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Communication

Structural Mass Spectrometry Probes the Inhibitor-Induced Allosteric Activation of CDK12/CDK13-Cyclin K Dissociation

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ABSTRACT: The rational design and development of effective inhibitors for cyclin-dependent kinases 12 and 13 (CDK12 and CDK13) are largely dependent on the understanding of the dynamic inhibition conformations but are difficult to be achieved by conventional characterization tools. Herein, we integrate the structural mass spectrometry (MS) methods of lysine reactivity profiling (LRP) and native MS (nMS) to systematically interrogate both the dynamic molecular interactions and overall protein assembly of CDK12/CDK13-cyclin K (CycK) complexes under the modulation of small molecule inhibitors. The essential structure insights, including inhibitor binding pocket, binding strength, interfacial molecular details, and dynamic conformation changes, can be derived from the complementary results of LRP and nMS. We find the inhibitor SR-4835 binding can greatly destabilize the CDK12/CDK13-CycK interactions in an unusual allosteric activation way, thereby providing a novel alternative for the kinase activity inhibition. Our results underscore the great potential of LRP combination with nMS for the evaluation and rational design of effective kinase inhibitors at the molecular level.

Cyclin-dependent kinase 12 and 13 (CDK12 and CDK13) serve important regulation roles in the transcription elongation and mRNA processing by phosphorylating the carboxy-terminal domain of RNA polymerase II in combination with cyclin K (CycK).[1−3] Recently, the aberrant expression (especially amplification) of CDK12 and CDK13 has been observed in many types of cancers, such as breast cancer and hepatocellular carcinoma, and targeted inhibition of CDK12 and CDK13 is demonstrated to be an important means of cancer therapy.[4−7] Most existing small molecule inhibitors of CDK12 and CDK13 are pan-inhibitors, and none of them have been approved by the FDA up to now.[8]

To date, the known small molecule inhibitors of CDK12/CDK13 are all ATP-competitive inhibitors developed through structural optimization of lead compounds, high-throughput screening, and structure−activity relationship (SAR) study.[9−11] These inhibitors have shown certain efficacy in cancer treatment models but have not been put into clinical usage mainly because of their drug resistance or poor pharmacokinetics.[12] Moreover, only a small fraction of the static structures of CDK12/CDK13-inhibitor complexes have been characterized by X-ray diffraction.[9,11,13,14] The dynamic conformations of CDK12/CDK13-CycK complexes modulated by inhibitors are difficult to be captured by conventional structural biology tools. The lack of conformational insights in the dynamic inhibition limits the rational design of highly selective and low-toxicity CDK inhibitors.

Recently, lysine reactivity profiling−mass spectrometry (LRP-MS) has been demonstrated as a useful structural MS strategy to derive the structural insights of functional protein complexes.[15] The interfacial molecular details and dynamic conformation changes involved in protein−protein, protein−ligand, and protein−nanomaterial interactions can be efficiently monitored by LRP-MS, thereby providing rich information, including interaction interfaces, binding pockets, and allosteric conformation modulations.[16−19] Moreover, native MS (nMS) with nondenaturing electrospray ionization (nESI) is emerging as a powerful tool for proteins and protein complexes.[20−24] Herein, we applied LRP-MS to characterize the dynamic molecular conformation insights of CDK12/CDK13-CycK complexes modulated by various small molecule inhibitors. Additionally, we utilized nMS to monitor the overall protein assembly changes in the complex and inhibitor interactions (Figure 1).

