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EDITOR'S CHOICE

WILEY

Caffeic acid phenethyl ester (CAPE) revisited: Covalent modulation of XPO1/CRM1 activities and implication for its mechanism of action

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Caffeic acid phenethyl ester (CAPE) is the bioactive constituent of propolis from honeybee hives and is well known for its anti-inflammatory, anticarcinogenic, antioxidant, and immunomodulatory properties. Herein, we revisited the cellular mechanism underlying the diverse biological effects of CAPE. We demonstrated that XPO1/CRM1, a major nuclear export receptor, is a cellular target of CAPE. Through nuclear export functional assay, we observed a clear shift of XPO1 cargo proteins from a cytoplasmic localization to nucleus when treated with CAPE. In particular, we showed that CAPE could specifically target the non-catalytic and conserved Cys^{528} of XPO1 through the means of mass spectrometric analysis. In addition, we demonstrated that the mutation of Cys^{528} residue in XPO1 could rescue the nuclear export defects caused by CAPE. Furthermore, we performed position-restraint molecular dynamics simulation to show that the Michael acceptor moiety of CAPE is the warhead to enable covalent binding with Cys^{528} residue of XPO1. The covalent modulation of nuclear export by CAPE may explain its diverse biological effects. Our findings may have general implications for further investigation of CAPE and its structural analogs.

KEYWORDS
caffeic acid phenethyl ester, covalent binding, nuclear export, XPO1/CRM1

1 | INTRODUCTION

Caffeic acid phenethyl ester, a polyphenolic compound isolated from honeybee propolis, is one of the most intensively studied nature-derived chemicals with a variety of biological and pharmacological effects.[1–3] Earlier studies demonstrated that caffeic acid phenethyl ester can induce apoptosis in breast,[4] prostate,[5] and leukemia[6] cancer cells by the activation of c-Jun N-terminal kinase, inhibition of NF-κb activities, and stimulation of P53 and P21 etc. A recent study[7] also showed that caffeic acid phenethyl ester can suppress the growth of melanoma by inhibiting PI3K/AKT/XIAP pathway. Furthermore, caffeic acid phenethyl ester is well known to have antimitogenic, antioxidant, anti-inflammatory, and immunomodulatory properties.[8,9] In the past decades, various possible mechanisms have been proposed for the biological effects of caffeic acid phenethyl ester.[10–12] However, the detailed biological mechanism of action is still a topic of much debate and remains to be delineated.

One of the most distinct structural features of CAPE is the electron-poor conjugated olefin moiety, also called as Michael acceptor. The unsaturated olefin moiety is critical

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for the biological activities of CAPE. For instance, prior studies showed that the saturated amide analogs of CAPE exerted much less or no potency compared to the parent compound.[13] Therefore, we hypothesized that the Michael acceptor structure endows CAPE with activities toward a nucleophilic residue such as cysteine via covalent bonding within the active site of a cellular target.

XPO1 (also referred as exportin 1 or CRM1) is the sole nuclear exporter of tumor suppressor proteins and pro-inflammatory cytokines such as P53, P21, P27, FOXO1, BRCA1, IkB, and RXRα.[14–16] The cargo proteins can bind with XPO1 through specific sequence signals referred as nuclear export signals (NESs). The elevated level of XPO1 has been implicated in many tumors and inflammatory disease states such that tumor suppressor proteins and anti-inflammatory proteins are excluded from nucleus to cytoplasm where they are unable to function.[17,18] The existing XPO1 inhibitors mediate nuclear export by covalently binding with the nucleophilic sulphydryl group of a conserved Cys^{528} residue within the NES-binding cleft of XPO1. For example, the unsaturated carbonyl group on the lactone ring of Leptomycin B (LMB), a natural XPO1 inhibitor discovered from *Streptomyces*, is deemed as the thiol-reactive warhead targeting XPO1.[19] The electrophilic α,β-unsaturated carbonyl group on the terminal δ-lactone ring of LMB functions as Michael acceptor which covalently binds with the nucleophilic sulphydryl group of Cys^{528} of CRM1. Our group recently found that curcumin, the major active component of turmeric plant, blocks nuclear export by covalent modulating XPO1 activities.[20] Similarly, the α,β-unsaturated carbonyl moiety of curcumin is the Michael acceptor moiety, which is accessible for nucleophilic attack by Cys^{528} of CRM1. In fact, the reversible or irreversible inhibition of CRM1 is rather dependent on the bond strength of the C–S bond formed during Michael addition reaction between the Michael acceptor moiety and Cys^{528} of CRM1. In addition, the stereochemistry of the Michael acceptor moiety within the inhibitor's structure is also crucial for the biological activities.

