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COMMUNICATION

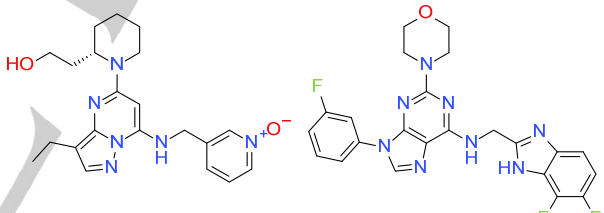
Structure Based Design of Selective Non-covalent CDK12 Inhibitors

Jeffrey W. Johannes,* Christopher R. Denz, Nancy Su, Allan Wu, Anna C. Impastato, Scott Mlynarski, Jeffrey G. Varnes, D. Bryan Prince, Justin Cidado, Ning Gao, Malcolm Haddrick, Natalie H. Jones, Shaobin Li, Xiuwei Li, Yang Liu, Toan B. Nguyen, Nichole O'Connell, Emma Rivers, Daniel W. Robbins, Ronald Tomlinson, Tieguang Yao, Xiahui Zhu, Andrew D. Ferguson, Michelle L. Lamb, John I. Manchester, Sylvie Guichard

Abstract: CDK12 knockdown via siRNA reduces transcription of DNA damage response genes and sensitizes BRCA wild type cells to PARP inhibition. To recapitulate this effect with a small molecule, we sought a potent, selective CDK12 inhibitor. Crystal structures and modeling informed hybridization between dinaciclib and SR-3029, resulting in lead compound **5**. Further structure guided optimization delivered a series of selective CDK12 inhibitors, including compound **7**. Profiling of this compound across CDK9, 7, 2 and 1 at high ATP concentration, single point kinase panel screening against 352 targets at 0.1 μM, and proteomics via kinase affinity matrix technology demonstrated the selectivity. This series of compounds inhibit phosphorylation of Ser2 on the C-terminal repeat domain of RNA pol II, consistent with CDK12 inhibition. These selective compounds were also acutely toxic to OV90 as well as THP1 cells.

Cyclin-dependent kinase (CDK) 12 forms a catalytically active enzyme complex upon association with cyclin K (CycK).[1] The CycK/CDK12 complex phosphorylates the C-terminal domain (CTD) heptapeptide repeat (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) of RNA pol II, specifically recognizing pSer7 and phosphorylating Ser2 and Ser5 of the CTD.[2] The CTD of RNA pol II recruits proteins for RNA processing, including the spliceosome for RNA splicing. It has been shown that siRNA-mediated knockdown of CDK12 reduced expression of a small number of genes, with a disproportional effect on long genes and genes with a high number of exons. Systematic analysis of these genes with reduced expression found that many of them were associated with DNA damage repair (DDR) networks, with BRCA1 as a key gene showing reduced expression.[3] In a related work, mutations to CDK12[4] found in ovarian cancer samples that impaired kinase activity had a detrimental effect on homologous repair, and tended not to co-occur with BRCA1 mutations.[5]

Table 1. Published pan-CDK inhibitor dinaciclib[16] and dual CK1δ/CK1ε inhibitor SR-3029.[17]



dinaciclib (**1**) SR-3029 (**2**)

	1	2
CDK12 Km* ATP IC_{50} (μM)	0.020	0.086
CDK12 high† ATP IC_{50} (μM)	0.050	>30
CDK9 Km ATP IC_{50} (μM)	<0.003	11.3
CDK7 Km ATP IC_{50} (μM)	0.042	>16.5
CDK2 Km ATP IC_{50} (μM)	<0.003	>30
CDK1 Km ATP IC_{50} (μM)	0.012	>30

* For Km assays [ATP] ranges from 10 to 50 μM (see SI for specific values used). † High assays [ATP] = 5000 μM.

Interest in targeting DNA damage repair mechanisms in tumors has grown following the approval of the PARP inhibitor olaparib.[6] PARP inhibitors are effective in tumors that harbour mutations to BRCA1, and are hence deficient in homologous repair (HR). Cells that are deficient in HR have difficulty in correctly repairing double strand breaks. PARP inhibitors which trap PARP1 on DNA cause excess production of double strand breaks, which is toxic to HR deficient cells.[7] The link between

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Supporting information for this article is given via a link at the end of the document.

