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A Triple Exon-Skipping Luciferase Reporter Assay Identifies A New CLK Inhibitor Pharmacophore

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Abstract

The splicing of pre-mRNA is a critical process in normal cells and is deregulated in cancer. Compounds that modulate this process have recently been shown to target a specific vulnerability in tumors. We have developed a novel cell-based assay that specifically activates luciferase in cells exposed to SF3B1 targeted compounds, such as sudemycin D6. This assay was used to screen a combined collection of approved drugs and bioactive compounds. This screening approach identified several active hits, the most potent of which were CGP-74514A and aminopurvalanol A, both have been reported to be cyclin-dependent kinases (CDKs) inhibitors. We found that these compounds, and their analogs, show significant cdc2-like kinase (CLK) inhibition and clear structure-activity relationships (SAR) at CLKs. We prepared a set of analogs and were able to ‘dial out’ the CDK activity and simultaneously developed CLK inhibitors with low nanomolar activity. Thus, we have demonstrated the utility of our exon-skipping assay and identified new molecules that exhibit potency and selectivity for CLK, as well as some structurally related dual CLK/CDK inhibitors.

Graphical abstract

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest

Supporting Information

All experimental details on the chemistry, screening methods and results, including examples of the MDM2-Luc curves and EC₅₀s for sudemycin D6, KH-CB19 and compounds **1**, **2**, **5–10**, and **13–15**. Additionally complete inhibition data for the 20-kinase profile of compounds **8**, **9**, and **14** is included.

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INTRODUCTION

The processing of pre-mRNA to mature mRNA in metazoans is a critical process for the development and normal functioning of cells. The pre-mRNA splicing process involves the removal of intervening sequences from pre-mRNA followed by the ligation of exons to form mature mRNA. This splicing process is catalyzed and regulated by a highly complex macromolecular protein-RNA complex called the spliceosome. The spliceosome is composed of five small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4, U5 and U6) and over 150 associated proteins.^{1, 2} The pre-mRNA maturation process includes alternative splicing (AS), which is the mechanism that allows for different forms of mature mRNAs to be generated from the same pre-mRNA. Commonly, alternative splicing patterns determine the inclusion or exclusion of portions of the coding sequence in the mRNA, giving rise to protein isoforms that differ in their peptide sequence. Alternative splicing is regulated by numerous spliceosomal transacting proteins, which are in turn regulated by cis-acting regulatory sites on pre-mRNA substrates.¹ Since pre-mRNAs for a given gene may contain many different exon and intron combinations, there are often a very large number of possible mRNAs that can lead to a correspondingly large set of proteins with different, even opposing, biological functions within the cell. The complexity of the spliceosome and the current scarcity of molecular-resolution X-ray structures complicates a rapid advancement in the understanding of many of the important functional mechanisms that are critical to the normal functioning of the cells of higher organisms.³ Because of the importance of splicing in normal organismal development, the spliceosome is increasingly being recognized as a major frontier for molecular biology and is now accepted as a valid oncology target.^{4, 5}

Interest in the spliceosome was dramatically bolstered when two independent groups reported that a pair of structurally divergent bacterial natural products, FR901464 and pladienolide, both target a similar site on the SF3B subunit of the spliceosome.^{6, 7} Subsequent to those initial discoveries the list of compounds that are known to target the SF3B subunit has grown to include additional bacterial natural products such as herboxidiene (GEX1A)⁸ (isolated from *Streptomyces* sp. A7847) and the thailanstatins (isolated from *Burkholderia thailandensis*).⁹ These bacterial fermentation products show cytotoxic IC₅₀s in the low nanomolar range in tumor cell lines and were reported to have a similar distinctive effect on the cell cycle in mammalian cell lines, which includes cell cycle arrest in the G1 and G2/M phases.¹⁰ Several of these natural products have also been reported to show potent antitumor activity *in vitro* and *in vivo*.^{11, 12} Work in this area led to the development of the semisynthetic pladienolide analog E7107 that entered Phase I clinical studies,^{7, 13, 14} without the benefit of many subsequent recent discoveries relevant to mechanism of action, tumor selectivity, and patient stratification.^{4, 5}

