Carcinoma-associated fibroblasts are fibroblasts activated by surrounding cancer cells. Carcinoma-associated fibroblasts exhibit enhanced cell migration, which plays an important role in cancer metastasis. Previously, we demonstrated enhanced migration of NIH3T3 fibroblasts when they were cultured in the presence of MCF7 breast cancer cells. Human fibroblasts displayed a similar phenomenon even when they were co-cultured with cancer cells other than MCF7 cells. In this study, we screened ~16,000 compounds from the RIKEN Natural Products Depository chemical library for inhibitors of enhanced NIH3T3 cell migration in the presence of MCF7. We identified NPD8733 as an inhibitor of cancer cell–enhanced fibroblast migration. This inhibition was observed not only in a wound-healing co-culture assay but also in a Transwell migration assay. Using NPD8733 and a structurally similar but inactive derivative, NPD8126, on immobilized beads, we found that NPD8733, but not NPD8126, specifically binds to valosin-containing protein (VCP)/p97, a member of the ATPase-associated with diverse cellular activities (AAA+) protein family. Using VCP truncation variants, we found that NPD8733 binds to the D1 domain of VCP. Because VCP’s D1 domain is important for its function, we concluded that NPD8733 may act on VCP by binding to this domain. siRNA-mediated silencing of VCP in NIH3T3 fibroblasts, but not in MCF7 cells, reduced the migration of the co-cultured NIH3T3 fibroblasts. These results indicate that MCF7 activates the migration of NIH3T3 cells through VCP and that NPD8733 binds VCP and thereby inhibits its activity.

The tumor microenvironment plays an important role in cancer metastasis, and fibroblasts occupy a major portion of this microenvironment. Fibroblasts are known to support the growth of cancer cells by secreting growth factors as well as extracellular matrix components (1, 2). Recently, researchers have suggested that cancer cells can also modulate the morphology and activity of fibroblasts through autocrine and paracrine signaling, thereby activating fibroblasts (3, 4). The fibroblasts that are activated by cancer cells are referred to as carcinoma-associated fibroblasts (CAFs) (5).

CAFs are also known to directly interact with cancer cells and guide them in the invasion process in different organs (4, 6). Thus, these heterogeneous CAFs are involved in a wide range of functions in cancer progression, including cancer initiation, metastasis, angiogenesis, and metabolic reprogramming (7). Emerging studies have shown that CAFs may contribute to the resistance of chemotherapy (8, 9). Thus, CAFs may be potential targets for anti-cancer therapy.

Although there are numerous proteins that are expressed in fibroblasts associated with cancer cells, such as α-smooth muscle actin, fibroblast activation protein, podoplanin, tenascin-C, and many more (10), a specific marker for activated fibroblasts is still lacking. The limited knowledge regarding a specific protein marker makes it difficult to develop a drug targeting activated fibroblasts. Most compounds that are being considered for targeting cancer stroma are not just restricted to CAFs (11–13). Therefore, identification of novel protein markers is essential to specifically target CAFs.

In our previous study, enhanced migration of NIH3T3 fibroblasts was observed when co-cultured with MCF7 breast cancer cells (14). The fibroblasts that show enhanced migration, a characteristic of CAFs, are referred to as activated fibroblasts. As MCF7 cancer cells are able to induce changes in NIH3T3 fibroblast cell migration when co-cultured, any small molecule that inhibits such a phenomenon may be an inhibitor of fibroblasts activated by cancer cells. Understanding the mechanism of fibroblast activation by cancer cells is intricate, as there are numerous signaling pathways involved. Thus, our previous study suggests that the small-molecule inhibitors of activated fibroblasts may provide insight into understanding the mechanism of fibroblast activation.
In this study, we screened ~16,000 small compounds from the RIKEN NPDepo chemical library using a previously established wound healing co-culture assay. Compound NPD8733 was identified as inhibiting the enhanced migration of fibroblasts when co-cultured with cancer cells. By using an in vitro pulldown assay and proteomics analysis, valosin-containing protein (VCP)/p97 was identified to be the target protein for NPD8733. VCP is a ubiquitously expressed protein that belongs to the ATPase-associated with diverse cellular activities (AAA+) protein family. VCP is involved in many cellular processes, such as protein degradation, apoptosis, and autophagy (15–17). To date, there are no reports defining the role of VCP in the activation of fibroblasts. NPD8733 will become a useful tool for understanding the mechanism of fibroblast activation through VCP.

