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Original Article

Proteasomal deubiquitinating enzyme USP14/UCHL5 inhibitor bAP15 suppresses endoplasmic reticulum stress-mediated apoptosis and tumor growth in human chondrosarcoma

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Abstract: Chondrosarcoma is a malignant bone tumor with limited systemic options. Here, using the small-molecule probe bAP15 to inhibit proteasome-associated deubiquitinating enzymes (DUBs) USP14 and UCHL5, we evaluated the therapeutic concept of DUB inhibition in human chondrosarcoma. Immunohistochemistry showed higher USP14/UCHL5 expression in chondrosarcoma than in normal cartilage controls. In vitro, bAP15 increased Annexin V/PI-positive apoptosis and cleavage of caspase-3/PARP. Under our experimental conditions (low sub-micromolar exposure, 24-48 h), bAP15 led to a predominant accumulation of cells in G1, accompanied by p21 upregulation and reduced PCNA and phospho-histone H3. bAP15 was associated with lower phosphorylation of AKT/ERK and with the induction of ER-stress markers (GRP78, CHOP, IRE1α, caspase-4). In vivo, bAP15 suppressed xenograft growth. Collectively, these data support proteasome-associated DUB inhibition as a potential strategy in chondrosarcoma, with bAP15 serving as a chemical tool to probe this target class.

Keywords: Chondrosarcoma, bAP15, proteasomal deubiquitinating enzyme, endoplasmic reticulum stress

Introduction

Chondrosarcoma is the second most common primary bone malignancy and is characterized by the production of cartilaginous matrix [1]. High-grade chondrosarcomas have a poor prognosis, as they rarely respond to conventional chemotherapy or radiation [1, 2]. Complete surgical resection remains the mainstay of treatment, but this is often not feasible for advanced or metastatic disease [3, 4]. The lack of effective systemic therapies underscores an urgent need for novel treatment strategies [5, 6].

The ubiquitin-proteasome system (UPS) is a fundamental pathway for protein turnover that is frequently dysregulated in cancer [7, 8]. Deubiquitinating enzymes (DUBs) remove ubiquitin chains from substrates, rescuing them from degradation [9, 10]. Many DUBs are aberrantly expressed in tumors and can function as

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oncogenes or support cancer cell survival [10, 11]. In particular, the proteasome-associated DUBs USP14 and UCHL5 (UCH37) remove ubiquitin from proteasome-bound substrates, thereby repressing proteasomal protein degradation and modulating substrate residence at the 26S proteasome [12]. Overactivity of these DUBs can stabilize oncoproteins and has been implicated in cancer progression [12, 13]. Inhibition of proteasomal DUB activity was first reported to have anti-cancer effects by D'Arcy et al., who identified bAP15 as a 19S proteasome DUB inhibitor that targets USP14 and UCHL5 [12]. Unlike proteasome catalytic inhibitors (such as bortezomib), bAP15 blocks the deubiquitination step, leading to accumulation of polyubiquitinated proteins and proteotoxic stress in cancer cells [12]. bAP15 (also known as VLX1500) has shown promising anti-tumor activity in preclinical models of multiple malignancies, including multiple myeloma and carcinoma, often inducing apoptosis via ER stress and oxidative stress pathways [14-16]. Additionally, proteasome DUB inhibition can sensitize tumors to immune-mediated killing; for instance, bAP15 enhanced TRAIL-induced apoptosis by natural killer cells in an ovarian cancer model [15].

Given the aggressive nature of chondrosarcoma and the overactivation of pro-survival pathways in this tumor [2, 4], we hypothesized that targeting UPS function via USP14/UCHL5 inhibition could be an effective therapeutic approach. To date, the roles of USP14 and UCHL5 in chondrosarcoma and the potential of their inhibition have not been explored. In this study, we first examined USP14 and UCHL5 expression in human chondrosarcoma tissues. We then evaluated the anti-cancer efficacy of bAP15 in chondrosarcoma cell lines, focusing on its ability to induce apoptosis, cell cycle arrest, and ER stress. Finally, we assessed the in vivo therapeutic impact of bAP15 in chondrosarcoma xenograft models. Our findings reveal that bAP15 effectively suppresses chondrosarcoma growth through multi-faceted mechanisms, providing a strong rationale for DUB-targeted therapy in this currently untreatable sarcoma.

Materials and methods

Reagents and cell culture

The primary anticancer drug, bAP15 (#HY-13-989), was purchased from MedChemExpress. All other chemicals and reagents were purchased from Sigma-Aldrich or Merck KGaA. Two human chondrosarcoma cell lines were utilized in this study: JJ012 (kindly provided by Dr. Sean P. Scully, School of Medicine, Miami University, USA) and SW1353 (obtained from the Biological Resource Collection and Research Center of Taiwan). SW1353 cells were cultured in RPMI-1640 medium, while JJ012 cells were cultured in L-15 medium. Both media were supplemented with 10% fetal bovine serum, and cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 [16, 17]. All cell culture media and supplements were purchased from Corning.

