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Xanthohumol Impairs Autophagosome Maturation through Direct Inhibition of Valosin-Containing Protein

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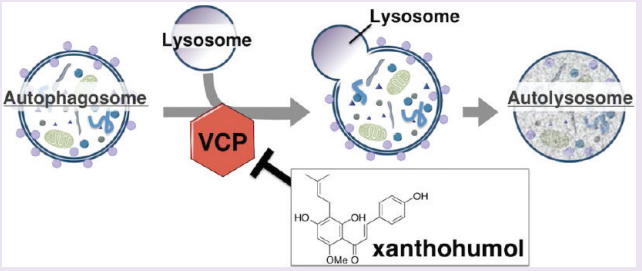
ABSTRACT: Autophagy is a bulk, nonspecific protein degradation pathway that is involved in the pathogenesis of cancer and neurodegenerative disease. Here, we observed that xanthohumol (XN), a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer, modulates autophagy. By using XN-immobilized beads, valosin-containing protein (VCP) was identified as a XN-binding protein. VCP has been reported to be an essential protein for autophagosome maturation. Using an *in vitro* pull down assay, we showed that XN bound directly to the N domain, which is known to mediate cofactor and substrate binding to VCP. These data indicated that XN inhibited the function of VCP, thereby allowing the impairment of autophagosome maturation and resulting in the accumulation of microtubule-associated protein 1 light chain 3-II (LC3-II). This is the first report demonstrating XN as a VCP inhibitor that binds directly to the N domain of VCP. Our finding that XN bound to and inactivated VCP not only reveals the molecular mechanism of XN-modulated autophagy but may also explain how XN exhibits various biological activities that have been reported previously.



Macroautophagy (herein referred to as autophagy) is an evolutionarily conserved pathway for degradation of intracellular components including organelles, which is critical for the maintenance of cellular homeostasis. Initially, the cytoplasmic components are sequestered by a unique membrane, referred to as an isolation membrane. Dynamic membrane organization is activated from small membrane particles to autophagosomes by the recruitment of autophagy related genes (ATGs) and microtubule-associated 1 light chain 3 (LC3).[1]

The next stage involves the fusion of autophagosomes with lysosomes and subsequent formation of autolysosomes. The inner membrane of the autophagosomes and the cytoplasm-derived materials contained in the autophagosomes are then degraded by lysosomal hydrolases.[2] The amino acids, which are produced by protein degradation, are then returned to the cytoplasm by lysosomal membrane permeases for reuse. Autophagy occurs in all cells at low basal levels under normal conditions to maintain homeostasis. It has been reported that aberrance of autophagy is involved in the pathogenesis of many diseases including neurodegenerative disease,[3,4] cancer,[5] muscle atrophy, and type 2 diabetes.[6]

Despite identification of more than 30 ATGs,[7,8] the molecular mechanism of autophagy is still not fully understood. Studying autophagy through chemical genetics could be an ideal approach to gaining a better understanding of autophagy signaling pathways. Most compounds that have been reported to be regulators of autophagy are distributed between two major groups. One group induces autophagy by inhibiting PI3K/Akt/mTOR signaling,[9] which is the major inhibitory signal that suppresses autophagy. The other group of regulators suppresses autophagy by inhibiting class III PI3K,[10] which is the homologue of yeast VPS34 and is required for the onset of autophagy.

In this study, we explored the mechanism of autophagy and identified additional small compounds that could modulate this process. This was done by screening for a small compound from an in-house natural product library using EGFP-LC3 stably expressing HeLa cells, and we identified xanthohumol (XN) as an autophagy modulator. Xanthohumol (30-[3,3-dimethyl allyl]-20,40,4-trihydroxy-60-methoxychalcone) is the principal prenylated chalcone of the female inflorescences of the hop plant ("hops"), an ingredient of beer.[11] Human exposure to XN is primarily through beer consumption. Several studies have reported on the potential health benefits of XN, including inhibition of diacylglycerol acyltransferase,[12,13] apoptosis induction,[14] NF-kappa B inhibition,[15] and ER stress induction.[16] However, there are no reports that show the relevance of XN to autophagy. Thus, to understand the mechanism by which XN modulates autophagy, we attempted to identify the target protein of XN responsible for the regulation of autophagy.