**Results**

**Screening of the RIKEN NPDepo chemical library for inhibitors of fibroblast migration accelerated by cancer cells**

We previously observed significantly enhanced migration of NIH3T3 fibroblasts when they were cultured in the presence of MCF7 breast cancer cells. This enhanced migration was also observed when a Transwell migration assay was used for co-culturing (14). Human fibroblasts also showed a similar phenomenon when co-cultured with cancer cells other than MCF7. We found that this enhanced migration was inhibited by a small-molecule ligand of an adaptor protein, β-arrestin, that activates the actin fiber–severing protein cofilin through its dephosphorylation (14).

In this study, we expanded the screening to identify additional small molecules that act as inhibitors of fibroblasts migration, which is normally increased by cancer cells. The initial screening for small-molecule inhibitors of accelerated fibroblast migration was performed in 96-well plates using 1 μg/ml of library compounds. The compounds that inhibited the enhanced migration of co-cultured NIH3T3 cells were selected. A similar screening was repeated twice using 24-well and 6-well plates. We identified five compounds, NPD8732, FSL0816 (procaterol), NPD6543, HTD1063, and NPD6330, that reproducibly inhibit the enhanced migration of co-cultured NIH3T3 cells and have no effect on the migration of NIH3T3 cells when cultured alone for 48 h (Fig. S1). These compounds showed no effects on cytotoxicity of either MCF7 or NIH3T3 cells, even at a high concentration, such as 10 μg/ml (Figs. S1 and S2, A and B). Additionally, we examined the effects of these compounds on MDA-MB-231 breast cancer cell migration. However, none of these compounds inhibited the migration of MDA-MB-231 cells (Fig. S2, C and D), indicating that these compounds specifically inhibit the enhanced migration of co-cultured NIH3T3 cells.

Among these five compounds, procaterol is a compound known to act as an agonist for the β2-adrenergic receptor, which is used in the treatment of asthma (18). Thus, we did not advance with the target identification study for this compound. The remaining compounds were examined for inhibition of migration of co-cultured NIH3T3 cells at 0.1, 0.3, 1, and 3 μg/ml to select potent compounds (Fig. S3). Compounds NPD8732, NPD6330, and HTD1063 were selected, whereas NPD6543 showed inhibitory activity only at a higher dose.

**NPD8733 inhibits the enhanced migration of co-cultured NIH3T3 cells in a dose-dependent manner**

To identify target proteins for these three potent compounds, we attempted to prepare compound-conjugated agarose beads using a photocross-linking technique (19). However, we could not obtain NPD8732 conjugated beads, as this compound was damaged by the 265-nm UV irradiation that is required for photocross-linking. Thus, we searched for compounds that were structurally similar to NPD8732 in the RIKEN NPDepo library and examined their ability to inhibit migration. We identified compound NPD8733 as inhibiting the enhanced migration of co-cultured NIH3T3 cells (Fig. 1). Significant inhibition of fibroblast migration, which was enhanced by co-cultured cancer cells, was observed in the presence of NPD8733 at a concentration of 3 μM or higher (Fig. 1, C and D). Migration of NIH3T3 cells alone was not affected by NPD8733 (Fig. 1E).

Similar inhibition of enhanced migration was also observed using a Transwell migration assay (Fig. 2). Only a small number of NIH3T3 cells migrated through the filter, whereas migration was enhanced more than six times when co-cultured with MCF7 cells. NPD8733 at 1 μM or higher inhibited this enhanced migration significantly, and at 9 μM, enhanced migration was completely abolished (Fig. 2, A and B). We confirmed that NPD8733 did not inhibit the migration of co-cultured NIH3T3 cells because of its cytotoxic effects on either NIH3T3 or MCF7 cells (Fig. 2C).

**NPD8733 binds to VCP**

To understand the mechanism behind the migratory inhibition of co-cultured NIH3T3 fibroblasts by NPD8733, NPD6330, and HTD1063, we sought to identify the target proteins of these compounds using pulldown analyses through small molecule–conjugated beads. Unfortunately, we could not identify proteins that specifically bound to the compounds NPD6330 and HTD1063 (data not shown), but we did find a protein that bound specifically to NPD8733-conjugated beads. To further analyze this protein, we prepared agarose beads conjugated with NPD8126, which is structurally similar to NPD8733 but does not have any effects on enhanced migration of co-cultured NIH3T3 cells (Fig. 3, A–C). The cell lysates from NIH3T3 cells co-cultured with MCF7 were incubated with naked beads as a control, NPD8126 beads, or NPD8733 beads for 3 h. Then the reacted beads were washed three times to remove unbound protein, and the proteins that co-precipitated with beads were eluted, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue (CBB). One protein band specifically co-precipitated with NPD8733 beads and was identified, using MALDI-TOF-MS, as VCP (Fig. 3D and Fig. S4).