Herein, we revisited the biological mechanism of CAPE by coupling of computational and experimental methods. We revealed that CAPE can enable nuclear retention of multiple tumor suppressor proteins, which leads to significant apoptosis in tumor cells. We showed that CAPE can directly target the cysteine residue in the NES-binding cleft of XPO1 by means of nuclear export functional assay and mass spectrometric analysis. In addition, we investigated the covalent interaction of CAPE with Cys^{528} residue of XPO1 by position-restraint molecular dynamics (MD) simulation. Our findings support the hypothesis that CAPE can mediate nuclear export by nucleophilic attack of the non-catalytic cysteine residue in the NES-binding cleft of XPO1. Our study may have general implications for the development of covalent inhibitors containing Michael acceptor functionality.

2 | MATERIALS AND METHODS

2.1 | Cell lines, culture condition, antibodies, and reagents

HeLa, MCF-7, SMMC-7721, and NCI-H460 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (CAS). All tumor cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). CAPE was obtained from TCI (Shanghai). Antibodies against RanBP1, p53, Cylin D1, Cox-2, β-actin, and HA tag were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa 488-conjugated anti-goat antibody and Alexa 594-conjugated anti-mouse antibody were obtained from Invitrogen. All other reagents, chemicals, and antibodies used for this study were obtained from Sigma-Aldrich (Shanghai) unless otherwise noted.

2.2 | Plasmids

The plasmid coding for NES-GFP was generated using annealed oligonucleotides corresponding to the NES of HIV-1 Rev. The resulting plasmid encodes for amino acids VPLQLPPLERLTLDCN linked to the C-terminal end of GFP. The HA-tagged wild-type XPO1/CRM1 and XPO1/CRM1 mutant Cys^{528} to Ser plasmids were kindly provided by Dr. Chang Chen from Institute of Biophysics, CAS.

2.3 | Cell growth inhibition assay

Tumor cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) rapid colorimetric assay. Tumor cells were seeded into 96-well plates and incubated overnight. The cells were then exposed with CAPE in concentrations of 0–100 μg/ml and incubated for up to 24, 48, and 72 hr. 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 hr. After incubation, the crystals were dissolved with 200 μl dimethyl sulfoxide. The multiwell plates were then measured at 570 nm (reference wavelength 630 nm) using a spectrophotometer (Tecan) according to well-established methods.

2.4 | Confocal fluorescence microscopy and protein localization experiments

For protein localization experiments, HeLa cells transfected with plasmids were plated in 96-well plates and exposed to CAPE at different concentrations. Protein localization was

