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Engineering chromosome region maintenance 1 fragments that bind to nuclear export signals

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Abstract

Chromosome region maintenance 1 (CRM1) exports nuclear export signal (NES) containing cargos from nucleus to cytoplasm and plays critical roles in cancer and viral infections. Biochemical and biophysical studies on this protein were often obstructed by its low purification yield and stability. With the help of PROSS server and NES protection strategy, we successfully designed three small fragments of CRM1, each made of four HEAT repeats and capable of binding to NESs in the absence of RanGTP. One of the fragments, C7, showed dramatically improved purification yield, thermostability, mechanostability, and resistance to protease digestion. We showed by isothermal titration that the protein kinase inhibitor NES binds to C7 at 1.18 μM affinity. Direct binding to C7 by several reported CRM1 inhibitors derived from plants were verified using pull-down assays. These fragments might be useful for the development of CRM1 inhibitors towards treatment of related diseases. The strategy applied here might help to tackle similar problems encountered in different fields.

KEYWORDS

CRM1, fragment, inhibitor, NES, nuclear export, protein engineering, stability, XPO1

1 | IMPACT STATEMENT

CRM1 exports nuclear export signal (NES) containing cargoes from nucleus to cytoplasm and is a drug target for diseases including cancers. Here, we designed three fragments of CRM1, which were more stable and robust than the full-length (FL) protein in different experiments. Our fragment and design strategy should be useful for the development of CRM1 inhibitors and tackling problems not limited to the field of nuclear transport.

2 | INTRODUCTION

Nuclear transport is an essential activity of eukaryotic cells that determines the subcellular localization of macromolecules such as proteins and nucleotides.[1] Among the different transport factors identified, CRM1 (chromosome region maintenance 1; also referred to as exportin1 or *Xpo1*) is a well-studied nuclear export factor that exports hundreds of cargoes from the nucleus to the cytoplasm.[2] CRM1 contains one large domain formed by 21 continuous HEAT repeats.[3] The arrangement of HEAT repeats establishes an overall superhelical shape, and a hydrophobic groove between repeat 11 and 12 on the outer surface.[4] All CRM1 cargoes contain NES sequences that bind to the hydrophobic groove on CRM1, which is also called the NES groove.[5,6] In the nucleus, CRM1 simultaneously binds to an NES cargo and a small GTPase Ran, forming a trimeric nuclear export complex.[7,8] When this complex reaches cytoplasm, it is dissembled by RanBP1/2 and RanGAP, allowing recycling of CRM1 and Ran for further rounds of export.[9,10]

Yuqin Lei and Qi An contributed equally to this study.

Since protein localization is crucial for its cellular function, deregulation of nuclear transport, or more specifically CRM1 is implicated in several diseases including cancer.[11,12] CRM1 inhibitors demonstrated promising potential in preclinical or clinical trials.[13] Several CRM1 inhibitors were derived from plants including, piperlongumine (PIP),[14] oridonin (ORI),[15] valtrate (VAL),[16] acetoxychavicol acetate (ACA),[16] goniothalamin,[17] plumbagin (PLU),[18] sulforaphene,[19] caffeic acid phenethyl ester,[20] and curcumin (CUR).[21] Though these reports all demonstrated inhibition of CRM1 cargo's export through cellular assays, none used purified proteins to demonstrate their direct binding such as using pull-down assays. This might be due to the fact that CRM1 protein is hard to express, purify, and manipulate (see Results). Improving those properties of CRM1 might help scientists to further investigate those inhibitors.

Here, we report the design and purification of CRM1 fragments that only contain HEAT repeats 10–13, which encompass the NES groove. One of the fragments, C7, showed more than 100 times yield, much enhanced stability, and wider window of applications. We tested several published plant inhibitors using C7 and found the results agreeing well with cellular nuclear export inhibition assays. Our construct might also facilitate the development of other CRM1 inhibitors including those undergoing clinical trials.

3 | RESULTS

3.1 | The design of CRM1 fragments

We tried several different approaches to design the CRM1 fragment, such as different truncations or adding tags to improve solubility. However, none of these methods yielded any appreciable soluble proteins, due to low expression or insolubility or heavy digestion (Figure S1). This is probably due to the intrinsic flexibility of the HEAT repeats and the presence of large hydrophobic surfaces (by the NES groove and the exposed surfaces due to truncation). In the end, we succeeded by PROSS[22] facilitated mutagenesis to improve protein stability and fusion of CRM1 468–657 with a cleavable NES sequence on the N or C terminus of the protein to shield the hydrophobic NES groove.