Clinical samples and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples of chondrosarcoma (n=6) and normal cartilage (n=3) were collected following informed consent under an IRB-approved protocol. Tissue sections were immunostained using primary antibodies against ubiquitin-specific protease 14 (USP14; #MA5-32821, Thermo Fisher Scientific) and ubiquitin C-terminal hydrolase L5 (UCHL5; #11527-1-AP, Proteintech Group). Immunoreactivity was independently evaluated by a board-certified pathologist (Dr. Lin W.C.). Representative images highlighting staining intensity for USP14 and UCHL5 were captured for analysis. To increase transparency, we note that "normal cartilage" samples were unmatched controls rather than patient-matched tumor-adjacent cartilage; this limitation is considered further in the Discussion. All experiments involving human participants were approved by the National Taiwan University College of Medicine Institutional Research Ethics Committee (No. 202309012RIND).

Apoptosis analysis

Apoptotic cell death was quantified using the Muse Annexin V & Dead Cell Kit (Merck Millipore) according to the manufacturer's instructions. After 48 h treatment with bAP15 (0-0.4 μM), JJ012 and SW1353 cells were harvested and incubated with Annexin V-FITC and propidium iodide (Muse apoptosis kits, Merck Millipore). Stained cells were analyzed on a Muse flow cytometry analyzer (Merck Millipore) to determine the percentages of early and late apoptotic cells. Detailed procedures were performed according to previously published protocols and manufacturer's instructions [18].

Cell cycle analysis

Cell cycle distribution was examined by propidium iodide (PI) staining and flow cytometry. Chondrosarcoma cells were treated with bAP-15 or DMSO for 48 h, then fixed in 70% ethanol at -20°C and stained with PI/RNase A solution (Merck Millipore). DNA content was analyzed on a Muse flow cytometry analyzer and the fraction of cells in G1, S, and G2/M phases was quantified. All detailed procedures were conducted following previously described methods and the manufacturer's guidelines [19].

Western blotting

Treated cells were lysed in RIPA buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors. Equal amounts of protein (30-50 μg) were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% BSA in TBST, membranes were incubated overnight at 4°C with primary antibodies. The antibodies used included cleaved-caspase-3 (#9661), cleaved-PARP (#5625), phospho-JNK (#9255), p21 (#2947), CDC25C (#4688), phospho-histone H3 (#53-348), phospho-AKT (Ser473), ERK1/2 (#9695), phospho-ERK1/2 (Thr202/Tyr204, #4370), IRE1α (#3294), GRP78 (Bip, #3183), and caspase-4 (#4450) from Cell Signaling Technology, as well as antibodies targeting total-JNK (#sc-571) and AKT1 (#sc-1618) obtained from Santa Cruz Biotechnology. In addition, the β-actin (#109639) antibody was purchased from GeneTex Biotechnology. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Protein signals were detected using an ECL substrate (Millipore) and visualized using a Bio-Rad imaging system. Quantitative densitometric analysis was performed with ImageJ software. Western blot analyses followed established standard protocols as previously described [18].

In vivo xenograft model

A total of 5×10^5 JJ012 or SW1353 cells were suspended in 200 μL serum-free medium, then mixed with an equal volume of Matrigel (BD Biosciences). This cell suspension was injected subcutaneously into the dorsal side of 8-week-old male nude mice from the National Laboratory Animal Center of Taiwan. Once tumors reached approximately 150 mm^3, mice were randomly assigned to either a bAP15 treatment group, receiving intraperitoneal injections of 5 mg/kg bAP15 dissolved in normal saline twice weekly (Monday and Thursday) for 5 weeks, or a control group administered with DMSO solution in physiological saline. Tumor volumes were measured twice weekly using calipers and calculated by multiplying the longest diameter (LD) by the square of the shortest diameter (SD), then dividing by two. All animal experiments adhered to ARRIVE guidelines and were approved by the Institutional Animal Care and Use Committee (No. 20240023) of National Taiwan University College of Medicine.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between groups were evaluated by one-way ANOVA with Tukey's post-hoc test or two-tailed unpaired Student's t-test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Overexpression of USP14 and UCHL5 in chondrosarcoma

To investigate potential therapeutic targets for chondrosarcoma, we first examined the expression of the deubiquitinating enzymes UCHL5 and USP14. Immunohistochemical (IHC) staining revealed significantly higher levels of UCHL5 and USP14 in clinical chondrosarcoma samples compared to normal cartilage tissues. Quantitative analysis of IHC staining confirmed that UCHL5 (**Figure 1A**) and USP14 (**Figure 1B**) expression was significantly higher in chondrosarcoma tissues (n=3) than in normal cartilage (n=3). These findings indicate a pivotal role for these DUBs in chondrosarcoma progression and underscore their promise as therapeutic targets.

bAP15 induces apoptosis in chondrosarcoma cells

Given the observed overexpression of UCHL5 and USP14 in tumors, we next tested the efficacy of their inhibitor, bAP15, on chondrosarcoma cell survival. Flow cytometric analysis with Annexin V/PI staining showed that treat-

