

**Covalent modification of Cys-239 in  $\beta$ -tubulin by small molecules as a strategy to promote tubulin heterodimer degradation**

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Running title: *A novel strategy for tubulin heterodimer degradation*

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**ABSTRACT**

Clinical microtubule-targeting drugs are functionally divided into microtubule-destabilizing and microtubule-stabilizing agents. Drugs from both classes achieve microtubule inhibition by binding different sites on tubulin and inhibiting or promoting polymerization with no concomitant effects on the protein levels of tubulin heterodimers. Here, we have identified a series of small molecules with diverse structures potentially representing a third class of novel tubulin inhibitors that promote degradation by covalent binding to Cys-239 of  $\beta$ -tubulin. The small molecules highlighted in this study include T0070907 (a peroxisome proliferator-activated receptor gamma inhibitor), T007-1 (a T0070907 derivative), T138067, *N,N'*-bis(2-iodoethyl)-*N,N'*-ethylene-bis(iodoacetamide) (EBI), and allyl isothiocyanate (AITC). Label-free quantitative proteomic analysis revealed that T007-1 promotes tubulin degradation with high selectivity. Mass spectrometry findings showed covalent binding of both T0070907 and T007-01 to Cys-239 of  $\beta$ -tubulin. Furthermore, T007-1 exerted a

degradative effect on tubulin isoforms possessing Cys-239 ( $\beta$ 2,  $\beta$ 4,  $\beta$ 5( $\beta$ )) but not those containing Ser-239 ( $\beta$ 3,  $\beta$ 6) or mutant  $\beta$ -tubulin with a C239S substitution. Three small molecules (T138067, EBI, and AITC) also reported to bind covalently to Cys-239 of  $\beta$ -tubulin similarly induced tubulin degradation. Our results strongly suggest that covalent modification of Cys-239 of  $\beta$ -tubulin by small molecules could serve as a novel strategy to promote tubulin heterodimer degradation. We propose that these small molecules represent a third novel class of tubulin inhibitor agents that exert their effects through degradation activity.

Microtubules are tubular structures formed by  $\alpha/\beta$  tubulin heterodimers that play important roles in multiple physiological activities of cells, such as migration, protein transport, and mitosis (1). Microtubules form spindles during mitosis, and disrupting their dynamic instability can prevent spindle formation. In view of the compelling

research findings, microtubules present highly attractive targets for anticancer drug design (1, 2). Microtubule inhibitors are functionally classified into two categories: microtubule-stabilizing and microtubule-destabilizing agents (3). The microtubule stabilizing agents mainly bind to the paclitaxel site or the laulimalide site of tubulin (4, 5), while the microtubule destabilizing agents bind to the colchicine, vinblastine, maytansine or pironetin sites (6). These agents achieve microtubule inhibition by binding different sites on tubulin and promoting or inhibiting polymerization with no concomitant effects on the protein levels of tubulin heterodimers (2). However, the commonly used clinical microtubule-targeting agents paclitaxel and vinblastine are susceptible to drug resistance (7, 8), emphasizing the urgent medical need to identify novel effective microtubule inhibitors with diverse mechanisms of action.

Targeting protein degradation is a known efficacious therapeutic strategy. A few examples are degradation of promyelocytic leukemia (PML)-retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) oncoprotein induced by arsenic trioxide or retinoic acid and that of estrogen receptors induced by selective estrogen receptor modulators (9–11). These earlier findings support the potential of tubulin degradation as a feasible anticancer approach. A number of small molecules have been identified that promote degradation of tubulin, such as T0070907, thymoquinone, isothiocyanates, and withaferin A (12–18), but their underlying mechanisms remain elusive at present.

In this study, we have identified five small molecules (T138067, *N,N'*-bis(2-ethyl-6-iodoacetamido)ethane-1,2-diamine (EBI), allyl isothiocyanate (AITC), T0070907, and T007-1, a T0070907 derivative) with diverse structures that promote tubulin degradation via covalent binding to Cys-239 of  $\beta$ -tubulin. Label-free quantitative

proteomic analysis revealed that T007-1 promotes tubulin degradation with high selectivity. Mass spectrometry findings demonstrated that both T007-1 and T0070907 bind covalently to Cys-239 of  $\beta$ -tubulin. Furthermore, T007-1 exerted a degradative effect on  $\beta$ 2,  $\beta$ 4, and  $\beta$ 5 ( $\beta$ ) tubulin isoforms, which possess a cysteine residue at position 239, but not  $\beta$ 3 and  $\beta$ 6 tubulin isoforms with serine at this position or mutant  $\beta$ -tubulin with a C239S substitution. The collective results suggest that this covalent binding activity of T007-1 accounts for its tubulin degradation effect. Notably, three small molecules (T138067, EBI, and AITC) reported to bind covalently to Cys-239 of  $\beta$ -tubulin also induced tubulin degradation while exerting no effect on mutant  $\beta$ -tubulin with C239S. All five small molecules identified in this study with totally different structures effectively promoted tubulin degradation by binding covalently to Cys-239 of  $\beta$ -tubulin. Based on the collective results, we propose that these small-molecule tubulin degradation agents exert their effects via covalent modification of Cys-239 of  $\beta$ -tubulin and constitute a third novel class of tubulin inhibitors.

## Results

### *T007-1 downregulates tubulin protein with high selectivity*

T0070907, a peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) inhibitor (Figure 1A), promotes proteasome-dependent tubulin degradation and exerts anticancer effects on colorectal carcinoma cells (13, 19). However, the mechanism underlying T0070907-mediated tubulin degradation remains to be established. To uncover the associated mechanisms and obtain anticancer lead compounds with improved activity, a series of T0070907 derivatives were synthesized. T007-1 (Figure 1A; synthesis described in supporting information), one of the T0070907 derivatives,

showed better anticancer activity than the parent compound against the human cervical adenocarcinoma cell line, HeLa, and human colon colorectal carcinoma cell line, Hct116 (Figure 1B). Label-free quantitative proteomic analysis of T007-1-treated HeLa cells showed that T007-1 induced suppression of the protein levels of  $\alpha$ - and  $\beta$ -tubulin isoforms with high specificity. The top four most downregulated proteins among the 1114 identified proteins were  $\alpha$ - and  $\beta$ -tubulin isoforms ( $\beta$ ,  $\beta$ 4B,  $\beta$ 8, and  $\alpha$ 1; Figure 1C, Supporting Information Set 1). Tubulin downregulation was confirmed via immunofluorescence staining of T007-1-treated HeLa cells. As shown in Figure 1D, HeLa cells treated with T007-1 showed little tubulin staining (green staining with anti- $\alpha$ -tubulin antibody). Immunoblot analysis revealed that T007-1 promotes  $\alpha$ -tubulin and  $\beta$ -tubulin degradation in HeLa and Hct116 cells in a dose-dependent manner (Figure 1E). Our results indicate that T007-1 mediates downregulation of tubulin with high selectivity.