Figure 1a shows the sequence alignment of three such proteins with the WT sequence. C5N and C6N both have C terminal cleavable NESs, though C6 contains more mutations designed by PROSS (21 vs. 34). NC7 contains a reverse NES from CPEB4 protein, a shorter linker, and totally 25 mutations (Figure 1b). NES groove residue mutations were rarely designed by PROSS and not selected in order to maintain the groove characteristics. As expected, the energy minimized structure (NC7) is highly similar to the WT protein in structure (Figure 1c). The hydrophobic surface areas of WT (truncated) protein were greatly reduced by mutagenesis and complementation with an NES (Figure 1c–e), suggesting that these proteins might be more stable and soluble

3.2 | Purification and characterization of CRM1 fragments

All three proteins could be easily expressed in *Escherichia coli* (*E. coli*) and purified by Ni-NTA beads, though a fraction of C5N was insoluble (Figure 2a). Before digestion, these proteins barely bound to NES proteins (Figure 2b), suggesting that the fused NESs folded correctly into the NES groove. After digestions by the respective protease, size exclusion chromatography was applied to remove the NES peptides. While a fraction of C5 proteins were aggregated, C6 and C7 were robustly homogenic, eluting at approximately monomer molecular weight size (Figure 2c). The NES-deleted proteins were able to bind GST-NES^{PKI}, which could be inhibited by CRM1 inhibitor KPT-330[23] similarly as the FL protein (Figure 2b), suggesting that the binding was specific. It should be noted that the binding of fragments required no Ran proteins, suggesting that the fragments were not autoinhibited as the WT protein.[24] Overall, we were able to generate three small fragments of CRM1 (around 200 residues) that bind to NES.

3.3 | Comparison of C7 with full-length CRM1

Since C7 contains less mutations than C6 and is slightly more stable than C5 (Figure 2), we focused on C7 for further analysis. The yield of C7 was estimated to be 100 mg/L, which was about 200 times higher than the FL human CRM1 protein (Figure S2). In addition, C7 exhibited much improved stability. First, it endured much higher heat denaturation temperature, while C7 is stable at 65°C, FL CRM1 heavily precipitated at 55°C (Figure 3a).

The protein C7 also survived much higher mechanical shear force. C7 remained soluble after 3 hr of shaking, while the majority of FL CRM1 precipitated after shaking for 1.5 hr (Figure 3b, top panel). Pull-down showed that the proteins remaining in the supernatant was active for both FL CRM1 and C7 (Figure 3b, bottom panel). In agreement, all our attempts to generate isothermal titration calorimetry (ITC) data using FL CRM1 failed due to heavy precipitation of the protein even at very low stirring speed (300 rpm). In contrast, we were able to show the binding of a PKI NES peptide to C7 using ITC (Figure 3c). The binding affinities were determined to be 1.18 μM, and the enthalpy change was −15.84 kcal/Mol. Overall, the fragment C7 was superior to the FL protein in several ways.