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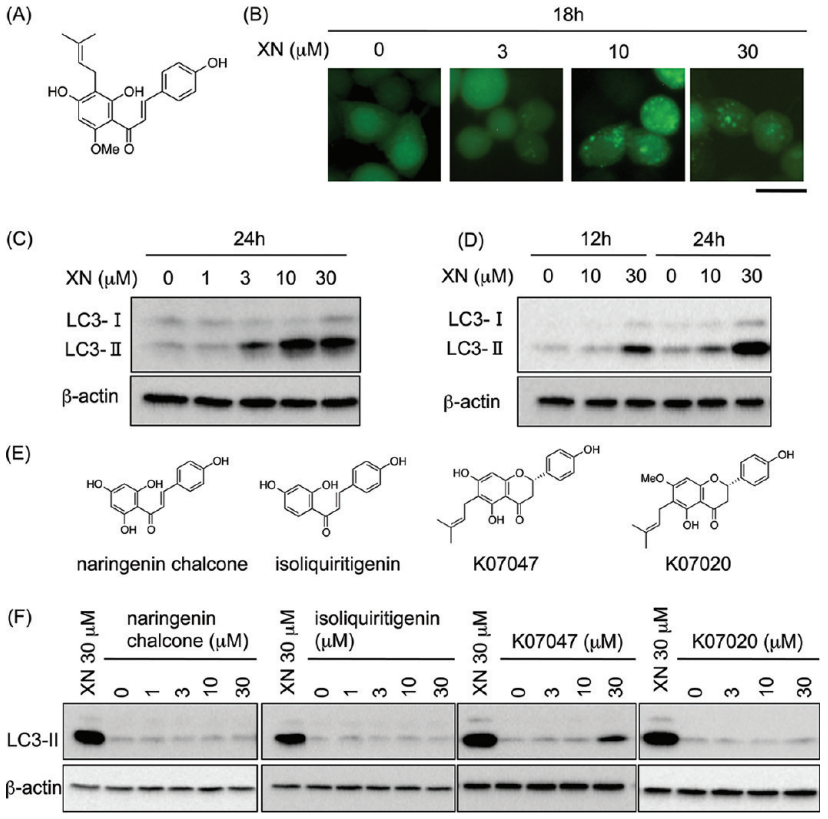

Figure 1. XN modulated autophagy. (A) Structure of xanthohumol (XN). (B) GFP-microtubule-associated protein 1 light chain 3 (LC3) stably expressing HeLa cells were treated with various concentrations of XN for 18 h. Cells were fixed with 3% (w/v) paraformaldehyde and observed under a fluorescence microscope (scale bar, 20 μm). (C) HeLa cells were treated with various concentrations of XN for 24 h. Cell lysates were immunoblotted with anti-LC3B antibody. β-Actin was immunoblotted as a loading control. (D) A431 cells were treated with various concentrations of XN for the indicated time. Cell lysates were immunoblotted with anti-LC3B antibody. β-Actin was immunoblotted as a loading control. (E) Structures of naringenin chalcone, isoliquiritigenin, K07047, and K07020. (F) A431 cells were treated with various concentrations of naringenin chalcone, isoliquiritigenin, K07047, or K07020 for 24 h. Cell lysates were immunoblotted with anti-LC3B antibody. β-Actin was immunoblotted as a loading control.

RESULTS AND DISCUSSION

Xanthohumol Inhibited Autophagosome Maturation.

In order to identify small compounds that could modulate autophagy and to explore the mechanism of autophagy through chemical genetics, we screened for a small compound from an in-house natural product library. As LC3-II is incorporated into the inner and outer surfaces of autophagosomes, the expression of a green fluorescence protein (GFP)-LC3 fusion protein can be used to identify GFP puncta representing autophagosomes.[17] Using this system to identify compounds that modulate autophagy, we searched for compounds that could increase the number of GFP-LC3 puncta in GFP-LC3 stably expressing human cervical carcinoma HeLa cells and found that xanthohumol (XN) showed this activity (Figure 1A). In untreated cells, GFP-LC3 was observed predominantly as diffuse green fluorescence in the cytoplasm. However, in XN-treated cells, characteristic punctate fluorescent patterns were observed, indicating that XN modulates autophagy in a dose-dependent manner, as shown in Figure 1B. Modulation of autophagy by XN was further confirmed by the detection of LC3-II, which is a phosphatidylethanolamine (PE) conjugated form of LC3, as a faster-migrating band when separated by SDS-PAGE and immunoblotted. As shown in Figure 1C, treatment of HeLa cells with XN for 24 h induced an increase in LC3-II levels in a dose-dependent manner. Similarly, XN increased LC3-II expression levels at 30 μM over 12−24 h in human epidermoid carcinoma A431 cells (Figure 1D). Next, we examined the effect of two other chalcones (naringenin chalcone and isoliquiritigenin) and two natural flavanones (K07047 and K07020) on LC3-II expression level. As a result, K07047 increased LC3-II levels weakly compared to XN, whereas naringenin chalcone, isoliquiritigenin, and K07020 did not increase LC3-II levels (Figure 1E,F)

The increase in LC3-II expression can be associated with either PE conjugation due to enhanced formation of autophagosomes or a block of LC3-II degradation due to impaired maturation of autophagosomes. To distinguish between these two possibilities, we detected expression levels of p62, a protein that is degraded by autophagy and accumulated when autophagy is impaired. Bafilomycin A1 (BMA) is known to prevent autophagosome maturation by inhibiting autophagosome-lysosome fusion[18] and caused an increase in the expression levels of p62 by inhibiting proteolytic degradation in autolysosomes, as shown in Figure 2A. Treatment with 30 μM XN for 24 h increased the expression levels of p62 as well. These data suggested that the increased LC3-II expression mediated by XN was a consequence of a block of autophagosome maturation. To further confirm that XN inhibited autophagosome maturation, we detected the localization of LC3 and lysosome in the presence of pepstatin A plus E64D, which are the lysosomal protease inhibitors, to inhibit the degradation of LC3 after fusion of autophagosome