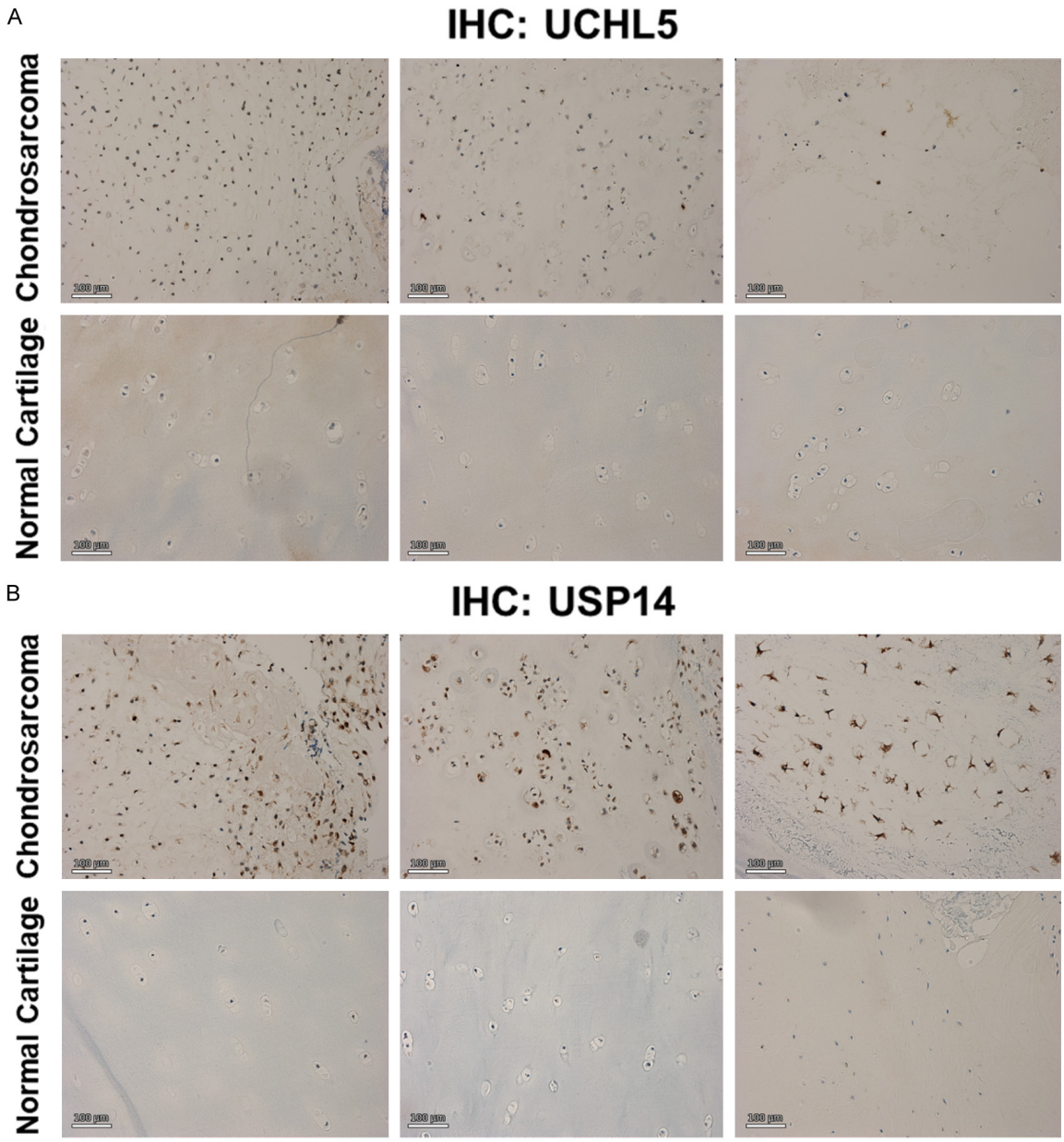

Figure 1. Overexpression of USP14 and UCHL5 in human chondrosarcoma tissues. A. Representative immunohistochemical staining for USP14 and UCHL5 in normal cartilage versus chondrosarcoma tissue. Chondrosarcoma samples show strong positive staining for USP14 and UCHL5 (brown) in tumor cells, whereas normal cartilage exhibits minimal staining. B. Quantification of IHC staining intensity confirming significantly higher USP14 and UCHL5 expression in chondrosarcoma tissues (n=3) compared to normal cartilage (n=3). Data are presented as mean ± SD. $P<0.05$.

ment with bAP15 (0, 0.2, 0.4 μM) for 48 hours induced apoptosis in JJ012 and SW1353 chondrosarcoma cells in a dose-dependent manner (**Figure 2A**). The percentage of Annexin V-positive (apoptotic) cells increased markedly with bAP15 treatment compared to vehicle controls. Consistently, Western blot analysis confirmed apoptosis induction: bAP15 treatment led to elevated levels of cleaved caspase-3 and cleaved PARP, as well as increased phosphorylation of c-Jun N-terminal kinase (JNK), an apoptosis-related stress kinase (**Figure 2B**). β-Actin was used as a loading control. These results demonstrate that bAP15 effectively trig-