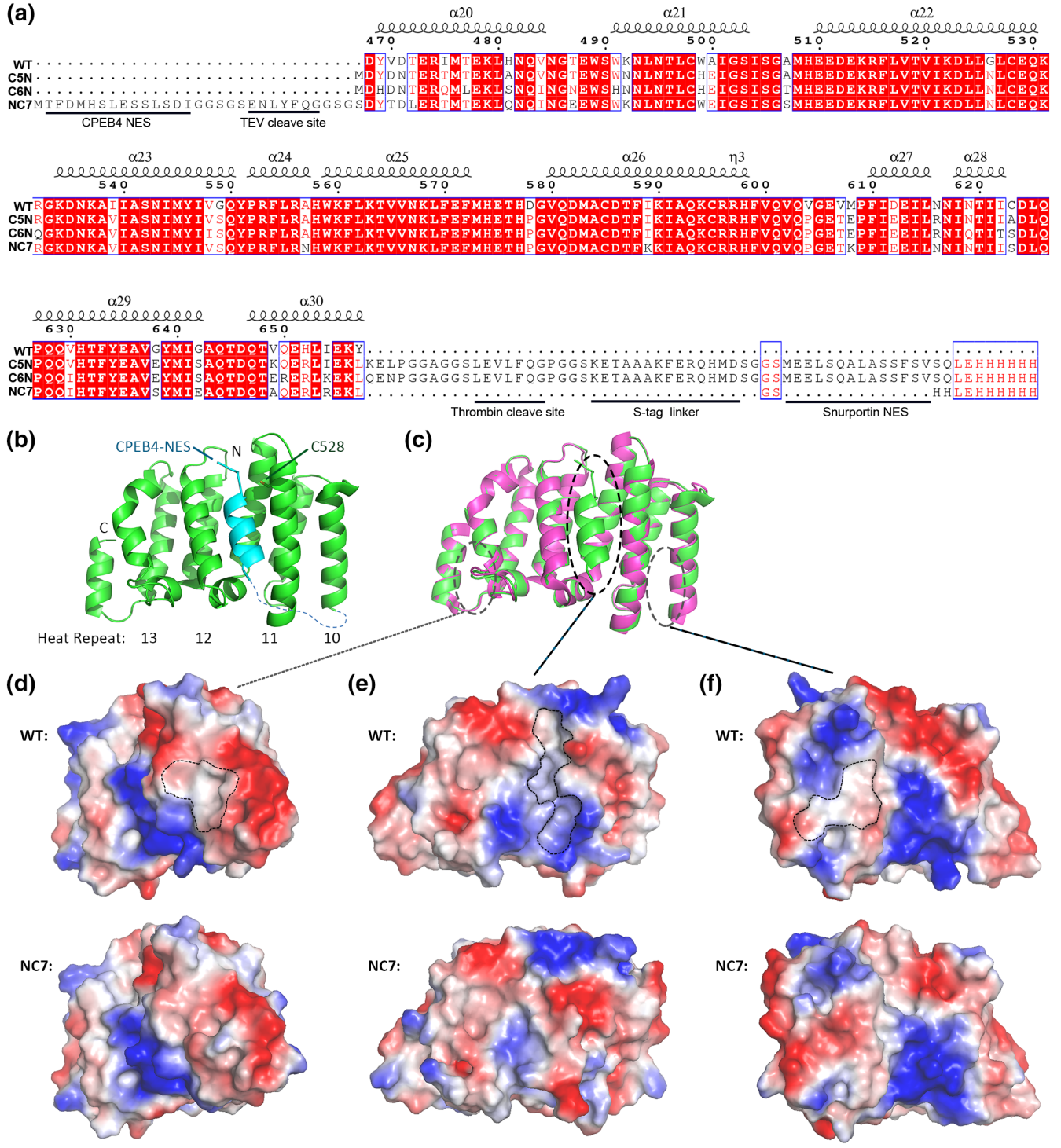

FIGURE 1 Design of short CRM1 fragments. (a) Sequence alignment of human chromosome region maintenance 1 (CRM1) WT, C5, C6, and C7. Secondary structure was shown above the alignment. The different features are labeled below the alignment. (b) Energy minimized model of NC7. CPEB4-nuclear export signal (NES) (cyan) and CRM1 HEAT repeats 10–13 (green) were connected by a flexible line (dotted line). C528 in the NES groove is shown as sticks. (c) Superimposition of WT (magenta, 453–657) and NC7 model. (d–f) Electrostatic surface potential map of circled regions in panel C. WT and NC7 are shown at top and bottom, respectively. Blue, red, and white colors represent basic, acidic, and hydrophobic surfaces, respectively

3.4 | The use of C7 in screening CRM1 inhibitors

After C7 was successfully purified and characterized, it was used to test the binding of published CRM1 inhibitors, focusing on the herbal molecules. PIP, ORI, VAL, ACA, PLU, and CUR were commercially obtained (Figure 4a). The structure and purity of the compounds (except VAL) were verified through ^{1}H NMR (Figure S3). C7 was incubated with 50 μM of each inhibitor for 1 h at 25°C and

