Comprehensive survey of CDK inhibitor selectivity in live cells with energy transfer probes

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ABSTRACT: Concerted multidisciplinary efforts have led to the development Cyclin Dependent Kinase inhibitors (CDKi's) as small molecule drugs and chemical probes of intracellular CDK function. However, conflicting data has been reported on the inhibitory potency of CDKi's and a systematic characterization of affinity and selectivity against intracellular CDKs is lacking. Toward this end, we have developed a panel of cell-permeable energy transfer probes to quantify target occupancy for all 21 CDKs in live, intact cells. Here we present the first comprehensive evaluation of intracellular isozyme potency and selectivity for a collection of 46 clinically-advanced CDKi's and tool molecules. We observed unexpected intracellular activity profiles for a number of CDKi's, offering avenues for repurposing of highly potent molecules as probes for previously unreported targets. We further evaluated mechanisms for achieving target selectivity through protracted target residence time under non-equilibrium cell culture conditions. Here we provide a broadly applicable method for evaluating the selectivity of chemical matter for CDKs in living cells, and present a refined set of tool molecules to study CDK function.

INTRODUCTION

Kinases represent the broadest class of intracellular enzymes in human cells, regulating critical nodes in signal transduction. As dysregulated kinase activity is common in a variety of cancers and immune diseases, small molecule kinase inhibitors have emerged as one of the most successful modalities for drug development in the 21st century.[1-2] For example, cyclin dependent kinases (CDKs) have been validated as oncogenic drivers in solid tumors.[3] The CDK family comprises 21 phosphotransfer enzymes with diverse cellular functions. CDK1, -2, -4 and -6 play key roles in the regulation of the eukaryotic cell cycle, while CDK7–9 and -19 are involved in regulation of gene transcription.[4-5] CDK activity is tightly regulated by intracellular protein-protein interactions, most critically with cyclin proteins. Many of the CDKs require heterodimerization with a cyclin protein to form an active enzyme.[6] This regulation is dynamic, as CDK/cyclin interactions oscillate depending on the cell cycle, providing a unique layer of complexity to intracellular signaling mediated by this kinase subfamily.[7] While knowledge of the regulatory role of the cell cycle and transcriptional CDKs has been extensively studied, the majority of the CDK family enzymes have unknown roles in cell physiology (most notably CDK5, -10, -11, 14–18, and -20). Nonetheless, the recent clinical advancement of dual CDK4/6 inhibitors for treatment of HER2 negative breast cancer has amplified broader interest in exploring the

therapeutic potential of the established and understudied CDKs with small molecule inhibitors.[8-9]

The vast majority of CDK inhibitors have been designed to occupy to the nucleotide co-substrate (ATP) binding pocket.[5, 9] As the catalytic pocket across the CDK enzyme family is highly conserved, the development CDK inhibitors (CDKi's) with isozyme selectivity is technically challenging. Moreover, the high concentration of intracellular ATP (varying between 1–10 mM and surpassing enzyme K_m by orders of magnitude) yields an unpredictable microenvironment for achieving competitive inhibition of CDK enzymatic activity.[10-11] While the CDK field is replete with ATP-competitive inhibitors with potent activity against the purified enzymes in cell-free biochemical assays, there remains a lack of well characterized inhibitors with potent and selective pharmacology against each of the CDKs in live cells. The dearth of robust target engagement assay technologies that allow for an assessment of CDKi potency and selectivity within intact, living cells has represented a key technical limitation. Cellular methods for evaluating CDK isozyme pharmacology are generally limited to substrate phosphorylation analyses (e.g. Western blot from cell extracts), but such approaches suffer from the redundancy of CDK phosphotransfer activity across known substrates. For example, although phosphorylation of Rb is a commonly used biomarker of CDK activity, the protein serves as a substrate for cell-cycle regulatory CDKs including CDK1, -2, -4, -5, and -6.[12-13] Beyond the cell-cycle-regulatory CDKs,[14] other isozymes in the family lack a known substrate for intracellular phosphorylation analysis. Thus, evaluating the intracellular pharmacology of individual CDKs represents a major challenge across the family and leaves the understanding of inhibitor selectivity incomplete. The pharmacological activity of CDKi's is predicated on their physical engagement with cellular targets. Accordingly, target engagement potency can correlate quantitatively with potency of intracellular kinase inhibition.[10-11, 15] Therefore, in the absence of isozyme-specific functional assays, cellular target engagement assays represent an ideal surrogate for evaluating inhibitor selectivity. Ideally, CDKi selectivity should be queried in a unified target engagement format, wherein occupancy is quantifiable in the presence of cyclin partners and other cellular factors that are known drivers in compound pharmacology for this kinase subfamily.

Here, we describe a comprehensive and systematic method to quantify target occupancy of CDKi's in live cells for the complete CDK family. We have then used this method to perform an evaluation of intracellular target engagement selectivity for 46 CDKi's comprising a collection of clinically-advanced compounds and recently published chemical tools. To evaluate CDK potency and selectivity in a physiological setting, we developed a panel of cell-permeable energy transfer probes that allow for quantitative evaluation of CDK/cyclin occupancy inside intact, living HEK-293 cells by Bioluminescence Resonance Energy Transfer (BRET). Our results identified small molecule CDKi's with strong isozyme selectivity within cells, supporting their use as chemical tools. In contrast, we determined that a number of previously reported "selective" CDKi's did not maintain their putative CDK selectivity profiles when evaluated in live cells. Surprisingly, a subset of this chemical matter, including a panel of well-studied clinically-advanced CDKi's, can be repurposed as chemical probes for understudied CDK isozymes. Real-time analysis of target occupancy also revealed that CDK inhibitors may show surprisingly durable inhibition (i.e., long residence time) in live cells, resulting in a remarkable shift in the selectivity profile over time. The methods described herein can be applied to evaluation of small molecule inhibitors of all CDK family members. This analysis can thus serve as an adaptable template to evaluate CDKi selectivity potential in a variety of cell types and experimental systems to support discovery of new medicines. The resulting comprehensive analysis of CDK inhibitor activity in living cells is intended to provide a key resource for optimizing drug candidates and selecting chemical probes for experimental pharmacology.