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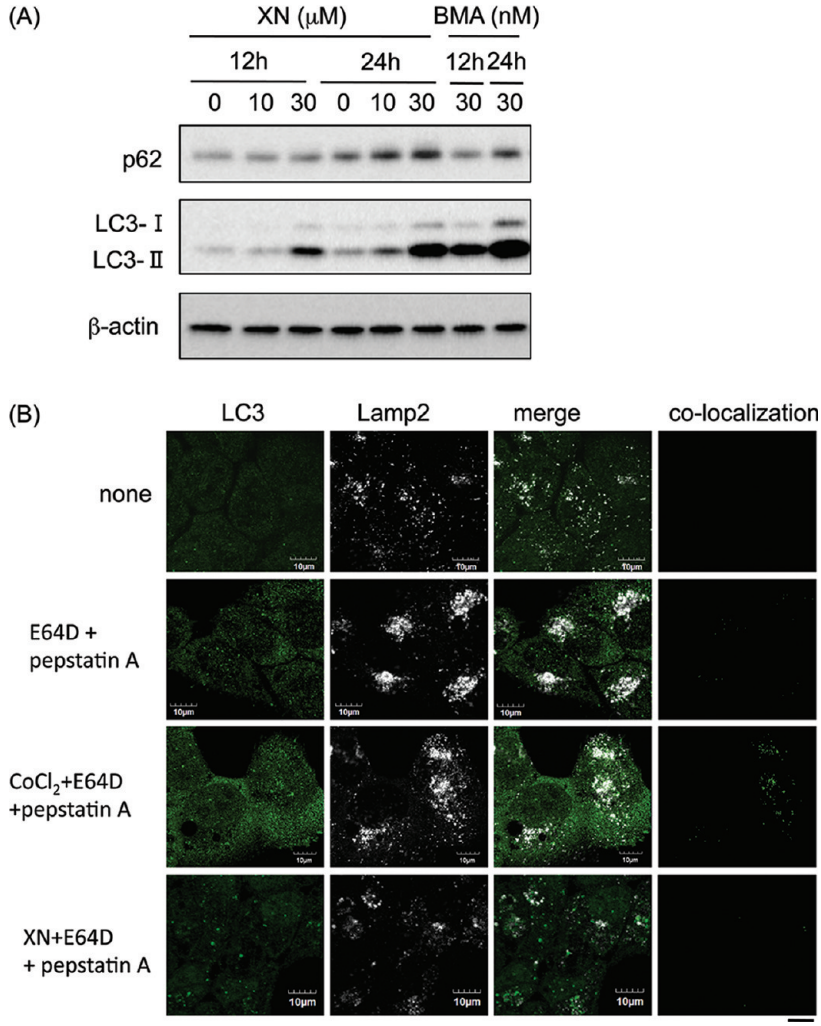

Figure 2. XN inhibited autophagosome maturation. (A) A431 cells were treated with various concentrations of XN or 30 nM bafilomycin A1 (BMA) for the indicated time. Cell lysates were immunoblotted with anti-p62 antibody. β-Actin was immunoblotted as a loading control. (B) A431 cells were treated with 30 μM XN or 0.3 mM $CoCl_2$ in the presence of 30 μM E64D and 30 μM pepstatin A for 24 h. Cells were then fixed with 3% (w/v) paraformaldehyde and immunostained with anti-LC3B and anti-lamp2 antibodies. The cells were observed under confocal microscopy (scale bar, 10 μm).

with lysosome. Because $CoCl_2$ are known to induce an increase in LC3-II expression levels by accelerating autophagosome formation,[19] we examined the effect of XN on the localization of LC3 and lysosome compared with the effect of $CoCl_2$. As shown in Figure 2B, $CoCl_2$ increased the number of LC3-positive puncta co-localizing with lysosome, whereas LC3-positive puncta increased by XN failed to co-localize with lysosome even in the presence of pepstatin A plus E64D. These data strongly indicated that XN impaired autophagosome maturation, resulting in increase in the level of LC3-II.

Identification of XN-Binding Proteins. To elucidate the underlying mechanism behind the suppression of autophagosome maturation induced by XN, we attempted to identify the cellular target protein of XN responsible for autophagy modulation. To this end, we used XN-immobilized agarose beads, which were prepared by a photocross-linking method.[20] A431 cell lysates were incubated for 3 h with XN-immobilized beads (XN beads) or control beads as a negative control. The reacted beads were washed, and the co-precipitated proteins were eluted, separated by SDS-PAGE, and stained with Coomassie brilliant blue (CBB). As shown in Figure 3A, four protein bands that specifically co-precipitated with XN beads were observed. Each protein band was identified by using MALDI-TOF-MS and LC−MS/MS as (i) valosin-containing protein (VCP), (ii) voltage-dependent anion channel (VDAC), (iii) prohibitin-2, and (iv) prohibitin.

