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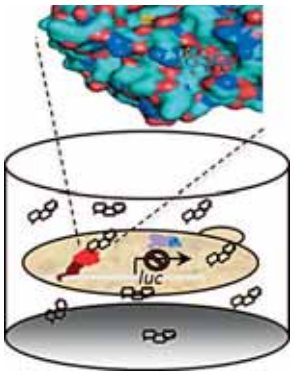
Table of Contents



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CHEMBIOCHEM Full Papers

Tampering with Cell Division by Using Small-Molecule Inhibitors of CDK–CKS Protein Interactions

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Cyclin-dependent kinases (CDKs) control many cellular processes and are considered important therapeutic targets. Large collections of inhibitors targeting CDK active sites have been discovered, but their use in chemical biology or drug development has been often hampered by their general lack of specificity. An alternative approach to develop more specific inhibitors is targeting protein interactions involving CDKs. CKS proteins interact with some CDKs and play important roles in cell division. We discovered two small-molecule inhibitors of CDK–CKS interactions. They bind to CDK2, do not inhibit its enzymatic activity, inhibit the proliferation of tumor cell lines, induce an increase in G1 and/or S-phase cell populations, and cause a decrease in CDK2, cyclin A, and $p27^{Kip1}$ levels. These molecules should help decipher the complex contributions of CDK–CKS complexes in the regulation of cell division, and they might present an interesting therapeutic potential.

Introduction

Cyclin-dependent kinases (CDKs) form a family of 20 protein kinases that play pivotal roles in the regulation of a variety of fundamental cellular processes, such as cell division, transcription, and mRNA splicing.[1] CDKs that regulate the transitions between the different phases of the cell cycle have been considered interesting therapeutic targets for cancer and other pathologies involving deregulated cell proliferation. Over the past 20 years a very large number of CDK small-molecule inhibitors have been discovered, and some have been tested in clinical trials.[2] Although some of the most recently discovered CDK inhibitors present quite promising therapeutic opportunities that might allow them to reach the market,[3] the toxicity of the vast majority of the inhibitors discovered so far has outweighed their therapeutic benefits, and none has obtained clinical approval. This toxicity is at least partly a result of the lack of specificity of these inhibitors, as they target ATP-binding sites, which are generally conserved within and even beyond the CDK family. For example, Roscovitine, one of the first CDK inhibitors tested in the clinic, efficiently inhibits ($IC_{50} < 1$ μM) five out of nine tested CDKs, targets a few protein kinases outside the CDK family, and even binds to pyridoxal kinase, a non-protein kinase.[4,5] The general lack of specificity of CDK inhibitors also represents a limitation for their application in chemical biology. Roscovitine and other inhibitors have been widely used to study the many functions of CDKs, but their loose specificity spectra undermine many results and oblige the development of elaborate, integrated approaches to gain reliable biological knowledge.[6] To obtain more specific molecules, a first approach is discovering ATP-noncompetitive CDK inhibitors that target allosteric sites.[7] Because the activity of CDKs is subject to tight spatial/temporal control mostly exerted by interactions with a variety of regulatory proteins, an alternative promising approach is discovering inhibitors of protein interactions involving CDKs. This approach was validated by the development of competitive peptides derived from alpha helix 5 of cyclin A; these proved to be efficient inhibitors of CDK2/cyclin A kinase activity and of tumor cell line proliferation.[8]

Protein–protein interactions have long been considered refractory to small-molecule inhibition because of the topologi-

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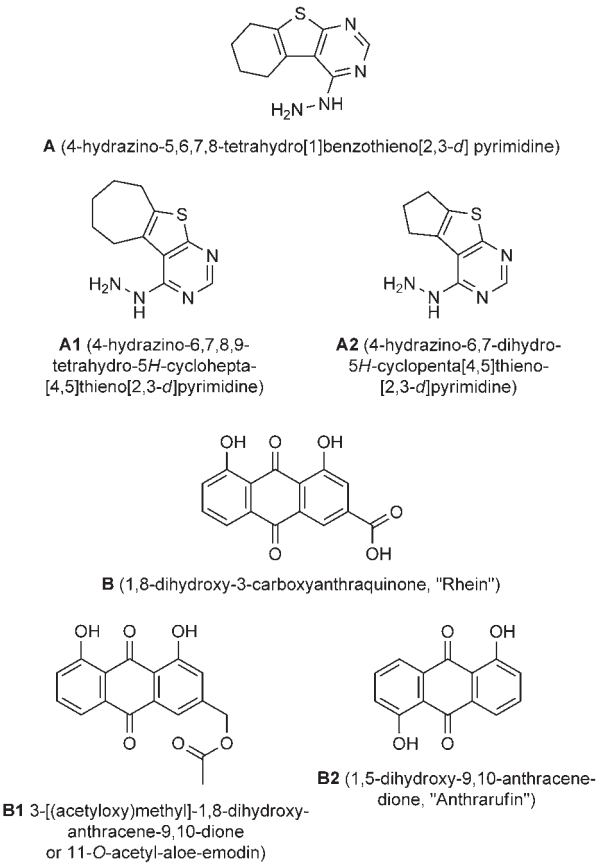
cal features of many binding interfaces. However, an increasing number of small-molecule inhibitors of protein–protein interactions are now being discovered and, because many of them are drug-like, protein–protein interactions are becoming a highly promising, yet still challenging, class of therapeutic targets.[9,10] A first proof of concept of CDK inhibition by a small molecule targeting a protein–protein interaction involved the discovery of a molecule that prevents complex formation between CDK5 and its activating protein p25, thereby efficiently disrupting kinase activity.[11] Our objective here was to target protein–protein interactions that regulate some of the functions of a subset of CDKs, without being strictly necessary for their enzymatic activity. We opted for the interactions between CDK and CKS proteins.