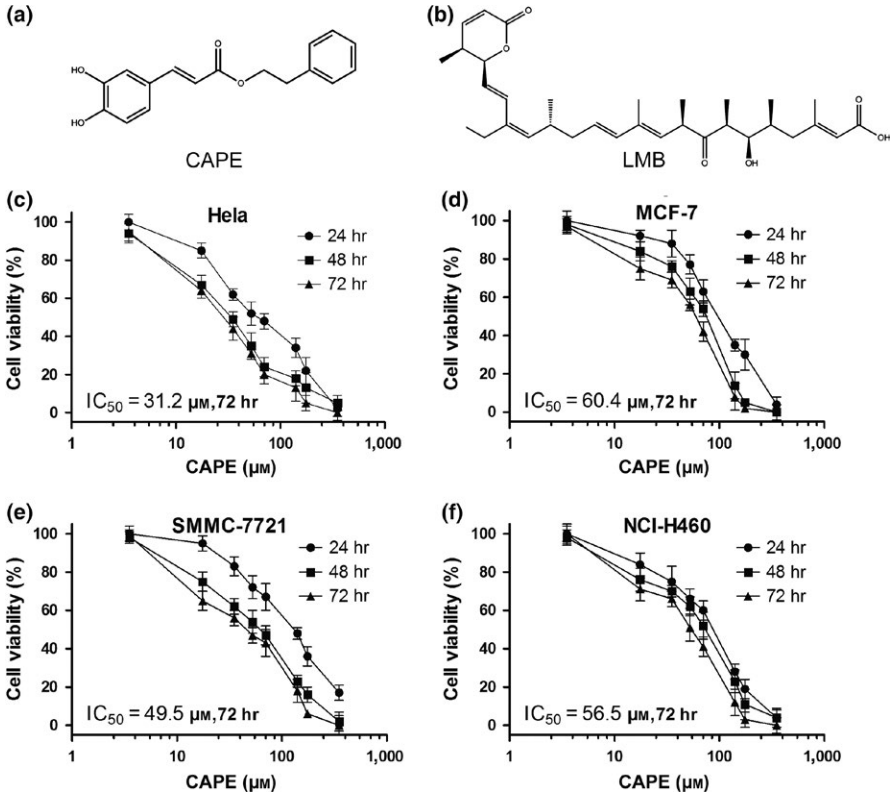

FIGURE 1 CAPE contains electrophilic Michael acceptor moiety and suppresses the growth of multiple tumor cell lines. (a) Chemical structure of CAPE. (b) Chemical structure of Leptomycin B (LMB). (c-f) Suppression of the cell growth of HeLa cells, MCF-7 cells, SMMC-7721 cells, and NCI-H460 cells by CAPE. Tumor cells were treated with the indicated concentrations of CAPE for 24, 48, and 72 hr. Cell proliferation was measured by MTT assay in triplicate wells

measured by verifying the cellular distribution of NES-GFP or FOXO1-GFP 2 hr after addition of CAPE. Nuclei was stained with 10 μg/ml Hoechst 33258 (Sigma-Aldrich), and photomicrographic images were recorded with the use of a confocal laser scanning microscope Fluoview FV10i (Olympus, Japan). Immunofluorescence was performed according to our prior established methods.

2.5 | Western Blot analysis

The preparation of cellular lysates, protein concentration determination, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis were performed according to our prior established methods. The proteins were visualized using Immobilon Western HRP detection substrate (Millipore). Images were recorded with a Luminescent Image Analyzer LAS-4000 system (Biorad, USA) and quantified with MULTI GAUGE software (Biorad).

2.6 | Mass spectrometry

A peptide containing Cys^{528} (amino acids sequence DLLGLCEQK, 523–531 of XPO1) and its derivative with Cys^{528} replaced by Ser were synthesized (GeneScript, Nanjing, China). One microgram of these peptides was treated with or without 10 μg of CAPE in 20 μl of buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50% methanol) at 37°C for 24 hr. The samples for mass spectrometry analysis were prepared as described in prior published methods. The samples were analyzed with a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, USA) in the delayed extraction mode.

2.7 | Computational modeling

Crystallographic structure of XPO1/CRM1 was obtained from the PDB bank (PDB code: 3GB8). For clarity purpose, only NES-binding cleft of XPO1 was used in all simulation. Molecular docking was performed using our newly developed in-house docking tool FIPSDock. The ligand and protein input structures in simulations were saved using PDBQT file format. Molecular dynamics simulation was performed using academic-free package Gromacs. The position-restraint molecular dynamics (MD) simulation was performed in OPLS/AA force field implemented in Gromacs. CAPE was parameterized following the standard protocol as described in Gromacs manual. All the simulations were performed on a high-performance DELL T7500 workstation running Linux operating system. All the molecular graphics were displayed and prepared using PyMol educational version.

2.8 | Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM version 5.00 for Windows in statistical analysis. $p < .05$ was considered significant.