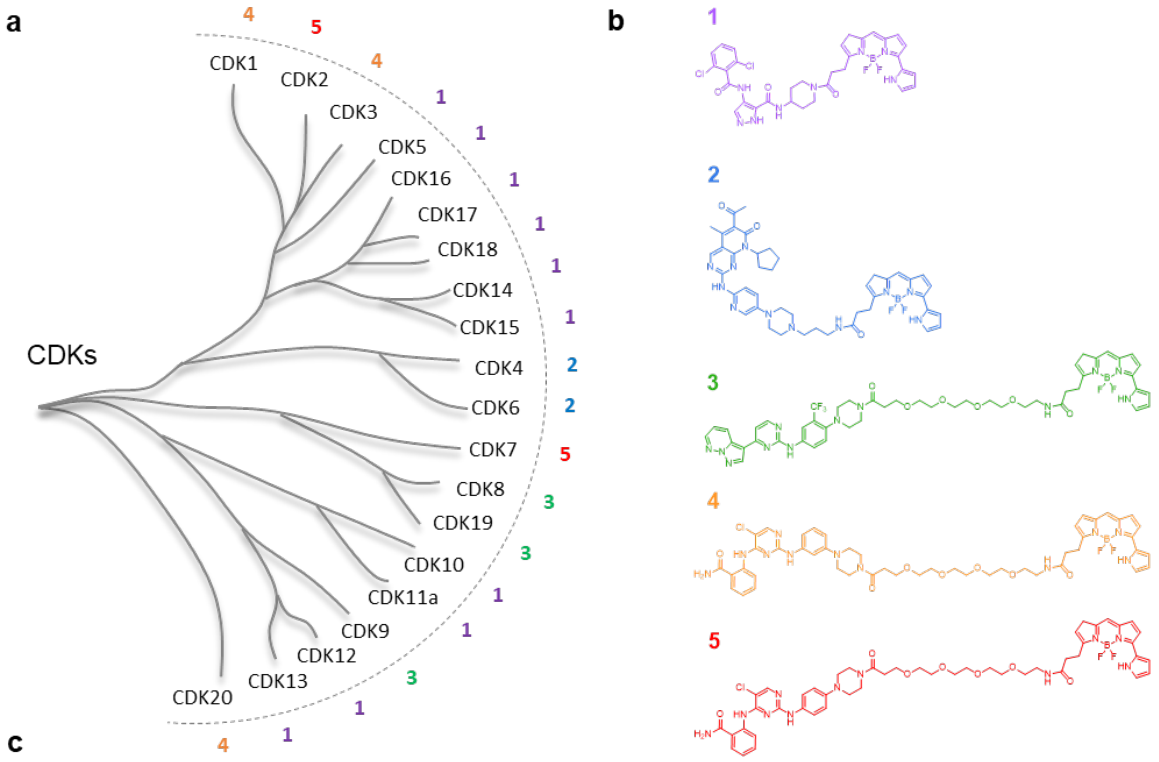
RESULTS AND DISCUSSION

Development of cell-permeable energy transfer probes for quantifying CDK inhibitor occupancy in live, intact cells.

To date, cell-free enzymatic or kinase binding assays have been used to successfully annotate the potency and selectivity of small molecule CDKi's.[16-17] Although robust and scalable in screening, these cell-free kinase assays do not query engagement in the presence of the cellular milieu (e.g., physiological ATP and the full complement of partners, and have therefore often revealed divergent pharmacology to that observed in cells.[10-11, 18] The disconnect between biochemical and cell-based kinase potency[10-11] has led to the development of more advanced techniques that allow for a systematic characterization of target occupancy in cell extracts via chemoproteomics[19] and photoaffinity probes.[20] Such methods represent key technological advancements for the CDK field, but are generally incompatible with intact

cell analyses. As kinase pharmacology is often impacted by the composite effect of the intracellular milieu, target occupancy measured in live cells may fundamentally differ from that observed in lysates or in purified systems.[10-11] For this reason, we elected to develop a robust, comprehensive, and scalable method to measure the intrinsic CDKi affinity and selectivity across all 21 family members within live cells.

Our groups previously collaborated to develop a collection of broad-spectrum energy transfer probes (NanoBRET tracers) to query target occupancy for 178 kinases in live, intact cells.[21] Although the probe set covered a large fraction of the kinome, coverage over the CDK family was limited to only four members.[21] To adapt the method to cover the CDK family comprehensively, we employed a two-fold strategy wherein novel energy transfer probes were developed either from known CDKi's or by optimization of known broad-spectrum ATP-competitive kinase inhibitors. Each tracer was optimized for binding to their target CDKs by installation of a functional group for dye conjugation as well as selection of the linker between the binding moiety and the fluorophore (Figure S1). Each bifunctional molecule was screened for binding across the entire panel of 21 CDKs (Table S2), after which those probes demonstrating the highest specific energy transfer signals for each individual CDK were selected for further characterization in dose-response experiments (Figure S2). This approach yielded 5 optimized energy transfer probes with sufficient performance to enable live-cell assays for 21 CDKs (Figure 1).



