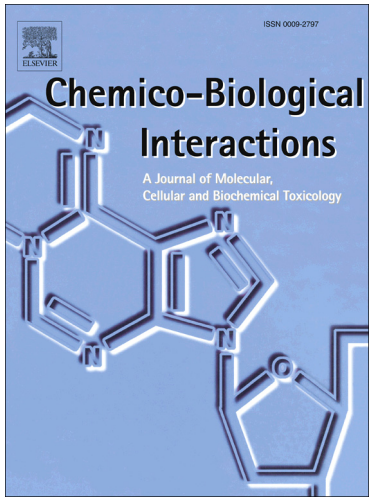

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Piperlongumine is a novel nuclear export inhibitor with potent anticancer activity

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Abstract

Piperlongumine is a natural compound recently identified to be toxic selectively to tumor cells *in vitro* and *in vivo*. However, the molecular mechanism underlying its anti-tumor action still remains unclear. In this report, we describe another novel mechanism by which piperlongumine mediates its anti-tumor effects. We found that piperlongumine is a novel nuclear export inhibitor. Piperlongumine could induce nuclear retention of tumor suppressor proteins and inhibit the interactions between CRM1 and these proteins. Piperlongumine could directly bind to the conserved Cys528 of CRM1 but not to a Cys528 mutant peptide. More importantly, cancer cells expressing mutant CRM1 (C528S) are resistant to piperlongumine, demonstrating the nuclear export inhibition via direct interaction with Cys528 of CRM1. The inhibition of nuclear export by piperlongumine may account for its therapeutic properties in cancer diseases. Our findings provide a good starting point for development of novel CRM1 inhibitors.

Key Words: piperlongumine, nuclear protein export, CRM1, anti-cancer

1. Introduction

Piperlongumine, a natural alkaloid of the Long pepper, has been used widely in Indian and Chinese traditional medicine [1]. Piperlongumine is an active compound exhibiting multiple reported pharmacological and biological activities including anti-microbial, anti-inflammation and platelet aggregation inhibition and [2-4]. Furthermore, piperlongumine has been reported to kill multiple types of cancer cells and has antitumor activities in a variety of animal models [5, 6]. Although piperlongumine was first discovered in 1967, major interest in it did not emerge until the 2011 publication in *Nature* by Raj et al [7]. This group reported that piperlongumine selectively kills multiple types of cancer cells and does not affect noncancerous cell types even at high doses. However, how piperlongumine acts as an anticancer agent is not yet clear.

To translate the promise of piperlongumine to cancer therapy and prevention, it is necessary to elucidate the biological mechanism of inhibiting tumor growth. It has been shown that anti-tumor activity of piperlongumine was mainly through suppressing constitutive NF-κB, AKT/mTOR and MAPK signal pathway in various cancer cells [8-10]. Piperlongumine also was reported to induce reactive oxygen species (ROS) generation resulting in apoptosis and cell cycle arrest [11]. More recently, piperlongumine was identified as a STAT3 inhibitor [12]. Unfortunately, its detailed biological mechanism of action is still in much debate and remains to be elucidated. A critical structural feature of piperlongumine is the electrophilic α,

β-unsaturated carbonyl group as a Michael acceptor moiety. This has raised the question that how the Michael acceptor unit of piperlongumine directly interacts with its cellular targets to exert biological activities.

Protein transport between nucleus and cytoplasm is critical for protein function, cell proliferation and survival [13]. The major nuclear exporter protein CRM1 (chromosome maintenance region 1), also known as exportin 1, is a key member of nuclear transport receptors [14]. CRM1 recognizes its export cargos through specific leucine-rich nuclear export signal (NES) consensus sequences. CRM1 cargos include nearly all major tumor suppressors and cell growth regulators, such as p53, p21, inhibitor of κB-α (IκB-α), survivin, FOXOs and nucleophosmin-1 (NPM1) [15-17]. CRM1-mediated export is increased in various cancers and high levels of CRM1 protein is associated with lower survival rates in the tumor patients [18]. The overexpression of CRM1 export receptor is linked to inactivation of tumor suppressor proteins, cancer development and resistance to chemotherapy. The nuclear retention of tumor suppressor proteins by inhibition CRM1 leads to restoration of their tumor suppressing activities [19]. Therefore, CRM1 is a promising therapeutic target for anti-tumor drug development. Most CRM1 inhibitors block the nuclear export by covalently binding to Cys528 in the NES groove of CRM1 [20, 21]. A well-known CRM1-specific inhibitor, leptomycin B (LMB), binds covalently to Cys528 of CRM1 by a Michael-type addition reaction [22]. The common structural feature of existing nuclear export inhibitors is the Michael acceptor unit as the thiol-reactive warhead.

In this report, for the first time, we demonstrate that piperlongumine is a direct inhibitor of CRM1. Piperlongumine inactivate CRM1-directed protein export by covalent modification of Cys528 in CRM1 through a Michael addition manner. Furthermore, our results are consistent with the pharmacological relevance of piperlongumine and could explain its cell growth modulatory and anti-tumor effects as previously reported. Our findings provide a type of natural product scaffold for the development of novel therapeutic agents.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

The Hela cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Piperlongumine was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Piperlongumine was dissolved in DMSO at a stock concentration of 10 mM and diluted in DMEM medium at the required concentration just before treatment. Antibodies against Actin, Foxo1, IκB-α, p53, p73, p21, HA tag were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa 488-conjugated donkey anti-rabbit antibody and Alexa 594-conjugated goat anti-mouse antibody were obtained from Invitrogen Life Technology (Invitrogen, Carlsbad, CA). The coding sequence for human CRM1 was PCR amplified and cloned into a pCDNA 3.1 vector containing a sequence coding for an HA tag. The C528S mutation was constructed by

site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions.