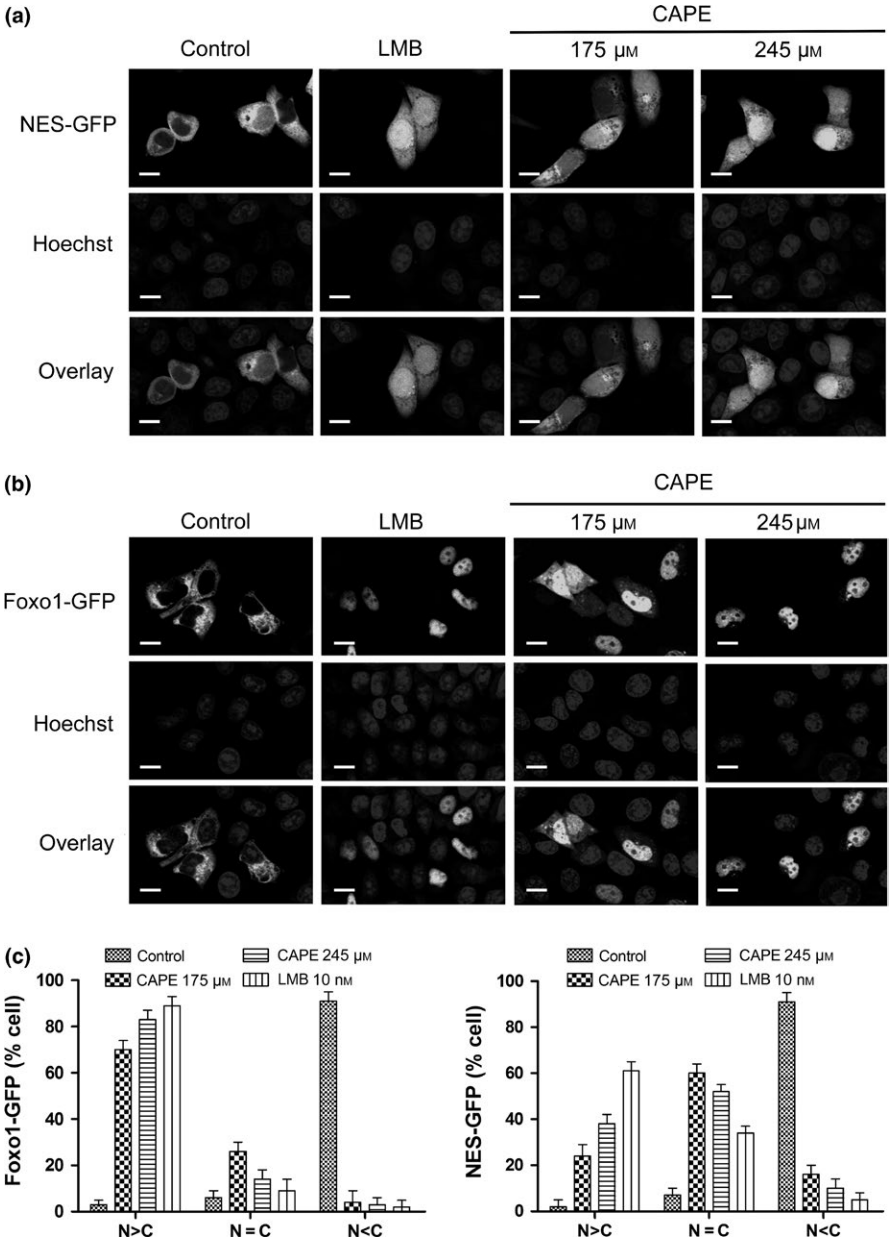

FIGURE 2 CAPE inhibits the nuclear export of XPO1 cargo proteins. (a) CAPE can cause nuclear retention of a NES-containing reporter protein. HeLa cells were transiently transfected with NES-GFP and treated for 2 hr with 10 nM LMB or indicated concentrations of CAPE. (b) Nuclear accumulation of FOXO1-GFP caused by CAPE treatment. Fixed cells were stained with Hoechst (blue), scale bar = 10 μm. (c) Quantification of FOXO1-GFP and NES-GFP cellular distribution. Cells were scored for predominant cytoplasmic (C > N), equal cytoplasmic, and nuclear (C = N) or predominant nuclear (C < N) distribution

3 | RESULTS

3.1 | CAPE suppresses tumor cells growth and nuclear export of cargo proteins

Consistent with prior studies, CAPE was found to suppress the growth of a number of tumor cell lines including HeLa, MCF-7, SMMC-7721, and NCI-H460. The estimated IC_{50} values ranged from 8.9 μg/ml in HeLa to 17.2 μg/ml in MCF-7 cell line (Figure 1). We then investigated the notable structural feature of electron-poor conjugated olefin moiety within CAPE. Next, we analyzed the subcellular localization of XPO1/CRM1 cargo proteins in both control cells and cells treated with CAPE. We used two artificial reporter proteins consisting of GFP (NES-GFP and FOXO1-GFP) as markers of XPO1 inhibition as described in previous published methods.[21] In control cells, both artificial reporter proteins were actively exported and localized mostly to the cytosol in a XPO1-dependent manner due to the presence of NES (Figure 2). In contrast, upon treatment with LMB, we found a strong accumulation of NES-GFP and FOXO1-GFP in the nucleus. Similarly, upon treatment with CAPE, we observed a clear and rapid shift of NES-GFP and FOXO1-GFP from a cytoplasmic localization to nucleus in a dose-dependent manner (Figure 2). These results implicated that CAPE functions as an XPO1 inhibitor.