Among these proteins, competition was observed only for VCP with 0.1−1 μmol XN as shown in Figure 3B. VCP has been reported to play a role in the maturation of autophagosomes.[21,22] VCP, also known as p97, is one of the best-characterized type II AAA (ATPases associated with diverse cellular activities) ATPases. VCP plays critical roles in a broad range of diverse cellular processes, including ER associated degradation *via* the ubiquitin-proteasome system,[23,24] cell cycle regulation,[25] and DNA repair.[26] Recently, it was reported that VCP is essential for autophagosome−lysosome fusion and formation of autolysosomes in human cell lines.[21,22] Therefore, we speculated that VCP might be the target of XN, and the binding of XN to VCP was confirmed by immunoblotting of co-precipitated protein from XN-beads using anti-VCP antibody (Figure 3C).

XN Bound Directly to the N Domain of VCP. Next, to determine whether XN could bind directly to VCP, we performed an *in vitro* binding assay using purified recombinant

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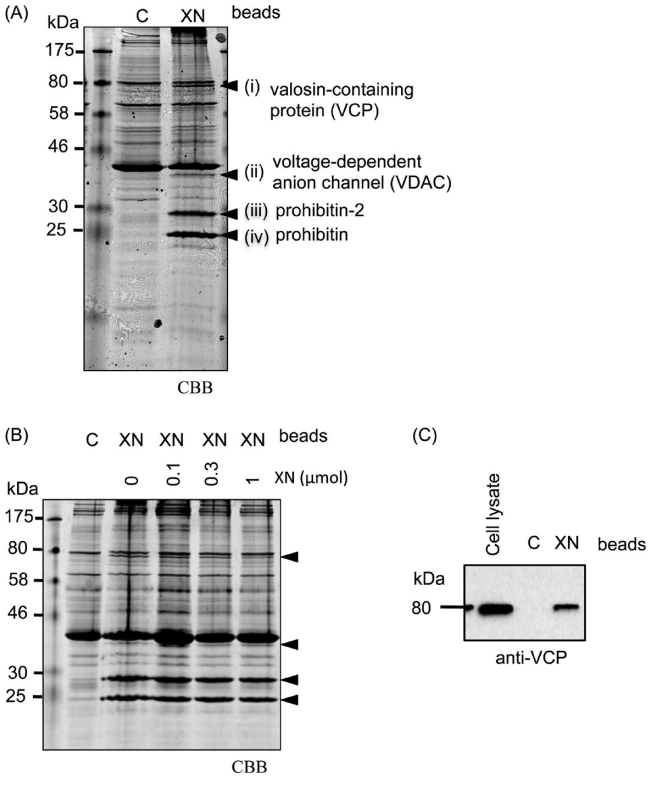

Figure 3. Identification of XN-binding proteins. (A) A431 cell lysates were incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were subjected to SDS-PAGE and stained by Coomassie brilliant blue (CBB). The co-precipitated proteins for XN beads were identified by using MALDI-TOF-MS and LC−MS/MS. (B) A431 cell lysates were preincubated with 0.1−1 μmol of XN as a competitor for 1 h and then incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were subjected to SDS-PAGE and stained by CBB. (C) A431 cell lysates were incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were immunoblotted with anti-valosin-containing protein (VCP) antibody.

GST-tagged VCP protein. Unlike GST, GST-VCP was co-precipitated only with XN-beads, as shown in Figure 4A. Moreover, competition was observed for VCP in the presence of 0.5 μmol of XN (Figure 4B), indicating that XN binds directly to VCP. On the other hand, competition was not observed for binding of XN-beads and VCP in the presence of XN analogues such as naringenin chalcone, isoliquiritigenin, K07047, and K07020 up to 0.5 μmol (Figure 4C), indicating that these analogues bind to VCP very weakly or fail to bind to VCP at least through the XN binding site. Because these analogues fail to induce LC3-II expression level or induce it very weakly, these observations further confirm the importance of the XN binding to VCP for impairment of autophagosome maturation. In addition, because naringenin chalcone and isoliquiritigenin did not bind to VCP, the prenyl and/or *O*-methyl group of XN is thought to be important for binding to VCP.

