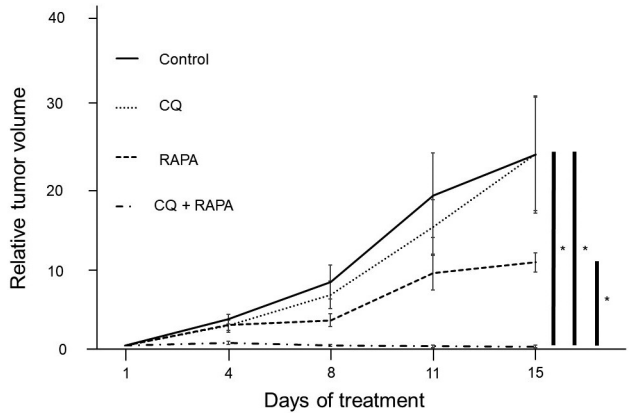

Figure 4. *Efficacy of drugs on the dedifferentiated liposarcoma patient-derived orthotopic xenograft model. Line graphs show tumor volume at the indicated times relative to that at the start of treatment. *p<0.05. Error bars: ±SEM. CQ, Chloroquine; RAPA, rapamycin.*

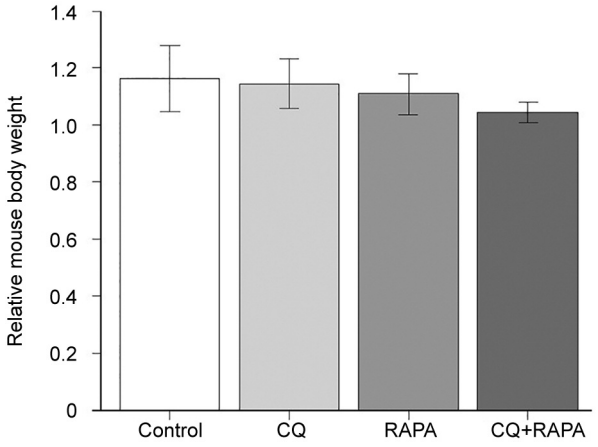

Figure 5. *Effect of drugs on relative mouse body weight. Bar graphs show body weight of mice from each group at day 15 relative to day 1 of treatment. Error bars: ±SD. CQ, Chloroquine; RAPA, rapamycin.*