2.2. Cell proliferation assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) rapid colorimetric assay [23]. Briefly, cells were seeded in quadruplicate on 96-well plates and incubated overnight under standard conditions to allow cell attachment. The cells were then treated with piperlongumine in concentrations of 0 to 100 μM and incubated for the indicated time. The MTT assay was performed by replacing the standard medium with 100 μL PBS containing 0.5 mg/mL MTT and incubating at 37°C for 4 hours. After incubation, the crystals were dissolved with 200 μL dimethyl sulfoxide. The multiwell plates were then measured at 490 nm using a spectrophotometer.

2.3. Immunofluorescence microscopy

Immunofluorescence microscopy assay assay was performed as described previously [20]. Hela cells were seeded onto black optical-bottom 96-well glass plates. Medium was removed and replaced with drug-containing medium. Following indicated treatments, cells were fixed for 20 min with 4% formaldehyde in PBS at room temperature. Next, cell membranes were permeabilized by treatment with 0.3% Triton X-100 in PBS for 20 minutes. After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h, cells were treated with primary antibodies (1:50 dilution) in

blocking buffer, followed by addition of fluorescent secondary antibodies Alexa 594 and Alexa 488 (1:200 dilution). After washing, cells were stained with 10 μg/mL Hoehst 33258. Photomicrographic images were acquired and analyzed using a confocal laser scanning microscope Fluoview FV10i (Olympus, Japan).

2.4. Nuclear transport functional assays

Inhibition of CRM1-mediated Foxo1-GFP and NES-GFP transport was monitored as described [24]. Hela cells were plated in 96-well plates and transfected with Foxo1-GFP or NES-GFP plasmids. 24 h after transfection, piperlongumine were added to the cells at different concentrations. Inhibition of the CRM1-mediated nucleocytoplasmic transport was measured by verifying cellular distribution of Foxo1-GFP or NES-GFP two hours after addition of piperlongumine. Next, cells were fixed for 20 min with 4% formaldehyde in PBS and stained with 10 μg/mL Hoehst 33258 (Sigma-Aldrich). Photomicrographic images were recorded with the use of a confocal laser scanning microscope Fluoview FV10i (Olympus, Japan).

2.5. Immunoprecipitation

Immunoprecipitation assay was performed as described previously [25]. Hela cells were seeded at 60 mm dish and treated with piperlongumine and incubated for 4 h. Then, cells were lysed in a cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and Protease inhibitor cocktail). After incubation, the lysed cell suspension was centrifuged. The supernatant was incubated

with anti-CRM1 antibody for 6 h and then with protein A-Sepharose 4B (Amershan Biosciences) for 12 h at 4 °C. Immune complexes were washed three times with lysis buffer and subjected to western blotting with antmi-p21, anti-p53, anti-p73 or anti-Foxo1 antibodies.

2.6. Western blotting

Cytoplasmic and nuclear cell extract from control and treated cells were used for Western blot analysis. The protein extracts were resolved by SDS-PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes. The membrane was blocked and incubated with relevant antibodies. The proteins then were detected by enhanced chemiluminescence on X-ray film with an ECL Western blotting detection kit (Amersham).

2.7. Streptavidin-biotin pull-down assay

Streptavidin-biotin pull-down assay was performed as described previously [20]. Synthesis methods of biotinylated piperlongumine are available in supplementary data. Hela cells were treated with biotinylated piperlongumine (50 μM) for 2 hours. Whole cell lysates were extracted from the cells and used for the assay. Streptavidin beads were added to the cell lysate and incubated at 4 °C overnight. After washing the beads with washing buffer, the captured protein was eluted form the beads by boiling them with SDS sample buffer and was analyzed by Western blotting.

2.8. Mass spectrometric analysis

Analysis of covalent attachment to the CRM1 peptide was performed as described in prior studies [24]. The peptide DLLGLCEQK (amino acids sequence 523-531 of CRM1) and its derivative with Cys528 substituted by Ser were synthesized (GeneScript, China). Piperlongumine (20 μg) was incubated with one microgram of each peptide in 20 μL of buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50% methanol) overnight at 37°C. After incubation, an aliquot of 0.5 μL of this solution was mixed with 0.5 μL of the matrix solution (10 mg/mL a-cyano-4-hydroxycinnamic acid in 1:1 acetonitrileywater containing 0.1% TFA). The mixed solution was deposited on a stainless steel sample plate, and then air-dried before analysis. This sample was analyzed with a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, USA) in the delayedextraction mode.

2.9 Data analysis

Data are means and standard deviations of three independent experiments. The results were statistical analyzed using a Student's *t* test and considered statistically significant at the $P < 0.05$ level.