CKS1 and 2 are two highly homologous proteins that interact with CDK1/2–cyclin complexes and play multiple evolutionary conserved roles in the regulation of the cell cycle and transcription.[12] Both proteins exert partially redundant functions, as revealed by the fact that either human gene can complement a yeast strain whose single *CKS1* gene is disrupted,[13] and the fact that *CKS1*$^{-/-}$ and *CKS2*$^{-/-}$ mice are viable,[14,15] whereas doubly nullizygous mice die extremely early during development.[16] Functional redundancy between CKS1 and CKS2 was observed in the control of mitosis, through a shared involvement in the transcriptional control of the mitotic regulators cyclin B1 and CDK1,[16] and in the recruitment of CDK–cyclin A complexes to the APC/C that directs cyclin A destruction.[17] It has also been observed in the control of the DNA damage S-phase checkpoint.[18] Both proteins interact with the mitochondrial single-stranded DNA-binding protein (mtSSB) and contribute to the replication of mitochondrial DNA.[19] However, CKS proteins also exert individual, specific functions. CKS1 is an essential partner of the SCF-Skp2 ubiquitin ligase for binding to p27^{Kip1} (and other cell cycle inhibitors) and addressing it to the proteasome.[14] CKS2 plays an essential, specific role in meiosis, as CKS1 is not expressed in germ-line cells.[15] Interestingly, CKS1 (and sometimes CKS2) is overexpressed in a variety of human malignancies.[12]

Here, we report the discovery and the characterization of small-molecule inhibitors of CDK–CKS protein interactions. We show that these molecules bind to CDK2, inhibit the division of tumor cells, and cause a decrease in CDK2, cyclin A, and p27^{Kip1} expression levels.



Scheme 1. Chemical structures of **A** and **B**, and of their active (**A1**, **B1**) and inactive (**A2**, **B2**) analogues.

Results

Discovery of small-molecule hits

We used a high-throughput, luminescence-based yeast two-hybrid screening assay performed in an *erg6* strain (enhanced cell permeability),[20] into which we introduced a firefly luciferase reporter gene.[21] We coexpressed LexA-CDK2 and B42-CKS1 as bait and prey proteins, respectively, and we optimized the screening setup (Figure S1 A and B in the Supporting Information). We screened 640 natural and synthetic small molecules from the French National Compound Library—a good sampling of the chemical space covered by the entire library. Upon completion of our screening (Figure S1 C), we retained two small-molecule hits (**A** and **B**; Scheme 1) that produced strong inhibition of the CDK2–CKS1 yeast two-hybrid interaction phenotype; CDK10–cyclin M[22] was used as a control. To further confirm the validity of these molecules we performed yeast two-hybrid dose–response assays on four different protein interactions (two CDK–CKS interactions and two unrelated control interactions). Both molecules produced concentration-dependent inhibition of the CDK2–CKS1 and CDK1–CKS1 interaction phenotypes, as expected from the structural resemblance between CDK2 and CDK1. However, the molecules produced less or no inhibition of two interactions phenotypes caused by unrelated protein pairs (CDK10/cyclin M and TGFβR–FKBP12; Figure 1).

Hit confirmation and identification of active and inactive analogues

To confirm the ability of the hits to inhibit the interaction between CDK2 and CKS1, we set up an in vitro protein interaction assay. We covalently coupled recombinant CKS1 protein to Sepharose beads, and we used this solid phase to capture a recombinant CDK2–cyclin A heterodimer, in absence or in presence of increasing concentrations of molecules pre-incubated separately with the solid phase and with the kinase.

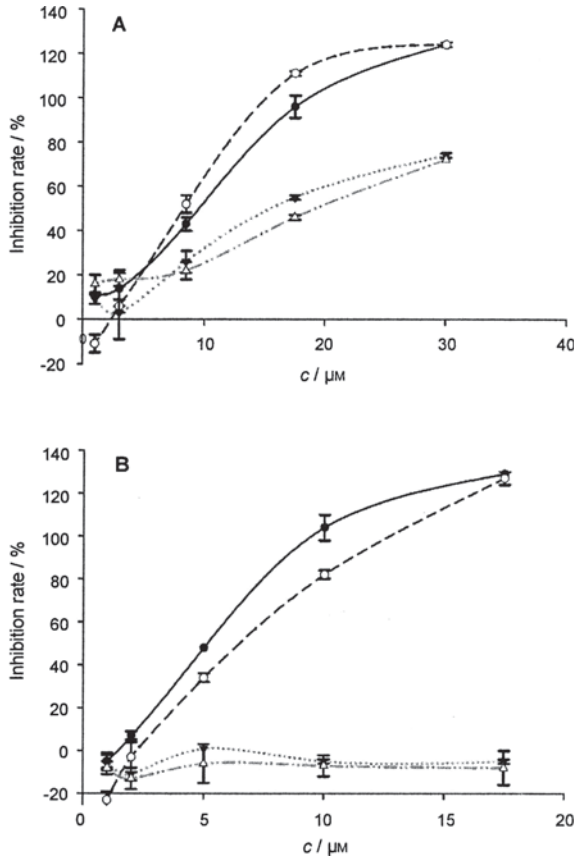


Figure 1. Inhibition of yeast two-hybrid interaction phenotypes by molecules **A** and **B**. LexA-CDK2/B42-CKS1 (●), LexA-CDK1/B42-CKS1 (○), LexA-CDK10/B42-cyclin M (▼) and LexA-TGFβR/B42-FKBP12 (△) interaction phenotypes in response to increasing concentrations of **A** or **B**. Inhibition rates were calculated from interaction phenotypes obtained with yeast treated with 1.5% DMSO ($n=2$).