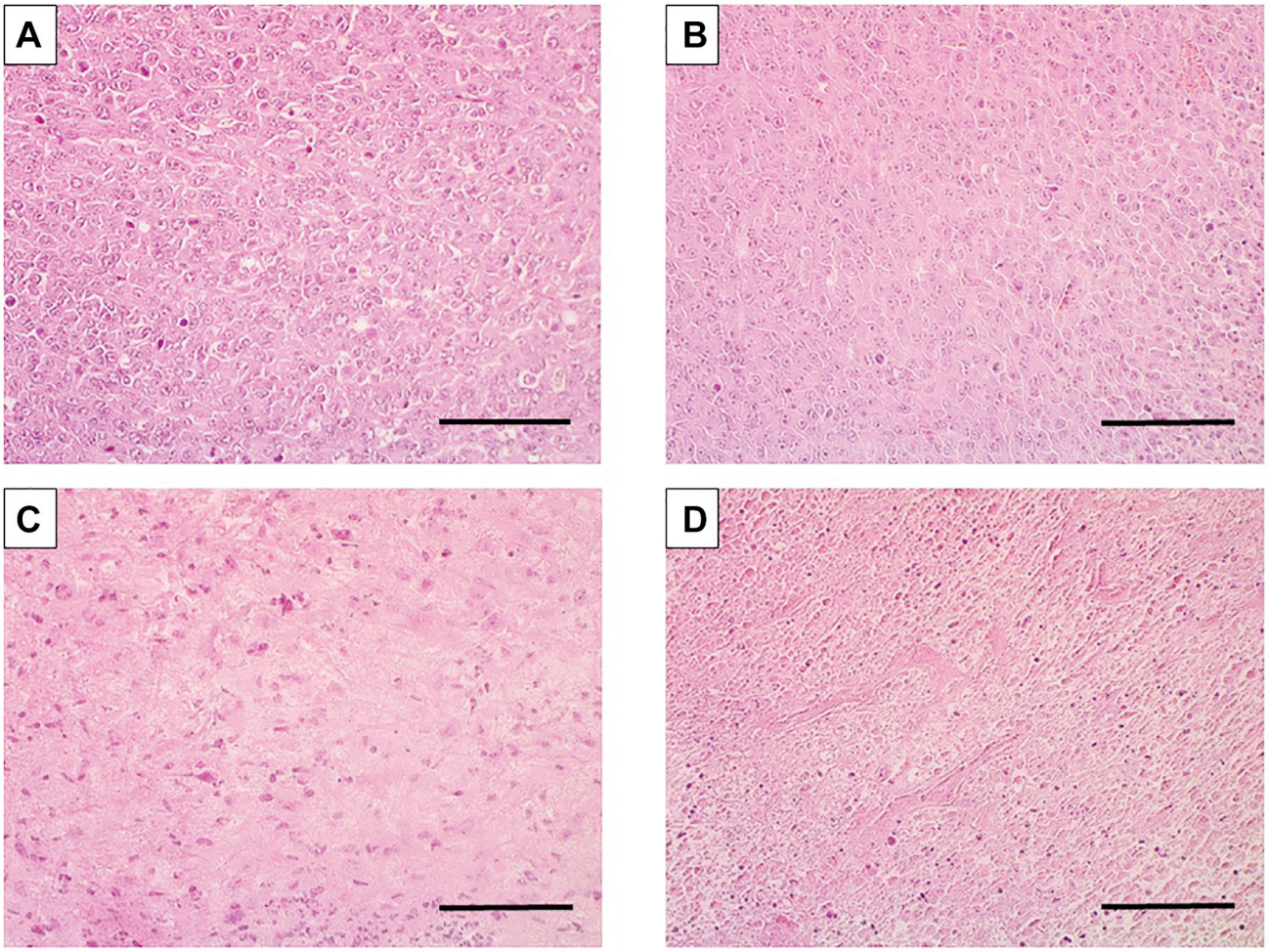

Figure 6. *Representative photomicrographs of H&E-stained dedifferentiated liposarcoma patient-derived orthotopic xenograft PDOX tissue sections. (A) Untreated control. (B) CQ-treated. (C) RAPA-treated. (D) Combination of CQ and RAPA. Magnification: 200×. Scale bar: 100 μm. CQ, Chloroquine; RAPA, rapamycin.*