To further confirm the binding of NPD8733 to VCP, we performed an in vitro pulldown assay using bacterial lysate expressing VCP. A pGEX-2T vector encoding a GST-tagged VCP was introduced into Escherichia coli BL21 cells, and the expression of the fusion protein was induced. This bacterial cell lysate was incubated with control beads or NPD8126-
NPD8733-bound beads for 3 h. The beads were then washed, and the co-precipitated proteins were eluted and examined using SDS-PAGE and Western blot analysis. Using CBB staining and an anti-GST antibody, we confirmed the specific binding of compound NPD8733 to the GST-VCP protein (Fig. 4A).

**NPD8733 binds directly to the D1 domain of VCP**

Sasazawa et al. (20) previously identified a small compound, xanthohumol, which binds to VCP and recognizes it to be autophagy-modulatory. They also showed that xanthohumol binds to the VCP N-terminal domain, which is a substrate and co-factor–binding domain. Using the same deletion constructs of recombinant GST-tagged VCP protein, we attempted to determine the binding domain for NPD8733 (Fig. 4B). E. coli BL21 cells were induced for the production of the GST-tagged WT VCP or three deletion mutants that lacked the N-terminal domain (GST-VCPΔN), the D1 domain (GST-VCPΔD1), or the D2 domain (GST-VCPΔD2). Then lysates from these cells were incubated with control beads or NPD8733-bound beads, and bound proteins were analyzed as above. As shown in Fig. 4C, GST-VCP, GST-VCPΔD2, and GST-VCPΔN were pulled down by NPD8733 beads but not by control beads, whereas GST-VCPΔD1 was not pulled down by NPD8733 beads, clearly indicating that NPD8733 binds to the D1 domain of VCP.

Because the D1 domain of VCP displays ATPase activity, we examined the effect of NPD8733 binding on ATPase activity. For this, we made GST-VCP construct S459* (the 459th Ser was changed to a stop codon) that contains only the D1 ATPase domain but not the D2 domain. Then we measured the ATPase activity of full-length and S459* GST-VCP (Fig. S5). As reported previously, both the D1 and D2 domains had ATPase activity, although that of D1 was weaker than that of D2. Next, we examined the effect of NPD8733 on these activities. NPD8733 had some inhibitory effects on the ATPase activity of GST-VCPs, although it was less potent than the known VCP ATPase inhibitor N,N'-dibenzylquinazoline-2,4-diamine. Actually, 50% inhibition of ATPase activity could not be obtained, not even in the presence of 100 μM NPD8733, which was more than 10 times higher concentration of the in vivo effective concentration. From these results, we concluded that NPD8733 binds to the D1 domain of VCP and that this binding has a modest effect on the ATPase activity of D1, but this effect cannot explain the inhibitory effect of fibroblast migration accelerated by cancer cells. This indicates that the binding of NPD8733 to the D1 domain of VCP inhibits VCP function in a way other than through its ATPase activity, which should be elucidated in the near future.

**VCP is essential for enhanced migration of co-cultured NIH3T3 cells**

As NPD8733 was identified to bind to VCP and inhibits enhanced migration of co-cultured NIH3T3 cells, we hypothesized that VCP may play an important role in migration. However, so far, there are no reports showing the significance of VCP in cell migration.

The expression level of endogenous VCP in NIH3T3 cells is similar to that in MCF7 cells (Fig. S6). Although the expression of VCP was not significantly changed by co-culturing, we examined the effects of VCP silencing in NIH3T3 or MCF7 cells on enhanced cell migration through co-cultures. We used two oli-
Inhibitor of VCP-dependent fibroblast migration

