An ubiquitin-binding molecule can work as an inhibitor of ubiquitin processing enzymes and ubiquitin receptors

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Abstract

The ubiquitin pathway plays a critical role in regulating diverse biological processes, and its dysregulation is associated with various diseases. Therefore, it is important to have a tool that can control the ubiquitin pathway in order to improve understanding of this pathway and to develop therapeutics against relevant diseases. We found that Chicago Sky Blue 6B binds directly to the β-groove, a major interacting surface of ubiquitin. Hence, it could successfully inhibit the enzymatic activity of ubiquitin processing enzymes and the binding of ubiquitin to the CXCR4, a cell surface ubiquitin receptor. Furthermore, we demonstrated that this ubiquitin binding chemical could effectively suppress the ubiquitin induced cancer cell migration by blocking ubiquitin-CXCR4 interaction. Current results suggest that ubiquitin binding molecules can be developed as inhibitors of ubiquitin-protein interactions, which will have the value not only in unveiling the biological role of ubiquitin but also in treating related diseases.

Introduction

As a crucial step in the post translational modification of proteins in eukaryotes, ubiquitin (Ub), a highly conserved 76 amino acid residue protein, attaches to the substrate proteins via an isopeptide bond between the C-terminal of ubiquitin and a lysine residue of the substrate by the activating enzyme E1, a conjugating enzyme E2, and a ligase E3 [1]. Similar to other signaling events such as phosphorylation, ubiquitination is reversible when deubiquitinase (DUB), a hydrolyzing enzyme, removes ubiquitin from its substrates [2]. In addition to attachments of a single ubiquitin to substrates, ubiquitin can also be attached to substrates as a polyubiquitin chain in which ubiquitin is linked via an isopeptide bond to one of seven lysine residues, or via a peptide bond to the N-terminal of another ubiquitin [2]. Accordingly, there are eight different types of polyubiquitin chains that all have distinctive structures and functions [2].

The best-studied function of ubiquitination is the ubiquitin-mediated proteasomal degradation of proteins, which regulates protein levels, thereby affecting the associated cellular processes. In addition to protein degradation, ubiquitination has also been directly involved in various cellular signaling events, including DNA damage repair [3], immunity [4-6], T-lymphocyte control [7], and cell division [8, 9]. Recent progress in ubiquitin biology revealed that various forms of unanchored ubiquitin are also involved in cellular signaling [10-12]. For example, unanchored extracellular ubiquitin is responsible for apoptosis, cell growth, immune modulation, and cancer progression through its binding to CXC chemokine receptor type 4 (CXCR4)[13-15]. Consequently, dysregulation of ubiquitin signaling is a cause of various diseases [16]. In particular, the development of cancer, neurodegenerative diseases and immune diseases are linked to malfunction of the ubiquitin-proteasome system [17, 18].

Therefore, the ubiquitin signaling pathway, and more specifically, the enzymes involved in both ubiquitination, deubiquitination and proteasome, have been considered a good therapeutic target,

and many inhibitors targeting specific enzymes in ubiquitin pathways have been developed as drug candidates for various diseases [16, 19]. In particular, Bortezomib, an ubiquitin proteasome system (UPS) inhibitor approved by the US FDA as a cancer drug, supports the druggability of the ubiquitin signaling [20, 21]. While the enzymes in the ubiquitin pathways have been recognized as druggable targets [22-24], ubiquitin, the main agent of this signaling pathway, has been generally considered as non-druggable. Hence, it has not attracted much attention. To the best of our knowledge, ubistatins are the only small molecules that block ubiquitin-protein interactions by binding directly to K48-linked poly-Ub chains [25]. It has been shown that ubistatins block cell cycle progressions through inhibition of the ubiquitin-dependent degradation of cell cycle components in cell extracts. However, the application of ubistatins, such as in therapeutics and intracellular probes, has not been demonstrated. Nevertheless, ubistatins present the possibility of direct binding to ubiquitin being a promising approach in modulating ubiquitin pathways.

In this study, we identified Chicago sky blue 6B (CSB6B) as a novel ubiquitin binding small molecules, which can act as an inhibitor that disrupts interactions between ubiquitin and ubiquitin binding domains that exist in ubiquitin processing enzyme or ubiquitin cell surface receptors in the ubiquitin pathway. Through dockings combined with molecular dynamic simulation and mutational analyses, we confirmed the binding mode of the inhibitor to ubiquitin. We further demonstrated that ubiquitin can be a druggable target for cancer therapies by showing that Ub-binding inhibitor can effectively block the extracellular ubiquitin induced cancer progression. Herein, we discuss mechanism of action of this chemical inhibitor and its potential for the inhibition of the druggable target ubiquitin, which could be used for functional studies of ubiquitin and for further therapeutic purpose.