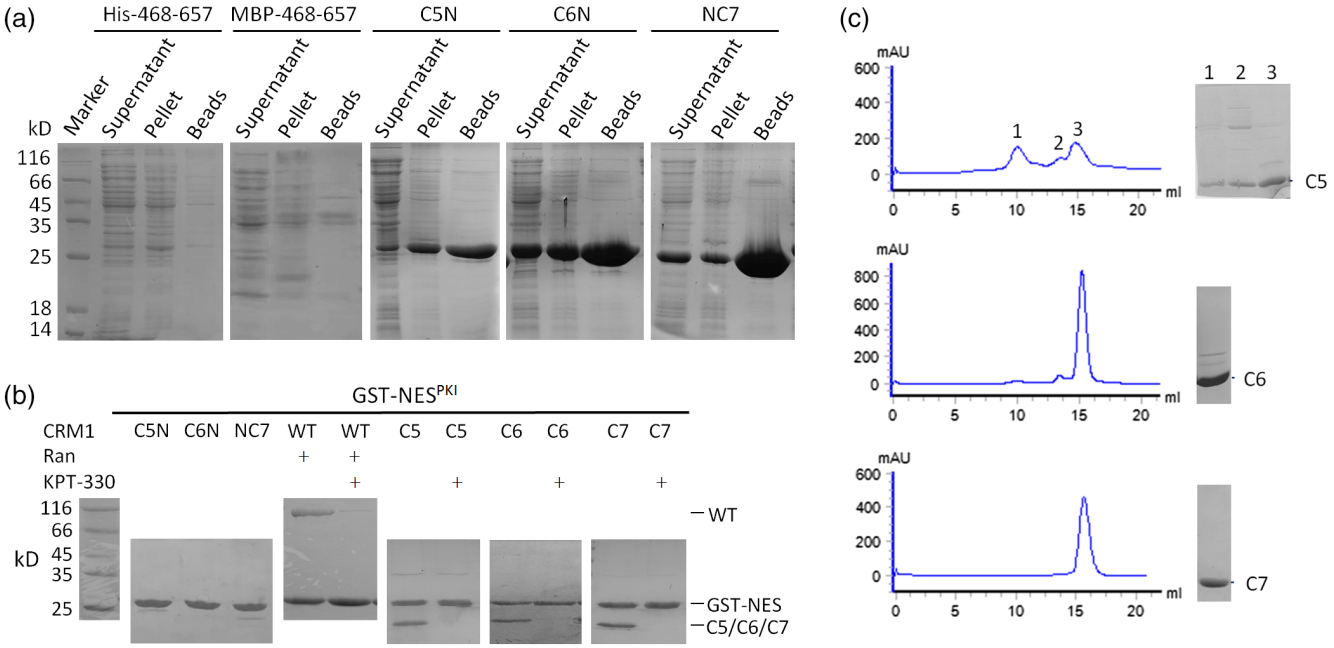

FIGURE 2 Expression, purification, and characterization of designed mutants. (a) Protein expression and purification yield comparison of different chromosome region maintenance 1 (CRM1) fragments. After lysis and centrifugation, supernatant and pellet were analyzed. "Beads" lane represent bound protein after wash. (b) GST-NES^{PKI} pull-down of digested or undigested WT or CRM1 fragments. KPT-330 is an inhibitor of CRM1. (c) Size exclusion profile of C5, C6, and C7 after protease digestion to cleave off bound NES (left panels). The peaks were analyzed by SDS-PAGE (right panels)

