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Title Page

Title:

The natural compound Withaferin A covalently binds to Cys-239 of β-Tubulin to promote tubulin degradation

Names of authors:

Jianhong Yang, Wei Yan, Yong Li, Lu Niu, Haoyu Ye, Lijuan Chen

Affiliations:

State Key Laboratory of Biotherapy and Cancer Center, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy and Cancer, Chengdu 610041, China

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Running Title Page

a) Running Title: WIT covalently interacts with Cys-239 of β-Tubulin

b) Corresponding author:

Address correspondence to: Lijuan Chen, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy and Cancer, Chengdu 610041, China. Tel.: 86-028-85502796; Fax:86-02885164060; Email:chenlijuan125@163.com

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Cys, cysteine; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFP, Green fluorescent protein; HSP90, heat shock protein 90; IKKβ, IκB Kinase β; PBS, phosphate buffered saline; PCR, Polymerase chain reaction; RIPA, Radioimmunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Abstract

Withaferin A (WIT) is a natural product possessing a wide range of pharmacological activities. Previous studies have reported covalent binding of WIT to tubulin and downregulation of tubulin protein levels although the underlying mechanisms remain to be established. In the current investigation, we showed that WIT induces downregulation of tubulin in a post-transcriptional manner, suggestive of direct and potent activity in tubulin degradation. The N,N′-Ethylenebis(iodoacetamide) assay and competitive binding experiments with four colchicine site-targeted tubulin inhibitors further revealed that WIT interacts with the colchicine site of tubulin to promote degradation. WIT irreversibly inhibited tubulin polymerization and mass spectrometry results disclosed binding to Cys239 and Cys303 sites of β-tubulin. Interestingly, WIT promoted degradation of the β-tubulin isoforms containing Cys239 (β2， β4 and β5(β)) but had no effect on those containing Ser239 (β3 and β6). Moreover, a C239S but not C303S mutation in β-tubulin completely abolished the degradation effect of WIT, suggesting that the Cys239-WIT covalent bond accounts for this activity. Our collective results clearly demonstrate that covalent interactions between WIT and Cys239 of β-tubulin promote tubulin degradation, supporting its potential utility as a therapeutic compound.

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Significance statement

Withaferin A, a natural product possessing a wide range of pharmacological activities, covalently binds to Cys239 of β-tubulin near the colchicine site and the WIT-Cys239 covalent bond accounts for WIT induced tubulin degradation, fully clarifying the underlying mechanisms and supporting its potential utility a therapeutic compound.

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Introduction

Withaferin A (WIT), a natural compound with a steroidal lactone structure isolated from *Acnistus arborescens*, *Withania somnifera* and other species of the *Solanaceae* family (Kupchan et al.,1969; Mohan et al., 2004), has a long history of use as traditional medicine in India. Multiple pharmacological activities of WIT have been identified to date, including cardioprotection, anti-diabetic, anti-inflammatory, immune regulation, anti-angiogenesis and anti-tumor effects (Mohan et al., 2004; Ravindran et al., 2015; Tekula et al., 2018; Lee et al.,2016; Maitra et al., 2009; Gambhir et al., 2015; Munagala et al., 2011). The presence of active groups, such as a ketone-containing unsaturated A ring, epoxide-containing B ring and unsaturated lactone ring in WIT, imparts strong biological activity (Gu et al., 2014; Siddique et al., 2014), through interactions with specific amino acid residues in target proteins to form covalent bonds. For example, an unsaturated α, β-double bond in an A ring has been shown to covalently bind cysteine through a Michael addition reaction. Additionally, the epoxide ring can react with a histidine, serine or threonine residue to form a covalent bond (Drahl et al., 2010). The double bond in ring A and epoxide in ring B are mainly responsible for cytotoxicity (Berghe, et al., 2012). Plurality of target proteins corresponding to the pharmacological activities of WIT has been reported. WIT reacts covalently with a cysteine residue in the majority of these targets, including Cys328 of vimentin (Bargagna-Mohan et al., 2007), Cys179 of IKKβ (Heyninck et al., 2014), Cys133 of Annexin II (Falsey et al., 2006) and a certain Cys at the N-terminus of HSP90 (Yu et al., 2010), highlighting its potential utility as a multi-target bioactive compound.

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Microtubules, tubular structures formed by the assembly of αβ-tubulin in a head-to-tail structure (Stanton et al., 2011), are a popular target for the development of anti-tumor drugs. For example, the commonly used chemotherapeutic drugs paclitaxel, vinblastine and eribulin mesylate (Halavan) exert their activities by targeting microtubules (Dumontet et al., 2010). Microtubules play important roles in diverse physiological activities of cells, including motility, intracellular protein transport and cell division (Stanton et al., 2011). The anti-tumor drugs targeting microtubules mainly inhibit tumor cell growth by suppressing cell division (Stanton et al., 2011). Six binding sites on the tubulin dimer have been identified for paclitaxel (Ojeda-Lopez et al., 2014), vinblastine (Gigant et al., 2005), colchicine (Ravelli et al., 2004), maytansine (Prota et al., 2014), laulimalide (Prota et al., 2014) and pironetin (Yang et al., 2016), respectively. Small molecules that bind different sites induce microtubule inhibition through distinct mechanisms. Compounds binding to paclitaxel or laulimalide sites promote tubulin polymerization while interactions with the other four sites lead to inhibition of polymerization (Yang et al., 2016; Na et al., 1982). Vinblastine-binding compounds display a unique feature in that they induce the formation of tubulin paracrystals (packing of unpolymerized tubulin in the cytoplasm) (Gigant et al., 2005). These diverse mechanisms of tubulin inhibition provide several options for microtubule drug development and facilitate comprehensive investigation of microtubule polymerization and depolymerization processes.