Materials and Methods

Sample preparation

Plasmids containing His_6-USP5, His_6-UCHL1, GST-E2-25K, His_6-Mms2, His_6-ubiquitin and ubiquitin mutants, or GST-ubiquitin-HA, were transformed into *E. coli* BL21 (*DE3*) cells. Proteins were expressed using IPTG and then purified by chromatographic methods. The fractions containing purified proteins were analyzed by SDS-PAGE, collected, concentrated to 5 mg/ml and stored in a -80°C deep freezer. FITC-labeling of ubiquitin using the FluoReporter FITC protein labeling kit (FITC1) was carried out following the manufacturer's instructions (Life Technologies).

Enzyme activity assay

For in vitro K48-polyubiquitination, 200 – 500 μM of mono-ubiquitin was mixed with 0.1 μM Uba1, 1 unit creatine-phosphokinase, 1 unit pyrophosphatase and 0.1 μM E2 – 25K in the buffer containing 50 mM Tris – HCl, PH 7.5, 5 mM creatine phosphate, 8 mM $MgCl_2$, 1 mM DTT, and 5 mM ATP. For K63-polyubiquitination, 0.1 μM Mms2/Ubc13 was used instead of E2 – 25K. For monitoring the inhibition of ubiquitination, 50 – 500 μM CSB6B was additionally treated in the reaction mixture. The reaction mixture was incubated at 37 ^oC for 4 h. The 5x SDS sampling buffer was added in order to stop the reaction. The results were analyzed by 15 % SDS – PAGE and western blot with ubiquitin specific antibody. Deubiquitination assays of USP5 and UCHL1 were performed by mixing 0.6 μM of USP5 or 8 μM of UCHL1 with 5 μM of GST-ubiquitin-HA in the absence or presence of 0 – 45 μM of CSB6B in the buffer containing 50 mM Tris HCl, PH 8.0, 100 mM NaCl, 10 mM DTT, at 37^oC for 3 h. The reaction was stopped by adding 5x SDS sampling buffer. Results were analyzed using the mobility shifts of GST-Ubiquitin-HA to GST-ubiquitin on 12.5 % SDS-PAGE stained with Coomassie Blue. The signal intensity was later measured by

ImageJ [26] and used to generate the IC50 curve fitting with build-in equations (log inhibitor vs. normalized response with Variable slope) from GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Wound healing and cell migration assays

H1299 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) containing 10 % Fetal Bovine Serum and 1 % penicillin/streptomycin in 12-well-plates, and incubated in a 5 % CO_2 incubator until cell confluency of 100 % was achieved. Would healing assays were performed by generating a scratch with a 10 µl tip and the medium was replaced with a serum-free DMEM in the presence or absence of CSB6B, AMD3100 or ubiquitin. The recovery region was visualized after 32 h. Cell migration assays was performed by using the Migration Assay Kit (cat # QCM512, MINIPORE). H1299 cells were cultured in serum-free DMEM pretreated with AMD3100 or CSB6B for 30 min and then loaded onto an upper chamber. The bottom chamber was also filled with drug-containing media and ubiquitin; the migrated cells in the bottom chamber were counted at 24 h post drug treatment.

Statistical analysis

Results were normalized by control value and represented as mean ± standard error of mean. All experiments were repeated at the minimum of three times, unless mentioned otherwise. Statistical comparisons between groups were determined with the Student's t-test. If p-values are less than 0.05, they are considered statistically significant and were indicated in the graph. The statistical significance of the normalized values is indicated using "$*$" (p <0.05), "$**$" (p<0.01), and "$***$"

(p<0.001).

Results and Discussion

Initially, we screened chemical libraries to identify the compounds that inhibit deubiquitination activity of the 293T cell lysates. Deubiquitination activity was examined by confirming the cleavage of human influenza hemagglutinin derived (HA) tag from glutathione *S*-transferase (GST)-ubiquitin-HA in SDS-PAGE (Fig. 1A), which is previously reported [27, 28]. The inhibitory activity of compounds in the library was then evaluated by observing the disappearance of the GST-ubiquitin band in the presence of the compounds. By such screening, Chicago Sky Blue 6B (CSB6B) was identified as a potent inhibit (Fig. 1A). Next, we tested the inhibitory activity of CSB6B on USP5, which belongs to the ubiquitin-specific protease (USP) superfamily, and on UCHL1, one of ubiquitin C-terminal hydrolases (UCH) (Fig. 1B). The IC50 values were calculated from the intensity of the scanned image (Fig. 1C): 6.5 ± 0.8 μM and 11.2 ± 1.4 μM for USP5 and UCHL1, respectively. USP5 has substrate preference for the unanchored K48 polyubiquitin chains, while UCHL1 cleaves the small C-terminal adducts of ubiquitin to generate the ubiquitin monomer [29]. The two enzymes had different substrate specificities, but they were commonly inhibited by CSB6B, suggesting that CSB6B is a non-specific inhibitor.