Inhibitor of VCP-dependent fibroblast migration

Figure 2. NPD8733 inhibits enhanced migration of NIH3T3 cells co-cultured with MCF7 cells using a Transwell migration assay. A, NIH3T3 cells and co-cultured NIH3T3 cells were grown without FCS in the upper chamber and were allowed to migrate toward the bottom chamber containing 10% FCS. Different concentrations of NPD8733 were added to both the upper and lower chambers. The membrane was stained with crystal violet after 24 h of migration, and images of the three different fields were captured with an inverted microscope. B, the percentages of fibroblasts that migrated while co-cultured or when cultured alone were quantified using ImageJ. The graph shows the percentage of fibroblasts migrating in co-culture compared with fibroblast cultured alone and was plotted against different doses of NPD8733. In the control, without compound, there was a significant difference in the migration of co-cultured fibroblasts compared with that of fibroblasts alone at 24 h (n = 3; ***, p < 0.001). Treatment of NPD8733 at 1 μM and higher showed a significant decrease in the migration of NIH3T3 cells co-cultured in the presence of MCF7 cells (n = 3; ***, p < 0.001). The red dots indicate the individual measurements. C, cell proliferation using WST-8 reagent was carried out in NIH3T3 and MCF7 cells cultured in 1% FCS for 48 h. NPD8733 treatment up to 9 μM did not inhibit the growth of NIH3T3 and MCF7 cells. The red dots indicate the individual measurements.

Discussion

This study shows that NPD8733 inhibits the enhanced migration of co-cultured NIH3T3 cells by binding to VCP, a ubiquitously expressed protein that belongs to the AAA+ protein family. VCP is involved in multiple cellular processes, such as protein degradation, apoptosis, and autophagy (15–17). Although there are no reports that show involvement of VCP in the activation of fibroblast migration, VCP is known to play an important role in osteosarcoma cell metastasis through proteasome-dependent degradation of 1kBa, thus activating NF-κB (21). VCP is also known to directly interact with and regulate p53 and NF-κB proteins, controlling the progression of non-small-cell lung carcinoma progression (22). It has also been reported that VCP inhibition by siRNA suppresses migration of the lung carcinoma cell line H1299 (22). Interestingly, Fu et al. (23) observed that VCP knockdown in colorectal cancer cells suppresses metastasis in vivo. These in vivo results are consistent with our finding that VCP has an important role in the migration of fibroblasts surrounding cancer cells, as silencing of VCP in fibroblasts inhibited the accelerated migration in co-cultures.

VCP, also referred to as p97, consists of four domains, including the N-terminal domain, the two ATPase domains D1 and D2, and the unstructured C-terminal tail (15, 24). The N-terminal domain and C-terminal domain are known to be important for binding to substrates and co-factors involved in a variety of cellular functions (15), whereas the D1 and D2 domains encode ATPase catalytic domains. Although both D1 and D2 can hydrolyze ATP, they differ in terms of activity. Song et al. (25) showed that the D2 domain shows significant ATPase activity, whereas the D1 domain shows heat-induced ATPase activity. We also detected ATPase activity in both the D1 and D2 domains. However, binding of NPD8733 to the D1 domain only modestly inhibited the ATPase activity, and the mechanism of inhibition by NPD8733 remains to be elucidated.

We have shown that NPD8733 bound to the D1 domain of VCP. The D1 domain of VCP has minor ATPase activity and is also known to be involved in oligomerization for hexamer formation, which occurs after ATP hydrolysis (15). This oligomerization has been reported to be essential for protein degradation upon endoplasmic reticulum (ER) stress (17).
Inhibitor of VCP-dependent fibroblast migration

Figure 3. NPD8733 binds to VCP. A, NIH3T3 cells alone or NIH3T3 cells co-cultured with MCF7 cells were seeded overnight in 6-well plates in 10% FCS. The next day, the cells were scratched, treated with 1 μg/ml NPD8126 (2.8 μM) and NPD8733 (3.2 μM), and cultured in medium containing 1% FCS. Migration was observed at 24 h. B, chemical structures of NPD8733 and NPD8126. C, the cell migration percentage for fibroblasts and co-cultured cells in A was calculated using ImageJ. The graph shows the percentage of fibroblast migration in co-culture relative to the control (fibroblast alone) at 24 h and was plotted against the results using NPD8126 and NPD8733. There was a significant difference in the migration of co-cultured fibroblasts compared with fibroblasts alone at 24 h (n = 3, ***, p < 0.001). NPD8733 but not NPD8126 treatment showed a significant decrease in NIH3T3 cell migration when co-cultured with MCF7 cells (n = 3, ***, p < 0.001). The red dots indicate the individual measurements. D, NIH3T3 and MCF7 cells were co-cultured at a 5:1 ratio, and their cell lysates were incubated with control beads, NPD8126 beads, or NPD8733 beads for 3 h. The reacted beads were washed, and the eluted proteins were separated using SDS-PAGE and stained with CBB. The protein specifically co-precipitated with NPD8733 beads was identified by MALDI-TOF-MS to be VCP (arrow). MW, molecular weight.