3. Results

3.1. Piperlongumine suppresses growth of cancer cells and CRM1-dependent nuclear export

Piperlongumine (Fig. 1A) induced a dose-dependent growth inhibition after indicated time of exposure. The calculated IC_{50} values was 16.3 μM after 24 h exposure to piperlongumine (Fig. 1B). We then analyzed the subcellular localization of CRM1 substrates in cells treated with piperlongumine. The artificial reporter protein consisting of GFP was fused to a canonical NES (amino acids 71-86 of Rev) and expressed in mammalian cells. NES-GFP is a canonical biomarker for CRM1 inhibition. In control cells, the NES-GFP protein was actively exported and localized mostly to the cytoplasm due to the presence of NES (Fig. 1D and E). In contrast, GFP has a nearly pancellular localization because it is capable of passing through the nuclear pore by free diffusion (data not shown). Notably, upon treatment of cells with either 10 nM of LMB or 25 μM of piperlongumine, almost all cells showed an approximately equal distribution between cytoplasm and nuclear compartment. These results implicate that nuclear protein export is suppressed by piperlongumine with a similar fashion to LMB.

We also analyzed the subcellular localization of CRM1 cargo proteins in the absence or presence of piperlongumine (Fig. 1C). Foxo1, a Forkhead transcription factor, is a tumor suppressor protein involving in negatively regulating cell survival [26]. Foxo1 is known to be exported from nucleus to cytosol in a CRM1 dependent manner. As shown in Fig.1C, Foxo1-GFP is found exclusively in cytosol in untreated cells. In contrast, treatment with LMB led to a strong accumulation of Foxo1-GFP in the nucleus. Similar to LMB, treatment with 25 μM piperlongumine also induced

rapid nuclear accumulation of Foxo1-GFP. These results further suggest that CRM1 may be a direct cellular target of piperlongumine.

3.2. Piperlongumine induces nuclear retention of major tumor suppressor proteins

To verify whether CRM1 inhibition by piperlongumine leads to nuclear accumulation of different tumor suppressor proteins, Western blot assays on piperlongumine-treated nuclear cell lysates were performed. As shown in Fig. 2, exposure of cancer cells to piperlongumine resulted in a progressive increase in the nuclear fraction of major tumor suppressor proteins (Foxo1, p21, p53 and IκB-α). On the other hand, cytosolic fraction of the tumor suppressor proteins were reduced. These results implicate that CRM1 inhibition leads to nuclear retention of major tumor suppressor proteins, which then leads to suppression of cancer cell growth.

3.3. Piperlongumine disrupts the interaction of CRM1 with tumor suppressor proteins

To verify whether piperlongumine directly disrupts the interaction of CRM1 with tumor suppressor proteins, we treated Hela cells with or without piperlongumine addition, then immunoprecipitation assays were performed. Cell lysates form untreated and piperlongumine-treated cells were pulled down with CRM1 antibody and probed with Foxo1, p21, p73 and IκB-α, respectively. In contrast to control cells, piperlongumine treatment resulted in a reduced interaction of CRM1 with these tumor suppressor proteins (Fig. 3).

3.4. Piperlongumine directly binds to Cysteine 528 of CRM1

To investigate whether piperlongumine could bind covalently to Cys528 of CRM1 in a similar manner with LMB, we performed a mass spectrometry analysis of a synthetic CRM1 peptide. Both wild type peptide containing Cys528 and mutant peptide containing Ser528 of CRM1 were synthesized and reacted with piperlongumine. CRM1 wild type peptide presented a major peak at m/z 1018.5842 (Fig. 4A). Upon incubation with piperlongumine, the major peak of CRM1 peptide was shifted to m/z 1335.5850 (Fig. 4B). The mass shift between the modified peptide and the parental one was 317, which corresponds to the molecular weight of piperlongumine, indicating that piperlongumine can bind covalently to CRM1 and potentially act as an inhibitor. However, no peak corresponding to a piperlongumine adduct could be observed for the sample derived from the mutant peptide treated with piperlongumine, suggesting that the active site Cys528 residue is the main target of modification by piperlongumine (Fig. 4C and D).

3.5. Mutation of CRM1 abolishes piperlongumine-induced nuclear export inhibition

To further prove piperlongumine regulates tumor suppressor proteins through CRM1 inhibition, we analyzed the subcellular localization of the reporter protein Foxo1-GFP in cells co-expressing either wild type or mutant CRM1 upon treatment with LMB or piperlongumine. Hela cells expressing wild type HA-CRM1 or HA-CRM1-C528S both showed a cytoplasmic localization of Foxo1-GFP, demonstrating that exogenous wild or mutant type CRM1 does not interfere with

nuclear export. Upon addition of LMB or piperlongumine to the cells, Foxo1-GFP localization was shifted towards nucleus in cells expressing wild-type CRM1 (Fig. 5A). Strikingly, cells expressing HA-CRM1-C528S mutant were resistant to LMB or piperlongumine treatment, since Foxo1-GFP localized predominantly to the cytoplasm in these cells (Fig. 5B). Interestingly, both CRM1-WT and CRM1-C528S were localized at nuclear membrane as well as within the nucleus (Fig. 5A and 5B). This nuclear distribution of CRM1 may be due to its interaction with cargo proteins within the nucleus. Upon treatment with LMB or piperlongumine, wild-type but not Cys528 mutant CRM1 was localized at the nuclear membrane. These data further demonstrate that piperlongumine is specifically targeting Cys528 of CRM1. We transiently transfected Hela cells with wild type or Cys528 mutant CRM1. The cells expressing HA-CRM1-C528S partially reverse the anti-proliferative effects of piperlongumine (Fig. 5C). Together, our data demonstrate that piperlongumine affects tumor suppressor proteins via suppressing CRM1 activity.

Next, we performed CRM1 pull-down assay with the use of biotinylated piperlongumine. CRM1 was pulled down from the lysates of Hela cells treated with biotin-piperlongumine. The binding between CRM1 and biotin-piperlongumine was prevented by addition of LMB (Fig. 5D). Furthermore, the amount of Cys528 mutant CRM1 pulled down with biotin-piperlongumine was sharply diminished (Fig. 5E). These results indicate that piperlongumine directly binds to Cys528 of CRM1 in cell.