Energy Transfer Complexes

CDK	1	2	3	4	5	6	7	8	9	10	11a	11b	12	13	14	15	16	17	18	19	20
Cyclin	E1	E1	E1	D1	R1[a]	D1	None	C	K	L2	L2	L2	K	K	Y	Y	Y	Y	Y	C	H
Probe	4	5	4	2	1	2	5	3	3	1	1	1	1	1	1	1	1	1	1	3	4

[a]CDK5R1 Regulator

Figure 1. Comprehensive energy transfer system to profile target engagement for all 21 human CDKs in live cells. CDK phylogenetic tree[4-5, 7] (a) and associated cell-permeable energy transfer probes (b) used to comprehensively profile CDK engagement in live cells. Key components for each CDK assay are summarized in panel (c) and described in full in Table S1.

The structure of the five new energy transfer probes is depicted in Figure 1B. Briefly, probe 1 was developed from promiscuous CDK inhibitor AT7519,[22-23] which enabled assays for 11 CDKs.

Robust assays for CDK4 and CDK6 were enabled by probe 2, which was developed from the FDA-approved drug palbociclib.[24] GW779439 was discovered during review of the published kinase inhibitor set 2 (PKIS2) dataset,[25] and probe 3 was developed from this scaffold to enable robust assays for CDK8, CDK9, and CDK19. Lastly, optimization of inhibitors based on the CTx-0294885[21] scaffold yielded probes 4 and 5, which collectively enabled assays for CDK1, CDK2, CDK3, CDK7, and CDK20.

The enzymatic activity of many CDKs was known to be modulated in cells by the specific cyclin or regulatory partner to which they are complexed. We therefore implemented an assay design that would allow evaluation of compound pharmacology based on CDK/cyclin interactions. Thus, in addition to evaluating the CDK-NanoLuc fusions in both N- and C-terminal orientations, we also defined the assay systems by co-expression of an excess of specific cyclins and regulatory partners. For the majority of the CDKs, co-expression of an excess of a known[7] cyclin partner potentiated the energy transfer signal, providing support that the CDK population was shifted toward the selected cyclin pair (Figures S3 and S4). Collectively, these 5 new energy transfer probes enabled assays for all 21 CDK family members in complex with an appropriate partner protein, facilitating the first exploration of target engagement for known CDKi's in live and intact cells.

Selection of chemical matter for evaluation of intracellular CDK selectivity

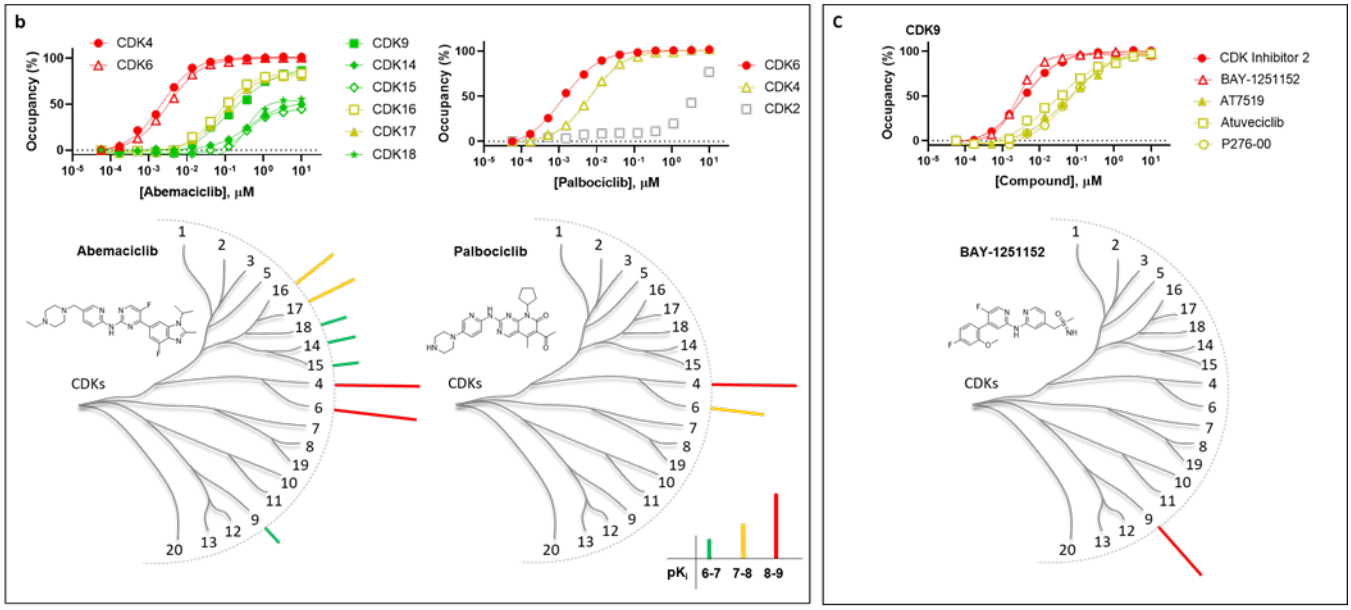
Despite the literature being rife with CDK inhibitors, very few CDKi's have been comprehensively profiled against the full CDK family in any assay format.[16-17, 19] As such, the chemical landscape of CDK inhibition has yet to be systematically defined. To define the CDK selectivity profiles in live cells, we assembled a set of 46 commercially-available CDKi's that represent broad chemical diversity (Table S3) that included the 3 FDA-approved CDK inhibitors, 18 drugs in advanced clinical trials, and many tool molecules that had been described in the literature.

