Suppression of Autophagy Sensitizes Osteosarcoma to mTOR Inhibition

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Research article

Keywords: mTOR, Autophagy, Osteosarcoma

Posted Date: July 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-41962/v1

Abstract

Aim

Osteosarcoma is some major health problem. We intended to investigate the role of Rapamycin and autophagy inhibition in the treatment of osteosarcoma.

Method

We conducted a series of in vitro studies using two osteosarcoma cell lines. Using genetic and pharmaceutical interventions we studied whether combined autophagy inhibition could sensitize osteosarcoma sales to a Rapamycin treatment. Proliferation, innovation, migration, and colony formation assays were performed.

Results

Osteosarcoma cells had low basal autophagy levels. Inhibition of mTOR only demonstrated moderate effects but induced increased autophagy levels, indicating possible resistance mechanism. Inhibition of both autophagy and mTOR axis synergistically inhibited proliferation, migration, invasion, and colony formation of osteosarcoma cells. The combination therapy induced apoptosis, which could be restored in part by NEC1.

Conclusion

Increased autophagy level was responsible for compromised effect of mTOR inhibition in osteosarcoma. Combination therapy using rapamycin and chloroquine held promise to the development of novel mortality.

Introduction

Osteosarcoma is the most common primary malignancy of bone in children[1]. The peak incidence is between 9 and 16 years and the most common sites are around the knee upper arm[2]. Typical symptoms include gradual onset of painful swelling, which however generally lacks specificity. Although early stage of osteosarcoma yields a good prognosis, this entity is rare and the majority is prone to be at advanced stage or metastatic with very poor survival[3].

Current treatment of osteosarcoma comprises complete surgical resection with adjuvant or neo-adjuvant therapies, most often the chemotherapy, which could entail long-term squeals in teenagers as the regime consists of multiple cytotoxic drugs[4]. Therefore in the era of precision medicine, targeted therapy specifically aiming at over-active pro-oncogenic pathways could bring hope to curing those diseases.

The mTOR axis functions as the signaling transduction hub in human cells and is frequently upregulated in a variety of cancers[5, 6]. The increased mTOR activity enables tumor cells to require constant energy supply for uncontrolled proliferation and metastasis[7]. Given its important role in cellular metabolism, targeted therapy using Rapamycin alone has been proven inadequate in multiple clinical scenarios, as resistance is soon gained to detour the mTOR signaling. One of the most critical mechanism for resistance is the activation of autophagy[8]. Via incubation of Ulk, mTOR constantly inhibits autophagic flux providing sales with more efficient energy supply[6]. When mTOR is inhibited, the inhibition of autophagy is removed and tumor cells could acquire less effective energy supply by degrading its cellular components to maintain survival[9]. Therefore, theoretically combined inhibition of mTOR and autophagy could more effectively inhibit tumor growth.

Thus far, there have been reports on the effect of Rapamycin in osteosarcoma, but there has been a dearth of reports on the combined mTOR/autophagy inhibition[10]. We intended to address the role of this combination in osteosarcoma in the current study, providing novel perspectives of treatments.

Materials And Methods

Cell lines and culture

The MG-63 and U-2 OS osteosarcoma cell lines were obtained from cell bank of Chinese Academy of Science and were cultured in DMEM media supplemented with 10% of fetal bovine serum. Establishment of stable ATG5 knockdown cell lines were conducted according to standard protocol[6]. Briefly, shRNAs for ATG5 were synthesized using sequences from TRC (http://www.broadinstitute.org/rnai/public/). Transduction of the vectors was performed by means of Fugene 6 and positive clones were selected with puromycin (1:5000).

Western blotting and Q-PCR

A Standard protocol for Western blotting was followed[11, 12]. Primary antibodies used in the current study included: phospho-S6, total S6, LC3, PARP, ATG5, Nrf2, KEAP2, and Actin used as internal reference, all purchased it from Cell Signaling. Proteins were incubated with primary antibody overnight and were subject to donkey-anti-rabbit antibody and finalized with ECL (SuperSignal West Pico). Quantitative PCR for expression level of NQO-1 was performed using sequences as follows: for NQO1, forward ATG TAT GAC AAA GGA CCC TTC C, reverse TCC CTT GCA GAG AGT ACA TGG; for GAPDH as internal reference, forward CTG ACT TCA ACA GCG ACA CC, reverse TGC TGT AGC CAA ATT CGT TGT. After total RNA was extracted from cells, reactions were conducted according to protocols by PrimeScript kit and samples were analyzed using the SYBR Green Premixkiet on ABI 7500 system.