4. Discussion

The natural compound piperlongumine has been shown to selectively inhibit tumor growth in many different types of cancers. However, the possible molecular mechanism involved in piperlongumine mediated cancer cell death is still poorly understood. To our knowledge, this is the first report that CRM1 is a direct cellular target of piperlongumine. The inhibition of nuclear export by piperlongumine may account for its therapeutic properties in tumor diseases.

Inhibition of nucleo-cytoplasmic transport by natural and synthetic products has been pursued as a therapeutic avenue in cancer based on a number of biologic observations [27]. CRM1 mediates the transit of proteins with a canonical leucine-rich hydrophobic NES out of the nucleus. Multiple tumor suppressor proteins, as well as CRM1, are mislocalized or expressed at supraphysiologic levels within cancer cells [15]. Targeted therapies work, at least in part, by forcing nuclear accumulation of tumor suppressor proteins which initiate cascades of pathways resulting in cell death. LMB is a classic nuclear export inhibitor that covalently attaches to the sulfhydryl group of cysteine at position of 528 in CRM1. However, LMB has very poor pharmaceutical properties and poor selectivity for cancer versus normal cells [28]. Piperlongumine can be administered orally and show good tolerability [7].

We also demonstrated that piperlongumine targets Cys528 of CRM1 similarly to LMB. Mass spectrometric analysis showed that piperlongumine could bind directly to

a CRM1-derived peptide containing Cys528, but not to a mutated peptide (Cys528Ser) lacking Cys. The calculated IC_{50} values was 16.3 μM after 24 h exposure to piperlongumine. This is consistent with the CRM1 inhibition activity of piperlongumine.

As CRM1 inhibition forces the nuclear localization of many tumor suppressor proteins, delineation of which specific growth regulators involved in piperlongumine-induced cytotoxicity is important [29]. We showed that piperlongumine could disrupt CRM1-Foxo1, CRM1-p21, CRM1-p73 and CRM1-IκB-α interaction, which then lead to nuclear retention of these tumor suppressor proteins. These results suggest that tumor suppressor proteins are essential components of the downstream signaling pathway of CRM1 inhibition in cancer cells. Notably, only a cytoplasmic localization of Foxo1 was observed in cells expressing CRM1-cys528 mutant when treated with piperlongumine. This has provided direct evidence that well-documented antitumor effects of piperlongumine are truly mediated by CRM1 inhibition.

Previous studies revealed that anti-cancer activity of piperlongumine may be through suppressing constitutive NF-κB signal pathway in cancer cells [30]. Piperlongumine inhibits NF-κB activity in cancer cells by blocking the degradation of IκB-α. However, the mechanism of suppressing IκB-α degradation is unclear. The nuclear retention of IκB-α could suppress its degradation which eventually may lead

to inhibition of NF-κB activity. Our results revealed that piperlongumine could inhibit degradation of IκB-α by inhibition of nuclear export.

In conclusion, the present study shows for the first time that piperlongumine could block CRM1 and thereby modulate nuclear traffic. In particular, we have provided strong evidence in support of the anti-cancer mechanism of piperlongumine which may involve nuclear retention of different tumor suppressor proteins. We anticipate that future development of low-toxicity CRM1 inhibitors may provide a novel approach for tumor target therapy. Our findings provide a good starting point for development of novel CRM1 inhibitors.

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Author contributions

M. N. and X. X. performed experiments, interpreted data and wrote the manuscript. Y. S. and L. Z. performed the immunofluorescence microscopy analysis. Y. Y. and F. Z. carried out the construction of plasmids. J. Q. performed Western blot assay. X. L. and K. X. designed the study, interpreted data and substantially contributed to critical

revisions. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared that no competing interests exist.

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Fig. 1. Piperlongumine inhibits cancer cell growth and CRM1-dependent nuclear export. (A) Structure of piperlongumine. (B) Inhibition of cell growth of Hela cells by piperlongumine. HeLa cells were treated with indicated concentrations of piperlongumine for 24, 48 and 72 h. Cell proliferation was measured by MTT assay in triplicate wells. (C) Piperlongumine causes nuclear accumulation of Foxo1-GFP protein. Hela cells were transiently transfected with Foxo1-GFP and treated for 2 h with 10 nM LMB or indicated concentrations of piperlongumine. Fixed cells were stained for Hoechst and analyzed by confocal microscopy. Images are representative of three independent experiments. (D) Piperlongumine inhibits nuclear export of NES-GFP. Hela cells were transiently transfected with NES-GFP and treated for 2 h with piperlongumine. Fixed cells were stained for Hoechst and analyzed by confocal microscopy. (E) Quantification of NES-GFP cellular distribution in (D). Cells were scored for predominant cytoplasmic (C > N), equal cytoplasmic and nuclear (C = N) or predominant nuclear (C < N) distribution of NES-GFP. More than 100 cells were scored.

Fig. 2. Piperlongumine induces nuclear retention of tumor suppressor proteins. Hela cells were treated with 30 μM piperlongumine for 4 h. Nuclear and cytosolic lysates were resolved using western blotting. The membranes were probed with Foxo1, p53, p21, IκB-α and Actin. Actin

was uses as a loading control for cytosolic lysates. Blots are representative of three independent experiments.