a

Target engagement potency for previously reported CDK4/6 inhibitors

Compound	IC_{50} for CDK/cyclin complexes (nM)[a]																			
	1	2	3	4	5	6	7	8	9	10	11a	11b	12	13	14	15	16	17	18	19	20
AMG 925		286		4		3									454	781	262				
Abemaciclib				3		1			209						358		60	71	327		
Milciclib				154	801	97	461									934	322	331			
ON123300				6		4	145								127		35	38	292		
Palbociclib				12		3															
Ribociclib				34		39															
Trilaciclib				5		5											516	457			

[a]CDK/cyclin pairings are described in Figure 1.



d

Target engagement potency for previously reported CDK9 inhibitors

Compound	IC_{50} for CDK/cyclin complexes (nM)[a]																			
	1	2	3	4	5	6	7	8	9	10	11a	11b	12	13	14	15	16	17	18	19	20
AT7519		580		725		695	125		121		145	82			728	462	100	48	500		
Atuveciclib									62												
BAY-1251152									3												
CDK inhibitor II							71		4	285	13	13				489	96	84	157		
P276-00				445		385			70												

[a]CDK/cyclin pairings are described in Figure 1.

Figure 2. A. Live cell engagement potency for CDK4/6 selective probes. Reported IC_{50} data are the mean of two independent experiments with values < 1μM. Blank cells represent IC_{50} values that failed to meet our criteria of potency (<1 μM) or occupancy (≥ 50% at 10 μM). B. Representative live cell target occupancy results measured for abemaciclib and palbociclib (upper). For palbociclib, CDK2 occupancy is included for comparative reference. Dendrogram-based illustration of engagement selectivity for abemaciclib and palbociclib against the complete CDK family (lower). C. Representative target occupancy results measured for CDK9 selective inhibitors (upper). Dendrogram-based illustration of engagement selectivity for BAY-1251152 (lower). D. Engagement potency for CDK9-selective inhibitors. Reported IC_{50} data are mean of two independent experiments. Blank cells represent IC_{50} values that failed to meet our criteria of potency (<1 μM) or occupancy (≥ 50% at 10 μM).

To assemble a comprehensive intracellular profile for all 46 CDKi's, each compound was initially profiled across all 21 CDK/cyclin complexes in live cells at 10 μM, using 50% occupancy as a cutoff for follow-up potency (IC_{50}) determination. Tables S4 and S5 summarize the potencies for all molecules conforming to stringent intracellular occupancy (≥ 50% at 10 μM) and potency (< 1μM) criteria.

Verification of CDK 4/6, CDK7, and CDK9 inhibitors as isozyme-selective chemical tools in living cells

CDK4/6: CDK4 and CDK6 are two closely related family members that control transition from G1 to S phase of the cell cycle, and are established oncogenic drivers in a variety of solid tumors.[26] Accordingly, drug development programs have yielded three dual CDK4/6 inhibitors (abemaciclib, palbociclib, and ribociclib) that have been FDA-approved for treatment of breast cancer. As validation of our approach, we generated the full CDK profile of these drugs and other known CDK4/6 inhibitors in our live cell energy transfer system to compare with their reported clinical pharmacology. Eight CDK4/6 inhibitors were evaluated against all 21 CDKs (Figure 2A). All three FDA approved CDKi's potently engaged CDK4/6 in the live cell assays. Abemaciclib showed a target engagement potency at CDK4/6 that agreed closely with its reported cellular potency in MCF-7 cells.[27] However, abemaciclib also showed collateral engagement of CDK2, -7, -9, and CDK14–18 (Figure 2B).[28] Palbociclib and ribociclib were more selective for CDK4/6, with > 100-fold selectivity against the remaining family (Figures 2 and S5). Other CDK4/6 inhibitors showed varying levels of cellular selectivity: AMG-925, ON123300, trilaciclib, and milciclib potently engaged CDK4/6 with > 10-fold selectivity over collaterally engaged CDKs (Figure S6).

CDK9: The role of CDK9 is in transcriptional regulation and its dysregulation has been implicated in a variety of human pathologies.[29] Our results demonstrate that BAY-1251152 and atuveciclib are inhibitors with strong and selective engagement to CDK9 in cells. Both compounds showed > 100-fold selectivity for CDK9 over other members of the family. Intracellular engagement assays also revealed that the pan-CDK inhibitors AT7519 and P276-00 engaged CDK9 with approximately 10-fold selectivity within the family (Figure 2D and Figure S7). Among the known CDK9 inhibitors, BAY-1251152 demonstrated the strongest target affinity and selectivity (Figure 2C) and is recommended as a tool compound for selective modulation of CDK9 function in cellular studies.

CDK7: CDK7 has been identified as a promising drug target due to its dual function in controlling the cell cycle and transcription, which has led to several inhibitors undergoing evaluation in clinical trials as anticancer therapies.[30-32] Molecules based on the pyrazolopyrimidine scaffold have been reported as potent inhibitors of CDK7. Surprisingly, in our analysis, pyrazolopyrimidine CT7001[33] showed potent engagement of CDK4 in addition to CDK7 (Figures 3A and 3B), with modest selectivity over CDK2 (407 nM), and negligible engagement (greater than 600 nM) with other members of the family. LDC4297,[34] a structurally related CDK7 inhibitor, also displayed engagement of CDK7, CDK2 and CDK4, but was less selective than originally reported and showed collateral activity on CDK1, -3, -5, and CDK14–18 (Figure S8). In our cellular analysis, the pyrazolopyrimidine BS-181 engaged CDK7 with modest potency (450 nM) with negligible occupancy at other family members at concentrations under 1 μM (Figures 3A and 3B). BS-181 should therefore be considered among the best-in class selective CDK7 probes in our panel.