We first quantified the labeling reactivity alternations of lysine residues of CDK12/CDK13-CycK complexes in the interactions with small molecule inhibitors, including THZ531, SR-4835, CDK12-IN-2, and CDK12-IN-3 (Figure 2a,b). The overall profiles of CDK12/CDK13 lysine reactivities were similar when binding with different inhibitors, which indicates these inhibitors share a similar inhibition molecular mechanism. However, although the kinase domains of CDK12 and CDK13 are highly conserved,[12] distinct profiles of lysine reactivities were observed because of their different structures.[14,25] Part of the lysine residues showed decreasing reactivities after inhibitor combination, which was attributed to the stabilization effects of inhibitors on their local structures via the enhanced proximal noncovalent interactions. The most

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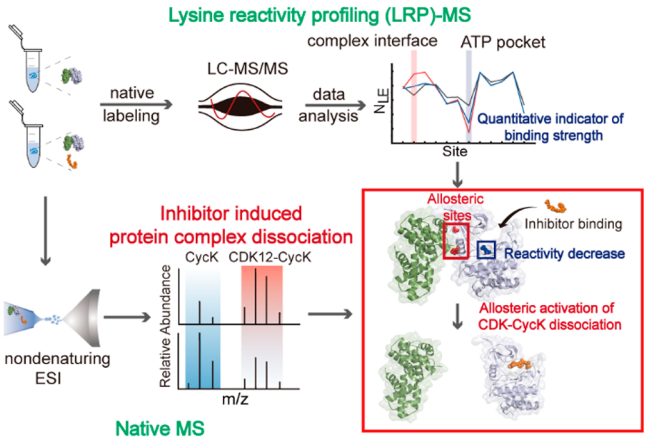

Figure 1. Schematic diagram of LRP and nMS probing the inhibitor-induced allosteric activation of CDK12/CDK13-CycK dissociation.

significant decrease in labeling reactivity was observed at CDK12 Lys874/CDK13 Lys852 located near the binding pocket of ATP (Figures 2c and S1), thereby demonstrating the inhibitors were all ATP-competitive and bound to an identical pocket. Interestingly, the reactivity alternations ($|\Delta N_{LE}|$) of CDK12 Lys874/CDK13 Lys852 induced by inhibitors exhibited good correlations with their inhibition IC_{50} (Figure 2d).

The $|\Delta N_{LE}|$ values of CDK12 Lys874 modulated by these four inhibitors were in the same order as CDK13 Lys852 (THZ531 > CDK12-IN-2 > SR-4835 > CDK12-IN-3). The $|\Delta N_{LE}|$ of covalent inhibitor THZ531 was significantly higher than those of the three noncovalent inhibitors. For noncovalent inhibitors, the same inhibitor binding to different proteins, as well as the same protein binding to different inhibitors, all followed the rule that a higher $|\Delta N_{LE}|$ corresponds to a smaller IC_{50} (Table S1). Thus, the $|\Delta N_{LE}|$ of hotspot lysine sites could be used as quantitative indicators to represent the binding strength and inhibition efficacy of inhibitors. On the basis of this, we can speculate that the IC_{50} values of SR-4835 and CDK12-IN-3 for CDK13 range from 99 to ~491 nM, which have not been clearly reported in the literature.[10,26]

Furthermore, the labeling efficiency of CDK12 Lys1021 was also significantly decreased ($|\Delta N_{LE}|$ > 10%) and was regulated by the four inhibitors with approximately equal magnitude (Figure 2a), thereby indicating a consistent allosteric modulation effect on this region. The crystal structure reveals the local region of CDK12 Lys1021 is a hinge structure that distinguishes CDK13.[9,13] Interestingly, the $|\Delta N_{LE}|$ of CDK13