CDK12 and BRCA1 leads to the hypothesis that synergy may be observed between CDK12 knockdown and PARP inhibition. Indeed, Bajrami et al. found that knockdown of CDK12 in OV90 ovarian cancer cells rendered the cells susceptible to the PARP inhibitor olaparib.[8]

These results suggest that a small molecule CDK12 inhibitor used in combination with a PARP inhibitor such as olaparib could be efficacious in BRCA1 wild type cells, and extend the utility of PARP inhibition beyond a BRCA1 mutant background.[9] To test this hypothesis and validate CDK12 as an oncology target we set out to discover a highly selective CDK12 small molecule inhibitor to ensure that the observed effects were due to the target of interest. Recently, Zhang et al. reported the discovery of THZ531, a small molecule covalent inhibitor of CDK12 that contains an acrylamide group that reacts with Cys1039 in CDK12.[10] THZ531 reduced the expression of DDR associated genes in a manner similar to siRNA CDK12 knockdown experiments.

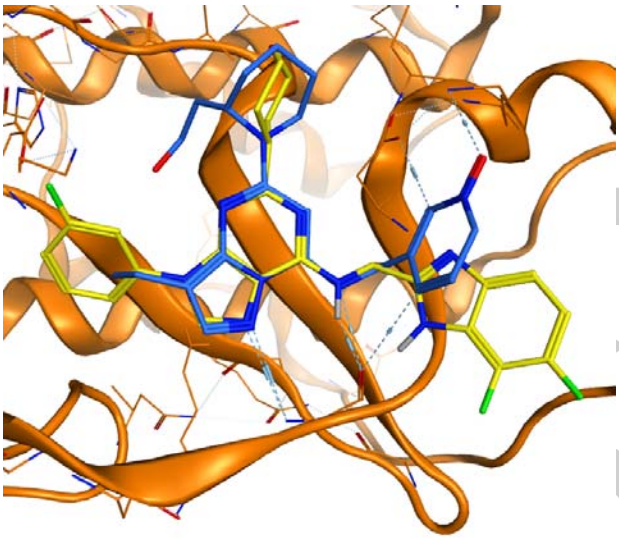


Figure 1. Overlay of dinaciclib in blue bound to CDK2 in orange (PDB 4KD1) with SR-3029 in yellow docked to CDK12 structure PDB 4CXA (CDK12 protein not shown for clarity).

CDK12 is a member of the cyclin dependent kinase (CDK) family of enzymes, which have been the subject of intense efforts across academia and the pharmaceutical industry[11] as oncology agents.[12,13] The family contains 20 members which can be divided into the cell cycle kinases (including CDK1,2,3,4, and 6) and the transcriptional CDKs (including CDK12,13, 9, 8, and 7).[14] CDK12 is very closely related and highly homologous to CDK13, and the ATP binding sites of these proteins are essentially identical,[15] however, knockdown of CDK13 affects different genes compared to CDK12, having effects on growth signalling pathways.[16] From the outset, we concluded that selectivity between CDK12 and CDK13 would be extremely difficult to obtain, so we initially focused on obtaining selectivity over CDK9 and CDK7 as transcriptional CDKs, and CDK2 and 1 as representative and well known examples of the cell cycle CDKs.

In order to screen and optimize for a selective non-covalent CDK12 inhibitor, we developed a battery of CDK enzyme assays using Caliper technology that could be run at low (Km) and high (5 mM) ATP concentrations for CDK12, 9, 7, 2, and 1. We felt that this panel covered both transcriptional and cell cycle CDKs sufficiently for screening. Our goal was to discover a compound with a CDK12 IC_{50} <500 nM at high ATP, with 10-fold high ATP selectivity to the other CDKs in the panel. Utilizing 5 mM ATP mimics the cellular concentration of ATP and provides an initial picture of kinase selectivity in a cellular context.

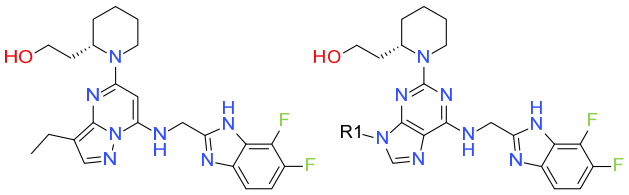
We initially screened a number of CDK family and other related Ser/Thr kinase inhibitors across this panel. One of our initial hits was dinaciclib (**1**, Table 1) a known CDK inhibitor in clinical trials in cancer.[17] Dinaciclib was very potent in our CDK12 assays at both Km (20 nM) and high ATP (50 nM). However, dinaciclib is a pan CDK inhibitor, showing potent inhibition of CDK9, 7, 2, and 1 at Km ATP. In the same screen, we identified SR-3029 (**2**, Table 1), a published CK1δ and CK1ε inhibitor,[18] as having some modest CDK12 inhibition at Km (86 nM). Unfortunately, this compound didn't show any inhibition of CDK12 at high ATP. Strikingly, SR-3029 did not inhibit CDK9 ,7, 2, nor 1 at Km ATP, demonstrating that selective CDK12 inhibition is possible within the CDK family.