CDK7 contains a reactive cysteine (C312) located outside the nucleotide pocket that can be targeted with covalent inhibitors. Gray and coworkers exploited this mechanism to develop THZ1, a potent covalent inhibitor of CDK7 with efficacy in multiple cell models.[35-36] We evaluated THZ1 at our standard 2 hour incubation time (Figures 3A and S8), as well as an extended 6 hour duration in live cells (Figure S9). Only modest selectivity of THZ1 was observed for CDK7 after two hours of incubation with cells (Figure 3A). Extended 6 hour incubation enhanced the engagement potency to CDK7 (Figure S9), matching closely with antiproliferative potency of THZ1 in Jurkat cells.[35] Thus, our findings corroborate time-dependent engagement of CDK7 by THZ1[36] and support its potential utility as a CDK7 tool compound after extended incubation times.

CDK1 and CDK2 inhibitors offer limited selectivity for their intended targets.

As critical modulators of cell cycle progression, CDK1 and CDK2 have been targets for development of cancer drugs.[3] We evaluated the intracellular selectivity of a number of molecules reported as selective CDK2 or dual CDK1/2 inhibitors. 10 inhibitors demonstrated intracellular affinity values for CDK1 and CDK2 below 100 nM, and a subset of these compounds showed single digit nM intracellular affinities (AZD5597, dinaciclib, BS-194, CDKI-73, and RGB-286638). Remarkably, all of the highest affinity CDK1/2 inhibitors collaterally engage other CDK family members. Comprehensive intracellular profiling of these potent CDK1/2 compounds revealed strong collateral engagement to other CDK's, most notably CDK14–18. CDKI-73 and RGB-286638 (Figure S10) engaged the closely related CDK16 and -17 with high affinity. Our data demonstrate that broad assessment of CDKi pharmacology in live cells is warranted, especially for compounds that advance to clinical development.

While high affinity CDKi's for CDK1/2 yielded strong engagement to other family members, some weaker affinity inhibitors also showed modest selectivity for CDK2 in cells. For example, NU6102 was selective for CDK2 over CDK4, with a relatively weak engagement of the remaining CDK family (Figure 3C). Thus, although CDK1/2 are two of the most highly studied family members, none of the inhibitors tested were both potent and selective for these isozymes in cells.

Repurposing CDK1/2 inhibitors as CDK8/19-selective chemical probes in cells

In our comprehensive live cell analysis, a number of the inhibitors in this study produced surprisingly strong engagement patterns to collateral CDKs. For example, potent inhibition of CDK14–18 was observed for several CDK1/2

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Target engagement potency for previously reported CDK7 inhibitors

Compound	IC_{50} for CDK/cyclin complexes (nM)[a]																			
	1	2	3	4	5	6	7	8	9	10	11a	11b	12	13	14	15	16	17	18	19	20
BS-181							450														
CT7001		407	504	33			13			645											
LDC4297	90	84	246	60	242	140	16					780				240	381	182	219		
THZ1							105			725	340										

[a] CDK/cyclin pairings are described in Figure 1.