Proliferation assay

In the current study, the crystal violet assay was used to profile the proliferation of osteosarcoma cells following and established protocol[6]. Briefly, cells were treated with Rapamycin or chloroquine at set time

point and were stained using crystal violet, which was later dissolved using methanol. Plates were subject to a plate reader detecting absorption at 540 nm of wavelength.

Migration and invasion assay

The upper chamber of Transwell inserts either coated or uncoated with Matrigel were used for invasion and migration assays, respectively. Cell were cultured inside the upper chamber for 72 hours and were later subject to crystal violet staining at the lower surface of the inserts. The penetrated cells were counted.

Colony formation assay

The colony formation assay was used to profile the anchorage-independent growth of tumor cells. Briefly, cells were cultured in the middle gel layer composed of mixed media and noble agar, with complete media on top and gel with higher percentage of agar below. The top layer was changed with fresh media every 3 days and colonies were stained using crystal violet after two weeks.

Statistical analysis

All assays were run in triplicates and all experiments were conducted in three biological replicates. The Students t-test was used to study the means between two groups. The P value of < 0.05 was accepted as statistically significant.

Results

Osteosarcoma has low basal autophagy level and high mTOR activity

In order to validate our speculation that combined inhibition provided synergistic effect, we studied basal levels of autophagy and mTOR activity in osteosarcoma cells. In the current study, we used HK2 kidney cells as negative control and RCC4 renal cell carcinoma cells as positive control. HK2 cells were reported to have both low autophagy and mTOR activity, where as RCC4 cells were known for high basal autophagy and mTOR activity[13]. We have shown that both osteosarcoma cell lines were characterized with a low basal autophagy and high basal mTOR activity, indicating an mTOR-dependent regulatory axis in osteosarcoma (Fig. 1A-B). Inhibition of mTOR activity using Rapamycin substantially increased autophagy in both osteosarcoma cells (Fig. 1C). By blocking ATG5, which was one off the key machineries of autophagic flux, we showed that autophagy was substantially inhibited in osteosarcoma cells (Fig. 1C-D). Here we have shown that increased autophagy subject to Rapamycin could be a mechanism for resistance in osteosarcoma cells.

Combined incubation of both mTOR and autophagy synergistically suppressed osteosarcoma

We then instigated it weather combination therapy using Ramapycin and pharmaceutical or genetic inhibition of autophagy could show synergy in osteosarcoma cells. We found that monotherapy with Rapamycin solely reached limited inhibitory effect with an inhibitory rate of ~ 10–30% pending different osteosarcoma cells (Fig. 2A-B). Nonetheless, combined inhibition using the shRNA against ATG5 and Rapamycin reached synergistic inhibition in cell proliferation (Fig. 2A-B). We further tested pharmaceutical inhibition of autophagy using chloroquine and found that pharmaceutical autophagy inhibition also reached synergistic effect in combination with Rapamycin (Fig. 2C-D).

Combined inhibition induced apoptosis and oxidative stress in osteosarcoma

We then investigated the possible mechanism of how combined inhibition could contribute to synergistic effect. Using staurosporine as a positive control, we demonstrated that combined inhibition induced cell apoptosis either pharmaceutically or genetically (Fig. 3A). Administration of the NEC1 in part restored the synergistic effect of combined inhibition (Fig. 3B). We have also shown that along with prolonged combined treatment time the NRF2 level gradually increased and KEAP1 level gradually decreased in osteosarcoma cells, indicating the induction of intracellular oxidative stress (Fig. 3C). As expected, we have shown gradual decrease of the NQO-1 expression level following prolonged treatment of combination therapy in osteosarcoma (Fig. 3D). Finally, we investigated the EMT and anchorage-independent growth of osteosarcoma cells and we discovered that combined inhibition synergistically inhibited cell invasion, migration and colony formation of both cell lines, further supporting its role as a novel treatment modality (Fig. 4A-C).