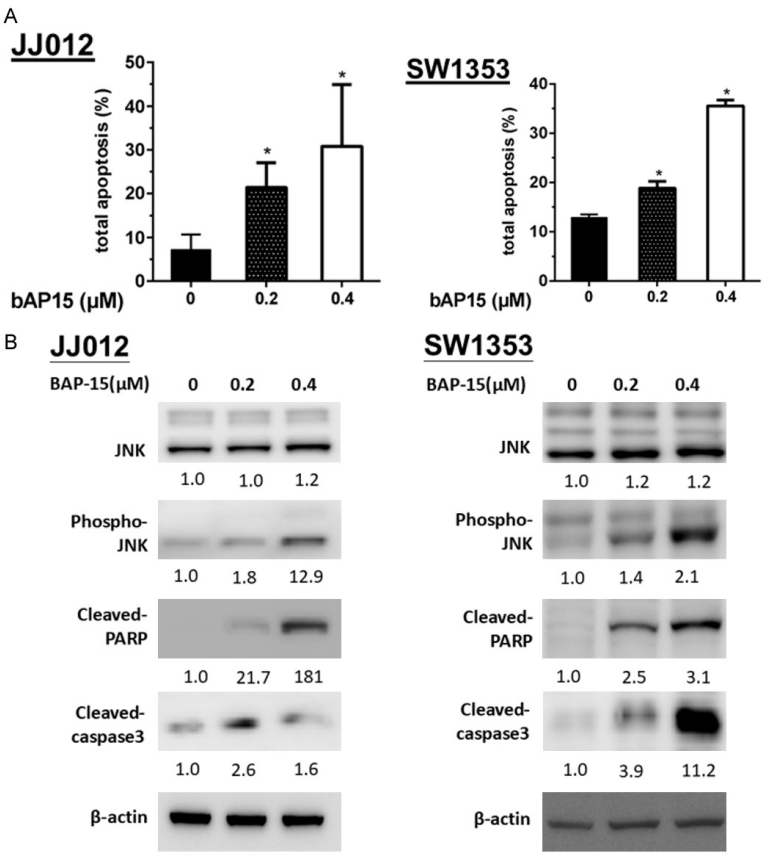

Figure 2. bAP15 induces apoptosis in chondrosarcoma cells in vitro. A. Flow cytometric analysis of apoptosis (Annexin V/PI staining) in JJ012 and SW1353 cells after 48 hours of bAP15 treatment (0, 0.2, 0.4 μM). bAP15 increases the proportion of Annexin V-positive (apoptotic) cells in a dose-dependent manner in both cell lines. Bar graphs indicate the percentage of early and late apoptotic cells at each dose, showing a significant increase in apoptosis compared to vehicle controls. B. Western blot analysis of apoptosis-related proteins in JJ012 and SW1353 cells treated with bAP15 for 48 hours. bAP15 treatment causes cleavage of caspase-3 and PARP and elevates phospho-JNK levels, confirming activation of apoptotic pathways. β-Actin serves as a loading control. Data are representative of three independent experiments. $P<0.05$ vs control.

gers apoptotic cell death in chondrosarcoma cells.

bAP15 leads to a predominant G1 accumulation under our conditions

To further elucidate bAP15's anti-tumor mechanisms, we analyzed its effect on cell cycle progression. Flow cytometry showed that, under the exposure conditions used here (0-0.4 μM; 48 h), bAP15 predominantly increased the G1 fraction, with concomitant decreases in S and/or G2/M, and increased sub-G1 at later time points (**Figure 3A**). This G1-phase enrichment suggests that bAP15 causes cell cycle arrest.

Supporting this, Western blotting showed that bAP15 increased the expression of p21, a cyclin-dependent kinase inhibitor, and reduced the levels of G1/S and G2/M regulatory proteins such as CDC25C, PCNA, and phospho-histone H3 (**Figure 3B**). These changes are consistent with a G1 phase arrest. Notably, the reduction of PCNA, a key DNA replication factor, suggests that USP14/UCHL5 inhibition by bAP15 may facilitate PCNA degradation via the ubiquitin-proteasome system, thus contributing to cell cycle blockade. β-Actin was used as a loading control for these experiments. Together, these data indicate that bAP15 not only induces apoptosis but also halts cell cycle progression in chondrosarcoma cells.

bAP15 suppresses AKT and ERK signaling

We next examined whether bAP15 affects major pro-survival signaling pathways in chondrosarcoma. The AKT and ERK pathways are critical mediators of cell survival, proliferation, and chemo-resistance in many cancers, including chondrosarcoma [2]. Western blot analysis revealed that bAP15 treatment led to a marked, dose-dependent reduction in the levels of phosphorylated AKT (Ser473) and phosphorylated ERK1/2 (Thr202/Tyr204) in both JJ012 and SW1353 cells (**Figure 4**). Importantly, total AKT and total ERK protein levels remained unchanged, indicating that bAP15 specifically diminished the active (phosphorylated) forms of these kinases. This suppression of AKT/ERK signaling likely removes important survival signals from chondrosarcoma cells, thereby facilitating apoptosis induction. These results suggest that bAP15's anti-tumor effects involve, in part, the inhibition of key oncogenic pathways.