Fig. 3. Piperlongumine disrupts the interaction of CRM1 with tumor suppressor proteins. Hela cells were treated with 30 μM piperlongumine for 4 h. Cell lysates were immunoprecipitated using CRM1 antibody, followed by western blotting with anti-Foxo1, anti-p21, anti-p73 and anti-IκB-α antibody, respectively. Bolts are representative of three independent experiments.

Fig. 4. Piperlongumine directly binds to Cys528 of a CRM1 peptide. Mass spectrometry was employed to measure the formation of adducts between compound and CRM1-derived peptide. Synthetic peptides containing Cys528 of CRM1 (A) or the mutant peptides in which Cys528 was substituted by Ser (C) were treated with piperlongumine (B, D) for 24 h and analyzed by MALDI-TOF MS.

Fig. 5. Piperlongumine is ineffective in cells expressing mutant CRM1. Hela cells co-transfected Foxo1-GFP with HA-tagged wild type CRM1 (A) or Cys528 mutant CRM1 (B). After treatment with 10 nM LMB or 30 μM piperlongumine for 2 h, cells were fixed and stained with antibodies against the HA tag. The cells were then analyzed by confocal microscopy. Images are representative of three independent experiments. (C) Growth inhibition assay in wild type and Cys528 mutant cells. Cells were seeded in 96-well plates and transiently transfected with HA-CRM1-WT or HA-CRM1-C528S plasmids. After treatment with piperlongumine at indicated concentrations for 48 h, cell proliferation was measured by MTT assay in triplicate wells. The sign

* indicates difference ($P < 0.05$) compared to control. (D) Hela cells were treated with biotinylated piperlongumine (50 μM) for 2 hours in the presence or absence of pretreatment with LMB (10 nM) for 1 hour. The whole cell lysates were subjected to pull-down analysis with the use of streptavidin beads. Captured proteins were analyzed by Western blotting. (E) Hela cells were transiently transfected with HA-CRM1-WT or HA-CRM1-C528S plasmids. Next, the cells were treated with biotinylated piperlongumine (50 μM) for 2 hours. The whole cell lysates were subjected to pull-down assay and were analyzed by Western blotting.