VCP is composed of a substrate and cofactor binding N domain followed by two AAA ATPase domains, termed D1 and D2, and forms a hexameric double-ring structure.[27,28] It has been demonstrated that both D1 and D2 domain contain Walker A and Walker B motifs that mediate ATP binding and hydrolysis, respectively. However, these two ATPase domains are not catalytically equivalent: D2 domain has the major ATPase activity at physiological temperatures, whereas D1 is involved in the regulation of heat-induced ATPase activity.[29] D1 also plays a major role in hexamerization.[30,31] To determine which domain in VCP is essential for interaction with XN, we prepared three GST-tagged VCP mutants that lacked the N-terminal domain (1−185) (GST-VCPΔN), D1 domain containing Walker A and Walker B motifs (186−348) (GST-VCPΔD1), and D2 domain containing Walker A and Walker B motifs (349−806) (GST-VCPΔD2) (Figure 4D). GST-VCPΔD1 and GST-VCPΔD2 were co-precipitated with XN beads, whereas GST-VCPΔ*N* was not, as shown in Figure 4E. These results indicated that XN bound to the N domain of VCP.

XN Inhibited VCP Function. Next, we examined whether this binding of XN to the N domain of VCP could inhibit VCP function. The structural alteration of the N domain of VCP has been reported to induce impaired maturation of autophagosome as well as impaired ER associated degradation (ERAD),[32] and loss of VCP-mediated ERAD activity leads to accumulation of unfolded protein in the ER, resulting in induction of ER stress.[33] Therefore, we examined the effect of XN on the expression of the ER stress markers CHOP and GRP78. As shown in Figure 5A, treatment with 30 μM XN for 12−24 h increased the protein levels of CHOP and GRP78 significantly in A431 cells, suggesting that XN inhibited VCP-mediated ERAD. On the other hand, Hirabayashi *et al.* reported that inhibition of VCP function by using dominant negative VCP induced cytoplasmic vacuolation.[34] These vacuoles are reported to be a result of abnormal budding and enlargement of the ER.[35] We also observed the presence of microscopic vacuoles not only in VCP knockdown A431 cells by using siRNA (Figure 5B) but also in XN-treated A431 cells (Figure 5C). The successful knockdown of VCP using siRNA and resultant up-regulation of LC3-II was confirmed by immunoblotting, as shown in Figure 5B right. Moreover, the XN analogue K07047, which modulated autophagy weakly, also induced vacuolization weakly compared with XN. On the other hand, other analogues including naringenin chalcone, isoliquiritigenin, and K07020, which had no effect on modulation of autophagy, did not induce vacuolization (Figures 1F and 5C). Taken together, these data indicated that XN bound to the N domain of VCP directly, thereby suppressing VCP function.

Apart from autophagy, XN has been reported to inhibit mitogen/antigen-induced T cell proliferation, development of cell-mediated cytotoxicity, and production of Th1 cytokines by inhibiting NF-κB.[36] Moreover, XN has been shown to inhibit the growth of a wide variety of human cancer cell lines by inhibiting proliferation and inducing apoptosis.[37,38] These previous observations regarding XN suggested the following two possibilities: one possible explanation is that various proteins were interfered with by XN and various biological phenomena were affected, and the other is that XN modulated a specific protein, which was involved in the various biological processes. Our finding that XN modulated the function of VCP may explain how XN exhibited the above-mentioned effects, because VCP is reported to play important roles in the degradation of IκB, resulting in enhancement of NF-κB signaling,[39,40] or because the expression level of VCP is correlated with progression, prognosis, and recurrence of certain types of cancer.[41,42]

Two types of VCP inhibitors have been reported in the literature. The first type of inhibitor is classified as a VCP