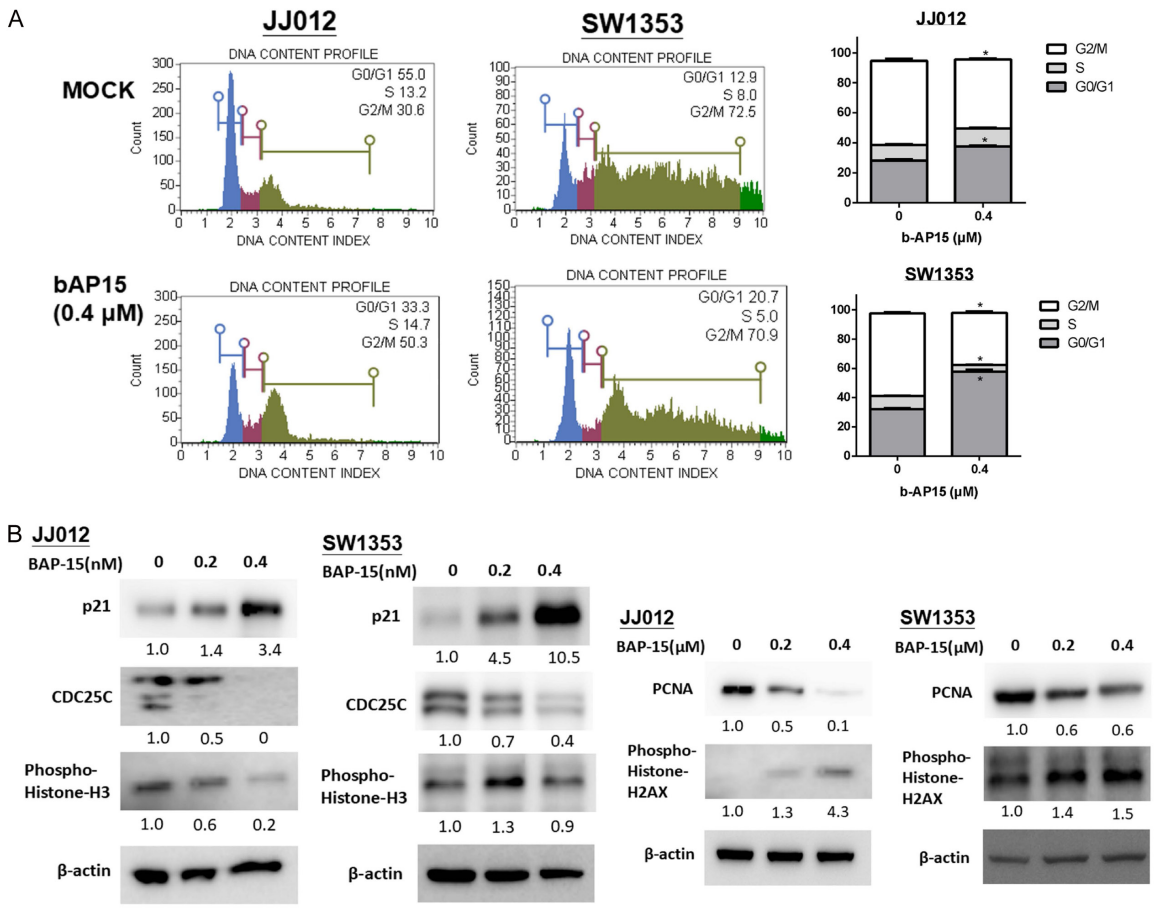

Figure 3. bAP15 induces G1 cell cycle arrest in chondrosarcoma cells. A. Cell cycle distribution of JJ012 and SW1353 cells treated with bAP15 (0, 0.2, 0.4 μM) for 48 hours. Propidium iodide DNA content analysis shows an increased G1 population and a corresponding decrease in S and G2/M populations in bAP15-treated cells, indicating G1 phase arrest. Bar graphs depict the percentage of cells in G1, S, and G2/M phases; $P<0.05$ compared to control for G1 fraction. B. Western blot analysis of cell cycle regulatory proteins in chondrosarcoma cells after bAP15 treatment. bAP15 upregulates p21 and downregulates CDC25C, PCNA, and phospho-histone H3, consistent with G1 arrest. β-Actin is shown as a loading control. These results demonstrate that bAP15 halts cell cycle progression at the G1 phase.

bAP15 triggers ER stress in chondrosarcoma cells

Proteasome inhibition is known to cause an accumulation of misfolded proteins in the endoplasmic reticulum, leading to ER stress and activation of the unfolded protein response [14]. We investigated whether bAP15 triggers ER stress in chondrosarcoma cells. Western blotting showed that bAP15 was associated with increased ER-stress markers (GRP78, CHOP, IRE1α, cleaved caspase-4), consistent with activation of ER-stress responses under our conditions - after 48 hours of bAP15 treatment in both JJ012 and SW1353 cells, compared to untreated cells (**Figure 5**). The induction of CHOP (a pro-apoptotic transcription factor) and caspase-4 (an ER stress-associated caspase) strongly suggests that unresolved ER stress contributes to bAP15-induced apoptosis. These findings identify ER stress activation as a key mechanism underlying the cytotoxic effect of bAP15 in chondrosarcoma cells.

bAP15 inhibits chondrosarcoma growth in vivo

To validate the therapeutic potential of bAP15 in vivo, we employed chondrosarcoma xenograft models. Nude mice bearing JJ012 or SW1353 tumor xenografts were treated with bAP15 (5 mg/kg, intraperitoneally, twice weekly) or vehicle control. Tumor growth was significantly inhibited in bAP15-treated mice compared to controls (**Figure 6**). By the end of the