Discussion

Autophagy plays a critical role in the progression and drug resistance of various cancers[14]. It has been reported that osteosarcoma Saos-2 cells lacking ATG4B, a cysteine proteinase that activates LC3B, have compromised autophagic machinery and fail to develop tumors in mouse models[15]. Small compounds like NSC185058 effectively inhibits ATG4B activity independent of mTOR activity and substantially halts the tumorigenesis of Saos-2 osteosarcoma. Such anti-tumor effect is conferred by reduction in ATG4B activity, as the compound had no effects on oncogenic protein kinases. This study proves that inhibition of autophagy itself could already show strong inhibition on osteosarcoma indicating that osteosarcoma is to certain extent addicted to autophagic process.

Strongly tied to autophagy, the signaling mTOR pathway also contributes to cell proliferation and chemoresistance of many cancers[16]. Rapamycin, as an inhibitor of mTOR, test now being used into targeted therapy in a variety of cancers. It has been reported that Rapamycin could induce autophagy in osteosarcoma and in another recent study investigating the small molecule inhibitor of autophagy Spautin-1 in the rapamycin-induced apoptosis in MG63 cells, authors reported that combined rapamycin and spautin-1 induced the apoptosis of MG63 cells[17].

Similar to the aforementioned results, our study also shows that rapamycin alone is not sufficient to inhibit osteosarcoma and to combined autophagy inhibitor offers synergistic effect. To date, this is the first to report bringing about chloroquine as the autophagy inhibitor in the combined therapy setting. Unlike Spautin-1, chloroquine has been used in clinical practice for a long time. The combination of chloroquine with rapalogs has been tested in multiple clinical trials[18–20].

In our study, we also found that combined therapy induced apoptosis in osteosarcoma cells, which was validated in another recent report. Autophagy has been suggested as a protective mechanism when cells are deprived of essential signaling transduction, such as mTOR axis. Inhibition of autophagy is thus largely dependent on the tumor context, as theoretically only cancers with low basal autophagy level and high basal mTOR activity could be synergistically inhibited by combined therapy. In our study, we have demonstrated basal autophagy level in osteosarcoma is low and subject of change of mTOR activity, giving the rationale for the combination therapy.

To sum up, Increased autophagy level was responsible for compromised effect of mTOR inhibition in osteosarcoma. Combination therapy using rapamycin and chloroquine held promise to the development of novel mortality.

Abbreviations

OS (osteosarcoma)

mTOR (mammalian target of rapamycin)

Declarations

Ethical Approval and Consent to participate:

Written informed consent was obtained from all participants and the study protocol conforms to the Ethical Review Waiver Policy of Wuxi No.2 People's Hospital, Nanjing Medical University after reviewed by the Ethical Committee of Wuxi No.2 People's Hospital, Nanjing Medical University.

Consent for publication:

Not applicable.

Availability of supporting data

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests:

None

Funding Acknowledgement

This study was supported by the grants from Wuxi Youth Talent Fund (QNRC081).

Authors' contributions:

Xiao Yang and Guoxin Zhu carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Zhengjie Yang carried out the immunoassays. MT participated in the sequence alignment. Ke Zeng and Lei Jin participated in the design of the study and performed the statistical analysis. Guoxin Zhu conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement: The authors declare no conflict of interest in preparing this article.