Earlier, Antony et al. (Antony et al., 2014) reported that WIT inhibits tubulin through covalent binding to a novel site at Cys303 of β-tubulin, (Antony et al., 2014)

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and exerts a degradation effect distinct from other conventional tubulin inhibitors. However, it remains to be established whether WIT-Cys303 binding is specific and degradation of tubulin is directly related to interactions with WIT, as Cys303 is located on the surface of β-tubulin. More importantly, we are yet to ascertain whether covalent bond formation between WIT and Cys303 is the direct cause of tubulin degradation. To identify potential novel tubulin binding sites, we conducted an in-depth study of the interactions between WIT and tubulin. Our data showed that WIT forms covalent bonds with Cys303 and Cys239 of β-tubulin. Moreover, the bond between WIT and Cys239, but not Cys303, was responsible for tubulin degradation, providing another example of a small molecule that acts as a covalent modifier of Cys239 in β-tubulin to promote tubulin degradation (Yang et al., 2019).

Materials and methods

Reagents. Colchicine, paclitaxel, vinblastine, nocodazole, plinabulin, combretastatin A4, N,N′-Ethylenebis(iodoacetamide) (EBI) and MG132 were purchased from Selleck Chemicals, WIT from Sigma, and Dihydrowithaferin A (DWIT) from BioCrick Biotech. Other conventional agents were obtained from Sigma or Kelun Pharmaceuticals. Purified tubulin was acquired from Cytoskeleton Inc and all antibodies were purchased from Abcam (GAPDH: ab181602; β-Actin: ab179467; α-Tubulin: ab7291; β-Tubulin: ab6046; β2-Tubulin: ab151318; β3-Tubulin: ab52623; β4-Tubulin: ab179509; β6-Tubulin: ab110592; p-H3 (Phosphorylation at ser10): ab5176).

Cell lines and cultures. HeLa and Hct116 cells were sourced from American Type Culture Collection. Both cell lines were cultured in Dulbecco's modified Eagle's

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medium supplemented with fetal bovine serum (10%) and double antibiotics (penicillin (100 units/ml) and streptomycin (100 μg/ml)). Cells were cultured at 37°C with 5% CO_2 in a humidified chamber.

Trypan blue exclusion. Cells were plated into 6-well plates and cultured for 24 h. After treatment with the specified compounds, cells were incubated for a further 48 h. Both alive and dead cells were collected, wash with PBS, and then stained with trypan blue dye (0.4 mg/ml in PBS) for 6 min. Then cells were counted with a hemocytometer to calculate the death ratio.

Western blot. Cells in six-well plates were harvested after treatment with the specified compounds and washed with PBS for 2 min. Collected cells were lysed with RIPA containing 5x loading buffer for 10 min with manual vibration every 2 min. Lysed samples were subjected to denaturation in boiling water for 10 min and preserved at -20℃ until experimental use. Equal amounts of samples (20 μg) were subjected to SDS-PAGE for separation and proteins on gels transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated with primary antibody for 12 h at 4℃ and washed with PBST (PBS containing 0.1% Tween-20) buffer for 30 min (3x10 min) before incubation with secondary antibody for 45 min at room temperature. PVDF membranes were re-washed with PBST buffer for 30 min, subjected to immunoreactivity analysis and visualized under a chemiluminescence imaging system (Tanon, Shanghai).

EBI assay. Purified tubulin (1 μM) was incubated with the specified compounds for 2 h and with 100 μM EBI for a further 2 h. Samples were mixed with 5x loading buffer

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and denatured in boiling water for 10 min. Equal amounts of samples were analyzed via western blot using β-tubulin antibody.

Mass spectrometry. Purified αβ-tubulin dimer (10 μM) and excess WIT (30 μM) were incubated for 3 h at room temperature. Unreacted WIT was removed via ultrafiltration. Samples were mixed with loading buffer and loaded on to SDS gels for electrophoresis, followed by staining with Coomassie brilliant blue. The tubulin band was excised, digested with trypsin and subjected to mass spectrometry analysis according to our published protocol (Yang et al., 2016).

Immunofluorescence staining. Cells on coverslips in 24-well plates were treated with high concentrations of compounds (10 μM paclitaxel, 10 μM colchicine, 10 μM vinblastine and 100 μM WIT) for 1 h. Following removal of the supernatant, cells were washed with PBS for 2 min before fixing with 50% methanol/50% acetone for 2 min. Next, cells were incubated with tubulin antibody at 4℃ overnight and washed with PBST for 20 min (4x5 min). After further incubation with secondary antibody and DAPI for 45 min at room temperature, cells were re-washed with PBST for 20 min (4x5min). The prepared samples were observed and imaged under an Olympus fluorescence microscope (Japan).

In vitro tubulin polymerization assay. This assay was performed according to Cytoskeleton, Inc. established protocol (https://www.cytoskeleton.com/pdf-storage/datasheets/bk006p.pdf). Briefly, purified tubulin (3 mg/ml) was incubated with the specified compounds at 4℃ for 1 min before transfer to a pre-cold microplate reader (Biotek, USA). Optical density values at 340 nm were measured once per minute.

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Quantitative PCR. Cells in six-well plates were treated with WIT for 16 h, collected, and washed with PBS. Total mRNA was obtained with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. A high-capacity cDNA reverse transcription kit (Applied Biosystems) was employed for reverse transcription of mRNA to DNA. Quantitative PCR detection of *α*-and *β-tubulin* levels was conducted following our previously established protocols (Yang et al., 2019).

Vector construction. The full-length wild type *TUBB* gene sequence containing the FLAG-tag at the C-terminus was synthesized by Genewiz (Suzhou, China). The gene was cloned into MSCV-IRES-GFP vector for co-expression of the target protein and GFP. Genes containing the C239S and C303S mutations were constructed using the Q5 site-directed mutagenesis kit (New England Biolabs E0554S).