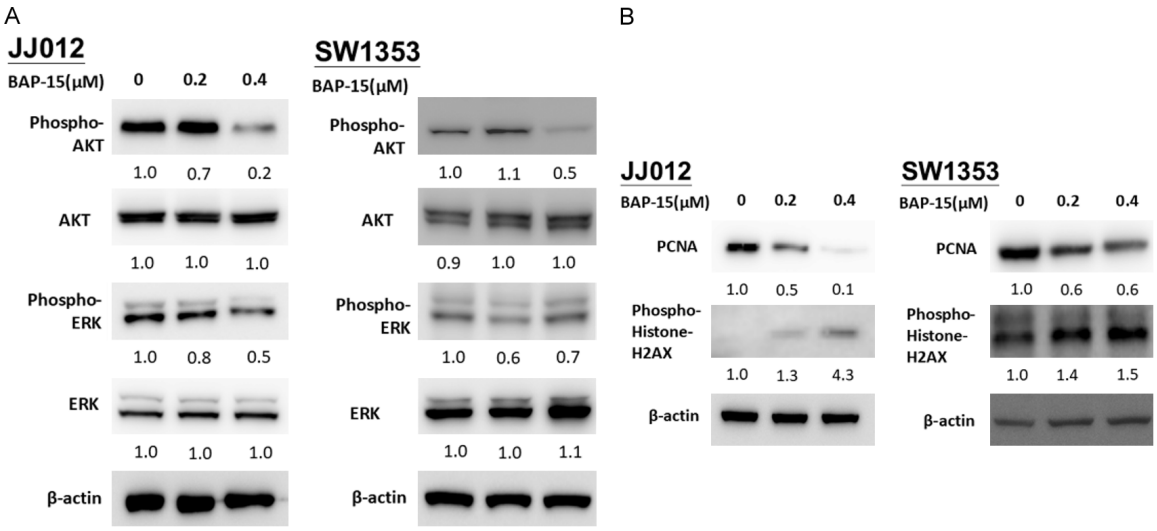

Figure 4. bAP15 suppresses AKT and ERK signaling in chondrosarcoma cells. Western blot analysis of AKT and ERK activation in JJ012 and SW1353 cells following 48-hour bAP15 treatment. bAP15 causes a dose-dependent reduction in phosphorylated AKT (Ser473) and ERK1/2 (Thr202/Tyr204) levels, while total AKT and ERK levels remain unchanged. Densitometric quantification of phospho/total ratios (relative to control) confirms significant suppression of AKT and ERK signaling by bAP15 treatment ($P<0.05$ vs 0 μM). The loss of AKT/ERK signaling likely contributes to the induction of apoptosis in bAP15-treated cells.

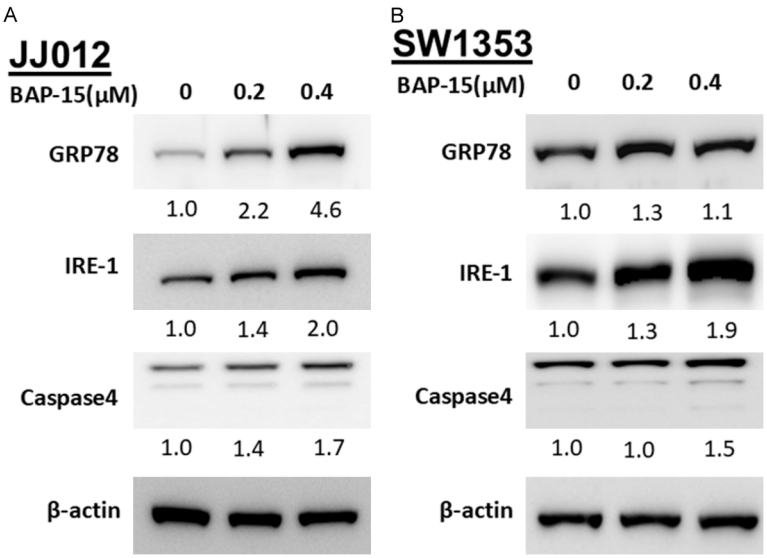

Figure 5. bAP15 triggers endoplasmic reticulum (ER) stress in chondrosarcoma cells. Western blots showing ER stress markers in JJ012 and SW1353 cells after 48 hours of bAP15 exposure. bAP15 upregulates IRE1α, GRP78 (BiP), CHOP, and cleaved caspase-4 compared to untreated controls, indicating activation of the unfolded protein response and ER stress-mediated apoptosis. β-Actin is included as a loading control. Densitometry confirms significant increases in these ER stress markers with bAP15 treatment ($P<0.05$ vs control).

treatment period, average tumor volumes in the bAP15 group were markedly lower than in the vehicle group for both JJ012 and SW1353 xenografts ($P<0.01$). No obvious toxicity (such as weight loss or behavioral changes) was observed in the bAP15-treated mice during the experiment (data not shown). These results confirm that bAP15 exhibits potent antitumor activity against chondrosarcoma in vivo.

Discussion

In the present study, we demonstrated that inhibition of proteasomal DUBs USP14 and UCHL5 by bAP15 effectively suppresses chondrosarcoma growth through multiple mechanisms, including induction of apoptosis, G1 cell cycle arrest, blockade of AKT/ERK signaling, and activation of ER stress. This multi-faceted anti-tumor activity highlights the central role of the ubiquitin-proteasome system in chondrosarcoma cell survival and suggests that DUB inhibitors like bAP15 could overcome the therapeutic resistance of this cancer.