When we explored the inhibitory mode of CSB6B, we found that CSB6B inhibits DUBs not by binding to enzymes but by binding directly to ubiquitin; this was confirmed by the mobility shift of ubiquitin upon CSB6B treatment on native polyacrylamide gel electrophoresis (PAGE) (Fig. 1D) as well as co-elution of ubiquitin/CSB6B in size exclusion chromatography (Fig. 1E). In order to investigate the binding mode of CSB6B to ubiquitin, the model of CSB6B-bound ubiquitin was constructed through docking, followed by energy minimization (Fig. 2A). To verify the

binding modes of CSB6B to ubiquitin, as suggested by docking analyses, we carried out a further investigation on the binding stability with molecular dynamics simulation (Fig. 2B). The minimum level of change in RMSD of atom positions for most of simulation period (50ns) represents stable interactions of CSB6B to ubiquitin in the current model (Fig. 2B). According to this model, CSB6B binds to ubiquitin by contacting the groove between β3–β4 (β–groove; residues 42–50) and by stabilizing the C-terminal motif (residues 68–74; Fig. 2AC). Many hydrophobic residues including I44, F45, A46, V70, L71 and L73 are involved in CSB6B binding, but charged residues, H68, Q49, R42, R72, and R74, flanking the hydrophobic patch also seems to strengthen the interaction by forming an electrostatic interaction with charged groups of CSB6B (Fig. 2B). Moreover, it appears that the CSB6B becomes bent along the convex protein surface in such a way that the contact of its aromatic rings with the hydrophobic patch of ubiquitin is maximized, thereby leading to stronger hydrophobic interactions (Fig. 2C).

To corroborate the suggested binding mode, we mutated key charged residues involved in binding and examined the binding affinity of mutated ubiquitin for CSB6B by using a fluorescence quenching assay. From this study, it was observed that the binding affinities of ubiquitin mutants H68 and R72A/R74C were reduced 1.6 fold (K_D =18.8 ± 1.5 μM), and 1.5-fold (K_D = 17.7 ± 1.5 μM), respectively, compared to that of wild type ubiquitin (K_D = 12.0 ± 0.7 μM). Therefore, electrostatic interaction from an individual residue seems to contribute to the CSB6B binding but is not substantial. These results also suggest that hydrophobic packing might be more crucial for the binding.

To investigate the binding specificity of chemical to the ubiquitin, we tested the binding of CSB6B to a small ubiquitin-like modifier 1 (SUMO1) which has structural fold similar to that of ubiquitin. However, CSB6B did not bind to SUMO1 as confirmed by independent migration of

SUMO1 and CSB6B in native PAGE (Supporting Information Fig. 1A). Although SUMO also has hydrophobic patches, which are likewise interaction sites to the partner proteins, those in SUMO1 are located on the opposite side of the molecule in comparison to those of ubiquitin. As it is, most hydrophobic residues involved in the CSB6B binding are overlapped with charged residues in SUMO: A46, Q49, H68, V70, R72, L73, and R74 in ubiquitin vs. E67, R70, E89, Y91, E93, Q94, and T95 in SUMO1 (Supporting Information Fig. 1B). These indicate that the binding of CSB6B to ubiquitin is specific among ubiquitin-like modifiers through the geometric fit, allowing proper hydrophobic interactions that are supplemented by electrostatic interactions.

The development of inhibitors of the ubiquitin signaling pathway is a potential strategy to decipher the complicated signaling events and also to treat several diseases associated with this pathway. Thus far, enzymes involved in the pathway, conjugation and ligation of ubiquitin, DUBs, and proteasomes had been targets of such research. However, ubiquitin had seldom been in the list of targets, except for the discovery of ubistatins, which are K48-specific inhibitors. In this study, we identified that ubiquitin residues comprising the hydrophobic patch for CSB6B binding are known to be crucial for the interaction of ubiquitin to its binding partners such as ubiquitin activating enzyme (UBA1), ubiquitin conjugating enzyme (E2-25k) and ubiquitin receptors such as CXCR4 [30]. Therefore, it is expected that ubiquitin binding to those binding partners can be blocked by CSB6B (Supporting Information Fig. 2). Accordingly, the activity of ubiquitin processing enzymes and the ubiquitin receptor signaling can be inhibited by CSB6B. To investigate these possibilities, we first examined the effect of CSB6B on E1-E2 enzymes by monitoring the formation of K48-linked polyubiquitin chains using E2 conjugating enzyme E2-25K and K63-linked polyubiquitin chains using the chain-specific conjugating complex Mmms2/Ubc13 with UBA1 as the common E1 enzyme. CSB6B clearly inhibited both K48 and K63 polyubiquitination in a concentration-dependent manner (Fig. 3A&B).