Figure 4. NPD8733 bound the D1 domain of VCP. A, E. coli BL21 cells were induced for expression of GST-tagged VCP, and their lysates were incubated with control beads (naked), NPD8126 beads, or NPD8733 beads for 3 h. The reacted beads were washed, and the eluted proteins were separated using SDS-PAGE and immunoblotted with anti-GST antibody. The membrane was stained with CBB. MW, molecular weight. B, schematic of GST-VCP WT, GST-VCP ΔN, GST-VCP ΔD1, and GST-VCP ΔD2. The two solid lines in D1 and D2 indicate Walker A and Walker B motifs in ATPase domains, respectively. C, E. coli BL21 cells were induced for expression of GST-VCP WT, GST-VCP ΔN, GST-VCP ΔD1, and GST-VCP ΔD2, and their lysates were incubated with control beads (naked) or NPD8733 beads for 3 h. Cells without vector construct (−) were used as a control. The reacted beads were washed, and the eluted proteins were separated using SDS-PAGE and immunoblotted with anti-GST antibody. The membrane was stained with Coomassie Brilliant Blue dye.
Wang et al. (26) identified that eeyarestatin I binds to the D1 domain of the VCP-associated deubiquitinating complex and affects protein homeostasis in the ER. ER stress signaling and protein homeostasis are known to be disrupted in cancer and are also involved in cancer progression processes such as invasion and angiogenesis (27). Martinez-Outschoorn et al. (28) have shown that MCF7 cells induce oxidative stress and down-regulation of plasma membrane caveolin-1 (CAV1) expression in stromal fibroblasts only when they were cultured together but not when cultured separately. Because VCP has been shown to be involved in down-regulation of CAV1 (29), it is possible that oxidative stress by co-cultured cancer cells might affect the activity of VCP in fibroblasts.

The mechanism of VCP function in cell migration is not yet clear. As the compound NPD8733 bound to the D1 domain, and this binding inhibited the accelerated migration of fibroblasts, it is possible that NPD8733 inhibits the function of VCP, which may be important for accelerated cell migration in co-cultures. We examined the effect of co-culture on the activity of NF-κBα because NF-κBα is known to be one important downstream targets of VCP (21). However, we could not see any increase in the DNA binding activity of NF-κBα by co-culturing (data not shown). The molecular mechanism of this downstream signaling pathway should be elucidated in future studies.

So far, several inhibitors have been reported to inhibit VCP function (30, 31). Previously, Sasazawa et al. (20) showed that xanthohumol is a VCP inhibitor by binding to the N-terminal domain of VCP. We examined the effects of xanthohumol in our co-culture system. However, we did not observe any significant differences in the migration of co-cultured NIH3T3 cells, indicating that the N-terminal domain of VCP is not crucial for the inhibition of fibroblast activation.

Because VCP is known to act in a wide range of cellular processes, such as proteasome-dependent degradation, autophagy, and cell cycle regulation, further investigation is required to understand the mechanism behind VCP effects on activated fibroblast migration. Although there are several studies indicating a role of VCP in cancer metastasis (22, 23), here we demonstrate for the first time that VCP might possess novel therapeutic potential for targeting activated fibroblasts.

**Experimental procedures**

**Compounds**

NPD8732, NPD8733, FSL0816, NPD6543, HTD1063, and NPD6330 were obtained from the RIKEN Natural Products Depository (NPDepo, Saitama, Japan). The compounds were dissolved in DMSO as a stock solution and were stored at −20 °C.

**Cell lines**

The mouse embryonic fibroblast cell line NIH3T3, the human breast cancer cell line MCF7, and MDA-MB-231 cells were supplemented with 10% fetal calf serum (FCS) (Nichirei Bioscience Inc., Tokyo, Japan), 50 units/ml penicillin (Invitrogen), and 50 μg/ml streptomycin (Invitrogen). They were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.
**Inhibitor of VCP-dependent fibroblast migration**

**Assays in cell culture**

A wound healing assay, cell proliferation assay, and Transwell migration assay were carried out as described previously (14). The seeding was performed in triplicate, and the results were analyzed accordingly.