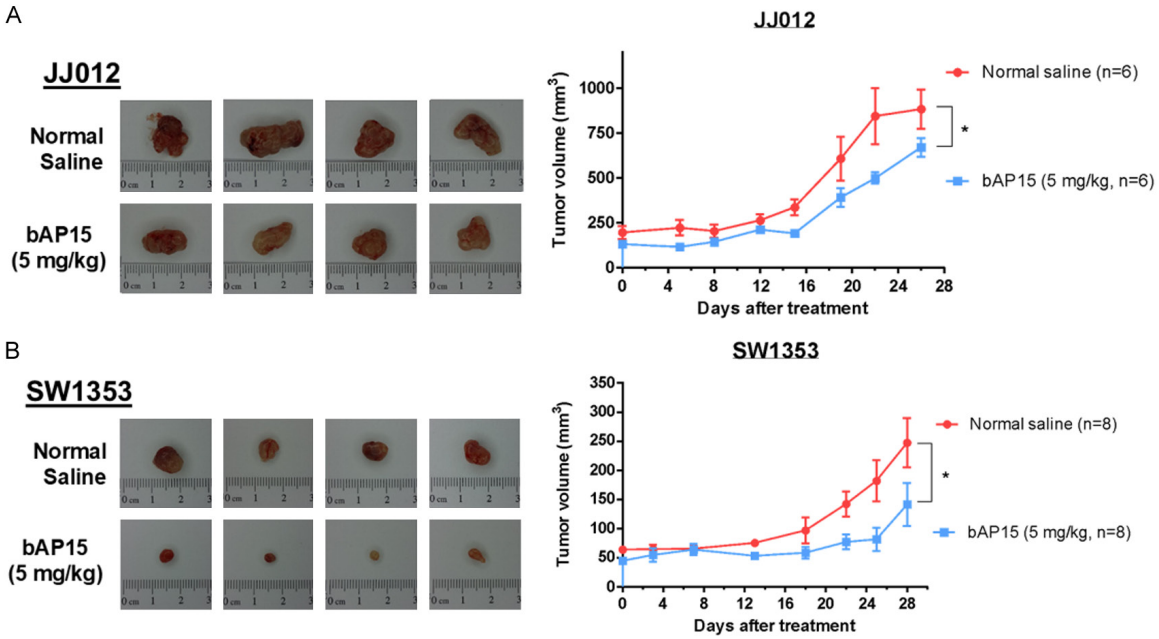

Figure 6. bAP15 suppresses chondrosarcoma tumor growth in vivo. Nude mice bearing JJ012 or SW1353 chondrosarcoma xenografts were treated with bAP15 (5 mg/kg, intraperitoneally, twice weekly) or vehicle control. A. Tumor growth curves for JJ012 xenografts show that bAP15 significantly inhibits tumor growth compared to vehicle-treated controls. B. Tumor growth curves for SW1353 xenografts similarly demonstrate tumor growth suppression by bAP15. Data are presented as mean tumor volume ± SEM. $P<0.01$ vs vehicle on the final day of treatment. No significant body weight loss or other toxicity was observed in the bAP15-treated mice, indicating that the treatment was well tolerated.

Notably, our findings are the first to establish a link between proteasome DUB inhibition and chondrosarcoma tumor suppression. Reports on proteasome-associated DUB inhibition have described both G1 and G2/M outcomes depending on cell type, genetic context (p53/p21/RB axis) [12], dose, and timing. Our data indicate a predominant G1 accumulation under the low sub-micromolar, 24-48 h conditions used here, which we now state explicitly to reconcile context-dependent differences with prior literature.

Consistent with our hypothesis, we found that USP14 and UCHL5 are overexpressed in chondrosarcoma tissues. UPS components are often upregulated in cancers to accommodate increased protein turnover and promote cell survival [8]. Prior studies have reported DUB overexpression in various malignancies and correlated it with aggressive behavior [10, 11]. Our IHC data suggest that chondrosarcoma cells similarly rely on heightened deubiquitinating activity. This provides a strong rationale for targeting USP14/UCHL5 in chondrosarcoma. Indeed, bAP15 was originally identified as a first-in-class inhibitor of proteasomal DUBs [12], and subsequent work showed it can trigger cancer cell death in multiple models [14, 15]. Our results extend those findings to chondrosarcoma, a tumor type not previously tested with DUB inhibitors.

Mechanistically, our data indicate that bAP15 induces a proteotoxic stress response in chondrosarcoma cells. We observed robust activation of ER stress markers (IRE1α, GRP78, CHOP) and caspase-4 following bAP15 treatment. This is in line with reports in other systems where proteasome DUB inhibition causes accumulation of undegraded proteins, leading to ER stress and apoptosis [5, 14]. CHOP upregulation is particularly significant, as it drives pro-apoptotic programs during unresolved ER stress. The concomitant phosphorylation of JNK we observed is another indicator of stress-induced apoptosis; JNK activation in response to proteasome inhibition has been noted in previous studies [14]. Additionally, bAP15's ability to reduce phosphorylated AKT and ERK in our models is noteworthy. The AKT and ERK pathways are known to promote chondrosarcoma

proliferation and chemoresistance [2]. Amphiregulin, for example, enhances doxorubicin resistance in chondrosarcoma via ERK activation [2]. Therefore, bAP15-mediated suppression of AKT/ERK signaling would remove critical survival signals, sensitize cells to apoptosis, and potentially improve response to other therapies. This dual impact on both proteostasis and signaling networks may explain the potent apoptosis observed with bAP15.