#### ***T007-1 promotes proteasome-dependent degradation of tubulin heterodimers***

To determine whether T007-1 affects  $\alpha$ -tubulin and  $\beta$ -tubulin mRNA expression, a quantitative PCR assay was performed. As shown in Figures 2A and 2B, T007-1 induced time-dependent downregulation of  $\alpha$ -tubulin and  $\beta$ -tubulin proteins in both HeLa and Hct116 cells but exerted no inhibitory effects on the corresponding mRNA levels. In fact,  $\alpha$ -tubulin and  $\beta$ -tubulin mRNA levels were increased following reduction of the corresponding protein levels in both cell lines treated with T007-1. Our results suggest that  $\alpha$ -tubulin and  $\beta$ -tubulin downregulation by T007-1 is a post-transcriptional event and gene expression patterns are negatively regulated in response to the levels of the corresponding proteins. As T0070907 is reported to promote  $\alpha$ -tubulin and  $\beta$ -tubulin degradation via

a proteasome-dependent pathway, we examined whether T007-1 induces degradation via a similar mechanism. Pretreatment with the proteasome inhibitor, MG132, completely blocked  $\alpha$ -tubulin and  $\beta$ -tubulin degradation by T007-1 (Figure 2C), confirming similar proteasome-dependent activity to the parent compound.

#### ***T007-1 induces G2/M cell cycle arrest and apoptosis in cancer cells***

Since conventional tubulin inhibitors, such as paclitaxel and colchicine, inhibit spindle formation and induce G2/M phase cell cycle arrest in cancer cells (20, 21), we further examined the effects of T007-1 on cell cycle arrest. As depicted in Figure 3A, T007-1 promoted cell cycle arrest at the G2/M phase in a dose-dependent manner in HeLa and Hct116 cells. Tubulin inhibitors are reported to promote apoptosis in cancer cells after induction of G2/M phase cell cycle arrest (22). Accordingly, we investigated the effects of T007-1 on two apoptosis marker proteins, cleaved caspase-3, and PARP. T007-1 promoted time-dependent cleavage of caspase-3 and PARP, clearly indicative of apoptosis induction (Figure 3B). T007-1-induced cleavage of caspase-3 and PARP was inhibited by the pan-caspase inhibitor, Z-VAD-FMK, further suggesting that the apoptotic pathway is caspase-dependent. Based on these findings, we conclude that T007-1 induces G2/M phase cell cycle arrest and apoptosis in cancer cells in a similar manner to conventional tubulin inhibitors.

#### ***T007-1 binds covalently to Cys-239 of $\beta$ -tubulin***

As T007-1 promotes tubulin degradation at the post-transcriptional stage, we examined whether the agent directly binds tubulin with the aid of a microscale thermophoresis assay. As shown in Figure 4A, both colchicine and T007-1 interacted with tubulin with  $K_D$  values of  $4.96 \pm 1.93 \mu\text{M}$  and  $0.39 \pm 0.11 \mu\text{M}$ , respectively. We further conducted an *in vitro* tubulin polymerization assay to determine the effects of

T007-1 on tubulin assembly by measuring the increase in absorbance of tubulin at 340 nm and 37°C using colchicine and paclitaxel as comparative agents. Paclitaxel clearly promoted while colchicine inhibited tubulin polymerization (Figure 4B). Analogous to colchicine, T007-1 inhibited tubulin polymerization in a dose-dependent manner. Earlier studies have shown that T0070907 binds covalently to Cys313 of PPAR $\gamma$ , resulting in inhibition of its activity (14). Since T007-1 is a T0070907 derivative, we speculated that this compound may similarly form covalent interactions with tubulin. T007-1 was incubated with purified tubulin heterodimers, digested with trypsin, and subjected to liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) for analysis. As expected, T007-1 (molecular mass of 474.5 Da) bound the 217-LTTPTYGDLNHLVSATMSGVTTCLR-241 peptide of  $\beta$ -tubulin (leading to mass shift from 2652 Da to 3089 Da with a 437 Da mass change in the modified tryptic peptide owing to loss of one molecule of HCl (36.5) to allow for the formation of the S–C bond). This finding indicates that the fluorophenyl of T007-1 is the key functional group in covalent binding to tubulin. Fragmentation of this peptide revealed that T007-1 attaches to Cys-239 in  $\beta$ -tubulin (Figure 4C). Using the same method, we showed that T0070907 similarly binds covalently to Cys-239 of  $\beta$ -tubulin (Figure 4D). Since Cys-239 is adjacent to the colchicine site, we additionally conducted a competition assay. The two colchicine binding site inhibitors (colchicine and plinabulin) suppressed tubulin degradation induced by T007-1 (Figure 4E), suggesting that binding of T007-1 to tubulin underlies its degradation activity.

***Covalent binding to Cys-239 accounts for tubulin degradation induced by T007-1***

We further investigated whether T007-1-induced

tubulin degradation is mediated through binding to Cys-239. Data obtained from label-free quantitative proteomic analysis showed no degradation activity of T007-1 on  $\beta$ 6-tubulin in which cysteine was substituted with serine at position 239 (Figure 5A). Immunoblot analysis consistently revealed that T007-1 specifically promotes degradation of  $\beta$ 2-,  $\beta$ 4-, and  $\beta$ 5-tubulin isoforms containing cysteine at position 239 while exerting no effect on  $\beta$ 3- and  $\beta$ 6-tubulin isoforms, which possess serine at this position (Figure 5B). These findings gave rise to the speculation that covalent modification of Cys-239 by T007-1 contributes to its tubulin degradation activity. To validate this theory, a FLAG tag was fused to the C-termini of wild-type and *C239S*  $\beta$ -tubulin genes, which were subsequently cloned into MSCV-IRES-GFP expression vectors. FLAG-wt or FLAG-C239S  $\beta$ -tubulin and GFP were co-expressed in HeLa cells. T007-1 clearly promoted FLAG-wt  $\beta$ -tubulin degradation while exerting no effect on FLAG-C239S  $\beta$ -tubulin (Figure 5C), confirming that Cys-239 is necessary for tubulin degradation induced by T007-1. As determined from LC-ESI MS/MS, fluorophenyl of T007-1 was the key functional group in covalent binding to Cys-239. We further synthesized a T007-1 derivative, 180422 (Figure 5D; the synthesis method is described in supporting information), lacking fluorophenyl that could not bind covalently to Cys-239 of  $\beta$ -tubulin. As expected, 180422 displayed loss of anti-proliferative and tubulin degradation activities (Figure 5E, F). Our collective data suggest that covalent modification of Cys-239 is a prerequisite for tubulin degradation by T007-1.

***Covalent modification of Cys-239 in  $\beta$ -tubulin by small molecules presents a novel tubulin degradation strategy***

Next, we investigated whether tubulin degradation is a common biochemical

consequence of covalent modification of Cys-239 in  $\beta$ -tubulin by small molecules. To this end, small molecules reported to form covalent interactions with Cys-239 of  $\beta$ -tubulin, such as T138067 and EBI (Figure 6A), were selected for analysis (23, 24). Similar to T0070907 and T007-1, T138067 and EBI promoted tubulin degradation in a dose-dependent manner (Figure 6B). Pretreatment with colchicine inhibited T138067- and EBI-induced tubulin degradation (Figure 6C). Furthermore, T138067 and EBI specifically promoted degradation of FLAG-wt  $\beta$ -tubulin and not that of FLAG-C239S  $\beta$ -tubulin (Figure 6D). These results support the theory that covalent modification of Cys-239 by T138067 and EBI plays a role in tubulin degradation by these two compounds.