Then, we investigated the inhibitory activity of CSB6B on the ubiquitin-protein interaction by examining the binding affinity of ubiquitin to CXCR4, a cell surface receptor that recognizes ubiquitin as a ligand [13-15]. Since ubiquitin binding to the cell surface would be mediated by CXCR4, the interaction of FITC-labeled ubiquitin with CXCR4 was assessed by measuring the fluorescence associated with human monocyte cells (THP-1) after treating CSB6B at various concentrations (Figs. 3C&D). Using this test, we confirmed a decrease in fluorescent signal associated with cells, in proportional to the concentration of CSB6B (Fig. 3C). In addition, we clearly observed that the binding of FITC-ubiquitin on cell surface can be blocked by CSB6B as well as AMD3100, an approved CXCR4 antagonist (Fig. 3D), implying that CSB6B inhibits ubiquitin-receptor binding in the same way that the chemical antagonist of CXCR4 does. Taken together, these results suggest that CSB6B has the potential to disrupt the interaction between ubiquitin and UBDs by binding to the major PPI recognition site of ubiquitin.

Owing to its four sulfonates, it can be expected that the permeability of the cell membrane to CSB6B was very low. The low permeability of CSB6B might lower its intracellular utility, but instead it gives a unique value to this class of compounds as an extracellular tool. Thus, we tested the effect of CSB6B in the extracellular space. It has been explored that the extracellular ubiquitin binds to CXCR4 as a ligand and turns on CXCR4 signaling, which results in enhanced AKT expression, AKT phosphorylation, and metastasis [13, 31]. In the ubiquitin-CXCR4 signaling process, it is known that hydrophobic residues of ubiquitin including Phe4 and Val70 directly bind CXCR4, and the C-terminal motif facilitates receptor activation [31]. Since CSB6B binding sites are overlap with CXCR4 binding and activation sites (Supporting Information Fig. 2), it was expected that CSB6B can effectively block the binding of ubiquitin to CXCR4 and inhibit the ubiquitin-CXCR4 signaling. Accordingly, the effects of CSB6B on the level of AKT phosphorylation and migration of H1299, a human non-small cell lung carcinoma cell line,

triggered by extracellular ubiquitin, were examined and compared to those of AMD3100 [15, 32]. Treatment of H1299 cells with ubiquitin in the cell culture media increased AKT expression and its phosphorylation, whereas co-treatment with AMD3100 or CSB6B reduced both to the control level (Fig. 4A). Moreover, ubiquitin-induced migration was abolished by CSB6B in the cell migration assay; this effect of CSB6B was comparable to that of AMD3100 (Fig. 4B). These results imply that CSB6B acts as a CXCR4 antagonist by directly binding to extracellular ubiquitin. Consistently, CSB6B effectively blocked wound healing caused by ubiquitin treatment (Fig. 4C&D) in a wound healing assay; this further supports the inhibitory action of CSB6B on the ubiquitin-CXCR4 interaction, similar to that of AMD3100.

In this study, we identified CSB6B as a DUB inhibitor (Fig. 1) and proposed that its binding to the hydrophobic patch of ubiquitin blocks the recognition of ubiquitin to numerous ubiquitin-binding or interacting molecules in signaling pathways (Fig. 2). We also proved that CSB6B effectively inhibits ubiquitin processing by showing its inhibitory activity on ubiquitin chain synthesis that is mediated by E1-E2 enzymes (Fig. 3AB). Finally, we demonstrated that CSB6B inhibits extracellular ubiquitin-mediated CXCR4 signaling (Fig. 4). This inhibitory activity is almost comparable to that of the approved CXCR4 inhibitor, AMD3100, which is a known specific inhibitor that binds to CXCR4. Thereby AMD3100 disrupts the binding of ligands, such as ubiquitin and SDF1α, to CXCR4 and subsequently blocks the CXCR4-mediated cancer cell migration [15]. Thus, the comparable activities of CSB6B and AMD3100 in the inhibition of the PI3K/AKT pathway, cell migration, and wound healing (Fig. 4) strongly suggest that CSB6B could be used as a selective inhibitor or chemical probe to modulate the activity of extracellular ubiquitin and to subsequently allow its functional study. Thus far, the functional study of extracellular ubiquitin has been extremely difficult because no method has been proven successful in selectively modulating the extracellular ubiquitin. Herein, for the first time, we propose a