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Figures

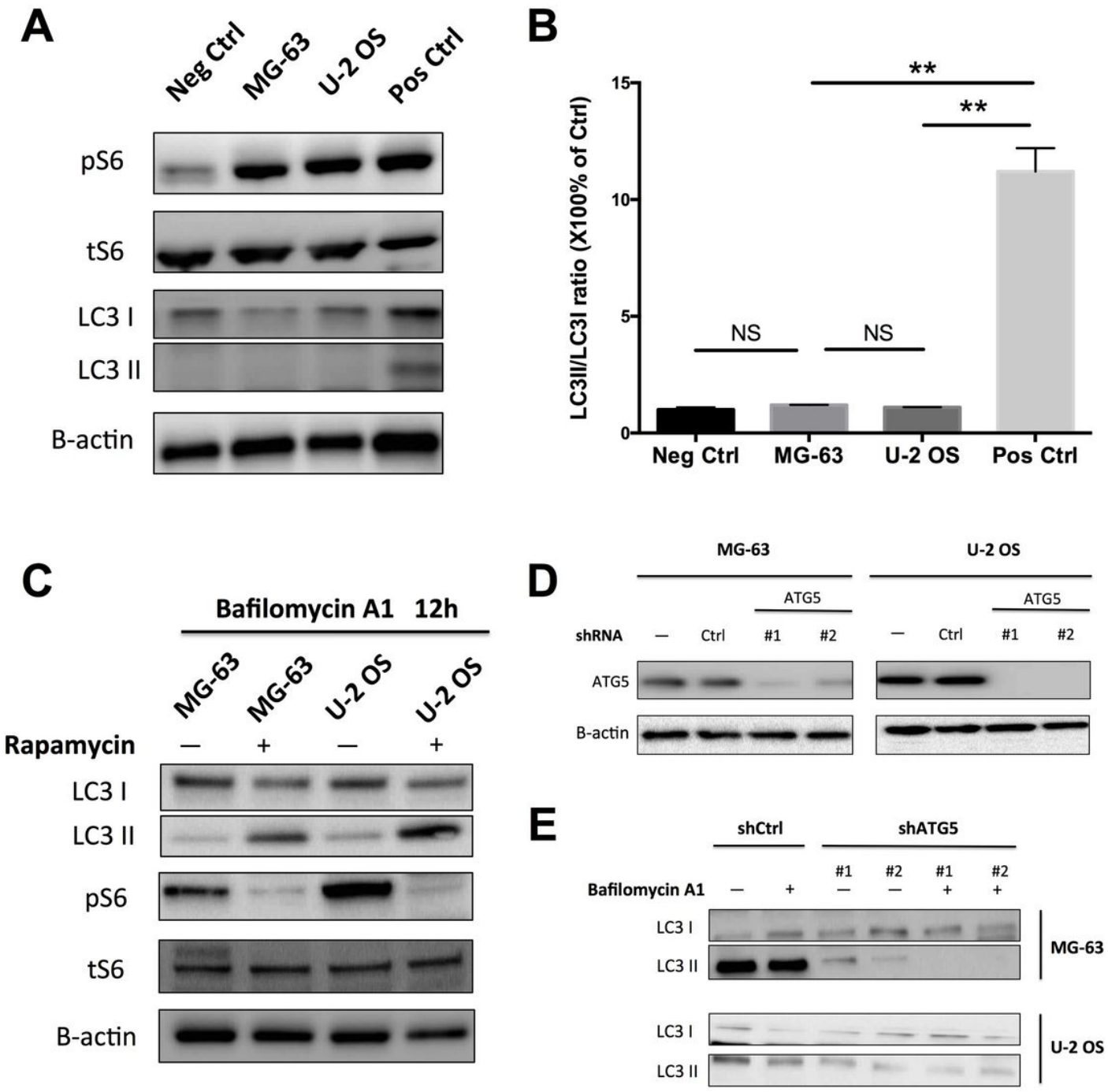

Figure 1

Low basal ontology level and high mTOR activity in osteosarcoma. A) Western belongs showing low basal autophagy level and high mTOR activity in osteosarcoma in relation to negative (HK2 cells) and positive (RCC4 cells) controls; B) autophagy level indicated by the LC3II to LC3I ratio; C) changes in autophagy and mTOR activity of osteosarcoma cells in response to Rapamycin (10 nM); D) Efficacy of shRNA against ATG5 in osteosarcoma cells; E) shATG5 substantially inhibited autophagy in osteosarcoma cells. (n = 3, **P < 0.01)