Both hits prevented the capture of the kinase by the CKS1 matrix. Molecule **A** produced maximum inhibition at the lowest concentration tested (10 μM); molecule **B** appeared less potent (partial and total inhibition at 10 and 30 μM, respectively). Interestingly, **B** at 50 μM was unable to disrupt a preformed complex between CKS1 and CDK2/cyclin A (Figure 2A). This phenomenon was observed with other small-molecule inhibitors of protein–protein interactions.[11,23]

We used this protein interaction assay to test chemical analogues. For each of the two confirmed hits, we identified an active and an inactive analogue, which we named **A1**/**B1** and **A2**/**B2**, respectively (Scheme 1 and Figure 2B). At 30 μM, all four active molecules produced complete (or almost complete) inhibition of the interaction, as judged from the residual CDK2 signal. This signal is comparable to that obtained with an affinity matrix made with a recombinant CKS1(E63Q) mutant, which no longer interacts with CDK2 but still maintains the general structure of the protein.[24] We also tested Meriolin, a potent CDK2 active-site inhibitor:[25] as expected from the distal position of the CKS1-binding interface relative to the active site,[24] a high concentration of Meriolin was unable to prevent the interaction between CKS1 and CDK2/cyclin A (Figure 2B).

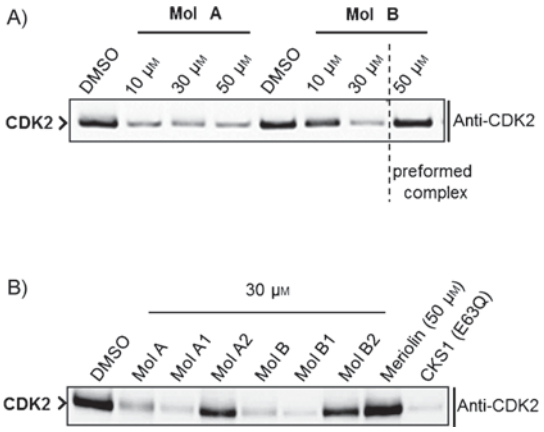


Figure 2. Hit confirmation by an in vitro protein interaction assay and identification of active and inactive analogues. A) Increasing concentrations of **A** or **B** were tested against the interaction between a CKS1–sepharose affinity matrix and recombinant CDK2/cyclin A kinase, after separate preincubation with each partner. Captured CDK2 was revealed by a western blot with an anti-CDK2 antibody. Molecule B was tested at 50 μM against a preformed CKS1-CDK2/cyclin A complex. B) Molecules **A** or **B** and their active and inactive analogues were tested at 30 μM as above. Meriolin, a potent CDK2 active-site inhibitor, was also tested at 50 μM. Background capture of CDK2 was determined by using an affinity matrix produced with a CKS1(E63Q) mutant protein, which is unable to bind to CDK2.

Taken together, these results confirm that both small-molecule hits inhibit the interaction between CDK2 and CKS1.

Quantification of molecule binding to CDK2 and prediction of the binding mode

Fluorescence experiments suggested that CDK2 was the target protein of our molecules (data not shown). To confirm and quantify the binding to CDK2, we performed microscale thermophoresis (MST) experiments,[26] in which we tested increasing concentrations of molecules against fluorescence-labeled recombinant CDK2. These experiments confirmed that **A**, **A1**, **B**, and **B1** bind to CDK2 and that **A2** and **B2** have very weak or undetectable binding (Table 1 and Figure S2).

Table 1. MST measurements of binding affinities of small molecules to CDK2.

Molecules	K_d [μM]	Molecules	K_d [μM]
A	28.3 ± 3	B	9.6 ± 0.2
A1	32.5 ± 6.7	B1	17.8 ± 2.8
A2	287 ± 8.5	B2	no binding detected

To analyze the likely modes of binding of the hit molecules, we performed docking experiments with AutoDock4[27] on the crystallographic structure of CDK2 in complex with CKS1.[24] We targeted the entire CKS1 binding surface of CDK2, and in order to probe possible allosteric bindings close to the protein–protein interaction interface, we extended the docking grid by 10 Å along the *x*-, *y*-, and *z*-axes. The docking results suggested that **A** and **B** are preferentially accommodated in two putative