3.2 | CAPE targets Cys^{528} within the NES-binding cleft of XPO1

LMB is known for its covalent binding with XPO1 by a Michael-type addition.[22] Therefore, we want to examine

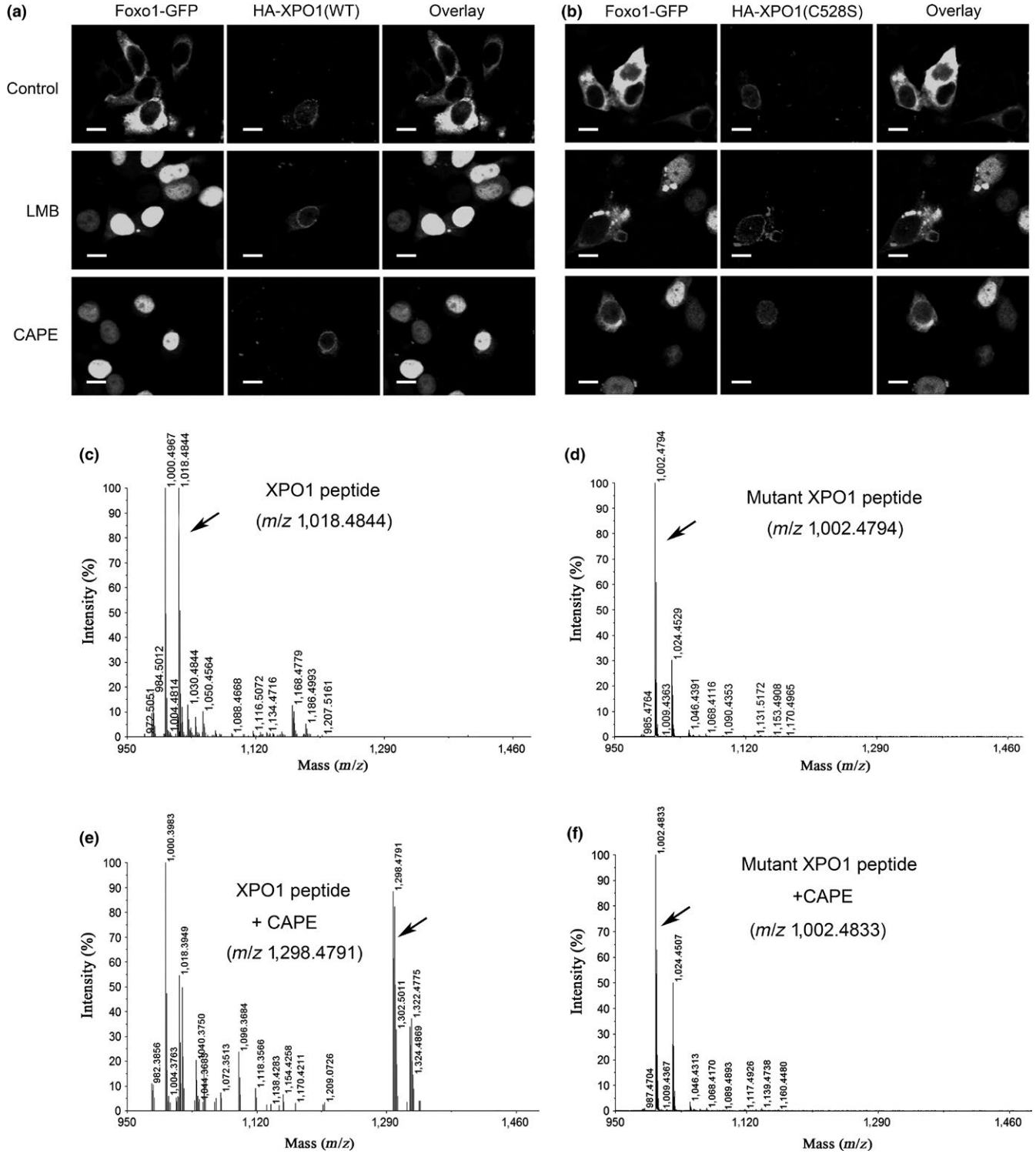

FIGURE 3 CAPE specifically targets cysteine 528 of XPO1. (a-b) HeLa cells co-transfected with plasmids coding for HA-tagged XPO1 (wild type or mutant, as indicated) and the reporter protein FOXO1-GFP. After treatment with 10 nM LMB or 100 μM CAPE for 2 hr, cells were fixed and stained with antibodies against the HA tag. Scale bar = 10 μm. (c-f) Synthetic peptides containing Cys^{528} of XPO1 or the mutant peptides in which Cys^{528} was substituted with Ser were treated with CAPE for 24 hr and analyzed by MALDI-TOF MS