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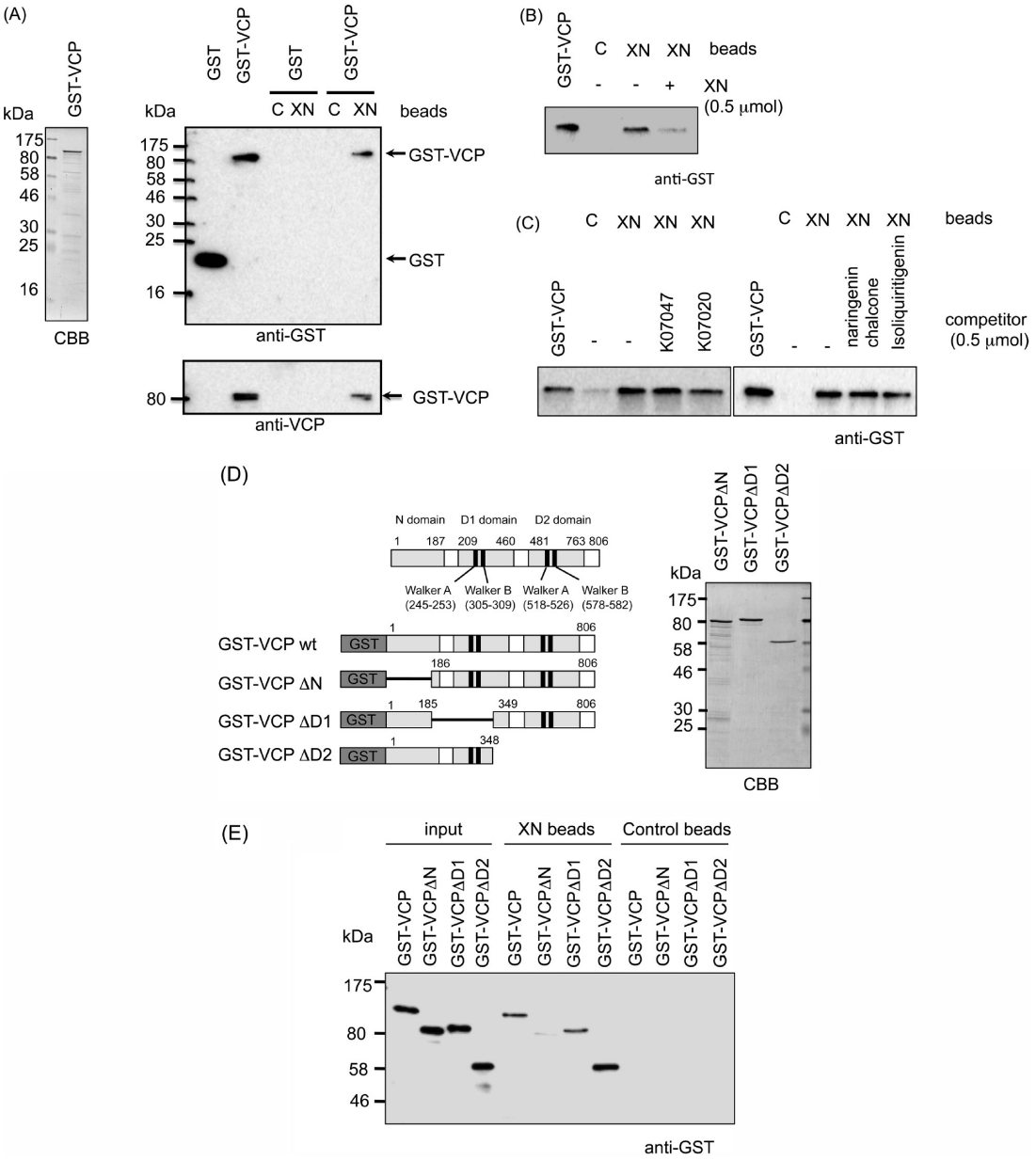

Figure 4. XN bound directly to the N domain of VCP. (A) (left) CBB staining of purified GST-VCP protein. (right) Purified GST and GST-tagged VCP were incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were immunoblotted with anti-GST and anti-VCP antibodies. (B) Purified GST-tagged VCP was preincubated with 0.5 μmol of XN as a competitor for 1 h and then incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were immunoblotted with anti-GST antibody. (C) Purified GST-tagged VCP was preincubated with 0.5 μmol of naringenin chalcone, isoliquiritigenin, K07047, or K07020 as a competitor for 1 h and then incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were immunoblotted with anti-GST antibody. (D) (left) Schematic illustration of GST-VCP, GST-VCPΔN, GST-VCPΔD1, and GST-VCPΔD2. (right) CBB staining of purified GST-VCPΔN, GST-VCPΔD1, and GST-VCPΔD2. (E) Purified GST-VCPΔN, GST-VCPΔD1, and GST-VCPΔD2 were incubated with control beads or XN beads for 3 h. The reacted beads were washed, and the eluted proteins were immunoblotted with anti-GST antibody.

ATPase inhibitor, which most likely binds to a site in the D2 ATPase domain. 2-Anilino-4-aryl-1,3-thiazoles were discovered by high-throughput screening (HTS) as inhibitors of VCP ATPase activity, and these were reported to inhibit VCP-associated protein degradation.[43] Syk inhibitor III was reported to be an irreversible inhibitor of VCP ATPase activity by interacting with Cys522 within the D2 ATPase domain of VCP and the ubiquitin-fused reporter protein.[44] N^2,N^4-Dibenzylquinazoline-2,4-diamine (DBeQ) was identified as a selective, potent, reversible, and ATP-competitive VCP inhibitor by screening a library of chemical compounds.[45] DBeQ blocks multiple processes that have been shown by siRNA to depend on VCP, including degradation of ubiquitin fusion degradation and ERAD as well as autophagosomal maturation. The second type of VCP inhibitor is Eeyarestatin I (Eer I), which binds to the D1 domain of VCP without affecting ATPase activity.[46] Eer I was found to directly associate with the ER membrane and VCP and inhibited VCP-associated deubiquitinating enzymes, thereby inhibiting VCP-dependent protein degradation. However, so far, VCP inhibitors that bind to the N domain of VCP have not yet been reported. Therefore, XN is the first example of such an inhibitor that binds to the N domain of VCP and