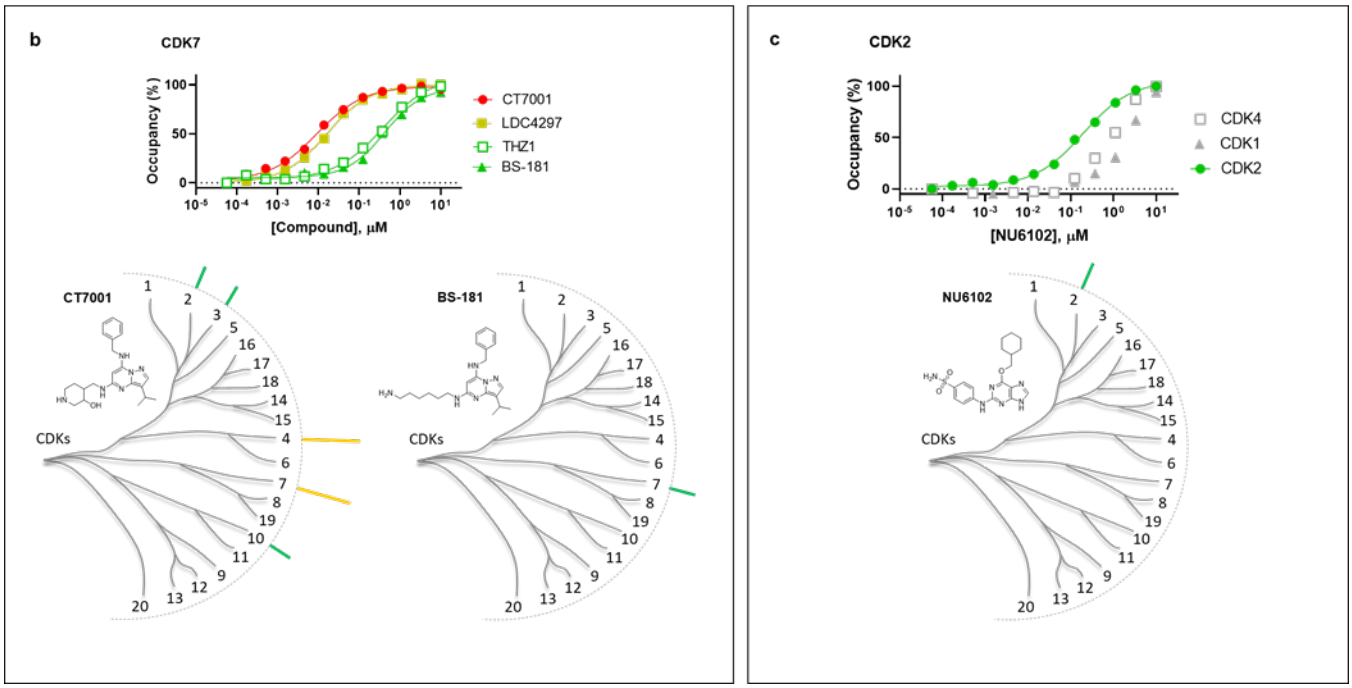


Figure 3. A. Live cell engagement potency for CDK7 selective probes. Reported IC_{50} data are the mean of two independent experiments. Blank cells represent IC_{50} values that failed to meet our criteria of potency (<1 μM) or occupancy (≥ 50% at 10 μM).B. Representative live cell target occupancy results measured for CDK7 selective inhibitors (upper). Dendrogram-based illustration of engagement selectivity for CT7001 and BS-181 against the complete CDK family (lower). C. Representative target occupancy results measured for NU6102 against CDK2 as well as putative targets CDK1, and CDK4 (upper). Dendrogram-based illustration of engagement selectivity for NU6102 (lower).

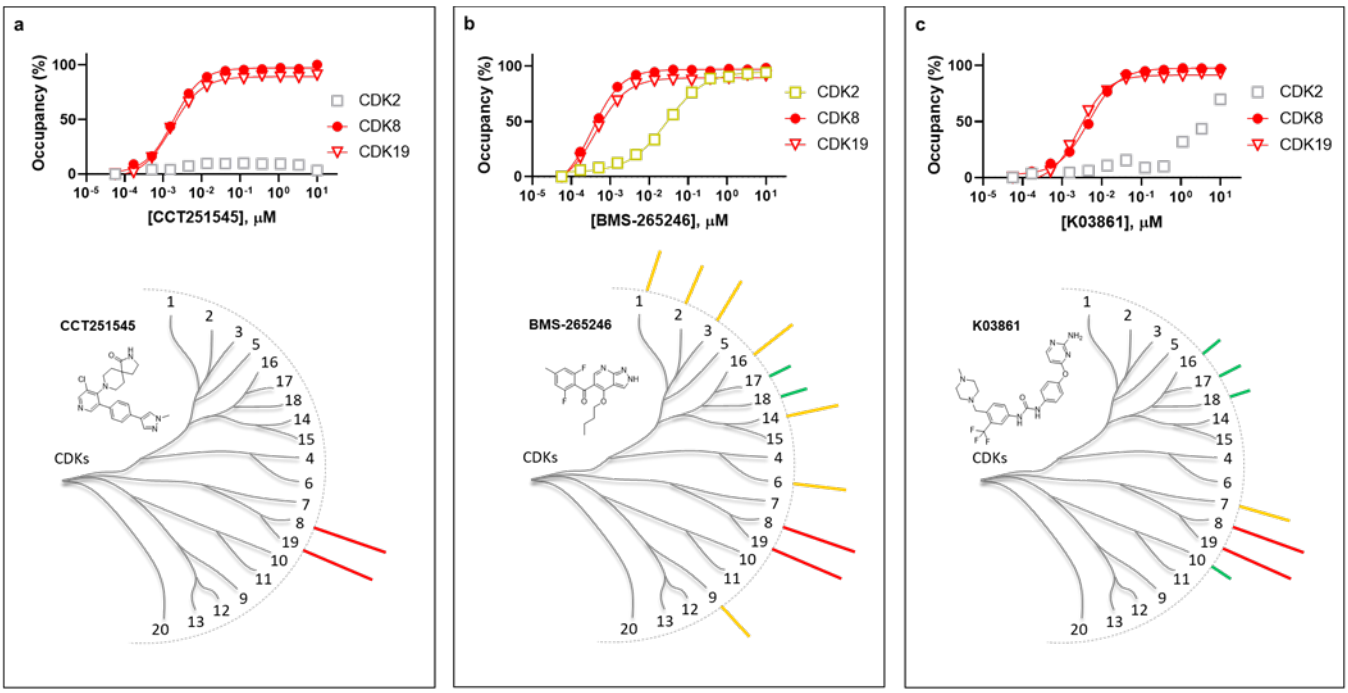
inhibitors. We therefore evaluated the possibility that a subset of CDKi's may engage these understudied CDKs with stronger intracellular affinity than their originally targeted family member, and if such molecules could be repurposed as probes for the lesser studied family member.

Across the panel of intracellular CDKs, the paralog kinases CDK8/19 showed the most distinct engagement profile. CDK8/19 are closely related but relatively understudied members of the CDK family that have been identified as components of the mediator complex involved in global regulation of transcription in eukaryotic cells[37] and are

potential oncogenes in a subset of solid tumors.[38] Recently, two chemical probes have been described for CDK8/19,[39-40] CCT251545 (and a related analog) potently inhibited downstream CDK8/19 activity biomarkers with single-digit nanomolar potency.[39-40] Our live cell occupancy results at CDK8 and CDK19 (2 nM and 4 nM, respectively) agreed closely with the these reported cellular potencies (Figures 4 and S11).