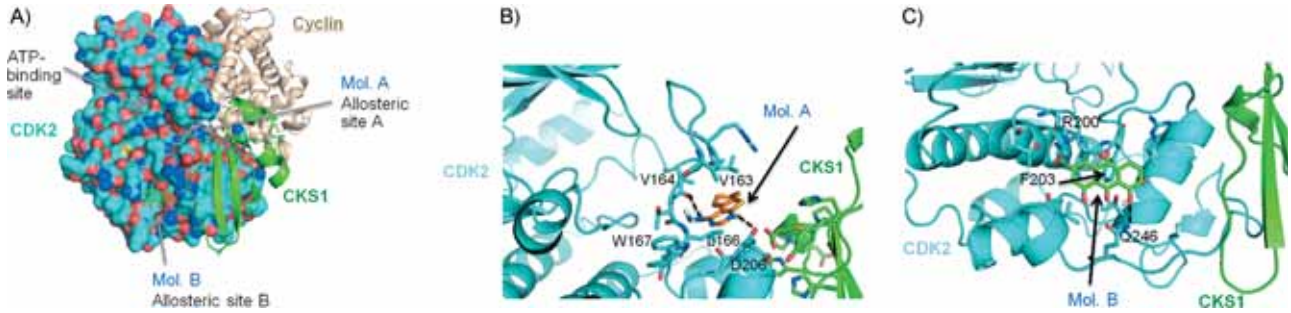


Figure 3. Molecular docking of the hit molecules with the crystal structure of the CDK2–CKS1 complex. A) General view of the structure of the CDK2–CKS1 complex (PDB ID: 1BUH), locating the enzymatic active site of CDK2, and the two putative allosteric sites bound by molecules **A** and **B**. The complex CDK2/cyclin A (PDB ID: 1JST) was superposed on the CDK2–CKS1 complex. B) Detail of the predicted binding site of **A**. C) Detail of the predicted binding site of **B**.

druggable pockets on CDK2 close to the CDK2/CKS1 interface. Molecule **A** was predicted to bind preferentially to a potential allosteric site (site A) located between the enzymatic active site and the CKS1 binding interface (Figure 3 A, B). For **B**, we found another potential allosteric binding site (site B) near the CKS1 binding interface (Figure 3 A, C).

Specificity studies

To examine whether the inhibitors specifically prevent CDK–CKS interactions, we set up another in vitro protein capture assay by using a GST–CDK2 recombinant protein non-covalently coupled to glutathione–agarose beads and soluble recombinant CKS1, CKS2, or cyclin A. In the absence of molecules, the GST–CDK2 solid phase efficiently captured CKS1 but not the CKS1(E63Q) mutant, thus validating the assay (Figure 4 B, C). The inhibitors and their respective analogues produced effects on the CDK2–CKS1 interaction similar to those observed with the capture assay of opposite orientation (Figure 4 A). As expected, we obtained comparable results against the interaction between CDK2 and CKS2 (Figure 4 A). Then, we tested the ability of the active molecules to prevent the interaction between CDK2 and cyclin A; this involves a binding interface that is distinct from the CKS1/2 binding interface. Neither **A** and **A1** nor **B** and **B1** inhibited the interaction with cyclin A (Figure 4 B, C).

We then examined the ability of the molecules to inhibit the in vitro kinase activity of CDK2/cyclin A. None of the molecules of the **B** series showed significant inhibition. Molecule **A** exhibited dose-dependent, weak inhibition of CDK2 kinase activity ($IC_{50} \approx 35$ μM), two orders of magnitude lower than that produced by bona fide CDK2 active site inhibitors such as Meriolin ($IC_{50} \approx 0.1$ μM).[25] Importantly, **A1**, which is equally potent to (if not more potent than) **A** at inhibiting CDK–CKS interactions, produced no inhibition of CDK2 kinase activity (Figure 4 D).

Finally, to rule out the possibility that the molecules inhibit CDK–CKS interactions by forming large aggregates, we performed two tests that have been shown to identify such molecules.[28] We repeated the pull-down experiments shown in Figure 2, in presence of 0.1% Triton X-100. This detergent did not affect the ability of **A**, **A1**, **B**, or **B1** to inhibit the interaction between CDK2 and CKS1 (Figure S3). Next, we performed dynamic light scattering (DLS) measurements on the active molecules, and we detected no sign of aggregation (Figure S4).

Taken together, these results indicate that the molecules produce specific inhibition of CDK–CKS protein interactions.

Bioactivity studies

Because inhibition of CDK–CKS interactions is expected to affect cell division, we examined the impact of the inhibitors on the proliferation of a human tumor cell line derived from a breast cancer. We treated MCF-7 cells with increasing concentrations of small molecules, and we measured cell viability after 48 h. Molecule **A** inhibited cell proliferation at 25 μM, and even more so at 50 μM. Molecule **A1** (equally if not more potent at inhibiting CDK–CKS interactions) was more potent at inhibiting cell proliferation, whereas the inactive analogue **A2** displayed anti-proliferative activity only at 50 μM (Figure 5 A). Molecule **B** weakly inhibited MCF-7 cell proliferation at 50 μM and was therefore not included in further studies. Its active analogue **B1** was more potent at inhibiting proliferation, whereas its inactive analogue **B2** was totally inactive (Figure 5 B). We obtained similar results with both molecule series on three other human tumor cell lines, derived from prostate (LNCaP), lung (A549), and bone (U2OS) cancers. Interestingly, untransformed human fibroblasts (HFF1) were much less responsive than any of the tumor cell lines (Figure S5).

In order to gain insight into the causes of this inhibition of cell proliferation, we performed flow cytometry experiments to examine cell-cycle profiles of MCF-7 cells treated with the molecules at 30 μM for 24 h. Molecule **A** caused a significant increase in the cumulated G1/S phase population and a significant decrease in the G2/M phase population. Its more active analogue **A1** caused an even higher increase in S phase, and a more significant decrease in G2/M phase. The inactive analogue A2 did not have any biologically significant effect. Molecule **B1** caused a significant increase in G1 phase and a significant decrease in S phase, unlike the inactive **B2** analogue, which did not have any biologically significant effect (Figures 5 C and S6).