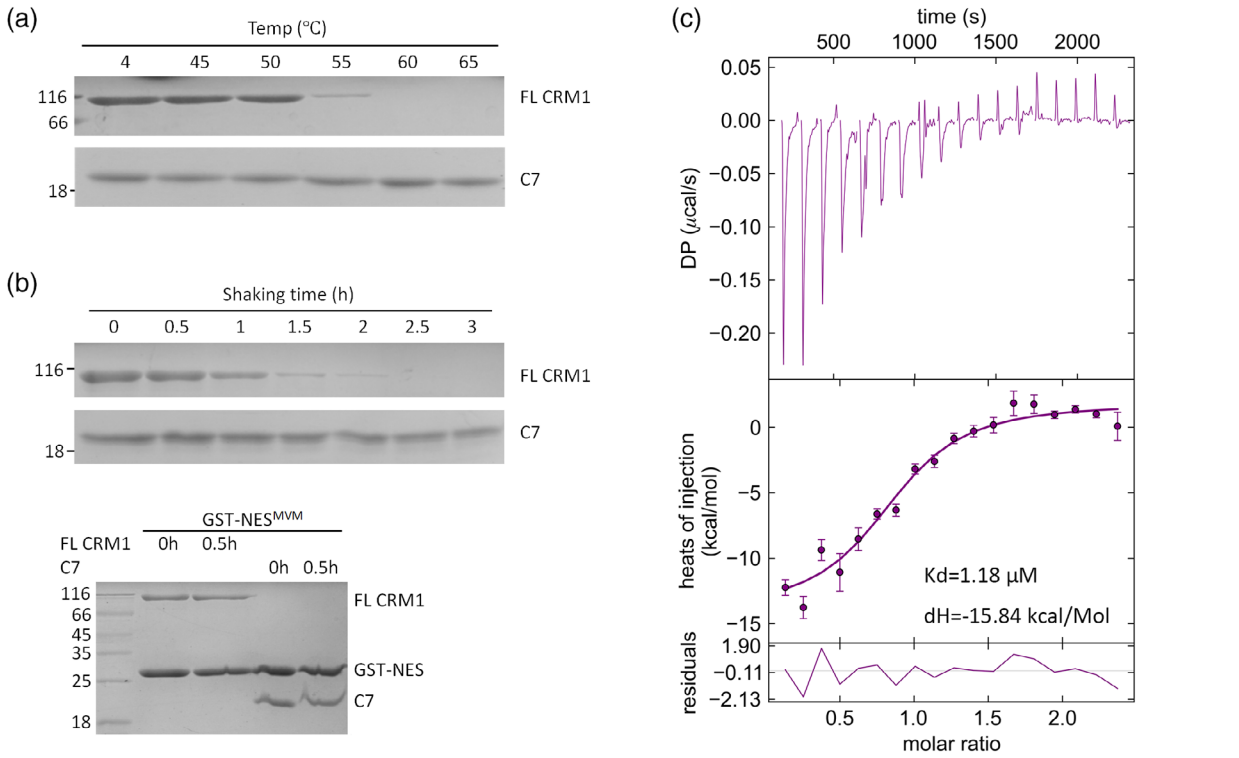

FIGURE 3 Thermostability and mechanostability comparison of C7 and FL CRM1, and isothermal titration profile of GST-NES^{PKI} to C7. (a) Supernatant of FL CRM1 or C7 (each 0.2 mg/mL) after incubation at indicated temperature for 2 min. (b) Supernatant of FL chromosome region maintenance 1 (CRM1) or C7 (each 0.2 mg/mL) after shaking in an Eppendorf tube for the indicated time period at 170 rpm. Stability experiments were performed in buffer containing 20 mM Tris pH 8.0, 200 mM NaCl. (c) Isothermal titration of GST-NES^{PKI} to CRM1. Fit values of K_d and ΔH are displayed in the figure, respectively. Error bars represents 95% confidence interval of measurements