With these two inhibitors in hand, one very potent but non-selective and the other very selective but lacking potency, we hypothesized that these two compounds adopt a common binding mode to CDK proteins. We docked SR-3029 to a published structure of CDK12 (PDB 4CXA)[19] and aligned and superposed this structure with PDB 4KD1, the crystallographically observed binding mode of dinaciclib bound to CDK2 (Figure 1).[20] Indeed the two compounds' heterocyclic hinge-binding cores of both compounds overlap, in a way which which perfectly aligns the peripheral groups. This model suggested that hybridization of the two compounds was possible.

At first, it was unclear which portions of dinaciclib conferred its exquisite potency and which moieties on SR-3029 gave high selectivity for CDK12. From reported SAR on the chemical series that culminated in dinaciclib, we knew that the hydroxyethyl piperidine group occupying the ribose pocket was very important for binding, but the effects of the core heterocycle or other substituents were unknown.

The first hybrid that we made was compound **3** (Table 2), which swapped the pyridine N-oxide solvent tail of dinaciclib for the difluorobenzimidazole solvent tail of SR-3029. This hybrid maintained similar potency compared to dinaciclib with improved CDK7 and CDK1 selectivity. Keeping this bezimidazole solvent tail fixed, we next explored the effects of other positions on potency and selectivity. Our first example of this is illustrated by compound **4** which compares the fluorophenyl group found in SR30-29 with the ethyl group of dinaciclib. Although compound **4** is highly selective, it has lost CDK potency across the panel, including potency at our primary target CDK12. Compound **5** is a matched pair comparing the pyrazolopyrimidine core of dinaciclib

with the purine core of SR-3029. The purine core further improved selectivity against CDK9, 7 and 2 with only a marginal loss of CDK12 activity. CDK enzyme potency and selectivity is significantly affected by the hydrophobic group occupying the selectivity pocket. Compound **5**, with a CDK12 high ATP potency of 103 nM, was quite selective against CDK7 and CDK1, had an acceptable margin to CDK9, but only a 3-fold margin to CDK2 at high ATP concentration.



3

R1 = 3-F-phenyl (**4**)
R1 = Et (**5**)

Table 2. Hybridization between dinaciclib and SR-3029 results in potent and selective lead compound **5**..

	3	**4**	**5**
CDK12 Km ATP IC_{50} (μM)	0.022	0.065	0.014
CDK12 high ATP IC_{50} (μM)	0.047	3.62	0.103
CDK9 high ATP IC_{50} (μM)	0.240	>30.0	1.09
CDK7 high ATP IC_{50} (μM)	21.2	>30	>30
CDK2 high ATP IC_{50} (μM)	0.040	>30	0.321
CDK1 high ATP IC_{50} (μM)	3.41	>30	28.6
Aq. Sol. pH 7.4 (μM)	9	3	39
LogD pH 7.4	4.1	>3.3	4.1

To understand the subtleties of how the compound binds and to confirm our hypothesized binding mode, we obtained a crystal structure of compound **5** bound to CDK12 in complex with cyclin K (Figure 2). Compound **5** binds to the backbone NH of hinge residue Met816 via the purine N-7, and the backbone carbonyl of Met816 via the NH at the purine 6-positon. The difluorobenzimidazole occupies the solvent channel, with the imidazole moiety engaging in hydrogen bonds to Tyr815 and Asp819, while also interacting with the Trp1036 of the C-terminal helix of CDK12. We posit that the benzimidazole drives selectivity, since Tyr815 is a Phe in CDK9, 7, 2, and 1 and the other CDKs completely lack a C-terminal helix containing the Trp. The hydroxyethyl piperidine attached to the purine core at C-2 occupies the ribose pocket. The ethyl group attached to purine core N-9 occupies the selectivity pocket, forming a hydrophobic interaction with Phe813. Examining the surface of the binding pocket (hydrophobic patches in green and hydrophilic patches in purple) we noticed that there was a considerable amount of empty space surrounding the ethyl group at N-9. We thought that expanding the steric size of the group at N-9 to fill this space would improve potency and possibly confer additional selectivity for CDK12.