Isothiocyanates, non-specific tubulin cysteine modifiers, reversibly bind to Cys-127, Cys-347, and Cys-376 of  $\alpha$ -tubulin and Cys-12, Cys-239, Cys-303, and Cys-354 of  $\beta$ -tubulin (12, 15, 16), and promote proteasome-dependent tubulin degradation in cancer cells (12, 15, 16). However, the specific cysteine residues responsible for the degradation effect remain to be established. Here, we examined the involvement of Cys-239 in isothiocyanate-induced tubulin degradation using one of the isothiocyanates, AITC (Figure 6E). Similar to T138067 and EBI, AITC promoted FLAG-wt  $\beta$ -tubulin degradation but had no effect on FLAG-C239S  $\beta$ -tubulin (Figure 6F). Our results clearly demonstrate that small molecules that covalently bind Cys-239 of  $\beta$ -tubulin promote tubulin degradation.

### **Discussion**

Tubulin inhibitors are widely used as chemotherapeutic agents for cancer therapy (25, 26), such as the microtubule-stabilizing agents paclitaxel and docetaxel and destabilizing agents vinblastine, vincristine, vinorelbine, and eribulin (27–30). These drugs inhibit or promote tubulin

polymerization to suppress tubulin dynamic instability, inhibit spindle formation during mitosis, and subsequently induce G2/M phase cell cycle arrest and apoptosis in cancer cells (20, 27) while exerting no effects on tubulin protein levels.

In this study, we have identified small molecules that promote tubulin degradation by covalently binding Cys-239 of  $\beta$ -tubulin. Although previous reports have shown that certain small molecules, for instance, T0070907, thymoquinone, isothiocyanates and withaferin A, promote tubulin degradation (12, 14, 15, 17, 18), the underlying mechanisms are currently unclear, such as whether these agents bind tubulin and the specific binding sites. Our study revealed that these agents might also promote tubulin degradation by covalently binding to Cys-239 of tubulinand, because they can all react with thiol groups in proteins (31, 32). In addition, covalent modifiers of Cys-239 of tubulin appeared to promote tubulin degradation with high selectivity. Using label-free quantitative proteomic analysis of HeLa cells treated with T007-1, we observed significant downregulation of only four among the 1114 identified proteins (protein ratios lower than 2/3, compared with controls) (Figure 1C), which were all tubulin isoforms. This high specificity implies that Cys-239 presents an ideal binding site for drug design based on tubulin degradation activity.

$\beta$ -Tubulin has several isoforms, including  $\beta$ 1- to  $\beta$ 6- and  $\beta$ 8-tubulin (33). Cys-239 has been identified in  $\beta$ 2-,  $\beta$ 4-,  $\beta$ 5-, and  $\beta$ 8-tubulin isoforms and Ser-239 in  $\beta$ 1-,  $\beta$ 3-, and  $\beta$ 6-tubulin isoforms. The  $\beta$ -tubulin isoforms show variable distribution in different cell types. For example, nerve cells express high levels of  $\beta$ 3-tubulin (34). In our study, T007-1 specifically promoted degradation of  $\beta$ 2-,  $\beta$ 4-,  $\beta$ 5-, and  $\beta$ 8-tubulin isoforms while exerting no effects on  $\beta$ 3- and  $\beta$ 6-tubulin isoforms. Therefore, drugs based on these tubulin degradation agents may show minimal

neurotoxicity.

Fluorophenyl of T007-1 is the key functional group mediating covalent bond formation. In the current study, compound 180422 without fluorophenyl showed total loss of anti-proliferative activity, implying that T007-1 acts through covalent binding and not non-covalent binding behavior since anti-proliferation activity is retained upon conventional non-covalent inhibitor binding at the colchicine site (21). This finding indicates that the fluorophenyl of T007-1 is important for both binding and degradation activities.

Cysteine residues play an important role in protein structural stability, and oxidation of even a single cysteine could cause protein unfolding and aggregation (35, 36). The key residue mediated by tubulin degradation agents is Cys-239. The underlying mechanism is proposed as covalent modification of Cys-239 resulting in tubulin unfolding and subsequent proteasome-dependent degradation of unfolded tubulin. However, this is only a theoretical assumption and requires further investigation.

Overall, our findings suggest that covalent modification of Cys-239 of  $\beta$ -tubulin by small molecules presents an effective strategy to promote tubulin heterodimer degradation. We propose that these small-molecule tubulin degradation agents represent a third novel class of tubulin inhibitors.

## **Experimental procedures**

### ***Reagents***

T0070907, EBI, MG132, plinabulin, colchicine, and paclitaxel were obtained from Selleck Chemicals. Z-VAD-FMK was purchased from MedChemExpress and T138067 from Medkoo Biosciences, Inc. T007-1 and 180422 were synthesized as described in supporting information. Penicillin, streptomycin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli-

um bromide), DMSO, DAPI (4,6-diamidino-2-phenylindole), and propidium iodide were obtained from Sigma and purified tubulin from Cytoskeleton Inc. All antibodies were purchased from Abcam. Other conventional reagents were acquired from Kelun Pharmaceutical.

### ***Cell lines and cultures***

The human cervical adenocarcinoma cell line, HeLa, and human colon colorectal carcinoma cell line, Hct116, were obtained from American Type Culture Collection and cultured with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. The temperature was set at 37°C with an atmosphere of 5% CO<sub>2</sub>. All cell lines were authenticated by short tandem repeat testing and free of mycoplasma.

### ***Cell viability detection***

Cells cultured in 96-well plates were treated with different concentrations of compounds for 72 h. Next, 20  $\mu$ l MTT (mg/ml) was added to each well and cultured for another 4 h. The supernatant was removed and 150  $\mu$ l DMSO added to each well. Plates were gently shaken for 10 min, and absorbance at 570 nm measured using a microplate reader (Biotek, USA).

### ***Label-free quantitative proteomic analysis***

Cells were incubated with or without T007-1 for 6 h, washed with phosphate-buffered saline (PBS), and lysed with radioimmunoprecipitation assay (RIPA) buffer (containing 2 mM phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail) for 30 min at 4°C. Samples were centrifuged at 13000 rpm for 30 min at 4°C. The supernatant fractions were collected and subjected to a BCA Protein Assay for determination of protein concentrations. Three biological replicates were examined for each experiment.

### ***Trypsin digestion***

Protein (300  $\mu$ g) was denatured at 100°C for

5 min. Samples were cooled to room temperature and further denatured using urea buffer (8 M urea, 150 mM Tris-HCl, pH 8.0). Subsequently, cysteine residues of protein were blocked with iodoacetamide (100  $\mu$ l of 50 mM) in urea buffer for 30 min at room temperature in the dark. Protein was digested with 4  $\mu$ g trypsin (Promega, Madison, WI, USA) at 37°C for 16–18 h, the resulting peptides collected and their concentrations determined at OD<sub>280</sub>.

#### *LC-ESI MS/MS*

Each sample (1  $\mu$ g) was loaded onto a Thermo Scientific EASY column for separation using a segmented gradient on an Easy-nLC nanoflow HPLC system (Thermo Fisher Scientific, CA, USA). The eluent was further analyzed with an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, CA, USA) in a positive ion mode. Mass spectra were acquired over a range of 350–2000 *m/z*. The maximum ion injection time was set at 50 ms for the survey scan and 150 ms for MS/MS scans for the 16 most intense signals in the acquired mass spectra.