Transfection experiments. HeLa cells were transiently transfected with the MSCV-IRES-GFP vector containing WT FLAG-β-tubulin, C239S FLAG-β-tubulin or C303S FLAG-β-tubulin for 24 h and treated with WIT for 16 h. Total protein was obtained and analyzed via western blot using FLAG and GFP antibodies. Expression of GAPDH was detected as a loading control.

Statistical analysis. Statistics results of multiple experiments were shown as means±95% confidence intervals (CI). Statistical differences were determined using an unpaired, non-parametric Student's t-test (Welch's). * $P<0.05$.

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Results

WIT promotes tubulin degradation in a post-transcriptional manner. WIT (Fig. 1A) has been shown to suppress the protein levels of tubulin (Antony et al., 2014) but the exact mechanism remains unclear at present. Western blot findings revealed that WIT downregulates α-and β-tubulin proteins in a concentration-dependent manner in HeLa and HCT116 cells (Fig. 1B). However, simultaneous q-PCR experiments disclosed no inhibitory effects of WIT on mRNA levels of α-and β-tubulin (Fig. 1C), indicating that WIT-induced downregulation is a post-transcriptional process. Conversely, WIT slightly promoted tubulin mRNA levels (Fig. 1C), which may be a result of negative regulation after tubulin protein suppression. Immunofluorescence results indicated that WIT could also inhibit tubulin polymerization in HeLa cells at concentrations that promote tubulin degradation (Fig. 1D), indicating the tubulin degradation and de-polymerization activities induced by WIT is initiated at almost identical concentration range. Upon pretreatment with the proteasome inhibitor, MG132, WIT no longer induced down-regulation of tubulin (Fig. 1E). Besides, immunoprecipitation results revealed that WIT could increase the ubiquitination level of both α- and β-tubulin (Fig. 1F). All these results confirmed WIT induces tubulin protein degradation in a ubiquitin-proteasome (post-transcriptional) dependent manner.

WIT binds to the colchicine site of beta-tubulin. All the tubulin inhibitors identified to date bind tubulin to promote or inhibit microtubule polymerization. For instance, paclitaxel acts as a tubulin polymerization agent while colchicine and vinblastine are tubulin depolymerization agents (Dumontet et al., 2010). Here, we investigated the

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effect of WIT on polymerization of tubulin *in vitro* using paclitaxel and colchicine as positive controls. As shown in Figure 2A, paclitaxel promoted polymerization of tubulin while colchicine exerted the opposite effect to a significant extent. WIT exerted an inhibitory effect on polymerization similar to that of colchicine, which was concentration-dependent, confirming direct interactions with tubulin. Colchicine and vinblastine sites are the two most commonly studied tubulin depolymerization sites in beta-tubulin and interacting compounds at the two sites have distinct effects on microtubule morphology. At high concentrations, compounds at the vinblastine site induce depolymerization of tubulin to further form paracrystals (packing of unpolymerized tubulin in the cytoplasm) that can be detected using immunofluorescence. However, compounds binding to the colchicine site have no such effect. Immunofluorescence results depicted in Figure 2B showed that high concentrations of paclitaxel markedly promoted tubulin polymerization, colchicine inhibited tubulin polymerization while vinblastine inhibited tubulin polymerization and significantly induced formation of tubulin paracrystals. Notably, the morphological changes of tubulin depolymerization caused by high concentrations of WIT were similar to those of colchicine, indicative of WIT binding at the colchicine site. Accordingly, we further investigated whether or not WIT interacts with the colchicine site with the aid of the N,N′-ethylenebis(iodoacetamide) (EBI) competition binding assay. One molecule of EBI is capable of simultaneously forming covalent bonds with cysteine residues at positions 239 and 354 near the β-tubulin colchicine site, thereby forming an EBI-β-tubulin complex that migrates faster than β-tubulin on SDS-PAGE

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(Fortin et al., 2010). As shown in Figure 2C, both colchicine and WIT inhibited formation of the EBI-β-tubulin complex, supporting the theory that WIT interacts with the colchicine site of β-tubulin. In competition experiments, pretreatment with colchicine, but not vinblastine, inhibited tubulin degradation by WIT (Fig. 2D), confirming that binding of WIT to the colchicine site accounts for tubulin degradation. To further validate this finding, we used a variety of colchicine site inhibitors, including nocodazole, plinabulin and combretastatin A4, for another competition experiment. Our data showed significant inhibition of WIT-induced tubulin degradation by these inhibitors (Fig. 2E). The collective results indicate that WIT binds the colchicine site of tubulin to promote degradation.

WIT exerts irreversible cellular effects. Reversibility of binding to tubulin plays an important role in determining the toxicity and effectiveness of microtubule inhibitors (Thomas et al., 2014; Yang et al, 2018). Generally, reversible inhibitors exert lower toxicity and irreversible inhibitors have better efficacy. Simultaneous reversibility can also be used to predict the mechanisms by which inhibitors bind to tubulin. For example, an irreversible mode of action such as that of cyclostreptin, usually indicates covalent binding to tubulin (Buey et al., 2007; Balaguer et al.,2019). Here, we examined the reversibility of WIT action in HeLa cells using the irreversible inhibitor, colchicine, and reversible inhibitor, colcemid, as positive controls (Yang et al, 2018; Yan et al., 2018). After treatment with inhibitors for 8 h, HeLa cells were washed and cultured for an additional 24 h. Cell microtubule morphology was detected via immunofluorescence at 0, 8, 16, 24, and 32 h time-points. As shown in Figure 3A, at the 0 h time-point,

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microtubules exhibited a normal network in the cytoplasm of HeLa cells. After 8 h of treatment with colchicine, colcemid or WIT, microtubule depolymerization was significantly enhanced and no polymerized microtubules were observed in the cytoplasm. All inhibitors were totally removed at the 8 h time-point. At 16 h, the colchicine and WIT-treated microtubes remained in a depolymerized state while colcemid-treated microtubules had begun to resume polymerization. Colcemid-treated microtubes continued to exhibit polymerization at the 24 h time-point while colchicine and WIT-treated tubulin remained in a depolymerized state. At the 32 h time-point, morphology of the microtubules in the colcemid treatment group had completely recovered to normal while no significant differences were observed in colchicine and WIT-treated microtubules between the 8 h and 32 h time-points. Simultaneous detection of the level of p-H3 on ser-10 (a G2/M phase arrest marker) (Ren et al., 2018) at the above time-points revealed similar results (Fig. 3B). These findings indicate that WIT behaves in a similar manner to colchicine as an irreversible microtubule inhibitor. Given its ability to form covalent bonds with target proteins, we further assessed whether WIT could bind covalently to the colchicine site of tubulin.