Figure 1

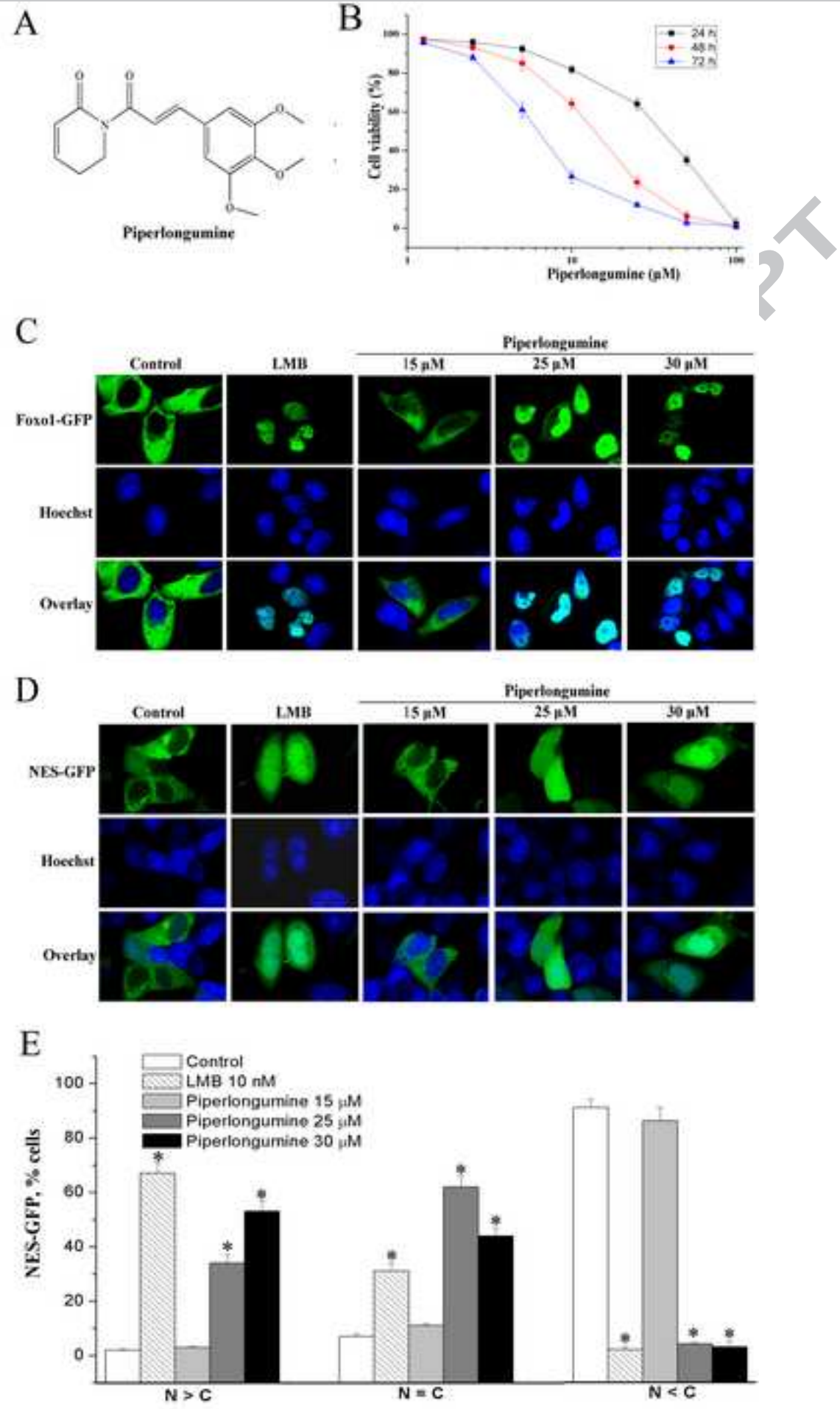


Figure 2

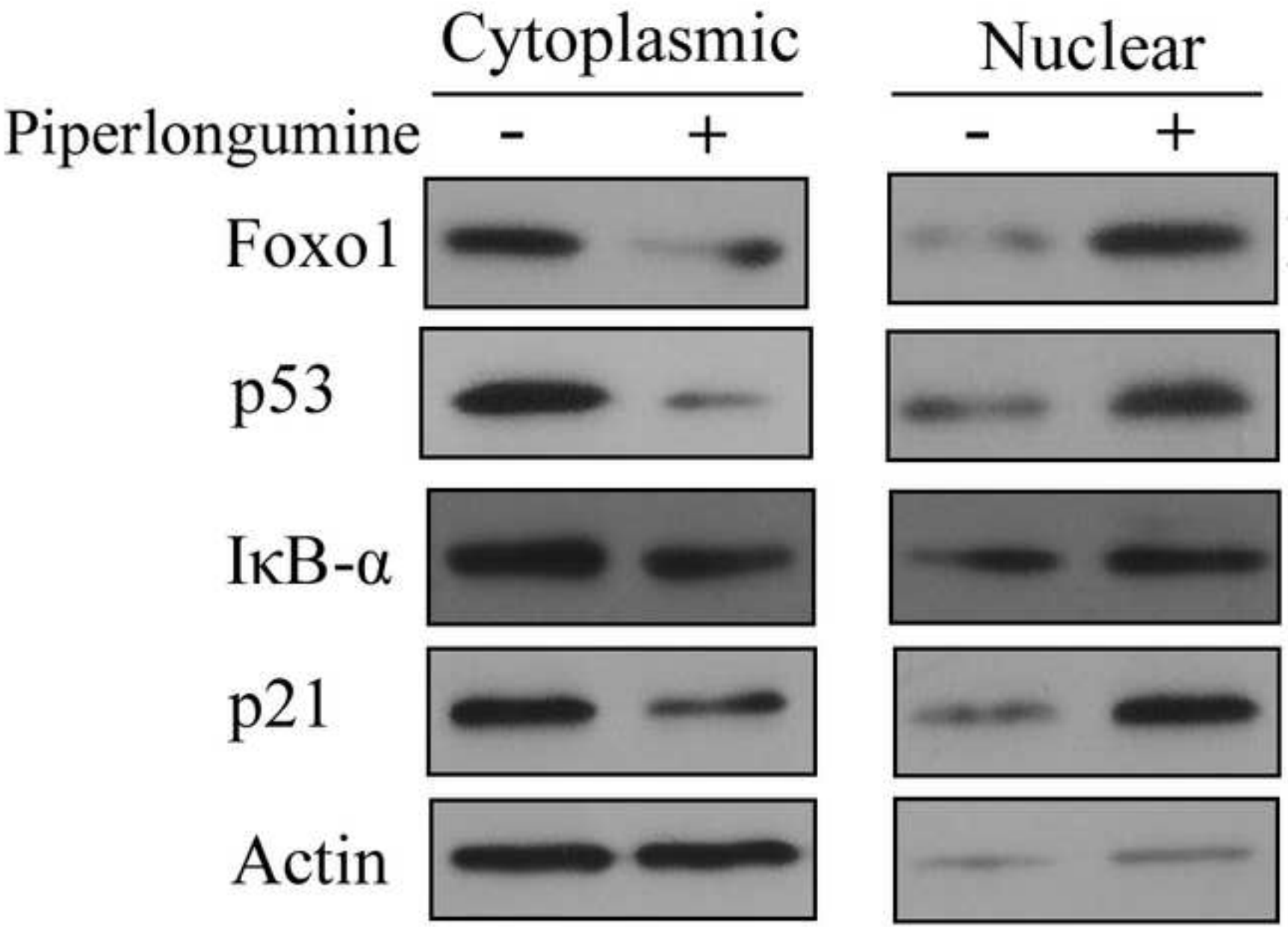


Figure 3

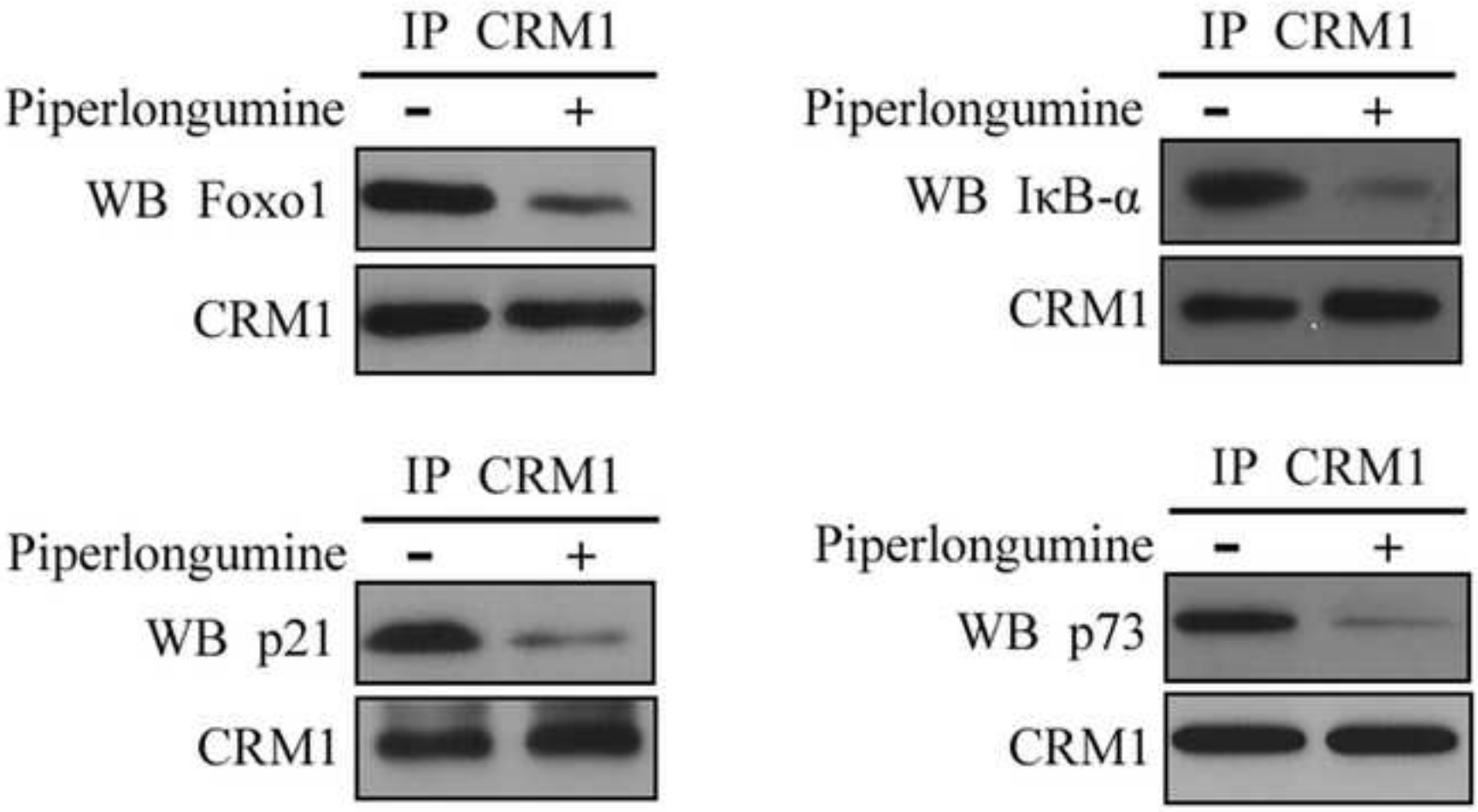


Figure 4