#### *Analysis of differentially abundant proteins*

The Andromeda peptide search engine was employed to identify proteins. Peptide and protein false discovery rates were estimated with the Self database, and the maximum protein and peptide-spectrum match false discovery rates set to 0.01. Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation as a variable modification. We calculated label-free quantification using MaxQuant. This value was obtained by dividing protein intensity by number of theoretically observable tryptic peptides between 5 and 30 amino acids and was highly correlated with protein abundance.

#### *Western blot*

Cells were collected and lysed in RIPA buffer. Equal amounts of total protein samples were loaded onto an SDS-PAGE system for separation

and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in 5% skimmed milk for 1 h and incubated with primary antibody at 4°C overnight. Next, membranes were washed three times with PBS with Tween-20 (3  $\times$  10 min) before incubation with secondary antibody for 45 min and re-washed with PBS containing Tween-20 (3  $\times$  10 min) before detection of immunoreactivity using enhanced chemiluminescence reagents (Millipore).

#### *Mass spectrometry*

Purified tubulin (20  $\mu$ M) was incubated with a slight excess of 25  $\mu$ M T0070907 or 25  $\mu$ M T007-1 for 3 h at room temperature. Excess compounds were removed via an ultrafiltration method and samples denatured via SDS-PAGE. Tubulin bands were excised for subsequent mass spectrometry, which was conducted by rigorously following a published protocol (6).

#### *Microscale thermophoresis assay*

Binding of T007-1 and colchicine to tubulin was detected with a microscale thermophoresis assay utilizing a Monolith NT.115 instrument (NanoTemper Technologies). Purified tubulin was labeled using a Monolith protein labeling kit RED-NHS (NanoTemper Technologies). Different concentrations (100  $\mu$ M to 3 nM) of T007-1 or colchicine were incubated with labeled tubulin (40 nM) in assay buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM GDP) for 10 min at 4°C. Samples were loaded into glass capillaries for detection.  $K_D$  values were obtained using NanoTemper software.

#### *Immunofluorescence staining*

Cover glasses were placed in the bottoms of six-well plates. Cells were seeded onto cover glasses and incubated for 24 h before treatment with T007-1 for 16 h. The culture medium was removed and washed with PBS for 2 min. Cells were incubated with 50% methanol and 50%

acetone for 2 min, followed by primary antibody for 4 h at room temperature. Next, cells were washed with PBS (4×5 min) and incubated with fluorescent secondary antibodies and DAPI for 45 min at room temperature, followed by re-washing with PBS (4×5 min). Images were acquired using a fluorescence microscope (Olympus, Japan).

#### ***In vitro tubulin polymerization assay***

Samples of purified tubulin (2 mg/ml) in protein expression medium buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM GTP) with 15% glycerol were incubated with different compounds at 4°C for 1 min, transferred to pre-warmed (37°C) 96-well plates and optical densities at 340 nm determined once per minute using a microplate reader (Biotek, USA) at 37°C.

#### ***Cell cycle analysis***

Cells were seeded onto six-well plates and incubated for 24 h before treatment with different concentrations of T007-1 for 16 h. Treated cells were washed with PBS, collected, and fixed in 70% ethanol at 4°C for 24 h. Fixed cells were washed three times with PBS, stained with 50 µg/ml propidium iodide for 30 min, and analyzed using a flow cytometer (BD FACSCalibur).

#### ***Quantitative PCR***

Cells were seeded onto six-well plates for 24 h before treatment with T007-1 for 2, 4, 8, or 16 h. Total mRNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's procedure. The mRNA content was determined using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription from mRNA to cDNA was conducted using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR analysis was conducted using *Taq* Universal SYBR Green

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Supermix (BIO-RAD) on a CFX96 real-time PCR system (BIO-RAD). Relative *α-tubulin* and *β-tubulin* mRNA levels were normalized to that of *GAPDH*. The primers employed were as follows: *α-tubulin*: forward primer, TCGATATTGAGCGTCCAACCT, reverse primer, CAAAGGCACGT TTGGCATAACA; *β-tubulin*: forward primer, TGGACTCTGTTCGCTCAGGT, reverse primer, TGCCTCCTTCCGTACCACAT; *GAPDH*: forward primer, GGAGCGAGATCCCTCCAAAAT, reverse primer, GGCTGTTGTCATACTT CTCATGG.

#### ***Vector construction***

Complete sequences of *TUBB* (β-tubulin) with FLAG tag fused to the C-terminus were synthesized by Genewie (Suzhou, China), containing *Bam*HI and *Pac*I restriction sites at all ends. The gene was cloned into MSCV-IRES-GFP expression vector co-expressing GFP and target proteins. *TUBB* containing the C239 mutation was constructed using a Q5 site-directed mutagenesis kit (NEB #E0554S).

#### ***Transfection experiments***

HeLa cells were seeded onto six-well plates and cultured for 24 h before transfection. Plasmid DNA (2.0 µg) was dissolved in 300 µl OptiMEM (Thermo) and incubated for 5 min. During this time, 7.5 µl Lipofectamine 2000 was added to 300 µL OptiMEM and incubated for 5 min. Next, plasmid DNA and Lipofectamine 2000 were mixed and incubated for 20 min before addition to HeLa cells in six-well plates for a 24 h incubation period. After addition of the appropriate compounds, cells were incubated for a further 16 h. Total protein was extracted and lysed with RIPA buffer before western blot analysis using FLAG and GFP antibodies. *GAPDH* was employed as the loading control.

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### **Conflict of interest**

The authors have no conflicts of interest to declare.

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### Footnotes

AITC, allyl isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; EBI, *N,N'*-ethylene-bis(iodoacetamide); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LC-ESI MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PML, promyelocytic leukemia; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; PVDF, polyvinylidene difluoride; RAR $\alpha$ , retinoic acid

receptor  $\alpha$ ; RIPA, radioimmunoprecipitation assay.

### Figure legends

**Figure 1.** T007-1 downregulates tubulin heterodimer proteins with high specificity. (A) Chemical structures of T0070907 and T007-1. (B) Effects of T0070907 and T007-1 on viability of HeLa and Hct116 cells determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values are shown as means  $\pm$  standard deviation (SD) of three independent experiments. (C) Label-free quantitative proteomic analysis of total proteins from HeLa cells treated with 3  $\mu$ M T007-1 for 6 h. The graph shows fold-changes of 1114 proteins between T007-1 and vehicle treatment groups versus  $P$  value ( $t$ -test; triplicate analysis). (D) Immunofluorescence of HeLa cells treated with or without 3  $\mu$ M T007-1 for 16 h (Green:  $\alpha$ -tubulin, Blue: nucleus). (E) Western blots of  $\alpha$ -tubulin and  $\beta$ -tubulin in HeLa and Hct116 cells treated with the indicated concentrations of T007-1 for 16 h using GAPDH as a loading control. The lower graph depicts protein levels of  $\alpha$ -tubulin and  $\beta$ -tubulin standardized to GAPDH levels. Quantitative data are presented as means  $\pm$  SD of three independent experiments.  $\alpha$ -Tub,  $\alpha$ -tubulin;  $\beta$ -Tub,  $\beta$ -tubulin.