WIT covalently binds to Cys303 and Cys239 of β-tubulin. A biomass spectrometry experiment was performed to determine the specific residues forming covalent bonds with WIT on β-tubulin. Purified αβ-tubulin dimer (10 μM) and excess WIT (30 μM) were incubated for 3 h at room temperature followed by removal of unbound WIT via ultrafiltration and SDS-PAGE. A 55 kD tubulin band was obtained, which was excised and subjected to mass spectrometry analysis. The data showed that two peptides were

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covalently modified by WIT (^{217}LTTPTYGDLNHLVSATMSGVTTCLR^{241} and ^{298}NMMAACDPR^{306}) in β-tubulin with a molecular weight increase of 470 kD (molecular weight of WIT), respectively. While the untreated peptides showed only a weight increase of 57 kD (Carbamidomethyl). The MS/MS fragment of WIT treated ^{217}LTTPTYGDLNHLVSATMSGVTTCLR^{241} peptide showed that all y-ions containing Cys239 were modified by WIT, supporting covalent binding to Cys239 (Fig. 4A). ^{298}NMMAACDPR^{306} peptide fragmentation data similarly revealed that WIT forms a covalent bond with Cys303 (Fig. 4B). Our results collectively demonstrate that one molecule of β-tubulin can simultaneously form covalent bonds with two molecules of WIT at Cys239 and Cys303 sites.

Covalent binding between WIT and Cys239 accounts for WIT-induced tubulin degradation. Multiple subtypes of tubulin exist, including β2-, β3-, β4-, β5 (β)- and β6-tubulin (Ernest, 2008). Among these isoforms, β3- and β6-tubulin contain Ser while β2-, β4- and β5 (β)-tubulin contain Cys at position 239. All the tubulin isoforms contain Cys303 (Fig.5A). In this study, we investigated the effects of WIT on β2-, β3-, β4- and β6 tubulin protein levels. Interestingly, WIT significantly promoted degradation of β2- and β4-tubulin containing cysteine at position 239 but had no effect on β3- and β6-tubulin levels (Fig. 5B), suggesting that covalent binding of WIT to tubulin Cys239 is the main factor underlying its degradation activity. For validating of this finding, FLAG-tagged (C-termini) WT *β-tubulin* or mutant *β-tubulin* (C239S and C303S) genes were cloned into MSCV-IRES-GFP expression vectors, which co-expressed tubulin and GFP in HeLa cells after transient transfection. WIT promoted degradation of WT β-

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tubulin and C303S β-tubulin but had no effect on C239S β-tubulin (Fig. 5C), suggesting that only Cys239 is required for WIT activity on tubulin. The unsaturated double bond in the A ring of WIT is liable to undergo a Michael addition reaction with the sulfhydryl group of cysteine (Maitra et al., 2009; Falsey et al., 2006; Yu et al., 2010; Antony et al., 2014). Accordingly, we further focused on the structure-activity relationship of WIT. DWIT is a structural analog of WIT (A. Khan et al., 2016), the only difference being the lack of an unsaturated double bond in ring A of DWIT (Fig. 5D). DWIT did not promote tubulin degradation (Figure 5E) or inhibit cell viability in HeLa cells (Figure 5F). Based on these results, we conclude that covalent bond formation between WIT and Cys239 is responsible for tubulin degradation.

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Discussion

Since 2007, the U.S. Food and Drug Administration has approved three anti-tumor drugs targeting microtubules, specifically, Ixabepilone, Cabazitaxel and Erbulin mesylate. Tubulin remains an important target for the development of stable and efficient anti-tumor drugs, investigation of mechanisms underlying tumor progression and clinical research (Dumontet et al., 2010). The development of microtubule-targeted drugs has attracted significant commercial investment. Functionally, tubulin binders can be divided into two categories: polymerization agents (such as paclitaxel) and depolymerization agents (such as vinblastine) (Ojeda-Lopez et al., 2014; Gigant et al., 2005; Ravelli et al., 2004). However, the tubulin inhibitors identified to date have no effects on tubulin protein levels. Previous experiments by our group showed that small molecules covalently bound to the Cys239 site, including T0070906, T007-1 and T138067, promoting tubulin degradation (Yang et al., 2019). These small molecules were collectively classified as the third type of tubulin inhibitor-tubulin degradation agent. In this study, we identified WIT as another naturally derived tubulin-degrading agent that also forms a covalent bond with cysteine at position 239 of β-tubulin. Tubulin degradation agents with distinct mechanisms of action should provide more options for the design of effective tubulin-based drugs and facilitate in-depth analysis of mechanisms underlying tubulin degradation.

Target protein degradation technology is a clinically effective treatment strategy, for instance, the application of fulvestrant-induced estrogen receptor degradation for breast cancer (Wu et al., 2005) and all-trans retinoic acid-induced retinoic acid receptor

degradation for promyelocytic leukemia therapy (Zhu et al., 1999). The newly developed proteolysis targeting chimera (PROTAC) protein degradation technology plays a significant part in modern anticancer drug design (Lai et al., 2017). However, PROTACs are made up of two small molecules which is an inherent disadvantage，for the molecular weight is usually large and pharmacokinetic data as well as drug-forming properties are poor (Lai et al., 2017). So far, no small molecule-based PROTAC has been developed for degradation of tubulin. Experiments from the current study support covalent combination with Cys239 as an effective strategy to promote tubulin degradation with good development prospects, since the molecular weight is small, and drugs with high-quality pharmacokinetic data could be designed.