potential molecule that has this quality. Moreover, as our results for the CXCR4 receptor suggest, CSB6B or its molecular scaffold could be utilized as a potential drug for controlling the epithelial mesenchymal transition (EMT) stage of cancer. In addition, using these results as a base, we demonstrated the druggability of ubiquitin. To the best of our knowledge, this study is the first in providing the novel method that develops the inhibitor which directly targets ubiquitin molecules; this may be used in functional studies of ubiquitin, particularly of extracellular ubiquitin. Such studies will provide a valuable insight into this complicated signaling system from numerous perspectives, other than those obtained from studies using conventional enzyme inhibitors. We expect that this will open up new avenues for the study on the biology of ubiquitin, especially of extracellular ubiquitin. Moreover, with the druggability of ubiquitin displayed, this inhibitor and its binding mode can potentially be utilized in the development of cancer therapeutics.

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Figure legends

Figure 1. Identification of CSB6B as a DUB inhibitor and an ubiquitin binder. (A) Inhibitory activity of CSB6B on cleavage of HA from substrate (GST-UB-HA) by the 293T total cell lysate. Lanes a-d; substrate only, substrate with cell lysate, substrate with cell lysate in the presence of a non-active compound, and substrate with cell lysate in the presence of CSB6B. **(B)** Inhibitory activity of CSB6B on USP5 and UCHL1. Cleavage of HA from substrate (GST-UB-HA) was monitored in SDS-PAGE in the presence of each DUB with varying concentrations of CSB6B (0-20 μM). (**C**) Inhibitory activities of CSB6B on USP5 and UCHL1 in Fig. 1B were quantified by band intensity in SDS-PAGE and % inhibition at each concentration of CSB6B is plotted. **(D)** Binding of CSB6B to ubiquitin was detected by native PAGE. Purified ubiquitin mixed with CSB6B at the indicated molar ratio, [CSB6B]:[Ub], was analyzed in the native PAGE. The mobility of CSB6B in the gel was monitored by the blue color of CSB6B (top). The same gel was Coomassie Blue stained to monitor the mobility of ubiquitin (bottom). **(E)** Size exclusion chromatography of CSB6B/ubiquitin mixture; gel filtration profile of CSB6B only (red) and a mixture of CSB6B and ubiquitin (blue) was monitored at 280 nm (left) for detection of protein and at 620 nm (right) for CSB6B.

Figure 2. The binding mode of CSB6B to ubiquitin. (A) Docking model of CSB6B/ubiquitin complex. CSB6B is drawn as a white stick model on the surface-filling model of ubiquitin with electrostatic potential. Ubiquitin residues close to CSB6B are drawn as pink sticks with labels. (B) RMSD graph over simulation times (50ns) indicates the stable interaction in the docking model of CSB6B/ubiquitin complex. (C) CSB6B/ubiquitin complex in three orientations. Ubiquitin and

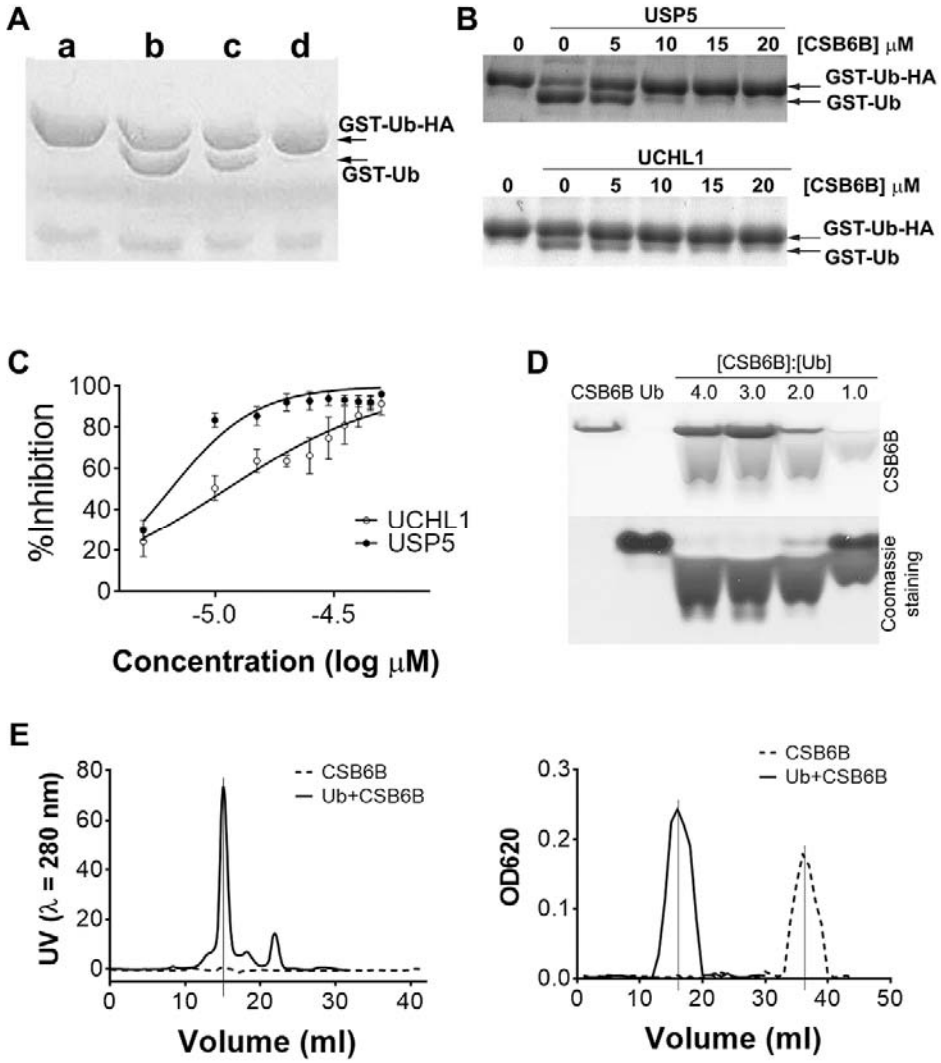
CSB6B are drawn as ribbon and stick models, respectively.