**Figure 2.** T007-1 promotes proteasome-dependent degradation of tubulin heterodimers. (A) Western blots of  $\alpha$ -tubulin and  $\beta$ -tubulin expression in HeLa and Hct116 cells treated with 3  $\mu$ M T007-1 for 0, 1, 2, 4, 8, and 16 h using GAPDH as a loading control. The right graph depicts protein levels of  $\alpha$ -tubulin and  $\beta$ -tubulin standardized to GAPDH levels. Values are presented as means  $\pm$  SD of three independent experiments. (B) Quantitative PCR analysis of  $\alpha$ -tubulin and  $\beta$ -tubulin mRNA in HeLa and Hct116 cells treated with 3  $\mu$ M T007-1 for 0, 1, 2, 4, 8, and 16 h. Relative expression levels were normalized to that of *GAPDH*. Values are presented as means  $\pm$  SD of three independent experiments. (C) HeLa cells were pretreated with 20  $\mu$ M MG132 for 1 h before treatment with or without 3  $\mu$ M T007-1 for 16 h and  $\alpha$ -tubulin and  $\beta$ -tubulin levels detected via western blot using GAPDH as a loading control. The right graph shows protein levels of  $\alpha$ -tubulin and  $\beta$ -tubulin standardized to GAPDH levels. Values are presented as means  $\pm$  SD of three independent experiments.  $\alpha$ -Tub,  $\alpha$ -tubulin;  $\beta$ -Tub,  $\beta$ -tubulin; Con, control; +, presence of the indicated compound; -, absence of the indicated compound.

**Figure 3.** T007-1 induces G2/M cell cycle arrest and apoptosis in cancer cells. (A) Cell cycle analysis of T007-1-treated cancer cells. HeLa and Hct116 cells were treated with the indicated concentrations of T007-1 for 16 h, stained with propidium iodide, and subjected to a flow cytometry for cell cycle analysis. Histograms show the proportions of G0/G1 (blue), S (dark purple), and G2/M (red) phase cells. The dark light curves represent original integral curves and fitting integral curves, respectively. The right graphs represent the percentage of G2/M phase cells. Values are shown as means  $\pm$  SD of three independent experiments. (B) HeLa cells were pretreated with or without the indicated concentrations of Z-VAD-FMK for 1 h before treatment with 3  $\mu$ M T007-1 for 12, 24, or 48 h. Protein levels of cleaved PARP and caspase-3 were detected via western blot using GAPDH as a loading control. The right graph depicts the protein levels of cleaved PARP and cleaved caspase 3 standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. Con, control; +, presence of the indicated compound; -, absence of the indicated compound.

**Figure 4.** T007-1 and T0070907 bind covalently to Cys-239 of  $\beta$ -tubulin. (A) Binding of T007-1 and colchicine to purified tubulin was determined with the microscale thermophoresis assay. Data points represent means  $\pm$  SD of three technical replicates each. (B) *In vitro* tubulin polymerization assay: optical density at 340 nm of purified tubulin incubated with the indicated compounds was detected once every min for 30 min at 37°C. This result is representative of two independent experiments. (C) MS/MS fragmentation pattern for T007-1 covalently modified peptide from  $\beta$ -tubulin indicating covalent binding of T007-1 to Cys-239. (D) MS/MS fragmentation pattern for T0070907 covalently modified peptide from  $\beta$ -tubulin, indicating covalent binding of T0070907 to Cys-239. (E) HeLa cells were pretreated with or without 3  $\mu$ M colchicine or 3  $\mu$ M plinabulin for 1 h before treatment with DMSO or 3  $\mu$ M T007-1 for 16 h and expression of  $\alpha$ -tubulin and  $\beta$ -tubulin detected via western blot using GAPDH as the loading control. The right graph shows the protein levels of  $\alpha$ -tubulin and  $\beta$ -tubulin standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments.  $\alpha$ -Tub,  $\alpha$ -tubulin;  $\beta$ -Tub,  $\beta$ -tubulin; Con, control; Pac, paclitaxel; Col, colchicine; PBL, plinabulin; +, presence of the indicated compound; –, absence of the indicated compound.

**Figure 5.** Covalent binding to Cys-239 accounts for tubulin heterodimer degradation induced by T007-1. (A) Fold-change in abundance of 1114 proteins comparing T007-1 to vehicle treatment groups, versus *P* value (*t*-test; triplicate analysis). The figure represents a replot of the same data in Figure 1C, and the red point represents  $\beta$ 6-tubulin. (B) Western blot analysis of expression of  $\beta$ 2-,  $\beta$ 3-,  $\beta$ 4-,  $\beta$ 5-, and  $\beta$ 6-tubulin isoforms in HeLa cells treated with different concentrations of T007-1. The right graph depicts the protein levels of  $\beta$ 2,  $\beta$ 3,  $\beta$ 4,  $\beta$ 5, and  $\beta$ 6-tubulin standardized to GAPDH. Values are shown as means  $\pm$  SD of three independent experiments. (C) HeLa cells were transiently transfected with MSCV-IRES-GFP vectors expressing both GFP and wild-type or C239S FLAG- $\beta$ -tubulin for 24 h, and subsequently treated with or without T007-1 for 16 h. FLAG-tubulin and GFP were detected via western blot using GAPDH as a loading control. The right graph shows protein levels of FLAG- $\beta$ -tubulin and GFP standardized to GAPDH. Values are shown as means  $\pm$  SD of three independent experiments. (D) Chemical structures of T007-1 and 180422. (E) Effects of T007-1 and 180422 on HeLa and Hct116 cell viability, determined with the MTT assay. Values are shown as means  $\pm$  SD of three independent experiments. (F) Western blot analysis of  $\beta$ -tubulin levels in HeLa cells treated with the indicated concentrations of T007-1 and 180422 for 16 h using GAPDH as a loading control. The right graph shows the  $\beta$ -tubulin protein level standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. Tub, tubulin; TubB,  $\beta$ -tubulin; Con, control; +, presence of the indicated compound; –, absence of the indicated compound.

**Figure 6.** Small molecules promote tubulin heterodimer degradation via covalent modification of Cys-239 in  $\beta$ -tubulin. (A) Chemical structures of T138067 and EBI. (B) Western blot analysis of  $\alpha$ / $\beta$ -tubulin expression in HeLa cells treated with the indicated concentrations of T138067 and EBI for 16 h using GAPDH as a loading control. The right graph shows  $\alpha$ -tubulin and  $\beta$ -tubulin protein levels standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. (C) HeLa cells were pretreated with 3  $\mu$ M colchicine for 1 h before treatment with DMSO, 1  $\mu$ M T138067 or

100  $\mu$ M EBI for 16 h and expression levels of  $\alpha$ -tubulin and  $\beta$ -tubulin detected via western blot using GAPDH as a loading control. The right graph shows protein levels of  $\alpha$ -tubulin and  $\beta$ -tubulin standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. (D) HeLa cells were transiently transfected with MSCV-IRES-GFP vectors expressing both GFP and wild-type or C239S FLAG- $\beta$ -tubulin for 24 h, followed by treatment with DMSO, 1  $\mu$ M T138067 or 100  $\mu$ M EBI for 16 h. FLAG-tubulin and GFP were detected via western blot using GAPDH as a loading control. The right graph depicts protein levels of FLAG- $\beta$ -tubulin and GFP standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. (E) Chemical structure of allyl isothiocyanate (AITC). (F) HeLa cells were transiently transfected with MSCV-IRES-GFP vectors expressing both GFP and wild-type or C239S FLAG- $\beta$ -tubulin for 24 h, and treated with the indicated concentrations of AITC for 16 h. FLAG-tubulin and GFP were detected via western blot using GAPDH as a loading control. The right graph shows the protein levels of FLAG- $\beta$ -tubulin and GFP standardized to GAPDH. Values are presented as means  $\pm$  SD of three independent experiments. T138, T138067; Col, colchicine; AITC, allyl isothiocyanate; EBI, *N,N'*-ethylene-bis(iodoacetamide);  $\alpha$ -Tub,  $\alpha$ -tubulin;  $\beta$ -Tub,  $\beta$ -tubulin; TubB,  $\beta$ -tubulin; +, presence of the indicated compound; -, absence of the indicated compound.