**Western blot analysis**

Western blot analysis was performed as follows. The cells were lysed in whole-cell extraction buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, and 50 mM Tris-Cl (pH 8.0)) containing a complete protease inhibitor mixture tablet (Roche Diagnostics). Next, 20 μg of cleared protein lysate was separated by 10% SDS-PAGE and then transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore, Billerica, MA). The blots were saturated with blocking buffer (5% skim milk in Tris-buffered saline with Tween 20) for 1 h at room temperature and incubated with the appropriate antibodies. The antibodies and final dilutions used for Western blot analysis were mouse anti-GST antibody (Santa Cruz Biotechnology, 1:1000) and mouse anti-VCP antibody (Abcam, ab11433, 1:1000), which is specific for both mouse and human. After washing in TBS-T, the membrane was incubated for 2 h at room temperature with horseradish peroxidase–goat anti-mouse IgG (GE Healthcare). Antibody binding was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

**Preparation of compound bound beads and pulldown experiments**

NPD8733 and NPD8126 beads were prepared using a method described previously (19). The co-cultured NIH3T3 cells and MCF7 cells (5:1; 2 × 10^5 and 0.4 × 10^6, respectively) were collected, washed with PBS, and resuspended in binding buffer (50 mM HEPES, 150 mM NaCl, and 1 mM EDTA (pH 7.5)) and a protease inhibitor mixture tablet (Roche Diagnostics). After cells were lysed by homogenization with sonication, the cell lysates were incubated with SDS-PAGE sample buffer. The eluted proteins were then subjected to SDS-PAGE and analyzed by staining with CBB and Western blot analyses.

**Silencing VCP expression using siRNA**

The siRNA double-stranded oligonucleotides were designed for VCP protein knockdown as described previously (30) (oligo 1, human VCP, sense 5'-GUAGGGUAUGAUGCAUUGU-3'; oligo 2, both human and mouse VCP, sense 5'-GGAGGUA-GAUAUGGAAUUU-3'; oligo 3, both human and mouse VCP, sense 5'-GAUAGAGGUUGCGGAUUU-3'; oligo 4, mouse VCP, sense 5'-GUAGCCUAUGAACAUGUUGU-3' (Dharmacon, GE Healthcare)). NIH3T3 and MCF7 cells were seeded in 6-well plates at 2 × 10^5 cells/well, grown for 16 h, and transfected with siRNA oligonucleotides at 30 pmol/well using Lipofectamine™ 2000 reagent (11668-027, Invitrogen) according to the manufacturer’s protocol. An equal amount of control siRNA (sc-37007, Santa Cruz Biotechnology) was used as the negative control. All experiments were carried out 24 h after transfection.

**Method for protein identification**

To identify the protein in a band of SDS-PAGE, we performed peptide mass fingerprinting with MALDI-TOF mass analysis as described previously (19). The protein-containing region was cut out and washed successively with water, decolorant (50% acetonitrile and 50 mM NH₄HCO₃), and acetonitrile. The samples were then dried in vacuo. An aqueous alkylation solution (100 mM iodoacetamide and 100 mM NH₄HCO₃) was added to the dried gels, and the mixture was incubated at 37 °C for 30 min. The gels were then washed with 100 mM NH₄HCO₃ solution, dehydrated with acetonitrile, and dried in vacuo. The dried gels were seeded in digestion buffer (10 mM Tris-HCl (pH 8.8) and 25 ng/ml trypsin) at 4 °C for 30 min and incubated at 37 °C overnight. Samples were analyzed using a Bruker Ultraflex mass spectrometer operated in positive ion reflector mode. α-Cyano-4-hydroxycinnamic acid (10 mg/ml) dissolved in 50% acetonitrile/0.1% TFA was used as the matrix. The picking list was generated by Biotools version 3.2 (Bruker Daltonik GmbH). The Mascot search program (Matrix Science Inc.) was used to search the Swiss-Prot database for peptide masses.

**Measurement of ATPase activity of VCP**

Full-length or truncated VCP was preincubated with test compounds in assay buffer (50 mM Tris (pH 7.4), 20 mM MgCl₂ and 1 mM EDTA) for 60 min and incubated in the presence of 100 nM ATP for another 60 min at room temperature. The remaining ATP levels after the reaction were measured by 10 μl
of Kinase Glo Plus reagent (Promega) and further incubation for 10 min at room temperature in the dark. The luminescence was read using a microplate reader (VarioSkAn Lux, Thermo Fisher Scientific). These values were subtracted from the ATP level without reaction, and the consumed ATP levels were calculated.

Statistics

All experiments were performed at least three times. Data are expressed as mean ± S.D. Data were subjected to one-way analysis of variance and Tukey’s honestly significant different post hoc test using R (R Foundation for Statistical Computing, Vienna, Austria).

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