Figure 3. The inhibitory activity of CSB6B on ubiquitin-protein interaction. (**A**) The inhibitory activity of CSB6B on K48 polyubiquitination (left) and on K63 polyubiquitination (right). The size of the ubiquitin bands in SDS-PAGE, determined by the immunoblotting using ubiquitin specific antibodies, represents the polymerization of ubiquitin chain. **(B)** The quantified inhibitory activity of CSB6B on K48 and K63 polymerization. The band intensity of monoubiquitin in **Fig.3A** was quantifed. **(C)** Inhibitory activity of CSB6B on the association of FITC-ubiquitin to THP-1 cells. Fluorescence intensity of FITC-ubiquitin from THP-1 cells was quantified, and the intensity reduction was plotted as a percentage inhibition. Error bars represent the standard deviation from at least three independent experiments. **(D)** Confocal images of THP-1 (Ctrl), THP-1 cells treated with FITC-labelled ubiquitin in the absence of chemicals (FITC-Ub), presence of AMD3100 (FITC-Ub+AMD) and CSB6B (FITC-Ub+CSB6B), respectively. DAPI staining and FITC-ubiquitin are indicated by blue and green colors, respectively.

Figure 4. Inhibitory activity of CSB6B on ubiquitin-dependent CXCR4 signaling and cancer cell migration. (**A**) The inhibitory activities of CSB6B and AMD3100 on the ubiquitin-induced phosphorylation of AKT. Phosphorylation levels of AKT and the amount of AKT were measured by immunoblotting with corresponding antibodies. (**B**) Inhibitory activities of CSB6B and AMD3100 on the ubiquitin-induced invasion activity of H1299 cells. H1299 cells were treated with 10 μM ubiquitin alone (Ub), in the presence of 1 μM AMD3100 (Ub+AMD) or 10 μM CSB6B (Ub+CSB6B). The migrated cells were counted after 24hr. **(C)** Wound healing assays were

performed after generating a scratch with a 10 μl tip in the same condition as **Fig. 4B**. The recovery region was visualized after 32 h. (**D**) Results of wound healing assay in **Fig. 4C** were quantified.

Figure 1.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Volume (ml)
OD620
0.3
0.2
0.1
0.0
0
10
20
30
40
50
CSB6B
Ub+CSB6B
Volume (ml)

Figure 2.