We also uncovered a number of compounds with unexpected selectivity for CDK8/19 (Figure 4). The CDK2 inhibitor K03861[41-42] engaged CDK8/19 in cells, with nearly 100-fold selectivity over other family members, including CDK2 (Figures 4C and 4D). K03861 is a type II inhibitor that stabilizes the inactive DFG-out conformation of CDK2.[41-42] CDK8/19 has been reported to adopt a similar inactive conformation to accommodate type II inhibitors.[43] However, intracellular engagement of type II inhibitors to CDK8/19 has not been reported previously. Our results identify K03861 as a selective chemical probe for CDK8/19, and further support the mechanism of type II inhibition as a strategy for potent and selective engagement of mediator kinases.

BMS-265246, known as a CDK1/2 inhibitor,[17] engaged CDK8/19 potently (1 and 2 nM, respectively) and with >10-fold selectivity index over CDK1/2 (Figures 4B and 4D). As an abandoned clinical asset, additional studies are warranted to evaluate the engagement of CDK8/19 as a mechanism of efficacy or adverse events.



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Target engagement potency

Compound	IC50 for CDK/cyclin complexes (nM)[a]																			
	1	2	3	4	5	6	7	8	9	10	11a	11b	12	13	14	15	16	17	18	19	20
BMS-265246	67	30	10			67	165	1							55		34			2	
CCT251545								2												4	
CCT251545 anolog								2												6	
K03861							68	4		420							253	181	276	2	

[a]CDK/cyclin pairings are described in Figure 1

Figure 4. Representative live cell engagement potency for CDK8/19 with compounds CCT251545 (A), BMS-265246 (B), and K03861 (C). CDK2 is included in each graph for comparative reference. D. Representative live cell engagement potency for CDK8/19-selective compounds. Reported IC_{50} data are the mean of two independent experiments. Blank cells represent IC_{50} values that failed to meet our criteria of potency (<1 μM) or occupancy (≥ 50% at 10 μM).

Our results demonstrate that CDK8 and CDK19 are collateral targets of a number of mischaracterized CDKi's in cells, and opportunities may exist for repurposing one or more of them as chemical probes of mediator kinase activity. Moreover, these results support that the composite effect of the intracellular

milieu has a strong influence on CDKi pharmacology.

Implications of intracellular residence time as a CDK selectivity determinant

For evaluation of CDKi selectivity, steady state analysis is standard practice. However, these equilibrium-based measurements may fail to accurately predict occupancy *in vivo*, where drug concentrations are highly dynamic.[44-45] In a dynamic open system, it is possible to achieve target selectivity via durable binding interactions that may not be evident under steady state conditions.[46] The residence time ($1/k_{off}$) of the target-ligand interaction is often a more accurate predictor of drug efficacy and pharmacodynamic effect.[44] It has been reported that some CDKi's display protracted residence time in a purified biochemical assays.[47] We therefore explored the possibility that CDKi's may yield durable engagement, and kinetic selectivity under simulated open system conditions in living cells.

To query residence time as a potential selectivity determinant, CDK2 and -6 were used as sentinel targets. Residence time was determined via pre-equilibration with each target/CDKi combination at a near saturating concentration (10-20 fold above K_d-apparent as determined above under equilibrium conditions). This condition was selected to ensure adequate target occupancy prior to compound washout. Residence time was then evaluated by a rapid compound washout procedure, followed by addition of energy transfer probe 1. Under these conditions, the rate of the energy transfer signal increase serves as a direct proxy for the loss of the target-CDKi interaction.[48-49]

In contrast to steady-state analysis (which yielded similar CDKi potencies for CDK2 and CDK6, Table S5), real-time analysis of CDK2 and CDK6 occupancy revealed a surprisingly wide range of target residence times for the inhibitor panel (Figure S12). The composite results revealed a subset of compounds with surprisingly durable engagement to either CDK2 or CDK6. In particular, the pan-CDKi RGB286638 engaged CDK2, -6, and -7 with similar equilibrium potencies (Figure 5A), but bound with robust durability to only CDK6 (Figure 5B). After 2 hours of occupancy analysis following compound washout, CDK6 remained > 50% occupied by RGB286638, while CDK2 and CDK7 were fully dissociated. This pattern was surprising, given the similar affinities observed for all three CDKs under steady-state conditions in cells (Figure 5A). Thus, RGB286638 is kinetically selective for CDK6 over CDK2 and CDK7 in living cells. This real-time readout of CDK occupancy may therefore support the development of CDKi's with superior target residence time in cells. Moreover, these preliminary results encourage broader assessment of cellular CDKi residence time as a selectivity determinant.