Figure 1

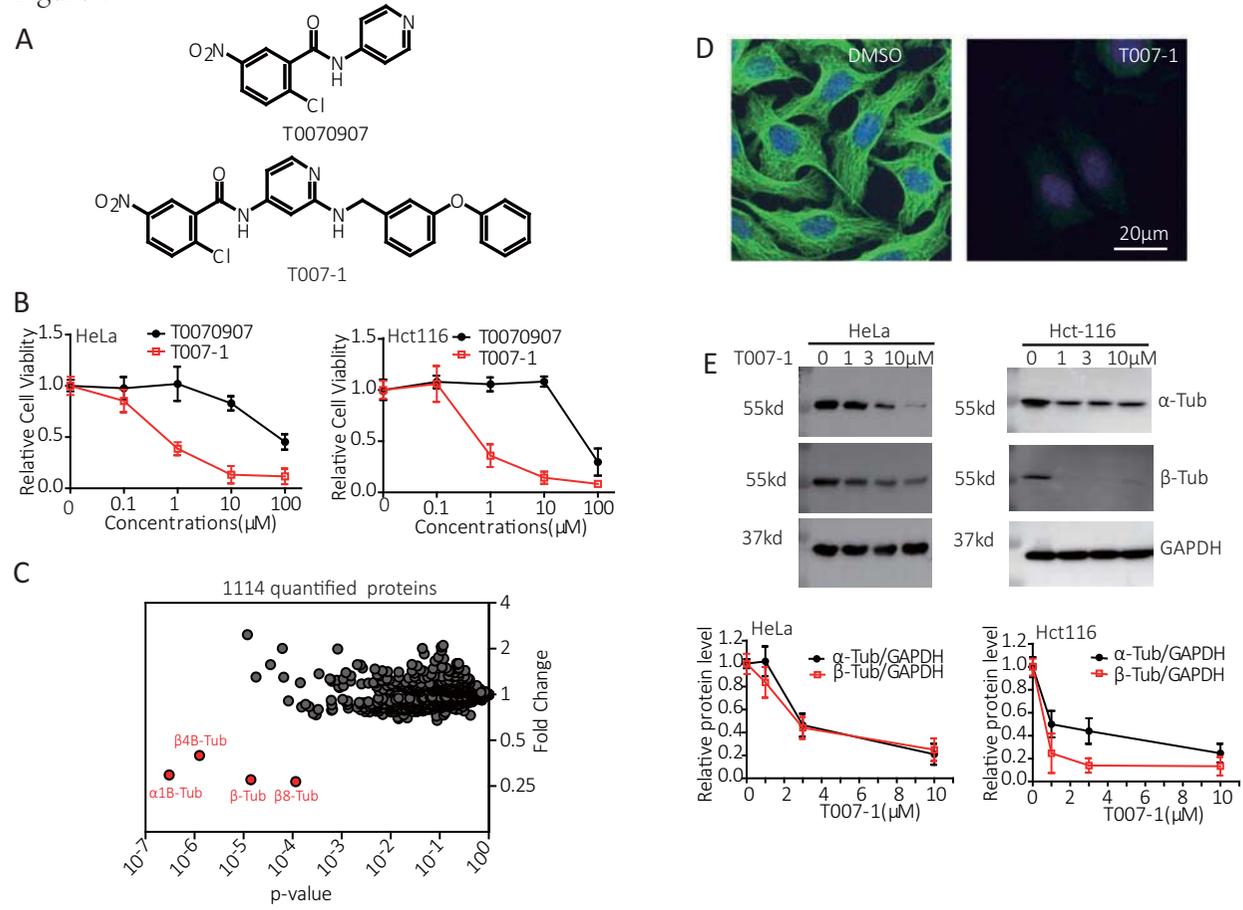


Figure 2

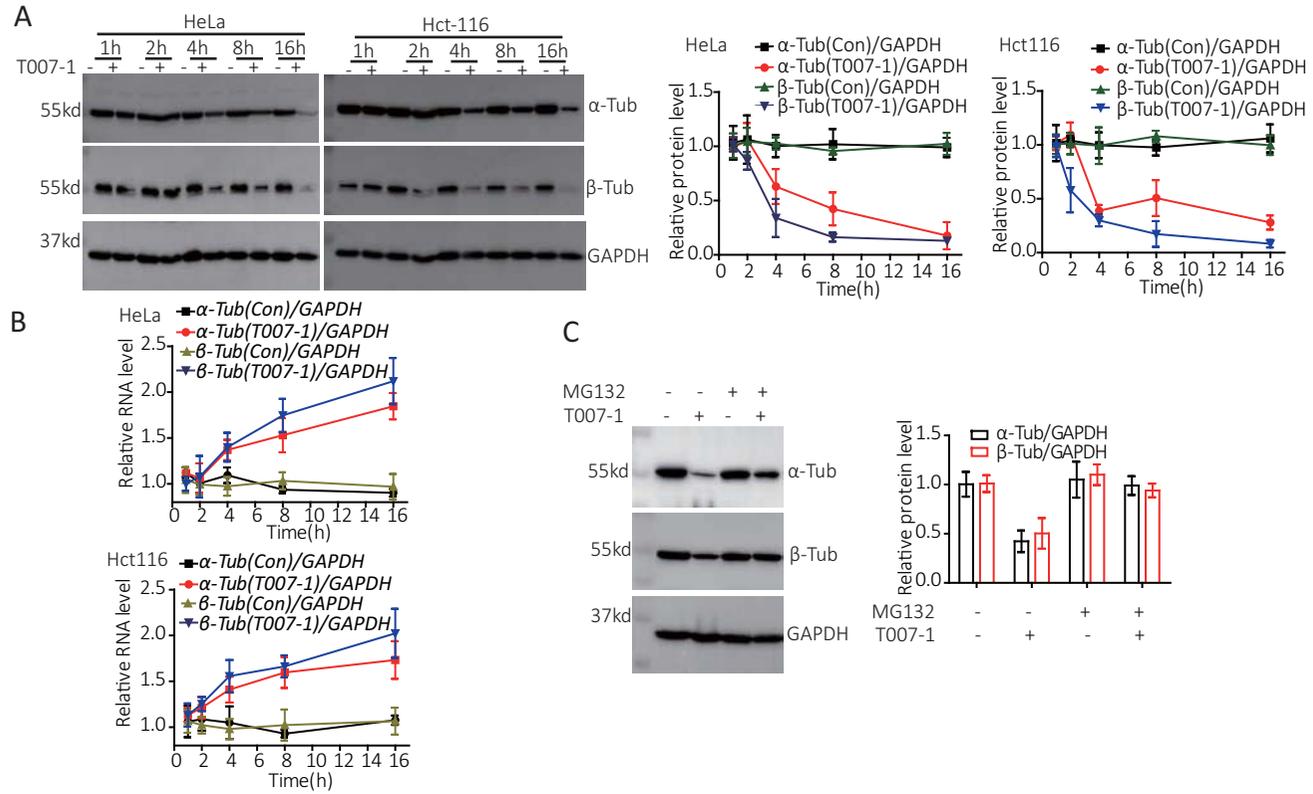


Figure 3