Figure 2

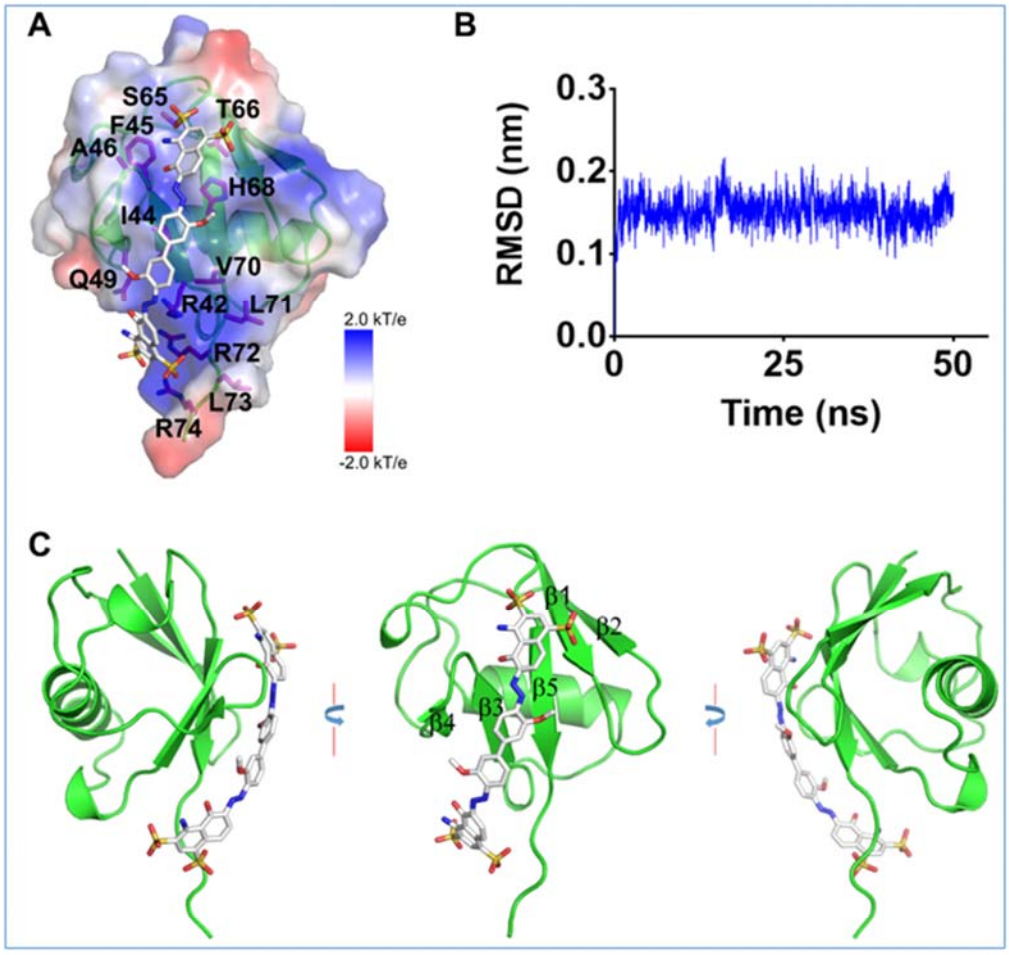


Figure 3.