whether CAPE also targets Cys^{528} of XPO1 as LMB does. As described previously, we have expressed an HA-tagged version of XPO1/CRM1, both as a wild-type protein and as a Cys528Ser substitution mutant. Hence, cells carrying a mutation at the corresponding Cys^{528} site are resistant to XPO1 inhibitors. We then analyzed the subcellular localization of FOXO1-GFP in HeLa cells co-expressing either wild-type or mutant XPO1 upon treatment with LMB or CAPE. Upon treatment with LMB or CAPE, FOXO1-GFP was retained in the nucleus in wild-type cells, whereas LMB or CAPE loses its

ability to retain FOXO1-GFP in Cys^{528} mutant (Figure 3a-b). These results demonstrate that the inhibition of nuclear export by CAPE is dependent on Cys^{528} of XPO1.

In addition, we synthesized peptide fragments of XPO1 containing either Cys (XPO1 wild-type peptide, WT) or Ser (XPO1 mutant peptide, MT) at the corresponding position of Cys^{528} in the NES-binding cleft of XPO1. The peptide fragments were treated with CAPE and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Mass spectrometry analysis revealed a mass peak at m/z 1018.4844. After the incubation with CAPE, an additional peak at m/z 1298.4791 was detected which corresponds to the covalent adduct of the peptide with CAPE (Figure 3c-f). However, no covalent adducts were detected for the sample derived from the mutant peptide treated with CAPE. These results further implicate that CAPE can covalently target Cys^{528} of XPO1.

3.3 | CAPE inhibits XPO1 protein activities

We analyzed the effects of CAPE on the expression level of crucial XPO1 cargo proteins related to cancer and inflammation (Figure 4). We first observed the upregulation of p53 after CAPE treatment at 10 μM in HeLa cells. In addition, we observed that the expression of pro-inflammatory and proliferative proteins COX-2 and Cylin D1 was reduced in a dose-dependent manner after treatment with CAPE (Figure 4). This is in line with prior reports that LMB could affect the expression of certain XPO1 cargo proteins in a similar manner.[23] Hence, our results suggest that CAPE functions as a nuclear export inhibitor.

3.4 | Position-restraint molecular dynamics simulation

We performed molecular docking in conjunction with position-restraint molecular dynamics (MD) simulations to analyze the possible covalent binding of CAPE with XPO1. A few crystallographic structures of ligand-bound XPO1 protein have been reported in recent years.[23–26] We assessed the possible binding modes by molecular docking followed by a 10 ns of position-restraint MD simulation in which the distance between the reactive Cys-sulfur atom and the unsaturated β-carbon atom on CAPE was restrained within 1.8Å. The MD simulation suggests that the backbone atoms of the XPO1 complexed with CAPE demonstrated a stable rmsd compared to the initial structure (Figure 5a). A closer analysis of the complex structure revealed that Leu^{525} can make putative hydrophobic contacts with the aromatic moiety of CAPE and Lys^{534} can form putative hydrogen bonding with CAPE (Figure 5b-c). By residue energy contribution analysis, the energy contribution of Lys^{534} is −36.11 kcal/mol, which mainly consists of hydrogen bonding interaction and electrostatic interactions. On the other hand, the energy contribution of Leu^{525} is −2.15 kcal/mol, which mainly consists of hydrophobic interaction. These contacts may contribute to the overall binding of CAPE with XPO1 in addition to the covalent bonding.