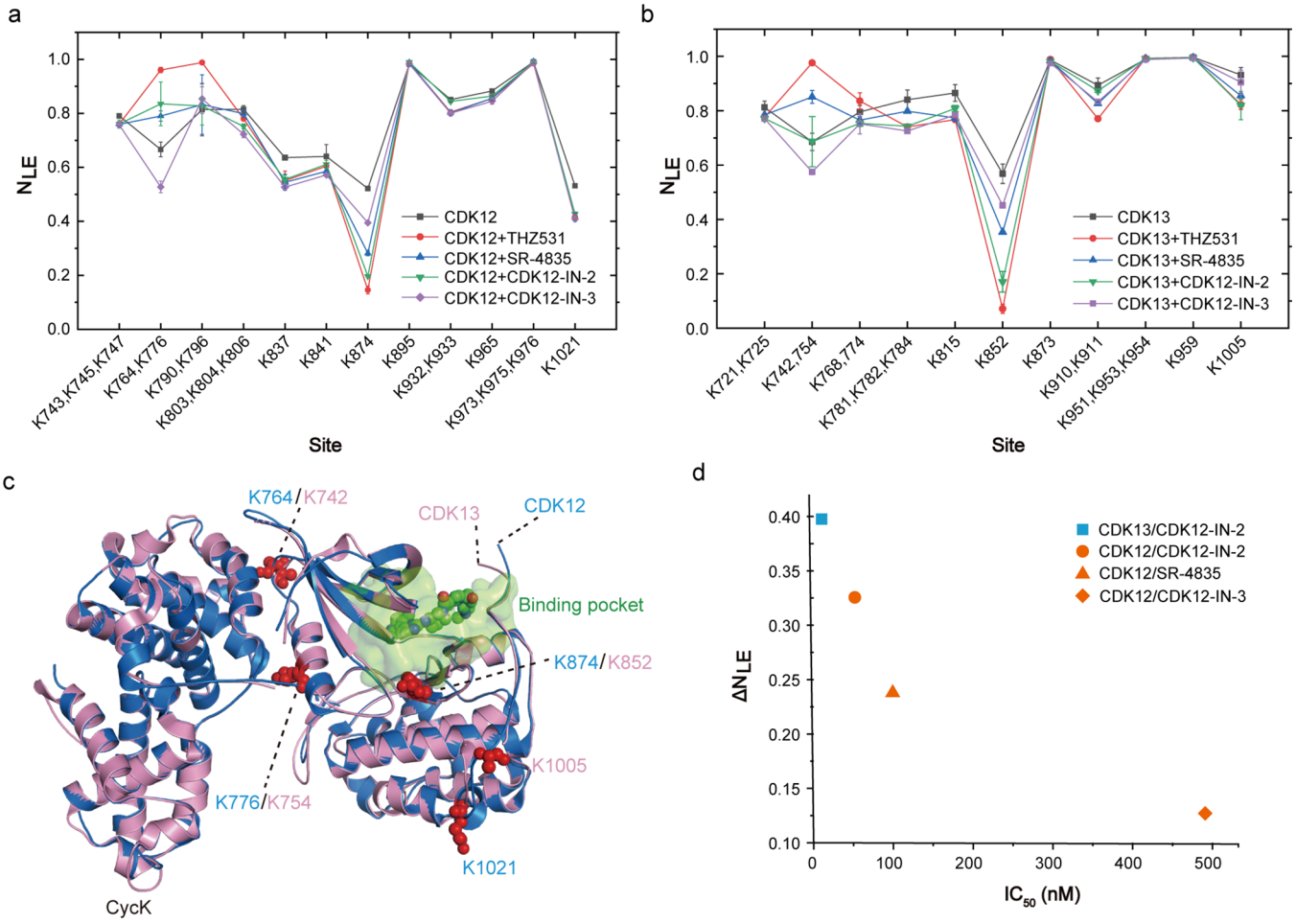

Figure 2. Quantitative comparison of the effects of different inhibitors on the lysine-normalized labeling reactivities (N_{LE}) of (a) CDK12-CycK and (b) CDK13-CycK complexes. (c) Positional relationships of CDK12 Lys874, CDK13 Lys852, Lys1005 (red), and the THZ531 binding pocket (green) (PDB: 5ACB, 7NXJ). (d) The correlation between the IC_{50} (nM) of different inhibitors against the corresponding reactivity alterations ($|\Delta N_{LE}|$) of CDK12 Lys874 and CDK13 Lys852.