To this end, we synthesized numerous analogs exploring substitutions at the N-9 position (varying R1 in Table 3). The CDK12 high ATP potency and CDK panel selectivity at high ATP was extremely sensitive to changes in the steric size and nature of the hydrophobic group at R1. For example, simple monofluorinaton of the ethyl group in compound **5** to give fluoroethyl analog **6** resulted in an almost 4-fold loss in CDK12 high ATP potency while having little impact on selectivity for CDK9 and CDK2 (still approximately 10-fold and 3-fold respectively).

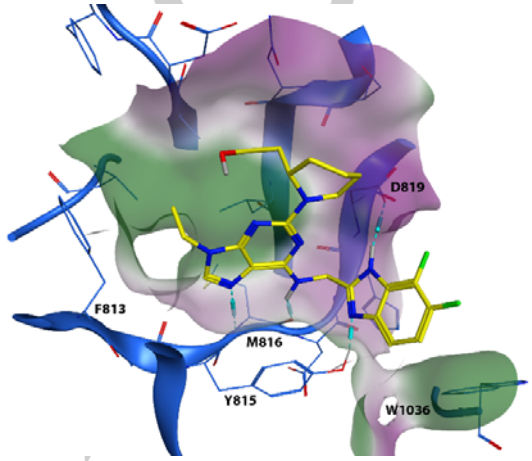


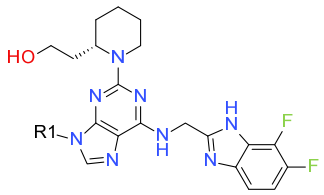
Figure 2. Crystal structure of compound **5** in yellow bound to CDK12 in blue (PDB 6B3E).

Introduction of branching at R1 with an isopropyl group improved the margin to CDK9 to approximately 20-fold at high ATP. Importantly, this branching also improved the gap between CDK12 and CDK2 to 5-fold. Overall, compound **7** is a 491 nM inhibitor of CDK12 at high ATP with improved selectivity to CDK9 and CDK2 compared to ethyl analog **5**. While the potency of compound **7** is reduced approximately 5-fold compared to **5**, the added selectivity is desired to support the use of the compound as an in vitro tool to validate small molecule inhibition of CDK12 as an oncology target. Difluoroethyl analog **8** showed a further reduction in CDK12 high ATP potency, highlighting the sensitivity to sterics at this position of the molecule. Compound **8** had a similar margin to CDK9 and CDK2 compared to compound **7**.

We selected **7** for further profiling to understand the selectivity of this compound across the kinome. The percent inhibition results when tested at a single concentration of 0.1 μM across a mixed panel of 352 ThermoFisher assays testing for kinase binding or kinase enzyme inhibition at various ATP levels are presented in Figure 3. Compound **7** is a selective inhibitor across the kinome. The top his in the panel are presented in the inset of Figure 3. Outside of the CDK family, inhibited kinases include IRAK1 (32%), PI4KB (29%), and TAOK1 (27%) at 0.1 μM compound concentration.[21] We also tested the kinase selectivity of compound **7** via affinity proteomics using a kinase affinity matrix.

In this experiment, CDK12 was the most significantly competed kinase off the solid matrix by compound **7**, followed by CDK13 and CDK9 to a lesser extent.[22] Compound **7** was also relatively clean against a diverse panel of human secondary pharmacology targets (see the supporting information table S5).

We next turned our attention to testing these compounds in our mechanism of action assay, which detects inhibition of phosphorylation of serine 2 (pSer2) on the C-terminal repeat domain of RNA pol II (Table 4). Selective compounds **7** and **8** inhibited Ser2 phosphorylation in MCF7 cells. Other potent but less selective CDK12 inhibitors such as dinaciclib (**1**) and compounds **3**, **5**, and **6** also inhibited pSer2. Importantly, a compound with weaker inhibition of CDK12 at high ATP, such as **4**, inhibited pSer2 to a lesser extent. Compound **2** is potent for neither CDK12 nor CDK9 at high ATP but still inhibits pSer2 with an IC_{50} 274 nM. Compound **2** was originally reported as a CK1δ and CK1ε inhibitor (ref. 18) and is highly selective for these kinases (see supporting information table S3 and S4). CK1 isoforms have been reported to associate with the CTD of RNA pol II,[23] and is possible that inhibition of CK1δ/ε kinase activity can inhibit phosphorylation of pSer2. Overall there is a good correlation between inhibition of CDK12 at high ATP and pSer2 inhibition.[24]