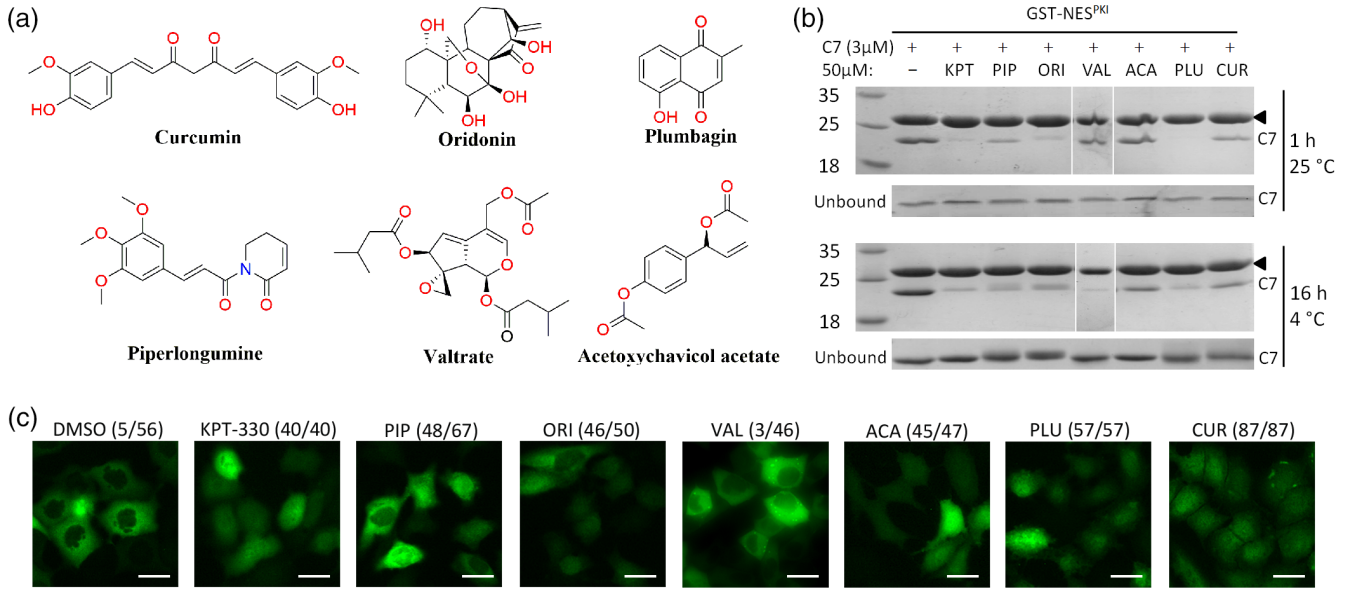

FIGURE 4 Binding to C7 by plant-derived chromosome region maintenance 1 (CRM1) inhibitors. (a) Chemical structures of plant-derived CRM1 inhibitors. (b) GST-NES pull-down of C7 in the presence of different inhibitors. Before incubating with GST-NES, C7 was incubated with each inhibitor (50 μM) for 1 h at RT or overnight at 4°C. The GST-NES bands were indicated with triangles. KPT stands for KPT-330. (c) GFP-NES-MBP-NLS nuclear export inhibition in HeLa cells by different inhibitors at 50 μM concentration (except KPT-330 which is performed at 10 μM). The numbers in the bracket are the numbers of cells that display inhibition of nuclear export and the total number of cells counted. Error bars are of 20 μm length

further incubated with GST-NES beads to evaluate the level of NES groove inhibition (Figure 4b). PLU and ORI showed almost complete inhibition, similar as the potent CRM1 inhibitor KPT. PIP, VAL, ACA, and CUR also inhibited CRM1 to different degrees. If this experiment was performed at 16 h at 4°C, the result was similar except that the levels of inhibition by VAL and ACA became slightly stronger, possibly due to longer incubation time. We also performed these two experiments with the WT protein, which showed very similar results, except that neither ACA nor CUR displayed any obvious inhibition (Figure S4).

To test the level of nuclear export inhibition in cells, we transfected HeLa cells with a GFP-NES-MBP-NLS construct that localizes to the cytoplasm when untreated. Upon treatment with KPT, all cells showed nuclear relocalization of cargoes (Figure 4c). When the plant inhibitors were applied at 50 μM concentration, PLU and CUR showed nuclear export inhibition in all cells. A fraction of PIP, ORI, and ACA treated cells showed nuclear export inhibition. VAL, however, did not show any inhibition of nuclear export when compared with the DMSO group.

4 | DISCUSSION

CRM1 is a relatively large and flexible protein, which contains 21 intimately connected HEAT repeats. The most conserved region in CRM1 is the NES groove, which is often of research focus.[25,26] However, our unpublished data showed that the FL protein is prone to degradation, of low purification yield and tends to lose activity during the process of purification/storage/manipulation. To simplify the purification and widen its biochemical/biophysical applications, we designed a fragment of CRM1 that binds NES, displaying much higher purification yield, thermostability and mechanostability. Our construct should be useful for future researches involving NES and CRM1 inhibitors. The approach that we undertook might be helpful to other similar works for reducing complexity and improving robustness.