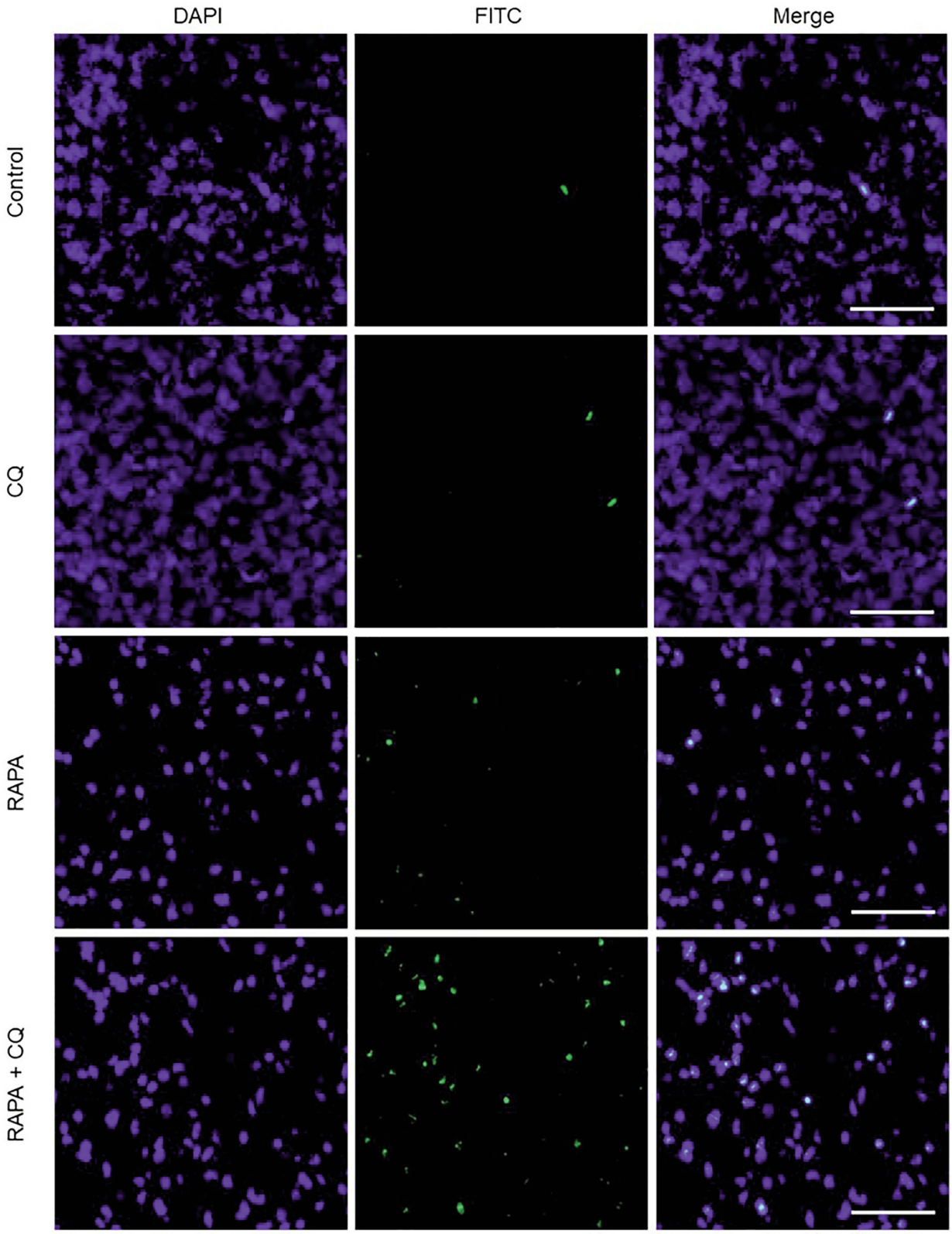

Figure 7. *TUNEL assay of apoptosis in the dedifferentiated liposarcoma patient-derived orthotopic xenograft tissue sections. 4',6-Diamidino-2-phenylindole (DAPI) (blue) indicates cell nuclei and fluorescein isothiocyanate (FITC) (green) indicates apoptotic cells. Magnification: 200×. Scale bar: 25 μm. CQ, Chloroquine; RAPA, rapamycin.*

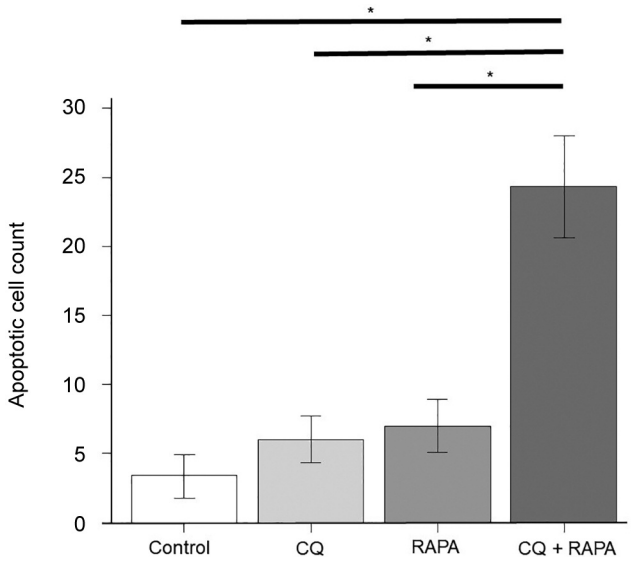

Figure 8. *Apoptotic cell count by TUNEL assay. Cells were counted from 6 high-power microscopic fields from 2 TUNEL-stained tissue sections. *p<0.05. Error bars: ±SEM. CQ, Chloroquine; RAPA, rapamycin.*

Our previous studies have shown the efficacy of combining mTOR inhibitors with other antitumor agents against the PDOX mouse model of osteosarcoma (14, 15). In the present study, a PDOX mouse model of DDPL was established and was used to evaluate the anticancer efficacy of the mTOR inhibitor RAPA and the antimalarial drug CQ. The results demonstrated that the combination of CQ and RAPA was highly effective against the PDOX model of DDLP.

The PI3K/AKT/mTOR signaling system is activated in STS such as liposarcoma, Ewing's sarcoma, rhabdomyosarcoma, and leiomyosarcoma (29-32). mTOR inhibitors are thought to have anticancer efficacy by reducing the activity of the signaling pathway and arresting the cell cycle in G_1 phase (33). In the present study, however, RAPA alone had no significant tumor-suppression effect.

CQ can induce apoptosis of cancer cells by inhibiting autophagy (17). Autophagy is an important physiological mechanism that controls the breakdown of proteins and organelles and maintains homeostasis (34). In cancer cells, autophagy promotes their proliferation by recycling accumulated metabolites and positively regulates cancer cell metabolism (35). CQ appears to accumulate in lysosomes and may inhibit proteolysis of proteins that are imported into the cell by endocytosis and induce apoptosis in cancer cells (17, 35). For example, knockdown of LYSET, which is involved in targeting catabolic enzymes into lysosomes, greatly inhibits proteolysis in lysosomes, depriving the cancer cell of essential amino acids, including methionine, to which DDLS is addicted (36), and was shown to inhibit tumor growth (37).

In the present study, CQ alone did not significantly increase the number of apoptosis-positive cells and had no tumor suppressive effect. However, the combination of CQ and RAPA was found to significantly increase apoptosis-positive cells and enhance tumor suppression.

The efficacy of combining autophagy inhibitors with antineoplastic agents has been previously studied, and there are several reports that the combination can enhance apoptosis induction (18-20, 38-44). However, there were no reports that the combination of CQ and RAPA induces apoptosis and enhances tumor suppression *in vivo*, especially in a PDOX mouse model of DDLS. The present study showed for the first time that the combination of CQ and RAPA is effective against DDLS i*n vivo*. This indicates CQ plus RAPA may be a potent clinical treatment for DDLS.

Conclusion

Combination therapy with CQ and RAPA, both FDA approved drugs, arrested a DDLS PDOX and demonstrated clinical potential for DDLS, a recalcitrant cancer. The present results suggest that treatment of DDLS patients with the combination of CQ and RAPA may be effective.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study. AntiCancer Inc. uses PDOX models for contract research.

Authors' Contributions

N.M. conceived the study, N.M., Y.A. Y. K. and K.O. performed the experiments and J.M. and R.M.H. provided scientific advice. N.M. wrote the paper and R.M.H. revised the paper.

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