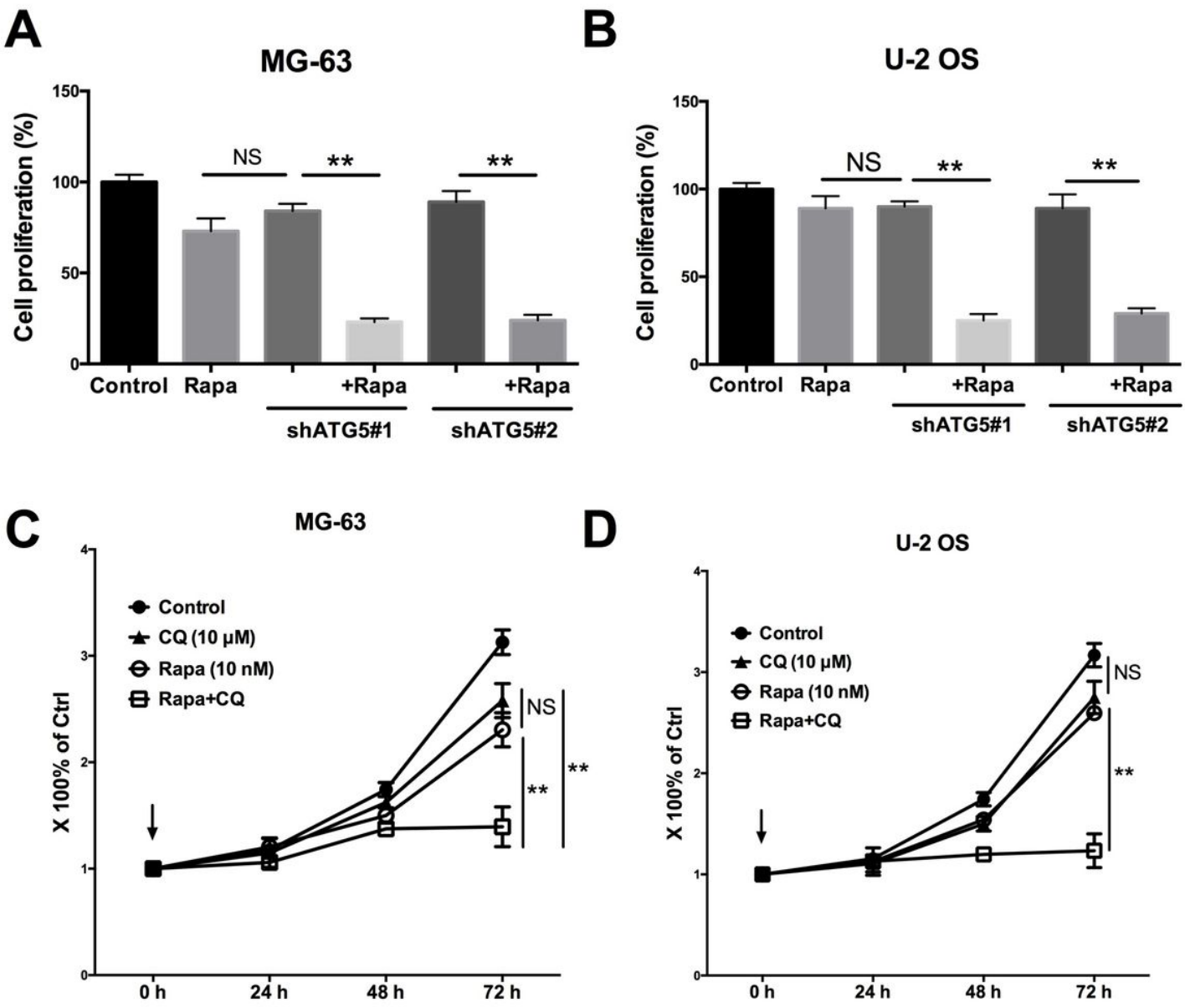

Figure 2

Combined autophagy and mTOR inhibition displayed synergistic effect. Genetic inhibition of autophagy in A) MG-63 and B) U-2 OS cells demonstrated synergistic effect with rapamycin (10 nM) in inhibiting proliferation at 72 h; Pharmaceutical inhibition of autophagy using chloroquine (10 μM) in C) MG-63 and D) U-2 OS cells demonstrated synergistic effect with rapamycin (10 nM) in inhibiting proliferation at 72 h. (n = 3, **P < 0.01)