Pull-down with C7 showed that all inhibitors could inhibit NES binding with CRM1 to some extent. However, ACA and CUR showed no NES inhibition in pull-down with FL CRM1. Cellular export inhibition assay showed that both ACA and CUR can inhibit nuclear export of cargo. Thus, the cellular data correlate better with the pull-down performed with C7 than FL CRM1. There are several explanations for this. First, the FL protein might precipitate on beads when incubated with chemicals, making it difficult to differentiate specific binding with nonspecific binding. Second, C7 is not autoinhibited, and might be more accessible to the inhibitors. Third, ACA and CUR might undergo chemical modifications in cells, and the metabolic intermediates might be more potent than the parent compound in nuclear export inhibition.

Among all the inhibitors tested, only VAL did not show any cellular export inhibition at 50 μM. VAL was reported

to inhibit nuclear export of viral nucleoprotein (NP) at 3.6 μM by cellular assay.[16] The discrepancy might rise from the different experimental setups—examining the export inhibition of NP cargo or transfected fluorescent cargo, respectively. There are other possible explanations, for example, VAL has low membrane permeability (different cells) or low stability (different culture medium) in our assay condition. Since we did show its direct binding to C7 and FL CRM1, it is probably OK to claim VAL as a CRM1 inhibitor. Thus, with the help of C7, we found that all tested plant inhibitors probably worked through direct inhibition of the NES groove, but not through other mechanisms such as allostery. The architecture of CRM1 is like a spring that can compress or extend.[27] Binding to other regions of CRM1 might be easily compensated by local/global adjustments, leaving the NES groove not inhibited. This implies that in order to be successful, future development of CRM1 inhibitors should probably focus on the NES groove.

5 | MATERIALS AND METHODS

5.1 | Source of chemicals

KPT, PIP, ORI, VAL, ACA, PLU, and CUR were purchased from Selleck (Shanghai, China), LKT Labs (Shanghai, China), MACKLIN (Shanghai, China), Desite (Chengdu, China), Abcam (Shanghai, China), Sigma (Shanghai, China), and J&K (Beijing, China), respectively. Stocks were prepared in DMSO at 100 mM and diluted in pull-down buffer or culture medium before use.

5.2 | Expression and purification of C5-C7

The CRM1 fragments were cloned into a pET-28a-based expression vector incorporating TEV or thrombin cleavable his-tag fusion. The plasmid was transformed into *E. coli* BL-21 (DE3) and grown in liquid broth medium. Expression of protein was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown overnight at 18°C. Cells were harvested and sonicated in lysis buffer (50 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol, 6 mM imidazole, and 1 mM PMSF). The protein was first purified on a Ni-NTA column and eluted in a buffer containing 50 mM Tris pH 8.0, 400 mM NaCl, 300 mM imidazole, and 10% glycerol. This was followed by enzyme digestion (1 mg:100 mg) to remove tag and fused NES. The digested protein was purified by a Superdex 200 increase gel filtration column on the ÄktaPure (GE Healthcare) using the gel filtration buffer (20 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, and 2 mM DTT). Eluted proteins were frozen at −80°C at 5–10 mg/mL.

5.3 | Isothermal titration calorimetry

ITC experiments were conducted at 20°C using ITC200 (Microcal) in a buffer containing 30 mM Tris pH 8.0, 200 mM NaCl, and 0.5 mM EDTA. GST-PKI NES (170 μM) was titrated into the sample cell containing 14 μM C7. Data were processed by NITPIC[28] and fitted by SEDPHAT.[29]

5.4 | Pull-down assay

To assess different interactions, GST-NES^{PKI} was immobilized on Glutathione sepharose beads. Soluble proteins at indicated concentrations were incubated with the immobilized proteins in a total volume of 500 μL for 1 hr at 4°C. After two washing steps, bound proteins were separated by SDS/PAGE and visualized by Coomassie Blue staining. Each experiment was repeated at least twice and checked for consistency. The pull-down buffer contains 20 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 0.005% Triton-X100.

5.5 | Cellular nuclear export inhibition assay

pEGFP-C1 plasmids were used to express EGFP-tagged NES-MBP-NLS, which localizes to the cytoplasm when transfected. HeLa cells were obtained from the Cell Bank of Chinese Academic of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% (vol/vol) fetal bovine serum (Biological Industries). Cells were transfected with TurboFect transfection reagent (Thermo Fisher) and treated for different plant inhibitors (50 μM) or controls (10 μM) for 2 hr, fixed and visualized by Olympus FV-1000 confocal microscope, and analyzed using NIH ImageJ software.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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