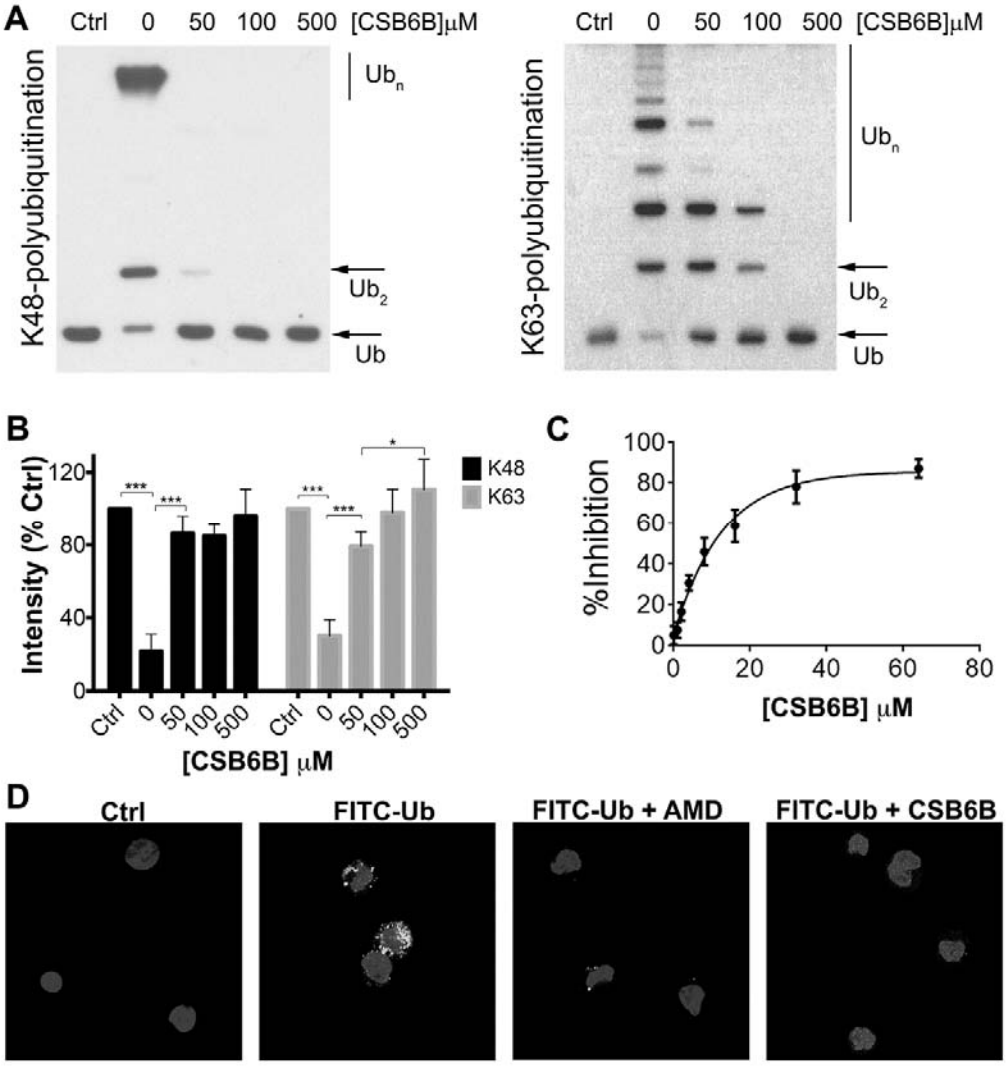
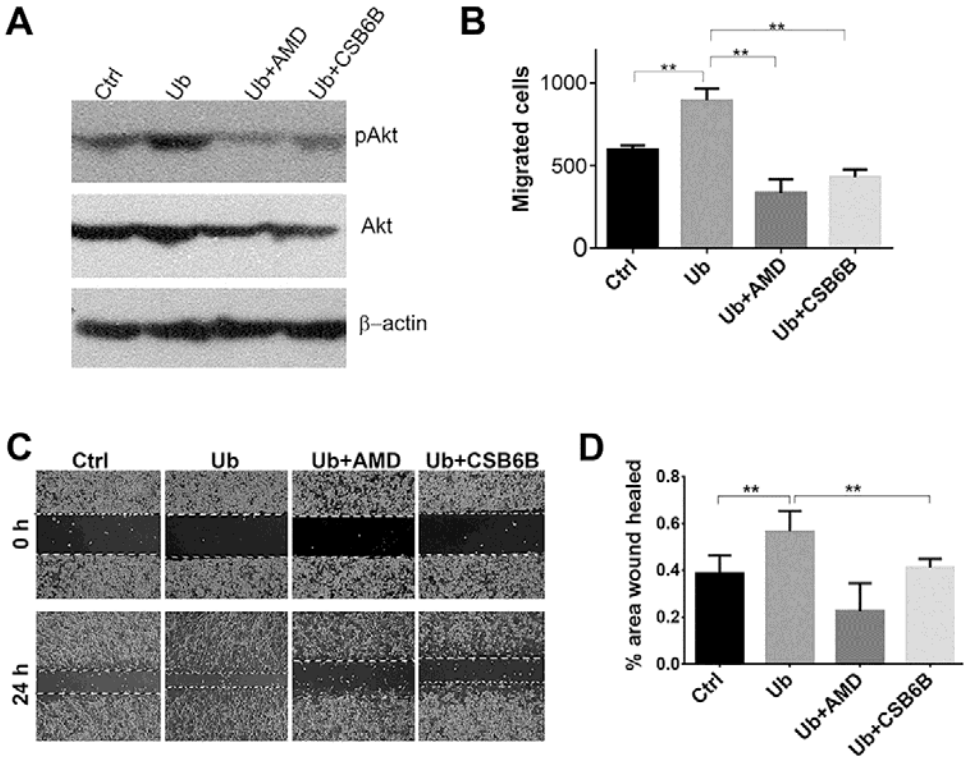

A
Ctrl 0 50 100 500 [CSB6B]μM
K48-polyubiquitination
Ub_n
Ub_2
Ub
Ctrl 0 50 100 500 [CSB6B]μM
K63-polyubiquitination
Ub_n
Ub_2
Ub
B
Intensity (% Ctrl)
120
80
40
0
K48
K63
***
***
***
***
*
Ctrl 0 50 100 500 Ctrl 0 50 100 500
[CSB6B] μM
C
%Inhibition
100
80
60
40
20
0
0 20 40 60 80
[CSB6B] μM
D
Ctrl
FITC-Ub
FITC-Ub + AMD
FITC-Ub + CSB6B

Figure 4.


A
Ctrl
Ub
Ub+AMD
Ub+CSB6B
pAkt
Akt
β–actin
B
Migrated cells
1000
500
0
**
**
**
Ctrl
Ub
Ub+AMD
Ub+CSB6B
C
Ctrl
Ub
Ub+AMD
Ub+CSB6B
0 h
24 h
D
% area wound healed
0.8
0.6
0.4
0.2
0.0
**
**
Ctrl
Ub
Ub+AMD
Ub+CSB6B