$R^1 = CH_2CH_2F$ (**6**)
R^1 = iPr (**7**)
$R^1 = CH_2CHF_2$ (**8**)

Table 3 Optimization of the purine N-9 substituent results in potent and selective lead compound **7**

	6	**7**	**8**
CDK12 Km ATP IC_{50} (μM)	0.017	0.031	0.023
CDK12 high ATP IC_{50} (μM)	0.389	0.491	0.845
CDK9 high ATP IC_{50} (μM)	4.94	9.09	19.6
CDK7 high ATP IC_{50} (μM)	>30	>30	>30
CDK2 high ATP IC_{50} (μM)	1.13	2.68	4.29
CDK1 high ATP IC_{50} (μM)	>30	>30	>30
Aq. Sol. pH 7.4 (μM)	53	18	50
LogD pH 7.4	3.7	4.6	>4.0

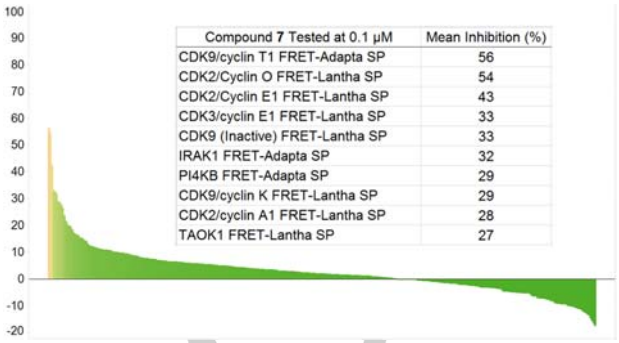


Figure 3. Percent inhibition results when tested at a single concentration of 0.1 μM across a mixed panel of 352 ThermoFisher assays testing for kinase binding or kinase enzyme inhibition at various ATP levels of compound **7**, with percent inhibition data for the top 10 hits listed in the inset table.

Table 4. CDK12 and CDK8 high ATP enzyme inhibition data compared to the cell assay results for inhibition of Ser2 phosphorylation in MCF7, 48 h growth inhibition in OV90 cells, and THP1 cell cytotoxicity.

Cmpd	CDK12 high ATP IC_{50} (μM)	CDK9 high ATP IC_{50} (μM)	MCF7 pSer2 IC_{50} (μM)	OV90 GI_{50} 48 h (μM)	THP-1 IC_{50} (μM)
3	0.047	0.240	0.012	0.104	
1	0.050	<0.010	<0.005	0.021	0.067
5	0.103	5.14	0.012	0.099	0.106
6	0.389	4.94	0.032	0.044	0.251
7	0.491	9.09	0.098	0.204	1.54
8	0.845	19.6	0.094	0.067	0.830
4	3.62	>30	0.336	0.373	2.58
2	>30	>30	0.274	0.321	2.17
9	>30		>3		>250
10	0.213	>30	0.039	0.102	0.093

‡ Ranked in order of CDK12 high ATP potency.

With selective compounds that can modulate pSer2 in cells, we next sought to see if these compounds were suitable for testing the hypothesis that a CDK12 enzyme inhibitor could synergize with a PARP inhibitor (such as olaparib) in a 14-day colony formation assay in OV90 cells. Prior to committing to a long-term assay, we tested this series of CDK12 inhibitors in a 48 h growth inhibition assay to check for acute toxicity to OV90 cells (Table 4). Interestingly, compounds **5**, **6**, **7**, and **8** were toxic to OV90 cells as single agents, with 48 h GI_{50}'s ranging from 21 to 373 nM. The growth inhibition IC_{50}'s correlated well with CDK12 inhibition and pSer2 inhibition, suggesting that CDK12 is driving this growth inhibitory effect. As a negative control, we tested ribociclib (**9**), a selective CDK4/6 inhibitor.[25] Compound **9** lacks CDK12 activity, is inactive in our pSer2 assay up to the highest

concentration tested, and is not toxic to THP1 cells (IC50 >250 μM). As a positive control, we included compound **10** (THZ531)[10] which is a potent inhibitor of CDK12 (213 nM) and lacks CDK9 inhibition. In our hands, THZ531 inhibited pSer2 at 39 nM, inhibited the growth of OV90 cells at 102 nM and had 93 nM IC_{50} against THP1 cells.