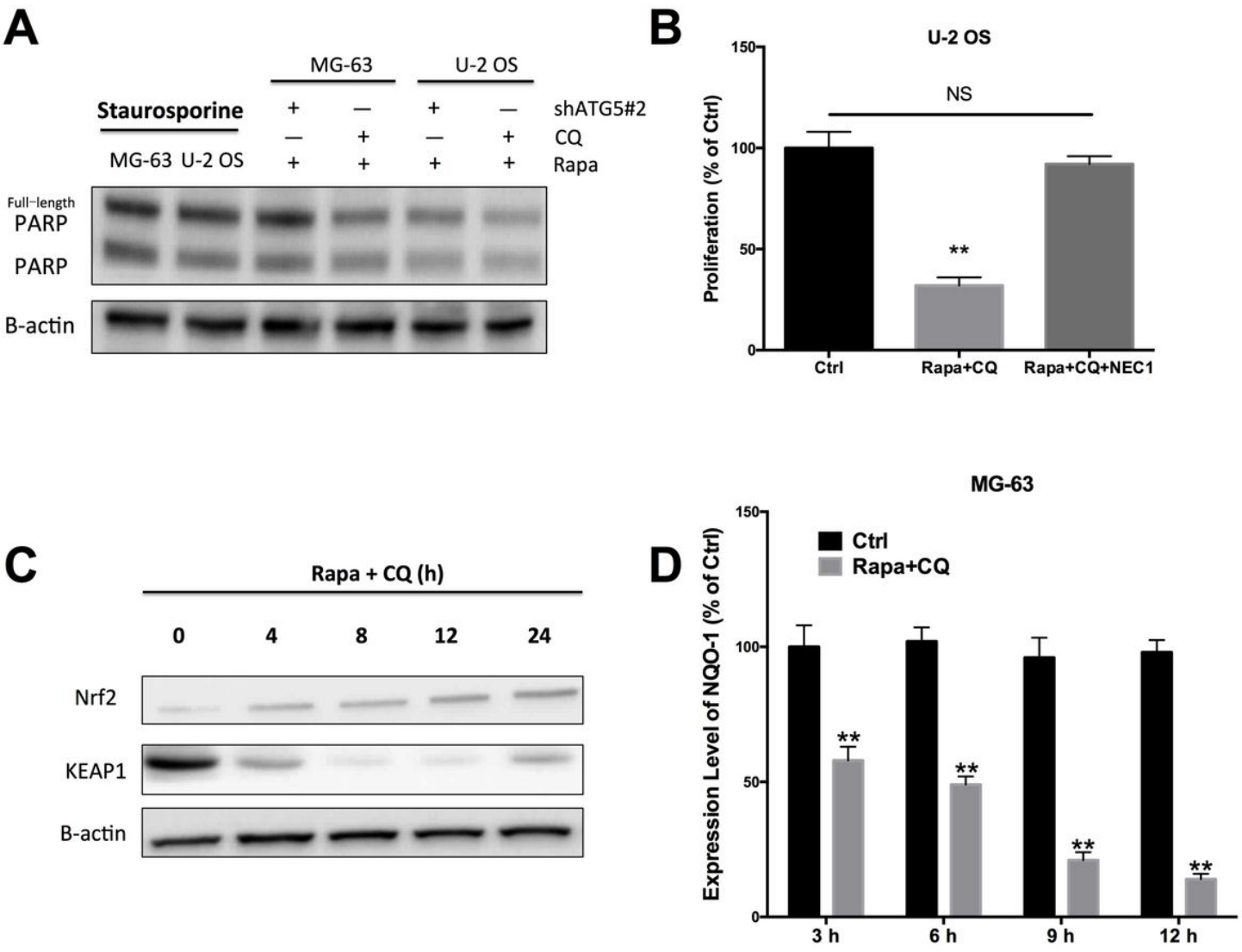

Figure 3

Combined autophagy and mTOR inhibition induced apoptosis. A) Both pharmaceutical and genetic inhibition of autophagy together with rapamycin induced apoptosis in osteosarcoma cells; B) NEC1 significantly restored inhibitory effect by combined therapy; C) Combined therapy induced increased Nrf2 and decreased KEAP1 level; D) Combined therapy induced decreased expression of NQO-1. (n = 3, **P < 0.01)