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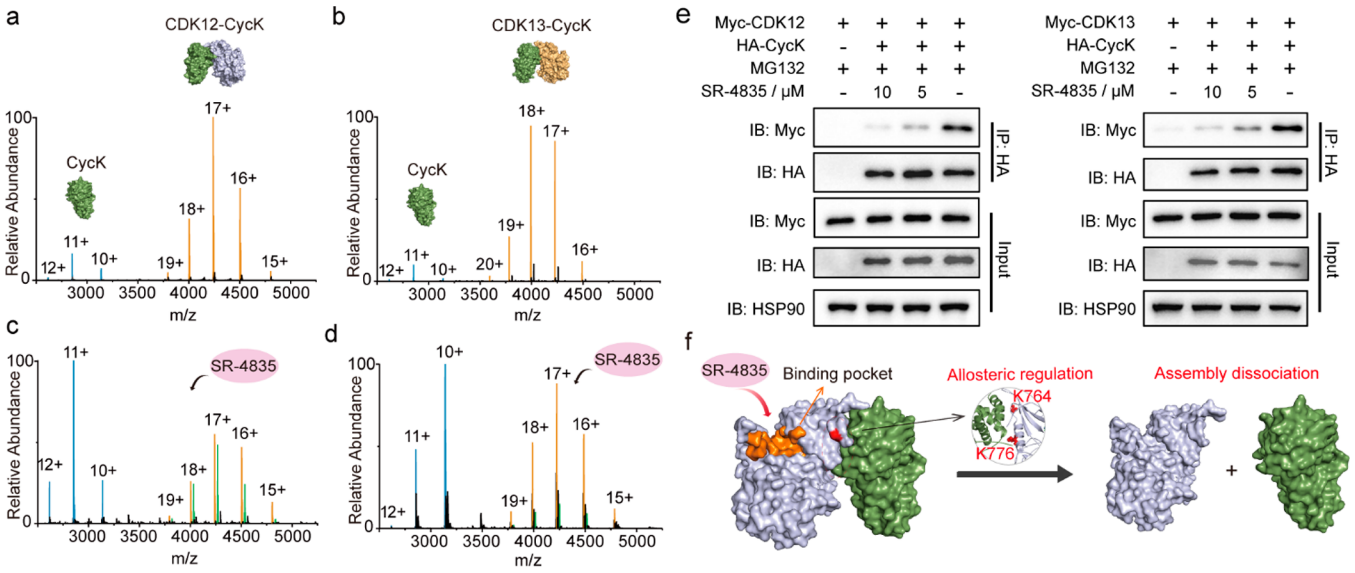

Figure 3. (a,b) nMS spectra of CDK12/CDK13-CycK protein complex samples. (c,d) nMS spectra of CDK12/CDK13-CycK protein complex samples in the presence of SR-4835 with a complex/inhibitor concentration ratio of 1:3. The peaks of CycK, CDK12/CDK13-CycK, and CDK12/CDK13-CycK-SR-4835 are highlighted in blue, orange, and green, respectively. (e) CDK12/CDK13-CycK complexes were destabilized by SR-4835 at the cellular level. Immunoblots of CDK12/CDK13-CycK levels for input and proteins immunoprecipitated with anti-HA magnetic beads from HEK-293T cells, which were transfected with indicated plasmids and treated with SR-4835 for 2 h. (f) Schematic diagram of SR-4835-induced allosteric activation of CDK12/CDK13-CycK dissociation (PDB: 5ACB).

Lys1005 locating in the C-terminal extension showed the same order as CDK13 Lys852 under the modulation of noncovalent inhibitors, although the overall magnitude was smaller (Figure 2b). Considering the end of the CDK13 C-terminal extension crosses the inhibitor binding pocket (Figure 2c),[25] the $|\Delta N_{LE}|$ of Lys1005 could be also used as a quantitative indicator for inhibitor binding strength analysis.