The unsaturated double bond of the A ring of WIT is important for covalent bond formation. Our studies clearly indicate that the unsaturated double bond is essential for WIT binding to tubulin, since DWIT devoid of the unsaturated double bond in the A ring showed total loss of tubulin degradation and anti-proliferation activity. Based on these findings, we conclude that the covalent bond is necessary for both binding and degradation activities.

Previous studies have additionally reported covalent binding of WIT to Cys303 located on the surface of β-tubulin (Antony et al., 2014). In this case, the unsaturated double bond of the A ring of WIT that can easily form a covalent bond is almost completely exposed and steric hindrance is small, allowing reaction with the cysteine residue on the protein surface, which leads to the possibility that the covalent bond between WIT and Cys303 is due to a non-specific reaction. Substitution of cysteine

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with serine at position 303 had no effect on the degradation activity of WIT, indicative of no pharmacological effect of the WIT-Cys303 bond. However, further studies are required to ascertain the potential biological effects of the WIT-Cys303 bond. Moreover, WIT covalently interacted with Cys239 of β-tubulin located near the colchicine site. A competition assay using colchicine site inhibitors, such as colchicine, nocodazole and plinabulin, blocked WIT-induced tubulin degradation, clearly indicating that WIT binding to the colchicine site accounts for its tubulin degradation activity. Most importantly, substitution of cysteine with serine at position 239 abolished the degradation activity of WIT, further supporting the theory that formation of the WIT-Cys239 bond is a prerequisite for WIT-mediated degradation. Of note, WIT also promotes degradation of α-tubulin. The underlying mechanism is proposed as both α- and β-tubulin are only stable in dimers in the cell and clearance of β-tubulin by WIT will cause lots of unstable monomeric α-tubulin, which could be easily degrade by proteasome. However, this is only a theoretical assumption and requires further investigation.

Our collective findings demonstrate that the natural compound, WIT, forms a covalent bond with Cys239 of β-tubulin to promote tubulin degradation, providing another example of a covalent modifier of Cys at position 239 of β-tubulin as an effective tubulin degradation agent with therapeutic potential.

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Authorship Contributions

Participated in research design: Jianhong Yang, and Lijuan Chen.

Conducted experiments: Jianhong Yang, Wei Yan, Yong Li, and Lu, Niu

Contributed new reagents or analytical tools: Jianhong Yang, Wei Yan, and Yong Li.

Performed data analysis: Jianhong Yang

Wrote or contributed to the writing of the manuscript: Jianhong Yang, Haoyu Ye, and Lijuan Chen.

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Footnotes

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Figure legends

Figure 1.WIT promotes tubulin degradation in a post-transcriptional manner. (A) Chemical structure of WIT. (B) HeLa and Hct116 cells were treated with increasing concentrations of WIT for 16 h and protein levels of α-and β-tubulin detected via western blot. The right image presents quantitative data on α-and β-tubulin proteins, values are presented as mean±95% CI of three independent experiments. (C) HeLa and Hct116 cells were treated with increasing concentrations of WIT for 16 h and mRNA levels of *α*-and *β-tubulin* detected via quantitative PCR, values are presented as mean±95% CI of three independent experiments. * $P<0.05$. (D) Cells were treated with different concentrations of WIT (1 μM, 3 μM, and 10 μM) for 16 h and microtubules were imaged using an Olympus fluorescence microscope. (E) HeLa cells were pretreated with or without 10 μM MG132 for 1 h before treatment with increasing concentrations of WIT for 16 h and protein levels of α-and β-tubulin detected via western blot. The right image presents quantitative data on α-and β-tubulin proteins, values are presented as mean±95% CI of three independent experiments. (F) Hela cells were incubated with MG132(10μM) for 1hour before treated with 0, 1, 3or 10μM WIT for 16hours, α- or β-tubulin were immunoprecipitated from the lysates, and ubiquitinylated α- or β-tubulin were detected with anti-ubiquitin antibodies. Results are representative of three independent experiments. α-Tub, α-tubulin; β-Tub, β-tubulin; Con, Control.

Figure 2. WIT binds to the colchicine site of β-tubulin. (A) Purified tubulin was incubated with the indicated compounds at 4℃ and OD values at 340 nm measured

once a minute. (B) Cells were treated with high concentrations of the specified compounds (10 μM paclitaxel, 10 μM colchicine, 10 μM vinblastine or 100 μM WIT) for 1 h and microtubules were imaged using an Olympus fluorescence microscope. (C) Purified tubulin (1 μM) was incubated with the specified compounds for 2 h before incubation with 100 μM EBI for another 2 h and subjected to western blot for detection of β-tubulin protein. The lower image depicts the EBI-β-tubulin protein level, values are presented as mean±95% CI of three independent experiments. (D) HeLa cells were pretreated with 3 μM colchicine or 3 μM vinblastine for 1 h before treatment with 3 μM WIT for 16 h. Protein levels of α-and β-tubulin were detected via western blot. The below image represents quantitative data on α-and β-tubulin proteins, values are presented as mean±95% CI of three independent experiments. (E) HeLa cells were pretreated with 3 μM nocodazole, 3 μM plinabulin or 3 μM combretastatin A4 for 1 h before treatment with 3 μM WIT for 16 h. Protein levels of α-and β-tubulin were detected via western blot. The below image represents quantitative data on α-and β-tubulin proteins, values are presented as mean±95% CI of three independent experiments. Con, control; Col, colchicine; PTX, paclitaxel; Vin, vinblastine; Noc, nocodazole; Pil, plinabulin; CA4, Combretastatin A4; α-Tub, α-tubulin; β-Tub, β-tubulin.