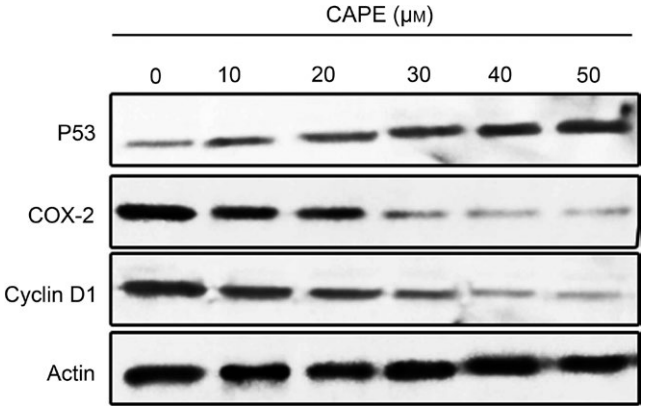

FIGURE 4 Effects of CAPE on crucial XPO1/CRM1 cargo proteins expression. HeLa cells were treated with vehicle or CAPE for 24 hr, and the whole-cell lysates were analyzed by immunoblotting for the presence of indicated proteins with corresponding antibodies

4 | DISCUSSION

CAPE is one of the major active components of propolis with strong antioxidant, antitumor, and antiproliferative activities. However, the mechanisms accounting for the physiological and pharmacological properties of CAPE are yet unclear. In this study, we demonstrated for the first time that CAPE functions as an inhibitor of nuclear export mediated by XPO1. Recently, a number of naturally occurring compounds have been reported as modulators of nuclear export by targeting the conservative Cys^{528} of XPO1. For instance, anguinomycin,[27] 15d-PGJ2,[28] goniothalamin,[29] ratjadone,[30] and curcumin have been identified as XPO1 inhibitors. Of note, CAPE shares a common structural feature of cysteine-reactive electrophiles[31] with these naturally occurring small molecules. Therefore, we hypothesized that CAPE may also function as a nuclear export inhibitor.

To support our hypothesis, we performed a series of computational and experimental studies. We confirmed that CAPE blocks nuclear export as LMB does by means of nuclear export functional assay. Importantly, we identified that CAPE specifically targets the Cys^{528} of XPO1 by measuring the translocation difference of FOXO1-GFP in wild-type and Cys^{528} mutant cells when treated with CAPE. This is further confirmed by mass spectrometric analysis of wild-type and Cys^{528} mutant peptide fragments treated with CAPE. It should be noted that during nuclear export functional assay experiments, we observed that the inhibition of nuclear

export disappears very quickly upon the removal of CAPE. Therefore, it is not likely that CAPE will undergo similar ester hydrolysis by CRM1 as it does to LMB. This is consistent with the weak inhibitory profile of CPAE as compared with LMB.

Further, we performed computational analysis to reveal that CAPE contains an electrophilic Michael acceptor structure which can react with the conservative thiol group in XPO1, thereby inhibiting XPO1 from associating with NES-containing cargo proteins. In addition, molecular dynamics simulation also revealed that CAPE could be well fitted into the NES-binding cleft of XPO1 and the electron-poor unsaturated olefin moiety of CAPE is also within the "reactive distance" of the sulfur atom of Cys^{528} to enable Michael addition reaction. This is evident from the LigPlot[32] diagram (Figure 5d) that CAPE is in the appropriate orientation with β-unsaturated carbon accessible for Michael addition reaction.

Our results demonstrate that the inhibition of nuclear export by CAPE is associated with a decrease in COX-2 and Cyclin D1 expression and an increase in markers of apoptosis including P53. This is consistent with the well-defined anti-inflammatory effects of CAPE, together with its effects on the apoptosis of multiple tumor cell lines. In addition, our results are in line with early studies that CAPE inhibits the activation of nuclear transcription factor NF-κB because COX-2 expression is regulated by NF-κB.

To date, a broad spectrum of biological properties in addition to antitumor and anti-inflammatory activities has been reported for CAPE. For example, CAPE was recently reported with inhibitory effects on replication of hepatitis C virus.[33] We believe that the inhibition of nuclear export by CAPE is a rather reasonable explanation for its various biological activities. Owing to the variety of cargo proteins transported by XPO1/CRM1 such as IκB-α, FOXOs, P53, P21, RXRα, Commd1, HIF1 as well as viral components responsible for viral infection cycles, CAPE is expected to have profound effects on disease-related gene expression, multiple intracellular pathways, and therefore cellular functions. Nevertheless, further investigation is warranted to pinpoint the detailed factors and pathways related to nucleocytoplasmic transport that contribute to the diverse biological effects of CAPE. The scaffold of CAPE is of pharmaceutical interest, and therefore, numerous CAPE derivatives[34,35] have been synthesized to