To determine the possible causes of the modified cell cycle profiles, we examined the impact of the molecules on the steady-state expression of CDK1 and 2, and of cyclins A2 and

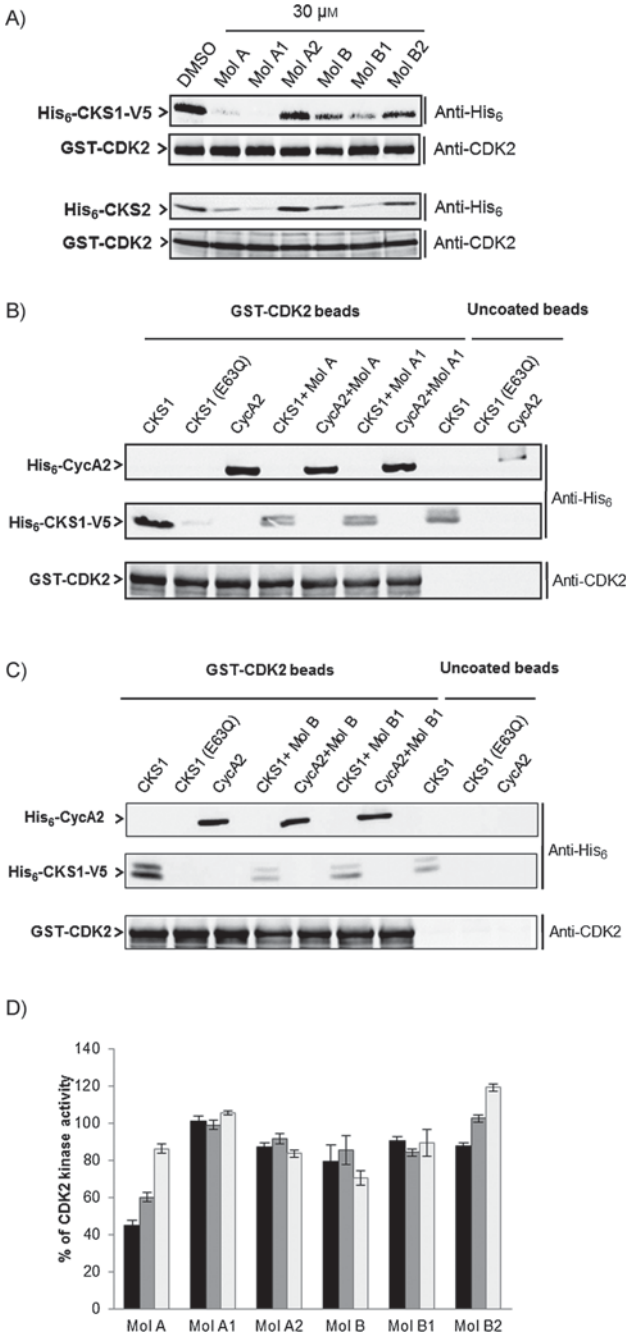


Figure 4. Testing CDK–CKS inhibitors against other protein interactions involving CDK2 and against CDK2/cyclin A in vitro kinase activity. A) Molecules were tested at 30 μM against the interaction between a GST–CDK2 affinity matrix and recombinant His_6-CKS1-V5 or His_6-CKS2 proteins. GST-CDK2 coupled to glutathione-agarose beads and captured CKS proteins were detected by a western blotting with antibodies against CDK2 and His_6 tag, respectively. B) and C) As above with recombinant His_6-CKS1V5, His_6-CKS1(E63Q)-V5 or His_6-cyclin A2 proteins. Background binding of CKS1, CKS1(E63Q) and cyclin A2 to glutathione agarose matrix was determined with uncoated beads. D) Molecules were tested at 10 (□), 25 (■), and 35 μM (■) against the in vitro kinase activity of recombinant purified CDK2/cyclin A, with Histone H1 as a substrate. Percentage kinase activity was determined relative to that measured in the presence of 0.1% DMSO ($n=3$).