Figure 3. WIT exerts irreversible cellular effects. (A) HeLa cells were treated with 10 μM colchicine, 10 μM colcemid or 100 μM WIT for 8 h. After the compounds were completely washed off, cells were cultured for an additional 24 h and microtubule morphology examined via immunofluorescence at 0, 8, 16, 24, and 32 h time-points.

(B) Western blot analysis of p-H3 expression was further examined at 0, 8, 16, 24, and 32 h. The right image depicts quantitative data on p-H3 expression, values are presented as mean±95% CI of three independent experiments.

Figure 4. WIT forms covalent bonds with Cys303 and Cys239 of β-tubulin. (A) Left: MS/MS fragmentation patterns of the untreated peptide "^{217}LTTPTYGDLNHLVSATMSGVTTCLR^{241}". Right: MS/MS fragmentation patterns of the covalently bound peptide "^{217}LTTPTYGDLNHLVSATMSGVTTCLR^{241}" indicate that WIT binds to Cys239. (B) Left: MS/MS fragmentation pattern of the untreated peptide "^{298}NMMAACDPR^{306}". Right: MS/MS fragmentation pattern of the covalently bound peptide "^{298}NMMAACDPR^{306}" indicate that WIT binds to Cys303.

Figure 5. Covalent binding between WIT and Cys239 accounts for WIT-induced tubulin degradation. (A) Selected amino acid sequences of β2-, β3-, β4-, β5 (β)-, and β6-tubulin isoforms. (B) HeLa cells were treated with increasing concentrations of WIT for 16 h and β2-, β3-, β4- and β6-tubulin protein levels detected via western blot. The right image depicts quantitative data on β2-, β3-, β4- and β6-tubulin protein expression, values are presented as mean±95% CI of three independent experiments. (C) HeLa cells were transiently transfected with MSCV-IRES-GFP vectors co-expressing GFP and WT-FLAG-β-Tubulin, C239S-FLAG-β-Tubulin or C303S-FLAG-β-Tubulin for 24 h. Cells were treated with or without 3 μM WIT for 16 h, and GFP and Flag-tubulin detected via western blot. The right image depicts quantitative data on GFP and Flag-tubulin, values are presented as mean±95% CI of three independent experiments. (D) Chemical structure of Dihydrowithaferin A (DWIT). (E) HeLa cells

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were treated with increasing concentrations of WIT or DWIT for 16 h and β-tubulin protein detected via western blot. The right image depicts quantitative data on β-tubulin protein, values are presented as mean±95% CI of three independent experiments. (F) HeLa cells were treated with increasing concentrations of WIT or DWIT for 48 h and cell viability assessed with the trypan blue exclusion assay, values are presented as mean±95% CI of three independent experiments. β-Tub, β-tubulin; β2-Tub, β2-tubulin; β3-Tub, β-3tubulin; β4-Tub, β4-tubulin; β6-Tub, β6-tubulin.