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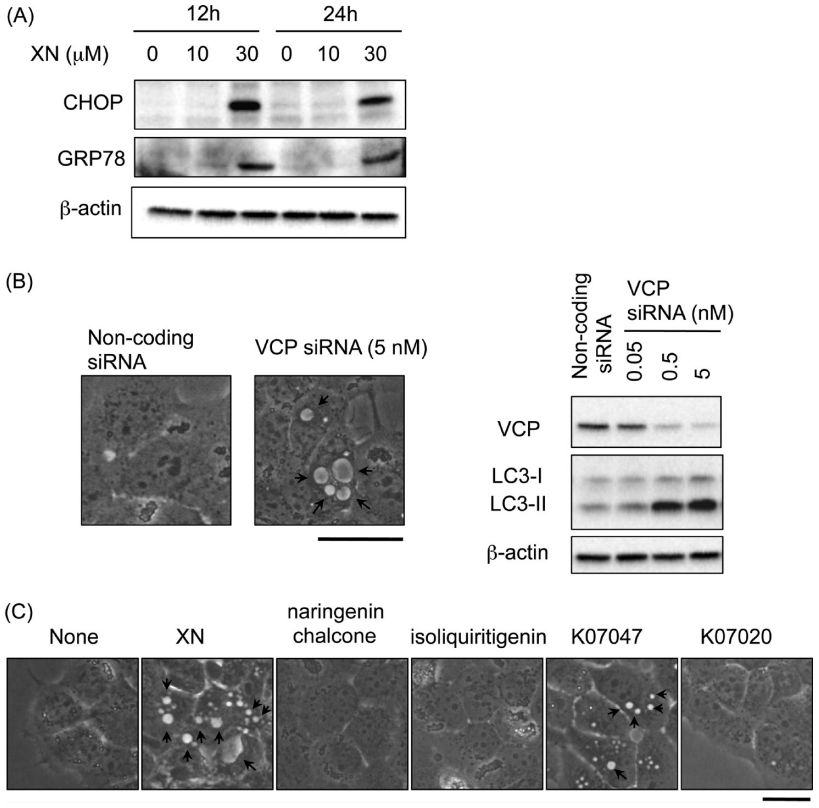

Figure 5. XN inhibited VCP function. (A) A431 cells were treated with various concentrations of XN for the indicated time. Cell lysates were immunoblotted with anti-CHOP and anti-GRP78 antibodies. β-Actin was immunoblotted as a loading control. (B) (left) A431 cells were observed under a microscope 72 h after transfection with non-coding siRNA or VCP siRNA (arrows, vacuoles; scale bar, 25 μm). (right) A431 cells were transfected with VCP siRNA or noncoding siRNA for 72 h. Cell lysates were immunoblotted with anti-VCP and anti-LC3B antibodies. β-Actin was immunoblotted as a loading control. (C) A431 cells were treated with 30 μM XN, naringenin chalcone, isoliquiritigenin, K07047, or K07020 for 24 h and then observed under microscope (arrows, vacuoles; scale bar, 25 μm).

inactivates VCP. Thus, XN is proposed to be a new class of VCP inhibitors, which may be used as a powerful tool for identifying the cofactor or substrate protein of VCP responsible for autophagy regulation.

METHODS

Reagents. Naringenin chalcone was obtained as a generous gift from Kikkoman Corporation. Isoliquiritigenin, E64D, and peptatin A were purchased from Sigma-Aldrich Co.

Cell Line. Human epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% (v/v) calf serum, 100 U mL^{-1} of penicillin G (Sigma-Aldrich Co.), and 0.1 mg mL^{-1} of kanamycin (Sigma-Aldrich Co.) at 37 °C in a 5% CO_2–95% air atmosphere. Human cervical carcinoma HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 100 U mL^{-1} of penicillin G, and 0.1 mg mL^{-1} of kanamycin at 37 °C in a 5% CO_2–95% air atmosphere. HeLa/GFP-LC3 stable cell lines were established as previously described.[47]

Isolation of XN from Hop and XN Analogues from Microbial Origin. XN was isolated from commercially available hop extract obtained from Hopsteiner. The extract (200 mg) was purified by using preparative octadecylsilyl (ODS) HPLC (UG 80, 20 mm, 250 mm; SHISEIDO) with 70% (v/v) aqueous MeOH to obtain pure XN (147 mg). The structures were identified by spectroscopic data (NMR and MS). UV (MeOH) λ_{max} (log ε) 369 (4.56); ESIMS m/z 355 $[M + H]^+$; ^{13}C NMR δ192.8, 165.5, 161.8, 161.2, 157.4, 142.0, 136.0, 130.3 (2C), 128.6, 125.5, 121.7, 116.2 (2C), 106.3, 106.2, 105.0, 56.1, 25.8, 21.6, and 17.9. K07020 and K07047 were isolated from 14 L of culture broths of *Streptomyces* sp. HK-803 and *Streptomyces spiroverticillatus* JC-8444 by UV absorption and mass spectra guided separation to afford 10.6 and 9.3 mg as a pale-yellow powder, respectively. The structures were identified by spectroscopic data (NMR and MS). K07020: UV (MeOH) λ_{max} (log ε) 226 (4.45), 288 nm (4.33); ESIMS m/z 353 $[M - H]^-$; ^{13}C NMR δ192.9, 164.6, 163.8, 161.8, 158.8, 131.6, 131.5, 128.9 (2C), 123.9, 116.2 (2C), 110.0, 105.7, 93.5, 80.0, 55.9, 46.2, 26.0, 22.7, and 17.9. K07047: UV (MeOH) λ_{max} (log ε) 226 (4.36), 293 nm (4.24); ESIMS m/z 339 $[M - H]^-$; ^{13}C NMR δ197.8, 165.9, 162.6, 162.5, 159.0, 131.5, 131.2, 129.0 (2C), 123.9, 116.3 (2C), 109.6, 103.2, 95.4, 80.4, 44.2, 25.9, 21.8, and 17.8.