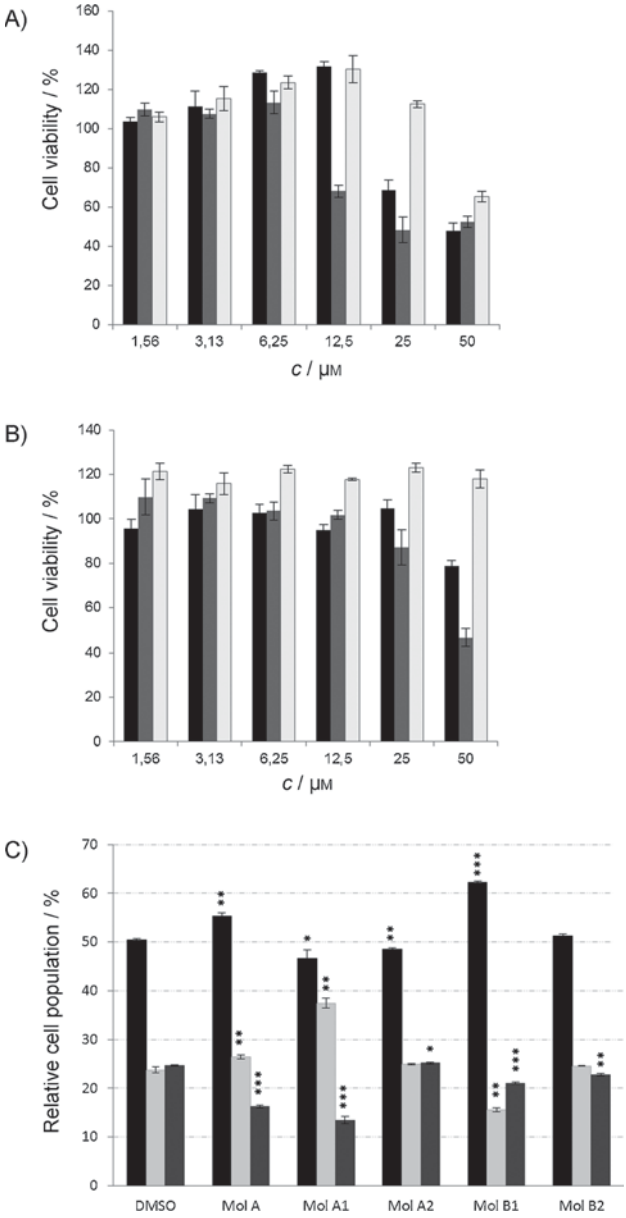


Figure 5. Anti-proliferative activity of the CDK–CKS inhibitors on MCF-7 tumor cells. Increasing concentrations of A) **A** (□), **A1** (■), and **A2** (■) or B) **B** (□), **B1** (■), and **B2** (■) were incubated with MCF-7 cells for 48 h, and viable cells were quantified by an MTS assay. Viability percentages were determined from DMSO-treated cells ($n=3$). C) Cell cycle analysis of MCF-7 cells treated with 30 μM molecules for 24 h. Cell populations in G1 (■), S (■), and G2/M phases (■) were determined by flow cytometry using propidium iodine to quantify DNA ($n=2$). A Student's *t*-test was performed against population percentages obtained with DMSO-treated cells (*$p \leq 0.05$; **$p \leq 0.01$).

B1. None of the tested molecules affected the expression levels of CDK1 and cyclin B1. However, **A**, **A1**, and **B1** induced a decrease in CDK2 and cyclin A2 expression, unlike the inactive analogues **A2** and **B2**. The expression level of CKS1 was unaffected by any of the tested molecules (Figure 6 A). Finally, we examined the impact of the molecules on the expression of $p27^{Kip1}$, whose degradation involves the SCF-Skp2-CKS1 complex but not CKS1–CDK. As could be expected for a specific inhibition of CKS functions exerted in the context of CDK complexes, none of the tested molecules caused an increase in

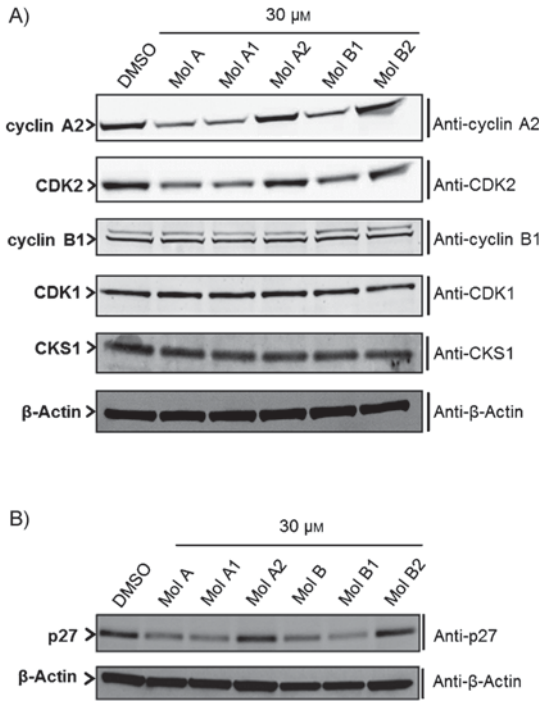


Figure 6. Effect of CDK–CKS inhibitors on expression levels of cell-cycle regulating proteins. A) MCF-7 cells were treated with 30 μM molecules for 48 h. Expression levels of the indicated proteins were determined by western blot with specific antibodies, on 30 μg total protein. An anti-β-actin antibody was used as control. B) As above but for $p27^{Kip1}$ expression levels.

$p27^{Kip1}$ levels; on the contrary, all four CDK–CKS inhibitors caused a decrease in $p27^{Kip1}$ levels (Figure 6B).

Discussion

We used a yeast two-hybrid screening assay that was originally developed to discover small-molecule mimics of peptide aptamers[29] and that was recently used to discover small-molecule inhibitors of a protein–protein interaction.[30] The results obtained from our small-scale screening confirmed that this assay represents a robust method to discover protein interaction inhibitors. They also provided another illustration of the usefulness of yeast two-hybrid methods in the discovery of small molecules.[31]

We identified and confirmed two very distinct small-molecule inhibitors of CDK–CKS protein interactions. Molecule **A** is 4-hydrazino-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine obtained by chemical synthesis; molecule **B** is a natural anthraquinone found in rhubarb (also known as Rhein).[32] Their respective molecular weights (220 and 284 Da) are notably smaller than those of most orthosteric inhibitors of protein interactions.[9,10] This might suggest that they target an allosteric site rather than the CDK–CKS binding interface, which does not present a conspicuous, druggable pocket.[24] In support of this hypothesis, our molecular docking study identified two putative allosteric sites on CDK2; one may accommodate **A** and the other may accommodate **B**. The different scaffolds of the two hit molecules support the idea that **A** and **B** likely bind to different sites.