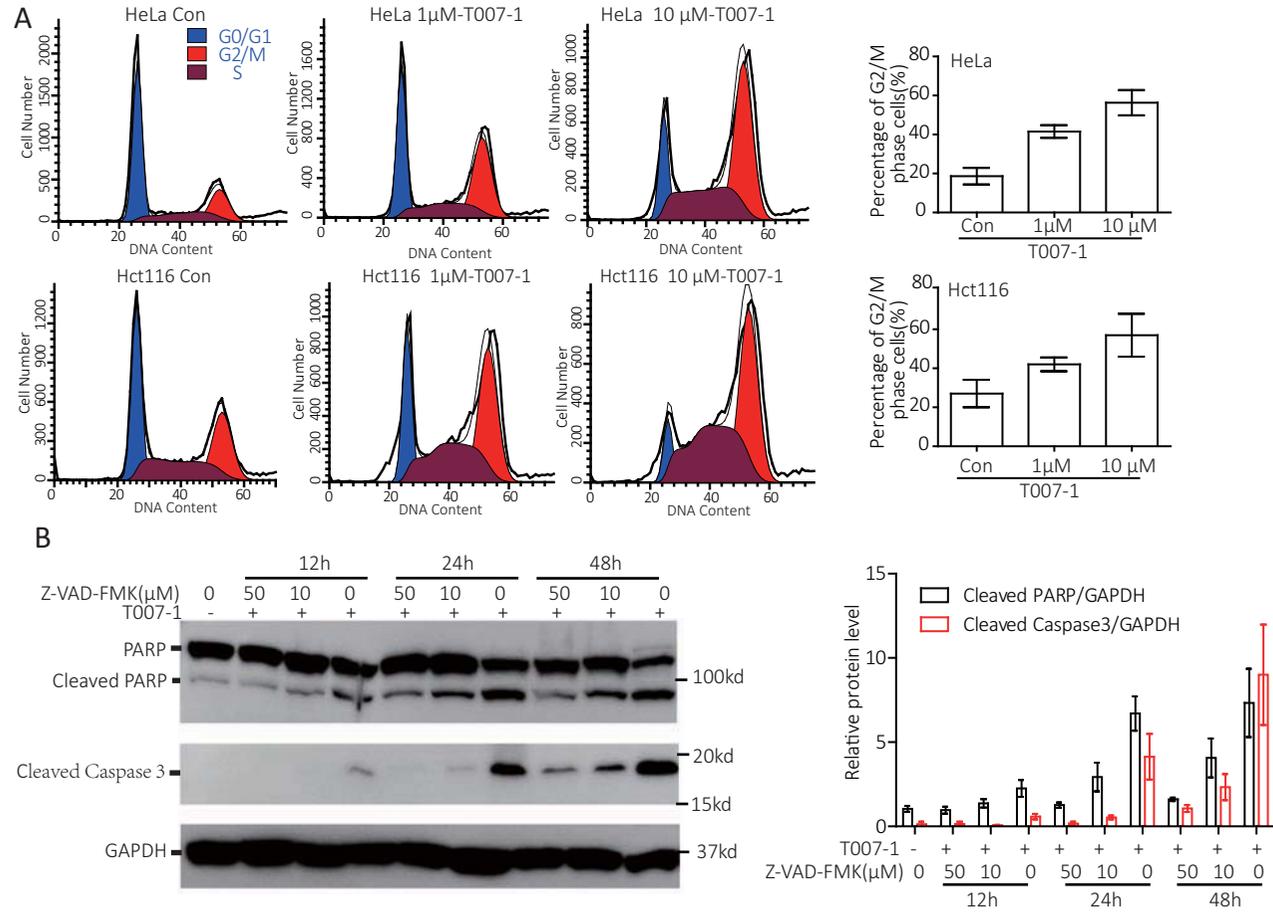


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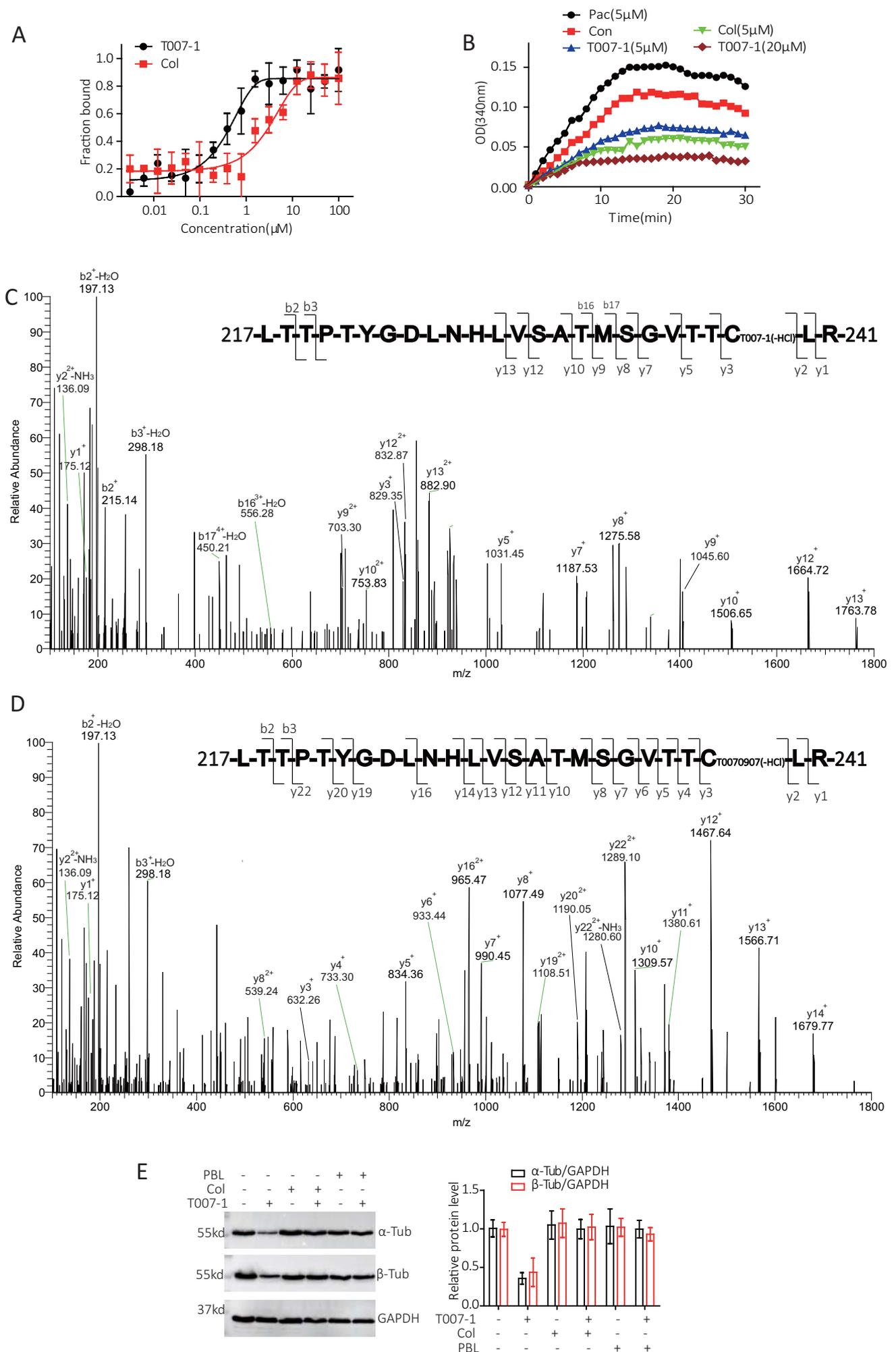