DNA Constructs. Human cDNA for VCP were amplified from A431 cell cDNA and subcloned into pGEX-2T (GE Healthcare UK Ltd.) to prepare GST fusion proteins in bacteria. Expression vectors encoding GST-fused VCP mutants (ΔN, 1–185 aa deletion; ΔD_1, 186–348 aa deletion; and ΔD_2, 349–806 aa deletion) were generated by PCR using pGEX-2T/VCP as a template.

Fluorescence Microscopy. For fluorescence microscopy, HeLa cells stably expressing GFP-LC3, which were grown on coverslips, were treated with chemicals for the indicated time at 37 °C. Cells were fixed with 3% (w/v) paraformaldehyde in PBS at RT. The cells were then washed with PBS and observed under a fluorescence microscope (Olympus).

Western blotting. Cells were lysed with RIPA buffer [25 mM HEPES, 1.5% (v/v) TX-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 100 mM Na_3VO_4, 0.1 mg mL^{-1} leupeptin, 1 mM PMSF; pH 7.8]. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore), and probed with specific antibodies. This was followed by detection using the ECL Western blotting detection system (Millipore) and LAS-1000 (Fuji Film). The primary antibodies used were as follows: anti-LC3B (L7543, Sigma-Aldrich Co.), anti-β-actin (AC-74, Sigma-Aldrich Co.), anti-p62 (5114, Cell Signaling Technology), anti-VCP (ab 11433, Abcam), anti-GST (B-14, Santa

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Cruz Biotechnology), anti-GRP78 (H-129, Santa Cruz Biotechnology), and anti-CHOP (MA1-250, Thermo Fisher Scientific Inc.) antibodies. The secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare UK Ltd.).

Immunofluorescent Microscopy. Immunofluorescent microscopy was carried out as previously described.[48] Fluorescence images were obtained using a confocal laser scanning microscope system FV1000 (Olympus).

Detection of Binding Proteins for XN Beads. XN beads were prepared as previously described.[20] A431 cells were harvested, washed with PBS, and then resuspended in binding buffer [50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, NP40 1% (v/v) and protease inhibitor cocktail tablets (Roche); pH 7.5]. After cells were lysed by homogenization with sonication, the insoluble material was removed by centrifugation, and the supernatant was collected as cell lysate. The cell lysate (3 mg of protein) was then incubated with XN beads (20 μL) for 3 h at 4 °C. The reacted beads were washed with binding buffer, and the binding proteins were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, and visualized by CBB staining. Identification of the proteins was performed using MALDI-TOF-MS and LC−MS/MS as previously described.[49]

***In Vitro* XN Beads Pull-Down Assay.** GST fusion proteins, which were expressed in the *Escherichia coli* BL21 strain and purified using Glutathione Sepharose 4B (GE Healthcare UK Ltd.), were incubated with XN beads in 1 mL of binding buffer for 3 h. The beads were washed with binding buffer and eluted with SDS-PAGE sample buffer. The eluted proteins were then subjected to SDS-PAGE. For the competition assay, each compound was added 1 h before incubation with XN beads.

RNA Interference. siRNA double-stranded oligonucleotides designed to interfere with the expression of VCP (sense 5′-UAGAACAGAACUCCCUUGGAAGGUG-3′; Invitrogen) and non-coding siRNA (Invitrogen) as a negative control were used. Reverse transfection was demonstrated by using HiPerFect (QIAGEN) according to the manufacturer's instructions. Briefly, A431 cells were trypsinized, resuspended in antibiotic-free medium, mixed with OPTI-MEM (Gibco) including siRNA and HiPerFect, and then seeded onto a 12-well plate. 72 h after transfection, cells were observed under microscope and lysed for Western blotting.

■ ASSOCIATED CONTENT

Accession Codes

Uni-Prot accession codes are described as following; valosion-containg protein (TERA_HUMAN), P55072; voltage-dependent anion channel (VDAC1_HUMAN), P21796; Prohibitin 2 (PHB2_HUMAN), Q99623; Prohibitin (PHB_HUMAN), P35232

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Notes

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