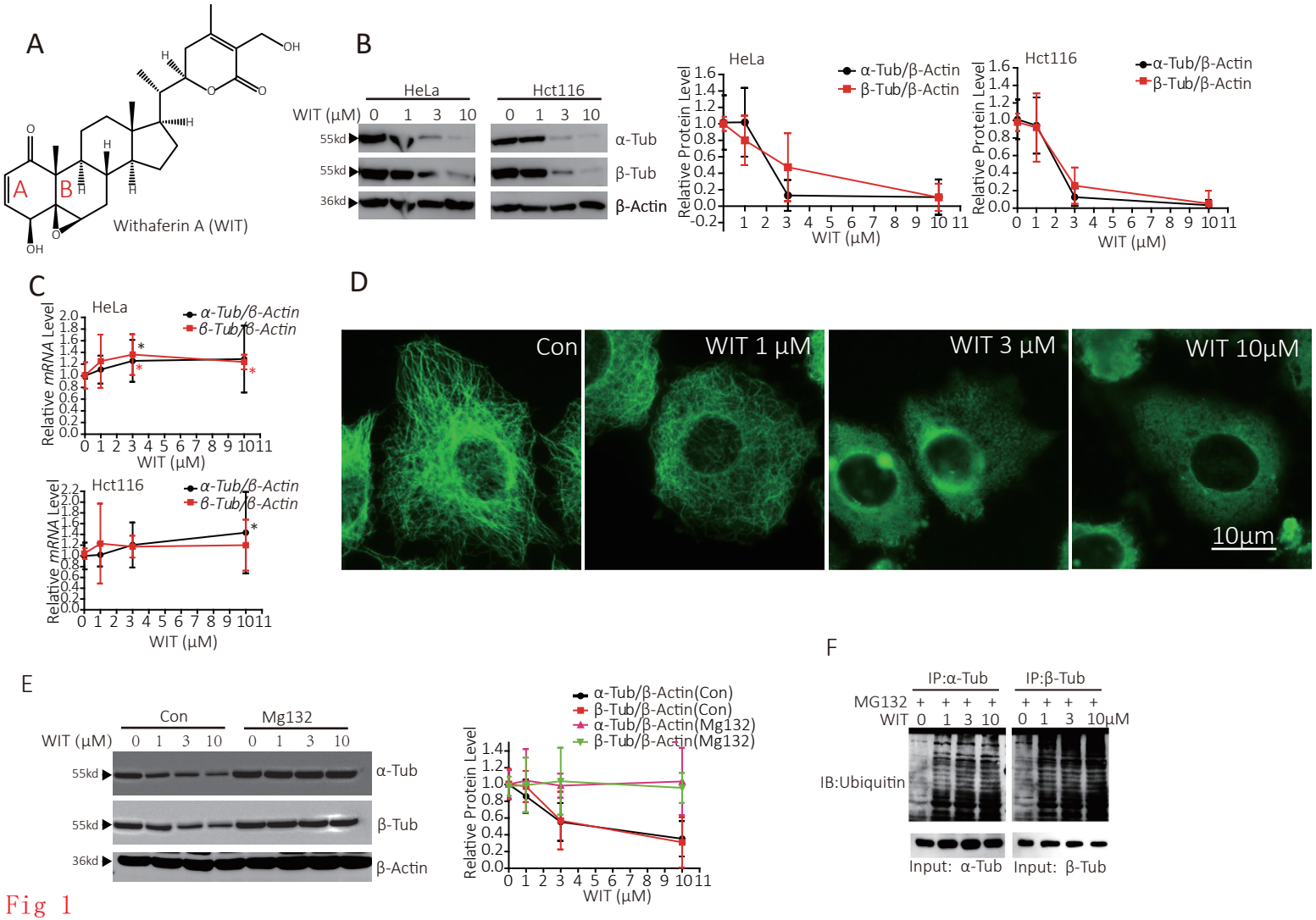

Fig 1

Fig. 2

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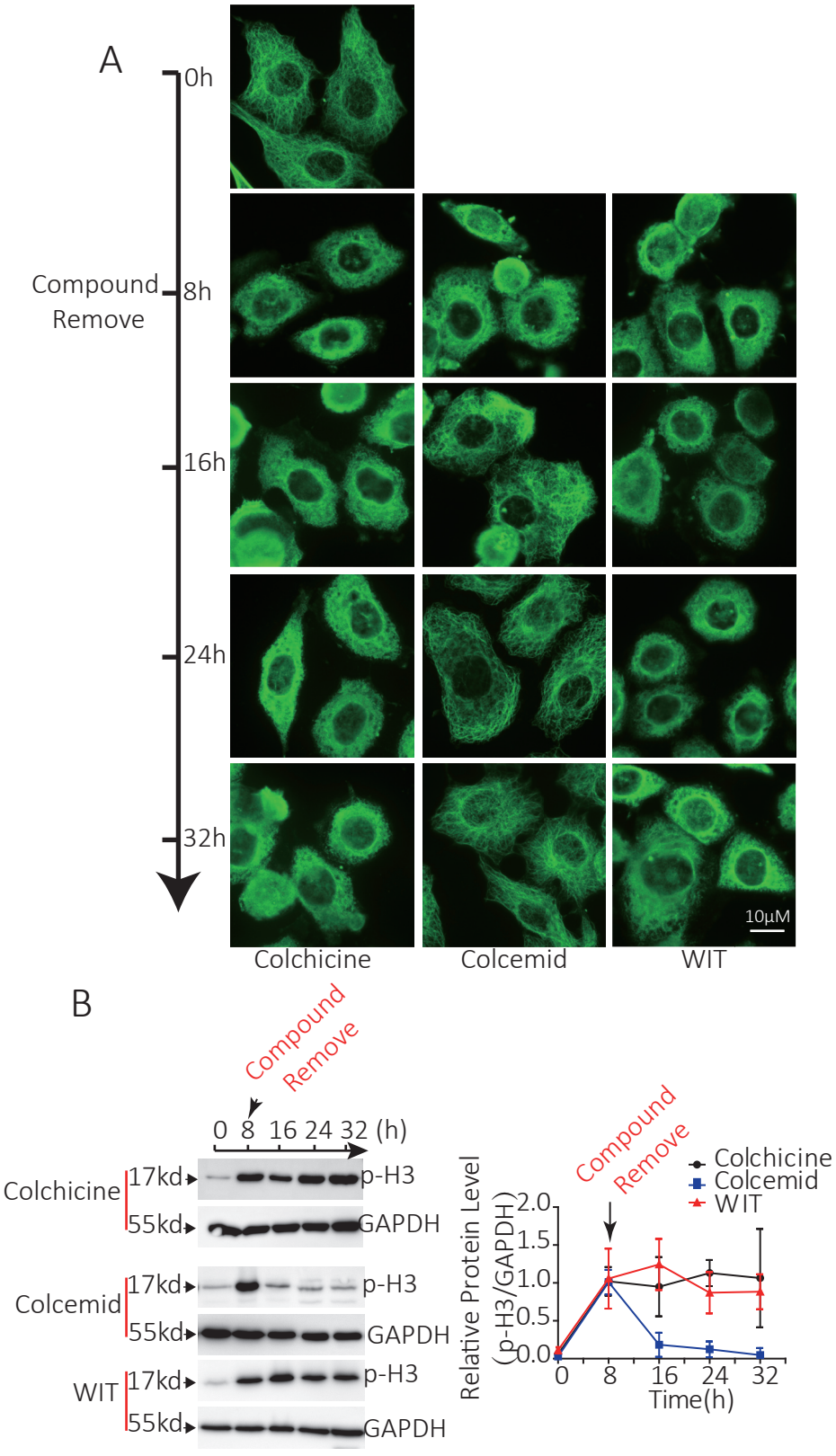