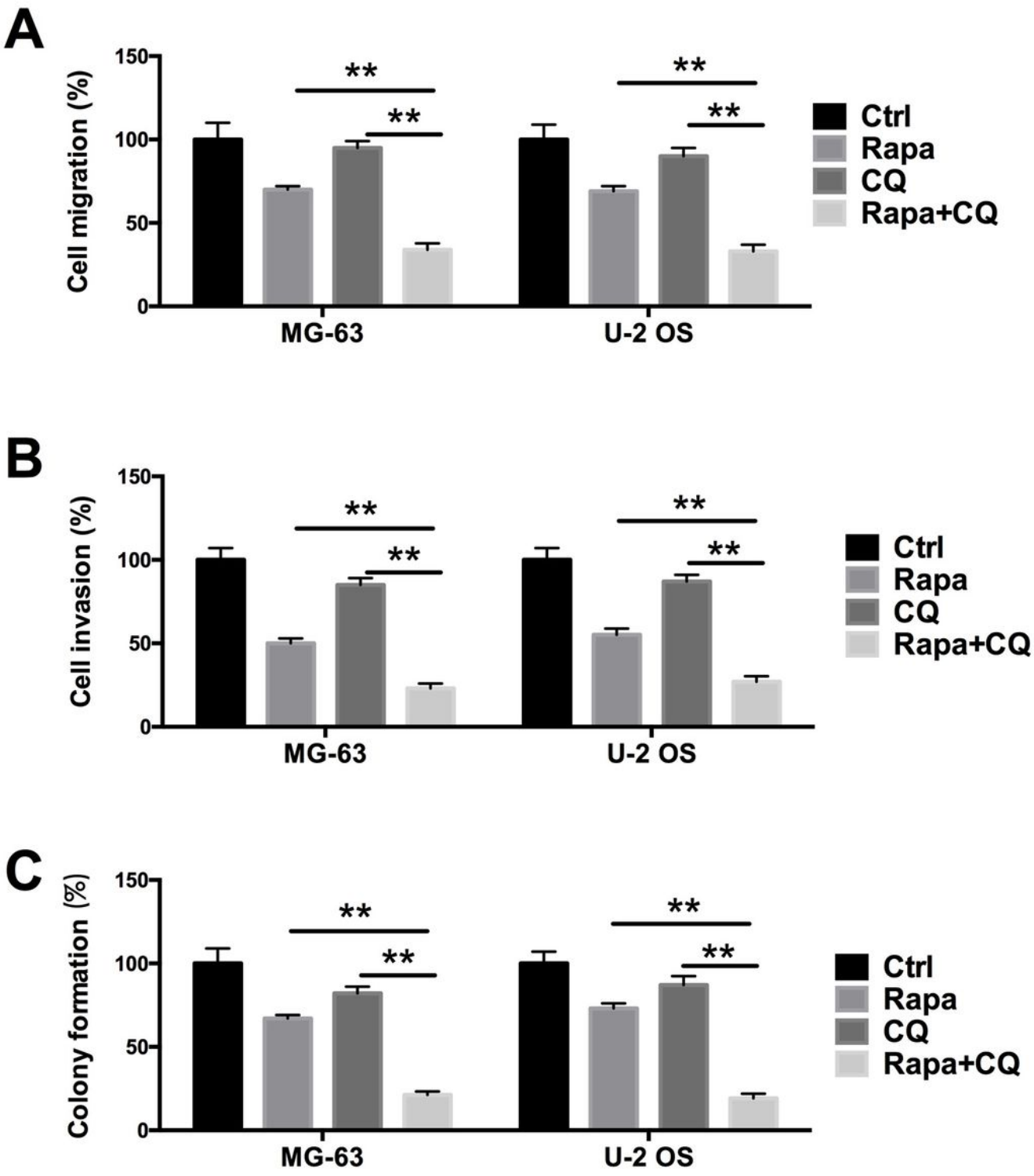

Figure 4

Combined autophagy and mTOR inhibition inhibited cell migration. Administration of both chloroquine (10 μM) and Rapamycin (10 nM) synergistically inhibited A) migration; B) invasion; and C) colony formation of both osteosarcoma cells.(n = 3, **P < 0.01)