Since the OV90 cell line does not harbor a BRCA mutation, we did not expect single agent efficacy with a CDK12 inhibitor a priori. Alternatively, it has been reported that modulation of pSer2 itself can have cytotoxic effects, as previously reviewed for CDK inhibitors.[26] In this vein, we hypothesized that these compounds might be toxic to blood cells, so we tested them in a 24 h cytotoxicity assay against THP1 cells, an AML cell line used routinely in discovery phase safety departments to obtain an early read on unwanted cytotoxicity. Indeed, this series of CDK12 inhibitors were toxic to THP1 cells. For example, highly potent CDK12 compound **5**, which was approximately 50-fold selective against CDK9, had a THP1 IC_{50} of 106 nM. More importantly, there was a relatively small difference between effects on pSer2 (12 nM) and OV90 growth inhibition (99 nM) and THP1 cytotoxicity for this compound. The compound with the biggest difference between pSer2 and OV90 effects and toxicity was compound **8**, a very selective, but slightly less active CDK12 inhibitor. Even in this case the margin was only approximately 10-fold.

From these data we surmised that selective CDK12 inhibition causes effects on the phosphorylation state of Ser2 of RNA pol II, and that this mechanism can have single agent toxic effects on cells. This hints at a possible therapeutic use for CDK12 inhibitors; however, the effects on pSer2 may carry the risk of a very tight therapeutic margin in vivo. The pioneering work by Johnson et al.[9] have demonstrated that it is possible to show synergy between dinaciclib (a CDK2, 9, and 12 inhibitor) with the PARP inhibitor veliparib in vivo in certain patient-derived tumor xenograft models. There may also be inherent differences between the biology of siRNA mediated knockdown and small molecule inhibition of CDK12. Small molecule inhibited CDK12 may keep cyclin-K sequestered via a CDK12:cyclinK interaction, while knockdown of CDK12 may create additional free cyclin-K.

In conclusion, we started with dinaciclib as a highly potent CDK12 inhibitor and SR-3029 as a weak but highly selective CDK12 inhibitor vs CDK9, 7, 2, and 1. Using a known crystal structure of dinaciclib bound to CDK2 and computational docking of SR-3029 to a CDK12 crystal structure, we hypothesized that hybridization of these two compounds might result in a potent and selective CDK12 inhibitor. We found that the difluorobenzimidazole moiety was key for CDK family selectivity, and that the purine core lent further selectivity for CDK12. Optimization of the purine N-9 group led to isopropyl compound **7** with 5-fold CDK2 and near 20-fold CDK9 selectivity. Lead compound **7** was profiled across a panel of 352 kinases at 0.1 μM followed by concentration response testing in 30 of the top hits, showing that **7** is a highly selective CDK12 inhibitor, and this result was confirmed by affinity proteomics using a kinase affinity matrix. This series of compounds showed potent inhibition of phosphorylation of Ser2 on the CTD repeat domain of RNA Pol II as well as growth inhibition of OV90 cells and acute cytotoxicity to THP1 cells.

Experimental Section

See supporting information for details of compound synthesis, crystallographic methods, computational methods, and enzyme and cell assay protocols.

Acknowledgements

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Keywords: CDK • kinase • transcription• selective • oncology

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Mix and Match: Structure guided hybridization of dinaciclib (**1**), a potent pan CDK inhibitor, with SR-3029 (**2**) a compound with weak, but selective CDK12 activity, resulted in lead compound **7**, a highly potent and selective CDK12 inhibitor. The selectivity of compound **7** amongst the CDK family and across the kinome, is supported by high ATP enzyme assays, single point kinase panel data, and affinity proteomics using kinase affinity matrix proteomics.

Jeffrey W. Johannes, Christopher R. Denz, Nancy Su, Allan Wu, Anna C. Impastato, Scott Mlynarski, Jeffrey G. Varnes, D. Bryan Prince, Justin Cidado, Ning Gao, Malcolm Haddrick, Natalie H. Jones, Shaobin Li, Xiuwei Li, Yang Liu, Toan B. Nguyen, Nichole O'Connell, Emma Rivers, Daniel W. Robbins, Ronald Tomlinson, Tieguang Yao, Xiahui Zhu, Andrew D. Ferguson, Michelle L. Lamb, John I. Manchester, Sylvie Guichard*

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Structure Based Design of Highly Selective Non-covalent CDK12 Inhibitors