Our specificity studies established that these molecules specifically inhibit CDK–CKS interactions, without affecting other CDK2–protein interactions (with cyclin A or a phosphorylation substrate), and without inhibiting CDK2 kinase activity. Moreover, we experimentally ruled out that these inhibitors act nonspecifically by forming high-molecular-weight aggregates.

For each inhibitor we identified an active and an inactive close analogue by using an in vitro protein–protein interaction assay. The overall congruency between the in vitro and cellular results for both sets of analogues supports the existence of a causal link between inhibition of cell division and inhibition of CDK–CKS interactions. In accordance with our results, Rhein has been previously shown to exert a weak anti-proliferative activity on various cancer cells, with molecular mechanisms that remain poorly described.[32] Our results indicate that the anti-proliferative activity of Rhein (and possibly other natural anthraquinones) is achieved at least in part by the disruption of CDK–CKS interactions.

We showed that the dominant effect of the inhibitors on the cell cycle is an increase in the G1 and/or S-phase population. This could be a consequence of, or result in, the decreased protein expression of CDK2 and cyclin A; these play a key role in the progress and completion of the S phase. Various reverse genetics approaches have pointed to multiple, complex roles of CKS proteins on cell-cycle control, and have led to different observations depending on the cellular models or the exerted perturbations. For example, the silencing of CKS1 in *CKS2*$^{-/-}$ MEF cells induced cell-cycle arrest in G2, whereas the silencing of both genes in HeLa cells causes apoptosis.[16] Silencing of CKS1 in MCF-7 cells caused an increase in the G2/M population and slowed G1 progression.[33] Our results offer a good illustration of the fact that (at least for highly connected proteins) modulating protein expression levels by classical reverse genetics approaches induces biological effects that can differ markedly from those induced by small-molecule inhibitors,[34] and even more so by small-molecules disrupting protein interactions.[35] In the present case study, inhibiting the expression of CKS proteins indiscriminately compromises all CKS functions, including those that are not exerted in the context of CDK complexes. For example, CKS1, independently of its interaction with a CDK addresses the SCF-Skp2 ubiquitin ligase to $p27^{Kip1}$.[14] Hence, contrary to our molecules, *CKS1* knockout or silencing induces an increase in $p27^{Kip1}$ levels.[14,33] In view of the pleiotropic functions of $p27^{Kip1}$ in the cell cycle and transcription regulation, it is not surprising that inhibiting CKS1 expression or targeting CKS1–CDK interactions produces quite different effects on the cell cycle.

The use of the molecules described here should contribute to distinguishing between those CKS functions that are exerted in the context of CDK-containing complexes and those that are exerted independently from them. To this end, other recently discovered small molecules inhibiting the interactions between Skp2-CKS1 and $p27^{Kip1}$,[36,37] or between Skp2 and CKS1[38] will also be useful.

CDKs interact with a variety of regulatory proteins and thus lend themselves to the discovery of other small-molecule inhibitors of protein–protein interactions that will help shed new light on their functions. To the best of our knowledge, this work brings the first demonstration that the inhibition of protein–protein interactions involving CDKs is a valid strategy to interfere with their role in cell cycle regulation, and therefore represents an attractive alternative to targeting CDK active sites.

Experimental Section

Plasmid constructions: See the Supporting Information.

Yeast two-hybrid screening assays: We performed yeast two-hybrid screening assays as previously described.[21] We used the pRAP-Col reporter plasmid (*2*μ replication origin, *URA3* marker, *8LexAop::luc* reporter gene), pEG202 or pHA bait plasmids (*2*μ or *CEN/ARS* replication origin, respectively, *HIS3* marker, directing constitutive expression of LexA fusion proteins under the control of an ADH promoter), and pLP3 or pLP5 prey plasmids (*2*μ or *CEN/ARS* replication origin, respectively, *LEU2* marker, directing inducible expression of B42 fusion proteins under the control of the *GAL1* promoter). We sequentially transformed an *erg6* strain with these plasmids. We performed the screening manually in 96-well plates (white, half area, flat bottom; PerkinElmer). We incubated yeast transformants for 4 h with small molecules (5 $\mu g\,mL^{-1}$, DMSO (1.5%)) and galactose (2%). We quantified luciferase activity with Dual-Glo luciferase assay reagents (Promega), and we measured the emitted light in a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany). Inhibition rates were calculated as follows: % inhibition $= 100-(\text{luc value}-m/M-m)\times 100$ (M: mean of signal with galactose and DMSO, m: mean of signal with DMSO without galactose). We calculated the Z' scores by applying the standard formula (see the Supporting Information).

Production and purification of recombinant proteins; pull-down assays: See the Supporting Information.

Thermophoresis experiments: For the molecules of the **A** series, we labeled GST-CDK2 recombinant protein with the NT-647 fluorophore according to the manufacturer's instructions (NanoTemper Technologies, München, Germany). We mixed GST-CDK2 (20 nM) with different concentrations of the molecules (9 nM to 300 μM), briefly incubated them at room temperature, loaded glass capillaries, and ran thermophoresis on a Monolith NT.115 machine (NanoTemper). For the molecules of the **B** series, we mixed unlabelled GST-CDK2 (500 nM) with different concentrations of the molecules (9 nM to 300 μM), briefly incubated them at room temperature, loaded glass capillaries, and ran thermophoresis on a Monolith NT.LabelFree (NanoTemper).

Molecular docking: See the Supporting Information.