Importantly, bAP15 caused a G1 cell cycle arrest in chondrosarcoma cells, as evidenced by p21 induction and downregulation of cell cycle regulators (CDC25C, PCNA, phospho-histone H3). Cell cycle arrest provides an opportunity for cells to attempt repair of stress; however, prolonged arrest combined with intense proteotoxic stress likely pushes cells toward apoptotic death. The induction of p21 by bAP15 could be p53-dependent or via p53-independent pathways (e.g., activation of JNK or ATF4 during ER stress). Chondrosarcomas commonly harbor mutations in IDH1/2 and other pathways but may retain wild-type p53 in many cases [3]. If p53 is functional, it could contribute to p21 upregulation and apoptosis in response to DUB inhibition. Regardless of p53 status, p21 upregulation here is consistent with previous findings that DUB inhibition can activate cell cycle checkpoints leading to growth arrest [13]. This cell cycle inhibition adds another layer to bAP15's anti-proliferative effect.

Our in vivo results are particularly encouraging. bAP15 significantly inhibited chondrosarcoma tumor growth in xenograft models without observable toxicity. This outcome aligns with prior studies using proteasome DUB inhibitors in vivo. For instance, bAP15 (also called VLX1500 in some studies) showed efficacy in reducing tumor burden in models of multiple myeloma and bladder cancer. In a rat chondrosarcoma model, an mTOR inhibitor (rapamycin) achieved tumor suppression [20], highlighting that chondrosarcomas are amenable to targeted therapies. Our work is the first to demonstrate *in vivo* antitumor activity by a UPS-targeting agent in chondrosarcoma. Notably, we used a relatively low-dose intermittent regimen of bAP15 (5 mg/kg twice weekly), yet observed clear tumor inhibition. This suggests that proteasome DUBs are viable therapeutic targets and that even partial, transient inhibition is sufficient to impair tumor growth. Future studies could explore optimal dosing schedules or delivery methods to maximize tumor exposure while minimizing systemic effects.

Comparative analysis with other treatments further underscores the potential of DUB inhibition in chondrosarcoma. Recently, other approaches such as neddylation inhibition and SIRT1 activation have been tested in chondrosarcoma models. The NEDD8-activating enzyme inhibitor MLN4924 (Pevonedistat) was shown to suppress chondrosarcoma growth and induce ER stress-mediated apoptosis, similar to bAP15's effects [16, 21]. Likewise, resveratrol, a SIRT1 activator, induced apoptosis and reduced tumor growth in chondrosarcoma via downregulating survival pathways [22]. These studies, together with our current findings, illustrate that chondrosarcoma cells can be effectively targeted by disrupting protein homeostasis or survival signaling. Notably, bAP15 may offer a more direct attack on proteostasis than resveratrol or even MLN4924, since it prevents removal of ubiquitin tags at the proteasome itself, leading to a broad accumulation of misfolded proteins.

Study scope and considerations. Our goal was to examine whether inhibiting proteasome-associated deubiquitinating enzymes (DUBs) is a tractable concept in chondrosarcoma, using bAP15 as a chemical probe of USP14/UCHL5. In keeping with this mechanistic focus, we used unmatched normal cartilage as reference controls and interpret these comparisons with appropriate caution; this is now clarified in the Methods. We also restricted exposure to a low sub-micromolar, 24-48 h window to prioritize pathway readouts under sub-cytotoxic conditions. While genetic perturbation of USP14/UCHL5 and branch-resolved UPR analyses (e.g., p-IRE1, p-PERK, ATF6(N)) would further refine mechanism, they are beyond the scope of the present report, and our conclusions are framed accordingly. Finally, bAP15 is used here as a chemical tool to interrogate proteasome-associated DUB activity rather than to nominate a clinical agent.

In summary, our study provides proof-of-concept that targeting proteasome-associated deubiquitinases is a promising therapeutic approach in chondrosarcoma. By inhibiting USP14 and UCHL5, bAP15 simultaneously trig-

gers proteotoxic stress and disables key survival circuits in chondrosarcoma cells, leading to potent anti-tumor effects. These findings open a new avenue for treatment of chondrosarcoma, for which current systemic therapies are inadequate. Further preclinical development, including pharmacokinetic optimization of bAP15 or next-generation DUB inhibitors (such as VLX1500), a derivative now in early clinical evaluation [23], is warranted. Ultimately, DUB-targeted therapy either alone or in combination with existing treatments could improve outcomes for patients with unresectable or advanced chondrosarcoma. Together, our findings support proteasome-associated DUB inhibition as a mechanistically grounded strategy in chondrosarcoma, with bAP15 providing proof-of-principle for targeting the USP14/UCHL5 axis.

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Disclosure of conflict of interest

None.

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