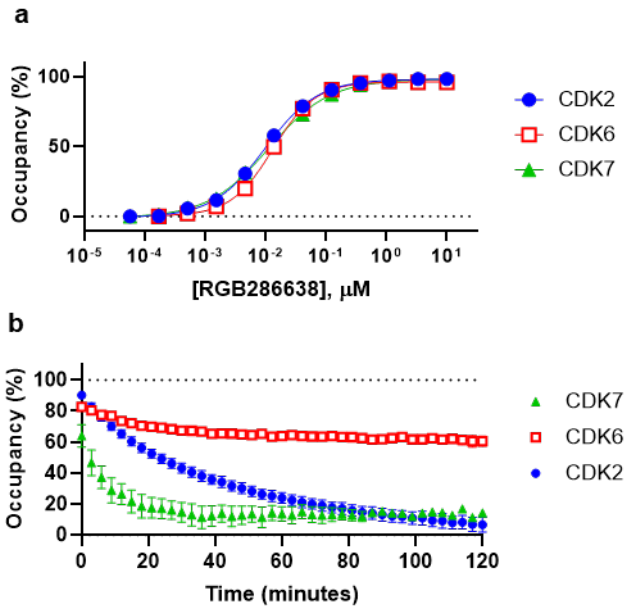

Figure 5. Pan-CDK inhibitor RGB286638 is kinetically-selective for CDK6 over CDK2/7 in cells. A. Similar equilibrium potency of RGB286638 is observed for CDKs 2, 6, and 7. Representative data are shown from experiments performed in duplicate. B. Residence time was measured by pretreating cells with compound (20-fold above the apparent K_d measured in panel A), followed by compound removal and introduction of energy transfer probe 1 at 1 μM. After two hours of real-time analysis, CDK6 is > 50% occupied while CDK2 and CDK7 are fully dissociated. Data are the mean ± S.E.M. of three independent experiments.

Toward a refined set of potent CDKi's for selective target engagement in live cells

Our goal was to develop a comprehensive CDK inhibition profile for a collection of 46 advanced CDK inhibitors. Our analysis cumulatively uncovered potent and selective inhibitors that have utility as selective tool molecules to modulate individual CDKs or paralog isozymes in live cells (Table 1). For CDK4/6 and CDK9, a subset of CDKi's showed high affinity and indexes of selectivity (< 10 nM). Furthermore, we identified several selective inhibitors of mediator kinases CDK8/19 including both type I and II inhibitors. For CDK2, -3, and -7, only modestly selective inhibitors with reduced intracellular potency were observed.

Despite profiling a wide array of chemotypes we were unable to identify selective tool

molecules for some CDKs. For CDK14-18, inhibitors showed strong potency, but with collateral engagement across other the CDK family members. For CDK20 no molecule showed > 50% engagement at 10 μM. Although our analysis failed to uncover selective modulators of these CDKs, the energy transfer based probes developed in this work can be used to identify and optimize potential tool molecules for these understudied but important family members.

CONCLUSIONS AND PERSPECTIVES

We have developed a panel of cell permeable energy transfer probes to enable the first quantitative evaluation of CDKi selectivity in intact cells. The method interrogates inhibitor selectivity under both closed system (equilibrium) and open system (non-equilibrium) conditions. We report a summary of intracellular target engagement potencies for 46 advanced CDKi including many with clinical activity (Tables S4 and S5). Although a portion of our intracellular analysis corroborated CDK profiles from cell-free biochemical systems, for many compounds, we observed a striking pattern of intracellular selectivity that diverged from the cell-free systems. Furthermore, intracellular occupancy measurements support avenues for repurposing of biochemically non-selective CDKi's as selective probes in live cells. We further extended the intracellular analysis of CDKi selectivity to a simulated open system, for evaluation of target residence time. These results support a potential disconnect between thermodynamic and kinetic selectivity for certain CDKi's, as well as a method to optimize kinetic selectivity within the CDK family.

As our compound panel represents only a fraction of known CDKi's, the results presented here suggest that comprehensive assessments of CDK target engagement are warranted as a standard practice for novel tool compounds and promising clinical leads. Based on many of these unexpected findings, an evaluation of CDKi selectivity in live cells may be warranted against a the broader kinome[11]. As CDKi selectivity patterns may be influenced by cellular context, this work-flow is designed to be readily adapted to evaluate target engagement in alternate cell models. This resource is therefore intended to serve as a template for querying intracellular selectivity for CDKi's as drug leads and chemical probes for experimental pharmacology.

Table 1. Best CDKi’s identified in this study for selective target engagement in live cells

CDK	Compound	On target potency	Collateral CDK (potency)
CDK2	NU6102	340 nM	ST[a]
CDK3	RO-3306	210 nM	CDK7 (685 nM)
CDK4/CDK6	Palbociclib	12 nM / 3 nM	ST[a]
CDK4/CDK6	Ribociclib	34 nM / 39 nM	ST[a]
CDK7	THZ1	105 nM	CDK11a (340 nM)
CDK7	BS-181	450 nM	ST[a]
CDK9	BAY-1251152	3 nM	ST[a]
CDK9	Atuveciclib	62 nM	ST[a]
CDK8/CDK19	CCT251545	2 nM / 2 nM	ST[a]
CDK8/CDK19	CCT251545 analog	2 nM / 6 nM	ST[a]
CDK8/CDK19	BMS-265246	1 nM / 2 nM	CDK3 (10 nM)
CDK8/CDK19	K08361	2 nM / 4 nM	CDK7 (68 nM)
CDK12	THZ531	770 nM	ST[a]

[a]Sub Threshold: no collateral CDKs were detected with potency below the cutoff of 1 μM.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information, including materials and method details, Tables S1–S5, Figures S1–S13, and NMR Spectra, is available free of charge at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / CIW and JVD drove the majority of the experimental designs and executions. CIW, JDV, TW, and MBR wrote the paper. ‡These authors contributed equally. (match statement to author names with a symbol)

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ABBREVIATIONS

CDK; Cyclin Dependent Kinase; CDKi, CDK inhibitor; BRET, Bioluminescence Resonance Energy Transfer; PKIS2, Published Kinase Inhibitor Set 2

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