Protein kinase assays: We incubated human recombinant CDK2/cyclin A (obtained from Aude Echalier, Centre de Biochimie Structurale, Montpellier, France) with small molecules at the indicated concentrations in buffer A (Tris·HCl (25 mM, pH 7.5), $MgCl_2$ (10 mM), EGTA (1 mM), DTT (1 mM), heparin (50 $\mu g\,mL^{-1}$)) containing histone H1 (1 $mg\,mL^{-1}$), ATP (15 μM) and [γ-^{33}P]ATP (15 μM, 3,000 $Ci\,mmol^{-1}$; 10 $mCi\,mL^{-1}$) in a final volume of 30 μL. After 30 min incubation at 30 °C, we stopped the reaction by using a MicroBeta FilterMate-96 Harvester (PerkinElmer) and P81 phosphocellulose papers (Whatman), which we washed in phosphoric acid (1%). We added scintillation fluid, and we measured the radioactivity in a counter with microplate robotic stackers (TopCount NXT, Packard). We calculated kinase activity as picomoles of phosphate incorporated during the 30 min incubation. We evaluated percentage kinase activity as the amount compared to that measured in the absence of inhibitors.

Mammalian cell cultures and viability assays: We grew human MCF7 cells in Dulbecco's Modified Eagle's Medium (DMEM, with L-glutamine, Gibco) supplemented with fetal bovine serum (10%; Invitrogen) at 37 °C, in humidified atmosphere containing CO_2 (5%).

We plated MCF7 cells (3×10^3 per well) in 96-well plates (CytoOne, USA Scientific). After 24 h, we treated the cells with various concentrations of molecules (in DMSO (0.1%)) and incubated them for 48 h. We assessed cell viability with a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega). Briefly, we removed the supernatant, added MTS salt solution to the cells, incubated them for 3 h at 37 °C in the dark, then measured the absorbance at 490 nm in an ELISA plate reader. We determined cell viability percentages relative to that of DMSO-treated cells.

Flow cytometry analysis: We grew MCF7 cells for 24 h in 6-well cell culture plates (CytoOne). Then, we treated them with small molecules (30 μM) for 24 h, washed them twice with PBS, harvested the cells by trypsinization, and washed the cells again with PBS. We fixed the cells (1×10^6) in PBS (0.3 mL)/cold ethanol (70%, 0.7 mL) for 1 h at 4 °C. We centrifuged the cells at 200 *g* for 5 min, washed them once with cold PBS, re-suspended the cell pellets in PI/RNase Staining Buffer (500 μL; BD Biosciences) and incubated them for 15 min at room temperature in the dark. We determined cell cycle profiles in a FACSCanto II flow cytometer with FACSDiva Software (BD Biosciences).

Whole-cell extract preparations: We grew MCF7 cells for 24 h in 6-well cell culture plates and treated them with small molecules (30 μM) for 48 h. We collected the cells by scraping in PBS, and we lysed them by sonication in lysis buffer (MOPS (25 mM, pH 7.2), β-glycerophosphate (60 mM), *p*-nitrophenylphosphate (15 mM), EGTA (15 mM), $MgCl_2$ (15 mM), sodium vanadate (1 mM), NaF (1 mM), phenylphosphate (1 mM), NP40 (0.1%), and protease inhibitor cocktail). We spun the lysates at 20000 *g*, (15 min, 4 °C), collected the supernatant, and determined the protein content by using a Bradford assay. We heat-denatured the proteins, and ran the samples (30 μg) on 12% Bis·Tris SDS-PAGE gels.

Western blot experiments: We transferred the proteins onto Hybond nitrocellulose membranes (GE Healthcare) and processed the blots according to standard procedures. We used the following primary antibodies: anti-CDK1 (1:500; ab8040, Abcam, Cambridge, UK), anti-CDK2 (1:500; sc-163, Santa Cruz Biotechnology), anti-cyclin A (1:500; sc-751, Santa Cruz Biotechnology), anti-cyclin B1 (1:500; sc-245, Santa Cruz Biotechnology) anti-CKS1 (1:500; 36–6800, Life Technologies), anti-$p27^{Kip1}$ (1:500; sc-528, Santa Cruz Biotechnology), anti-His HRP conjugate (1:1000; 71840, Merck Millipore), anti-actin (1:5000; CP01-1EA, Merck Millipore). We used anti-mouse (1:3000; 170-6516 Bio-Rad) or anti-rabbit (1:5000; 172-1019 Bio-Rad) HRP-conjugated secondary antibodies. We visualized the blots by using the enhanced chemiluminescence kits Pierce ECL Western Blotting Substrate or SuperSignal West Femto (Thermo Scientific).

Synthesis of compounds of the A-series: The synthesis of **A1** was as previously described.[39] Briefly, **A1** was prepared from 4-chloro-6,7,8,9-tetrahydro-5*H*-cyclohepta-[4,5]-thieno[2,3-*d*]pyrimidine (0.5 g, 2.37 mmol) and hydrazine monohydrate (0.6 mL). The mix-

ture was stirred under reflux in ethanol (15 mL) for 5 h. After cooling to room temperature, the resulting yellow precipitate (**A1**: 4-hydrazino-6,7,8,9-tetrahydro-5*H*-cyclohepta[4,5]thieno[2,3-*d*]pyrimidine (0.38 g, 77%) was filtered and washed with a solution of ethanol. Compounds **A** (4-hydrazino-5,6,7,8-tetrahydro[1]benzothieno-[2,3-*d*]pyrimidine) and **A2** (4-hydrazino-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3*d*] pyrimidine) were obtained by the same method from 4-chloro-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine and 4-chloro-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine, respectively.

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