Figure 5

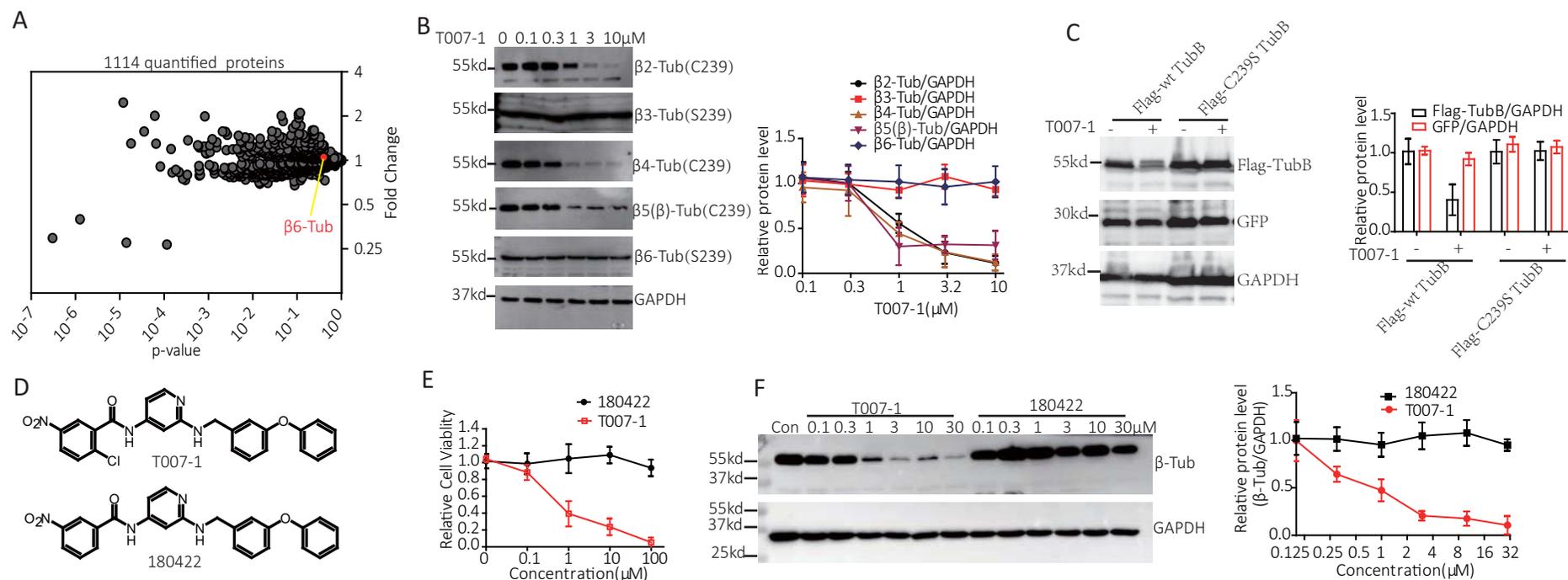
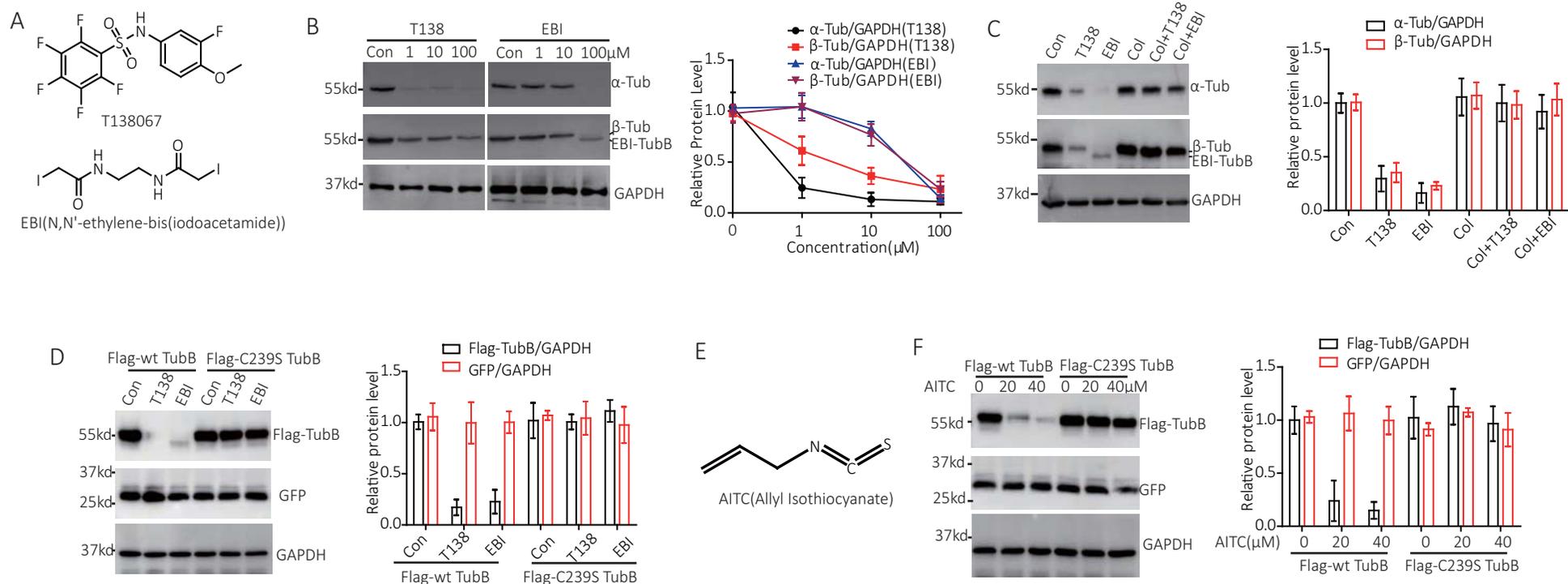


Figure 6



**Covalent modification of Cys-239 in  $\beta$ -tubulin by small molecules as a strategy to promote tubulin heterodimer degradation**

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