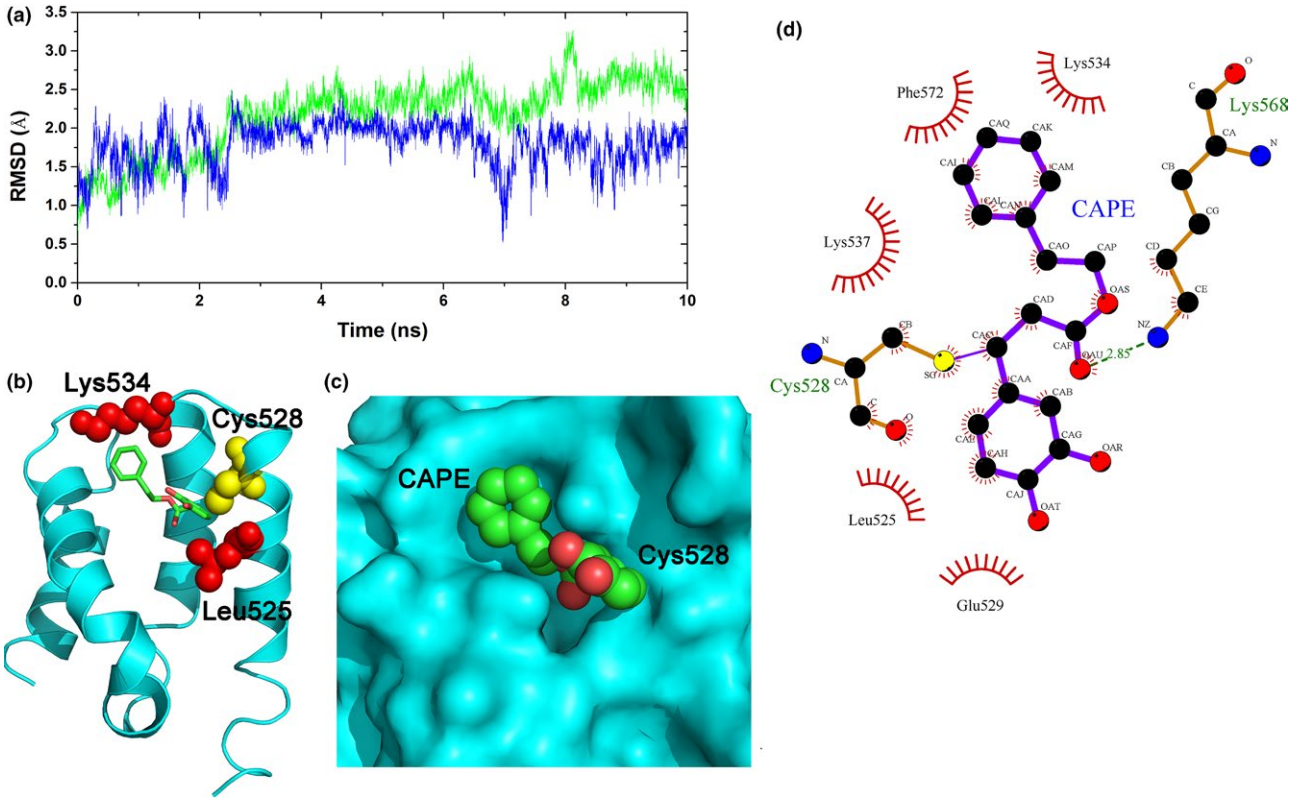

FIGURE 5 Computational analysis of CAPE binding in the NES-cleft of XPO1. (a) 10 ns molecular dynamics (MD) trajectory of CAPE binding with XPO1. The horizontal axis represents time, and the vertical axis indicates rmsd value. The values for the backbone atoms of the protein and the binding complex are represented in green and blue lines, respectively. (b) Pose of CAPE in the NES-binding cleft of XPO1 after MD simulation. Critical residues including Cys^{528}, Leu^{525}, and Lys^{534} are depicted in color spheres. CAPE is depicted in green stick model. (c) The snug-fit-in model of CAPE in the NES-binding cleft of XPO1 (PDB code: 3GB8). CAPE is depicted in CPK model, and Cys^{528} is highlighted in yellow color. (d) The LigPlot diagram of CAPE in the NES-binding cleft of CRM1

evaluate the efficacy against human disorders. Hence, our study may have general implications for the practice of drug design and discovery based on CAPE. Furthermore, our work may represent a strategy for the investigation of natural products of biomedical importance with electrophilic Michael acceptor moiety.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

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