More strikingly, distinct $|\Delta N_{LE}|$ values across different inhibitors were observed at the conserved sequence region of CDK12 Lys764-Lys776/CDK13 Lys742-Lys754 (Figure 2a,b), which locates at the CDK-CycK binding interface and far away from the binding pocket of ATP (Figure 2c). The lysine labeling reactivities in this conserved region increased significantly when THZ531 and SR-4835 were bound to both CDK12 and CDK13 (Figure S1), while the other inhibitors mostly induced opposite effects. The lysine reactivity increase is a signature of the interfacial interaction decrease or solvent-accessible surface area (SASA) increase. Recently, Dieter et al. reported the SR-4835 binding to CDK12-CycK complex had a molecular glue-like effect that induced the degradation of CycK, but THZ531 did not exhibit a similar effect.[27] In the molecular glue-like effect, SR-4835 was proposed to promote the interaction between CycK and CUL4 adaptor protein DDB1 via CDK12 to form a trimer complex, which further performed ubiquitination and degradation of CycK. On the basis of the lysine reactivity increase in the CDK12-CycK interfacial region of Lys764-Lys776, we speculated that the SR-4835 binding can weaken the CDK12-CycK interaction via an allosteric conformation regulation. Then, the release of CycK facilitates its degradation by the ubiquitin pathway.[28]

We further applied nMS to explore the overall assembly of CDK12/CDK13-CycK complexes with or without SR-4835 binding. For the CDK12-CycK sample, the protein complex could be detected with high peak intensity, and the MS peaks of CycK were relatively low (Figure 3a). According to a previous study, CDK12 was not soluble without binding to CycK,[29] so CDK12 was not detected in the nMS. The nMS results of CDK13-CycK were similar to the CDK12-CycK complex (Figure 3b). After the addition of SR-4835 to both complex solutions, a sharp decrease of protein complex peaks was observed in nMS, while the intensities of CycK peaks were greatly increased (Figure 3c,d). This effect was clearly enhanced with the increase of SR-4835 concentration (Figure S2). The nMS results clearly demonstrated the allosteric conformation regulation of SR-4835 will disrupt the CDK12/CDK13-CycK interaction and induce the dissociation of protein complexes. Although THZ531 binding increased the labeling reactivities of interfacial lysines, CDK12/CDK13-CycK dissociation was not observed in nMS characterization (Figure S3), thereby demonstrating the high complementarity of LRP and nMS. Furthermore, we performed validation by immunoprecipitation assay at the cellular level. The interactions between CycK and CDK12/CDK13 were significantly reduced by SR-4835 binding in a dose-dependent manner (Figure 3e). However, the same phenomenon of CDK12/CDK13-CycK destabilization could not be observed when treated by THZ531, which is consistent with the nMS results *in vitro* (Figure S4). Thus, we concluded that the SR-4835 binding activated the dissociation of CDK12-CycK and CDK13-CycK complexes. As the free CDK12/CDK13 does not have kinase activity, thus, this allosteric activation of CDK-CycK dissociation may be a novel mechanism for the SR-4835 inhibition (Figure 3f). Recently, Zhang et al. reported the non-ATP competitive inhibitor homoharringtonine can disrupt the CDK2-cyclin A (CycA) interaction via targeting the druggable pocket located at the interface.[30] Our structural MS results further demonstrate the ATP-competitive SR-4835 can also disrupt the CDK12/CDK13-CycK interactions via allosteric conformation modulations.

In summary, we integrated the structural MS methods LRP-MS and nMS to explore the molecular interactions between

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CDK12/CDK13-CycK and small molecule inhibitors. The LRP-MS analysis can provide the molecular interaction details, including inhibitor binding sites and allosteric conformation modulations, while nMS can monitor the overall assembly changes of the protein complex under inhibitor binding. We demonstrated the labeling reactivity alterations of hotspots CDK12 Lys874/CDK13 Lys852 could be utilized as indicators for evaluating the binding strength and inhibition efficacy of different ATP-competitive inhibitors. What is striking is that both the LRP-MS and nMS results indicated the SR-4835 binding induced unusual allosteric activation of CDK12/CDK13-CycK dissociation and kinase activity loss, which paves the way for the rational design of novel inhibitors in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c01697.

Representative spectra of LRP-MS, nMS spectra of CDK12/CDK13-CycK protein complex samples with different SR-4835/THZ531 concentrations, and IC_{50} values of inhibitors for CDK12/CDK13 reported in the literature (PDF)

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Notes

The authors declare no competing financial interest.

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