Fig. 3

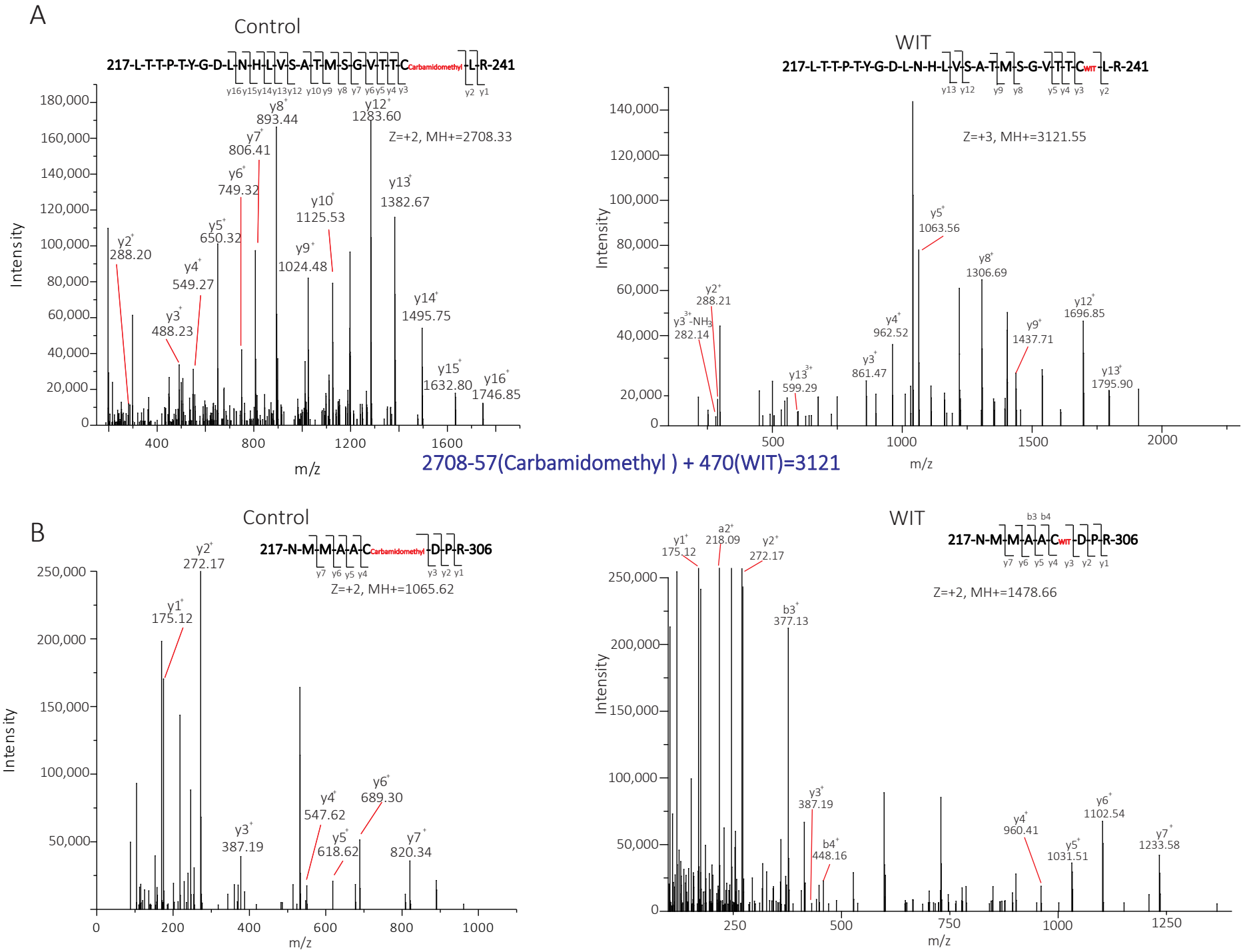

Fig 4

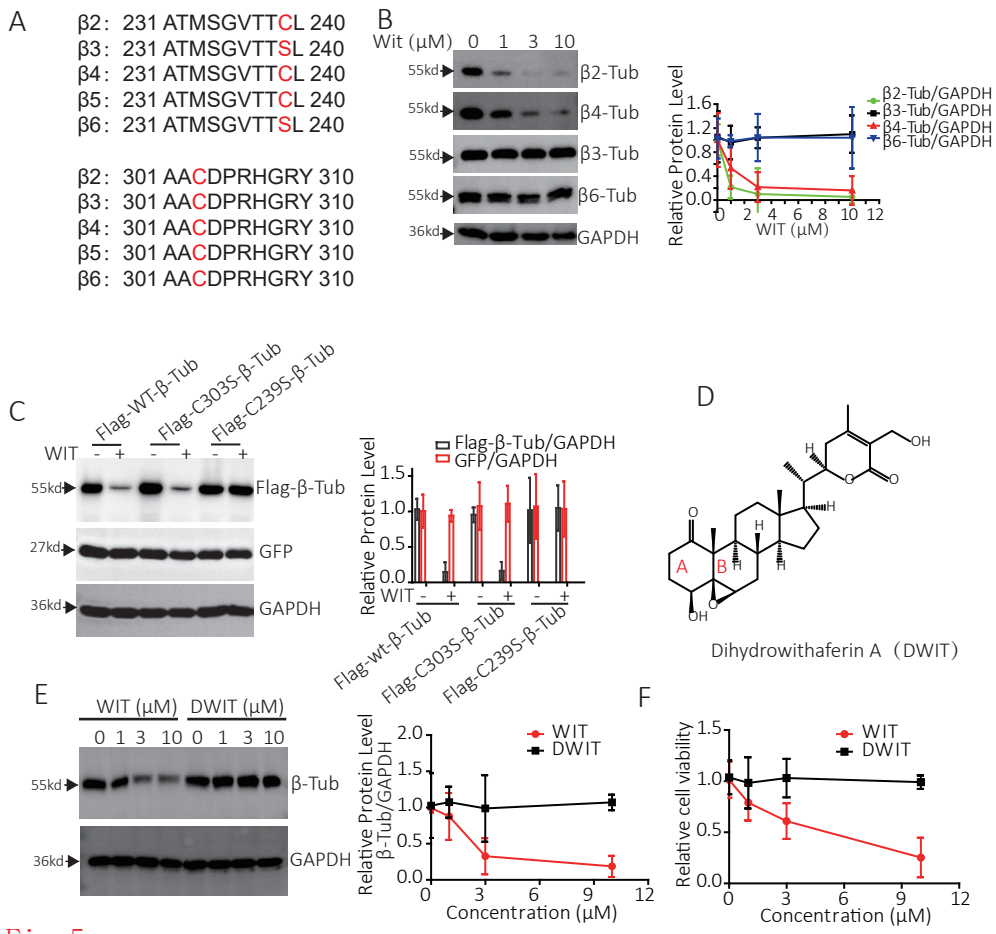

Fig. 5