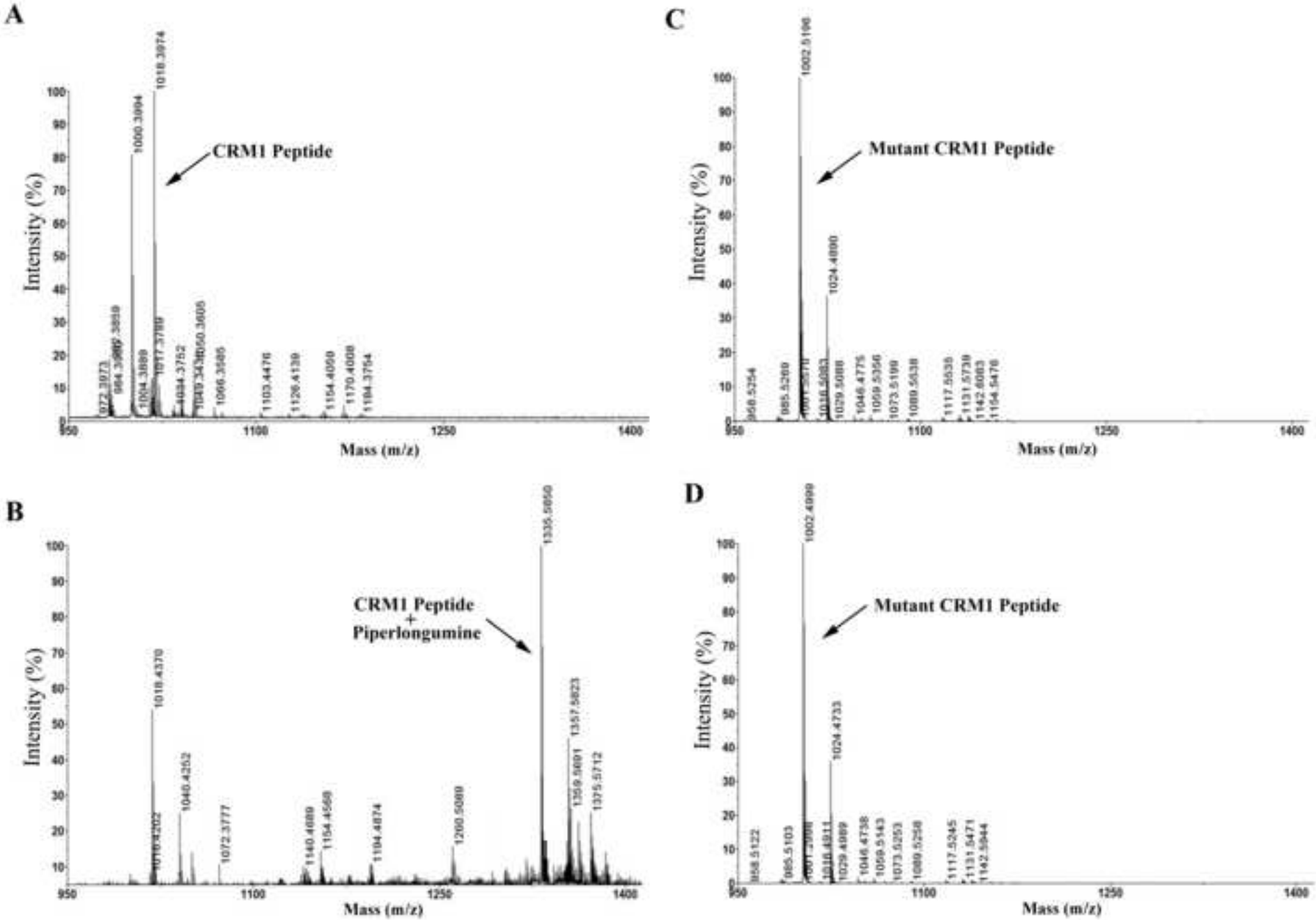
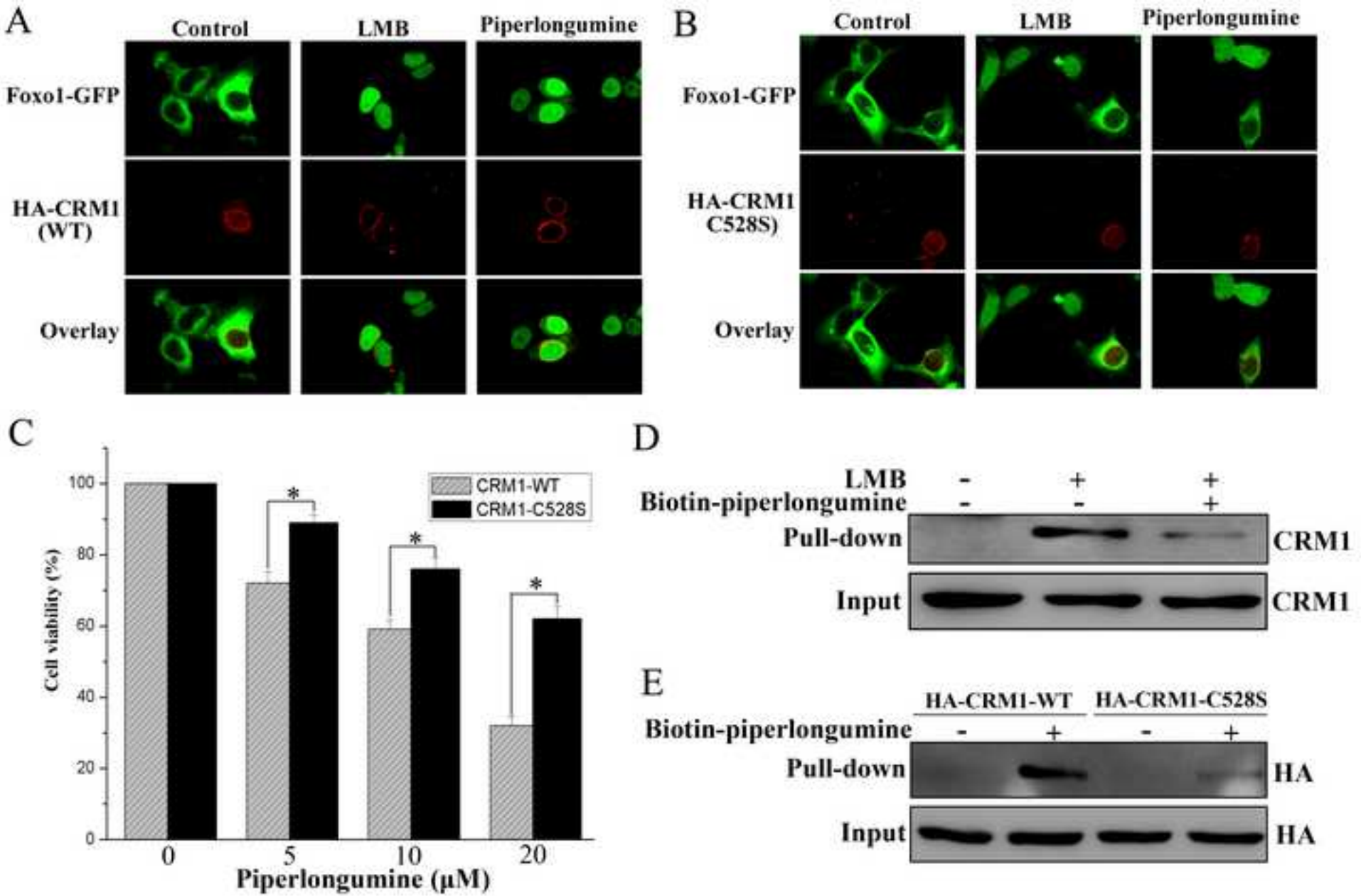


Figure 5



Highlights

- Piperlongumine is a novel inhibitor of CRM1
- Piperlongumine directly binds to cysteine 528 of CRM1
- Piperlongumine inhibits the interaction between CRM1 and tumor suppressor proteins.
- Piperlongumine suppresses the proliferation of cancer cells via inhibiting CRM1