As part of our effort to develop a class of drug-like synthetic spliceosome modulators our group has previously reported the design and synthesis of FR analogs that contain only 3 chiral centers (the sudemycins),^{15, 20–22} pladienolide analogs,²³ and several herboxidiene analogs²⁴ all of which are active compounds that effectively modulate alternative splicing.²² Also, new details regarding the mechanism of action of SF3B1 targeted agents have been elucidated.²⁵ Additionally results of the genome-wide array analysis of sudemycin treated tumor cells, which shows that sudemycins cause a rapid wide-ranging change in alternative

pre-mRNA splicing and that a biotin-labeled sudemycin probe directly interacts with the SF3B1 protein have been reported.²⁶ This collaborative project ultimately led to sudemycin D6 (SD6),¹⁵ which is currently in preclinical development as an anticancer agent (see Figure 1). Several groups have independently discovered new diverse small molecule structural classes that effect pre-mRNA splicing, using a range of screening strategies (see Figure 1). These compounds include TG-003,¹⁶ KH-CB19,¹⁷ Araki Cpd-2,¹⁸ and Madrasin¹⁹ (see Figure 1). Compounds KH-CB19 and the Araki Cpd-2 have been reported to be highly selective inhibitors of the of cdc2-like kinase (CLK) family,^{17, 18} while the molecular target of Madrasin has not been reported.¹⁹

In parallel to the work described above, strong evidence has continued to mount that aberrant splicing of pre-mRNA is a driver of tumorigenesis²⁷ and that the spliceosome is a valid target for cancer therapy.^{4, 5} Recent groundbreaking discoveries have identified recurrent mutations in SF3B1 (and/or other splicing factors) in multiple forms of cancer including: myelodysplastic syndromes (MDS),^{14,28} chronic lymphocytic leukemia (CLL),²⁹ acute myeloid leukemia (AML),^{30,31} breast cancer,^{32, 33} lung adenocarcinoma,³⁴ and uveal melanoma.³⁵ These genetic studies have also fueled complementary research in the therapeutic significance of spliceosome recurrent mutations. Very recently the selective sensitivity of tumors to agents that target SF3B1 have also been linked to overexpression of MYC.^{36, 37} Thus the collective progress in natural product screening, target identification, spliceosome related medicinal chemistry, and high-throughput transcriptome sequencing has led to a remarkable convergence of independent research areas, which have simultaneously identified new oncology drug targets and new small-molecule therapeutic leads.

RESULTS AND DISCUSSION

We have previously demonstrated that the SF3B1 targeted splicing modulator drugs potently induce exon skipping in MDM2 pre-mRNA.^{22, 24} More specifically, splicing of MDM2 is altered in response to treatment with the sudemycins, and other SF3B1 active agents, such that exons 4–11 are skipped.²² Based on these observations, we designed an exon-skipping MDM2-Luc reporter (see Figure 2).³⁸ The principal strategy in the design of this reporter is that, when expressed, cellular luminescence is dependent on the production of a correctly spliced full-length luciferase transcript due to the drug induced skipping of MDM2 cassette exons 4, 10, and 11, which interrupt the luciferase gene in this construct in the absence of a splicing modulator drug (see Figure 2). We have previously reported the use of this construct as a reporter for the real time pharmacodynamics of SF3B1 targeted agents *in vivo*, via a stable reporter cell line.³⁸ Using a stable cell line expressing this reporter, luminescent signals are generated in a dose-dependent fashion in the presence of splicing modulators (such as the sudemycins, herboxidiene and pladienolide B).³⁸ We have used this assay to screen a small molecule library (composed of 830 known drugs and 4,359 bioactive compounds)³⁹ and identified several active ‘hit’ compounds. These hits were then subjected to dose-response assays in the MDM2-Luc exon-skipping assay and those actives were then confirmed in an orthogonal PCR-based MDM2 alternative splicing assay in Rh-18 cells (see Supporting Information).²² Two structurally related ‘hits’ demonstrated activity in both of these screens that is similar (but less potent) to that observed for SF3B1 targeted agents such

as SD6 (see Supporting Information). The most potent of these confirmed hits are shown in Figure 3 below.

These two confirmed hits, CGP-74514A (**1**)⁴⁰ and aminopurvalanol A (**2**)⁴¹ are known to inhibit cyclin-dependent kinases (CDKs), and in particular CDK1.⁴¹ Compound **1** was reportedly optimized for selective CDK1 activity through a significant analoging effort.⁴⁰ However, structurally similar compounds have been reported to have a very broad spectrum of biological activities,⁴¹ which indicates that this inhibitor class may also inhibit other important protein kinases. In order to narrow down the possible identity of the target class that led to exon-skipping in our assay we then screened additional commercially available selective kinase inhibitor tool compounds that have shown cell-based activity.⁴² The compounds that we evaluated included the CDK selective clinical compound dinaciclib (a nanomolar inhibitor of CDK1, CDK2, CDK5, and CDK9),⁴³ SRPIN340 (selective serine arginine protein kinase (SRPK) 1 inhibitor ($K_i = 0.89 \mu\text{M}$; this compound inhibits SRPK1 and SRPK2 but does not significantly inhibit other SRPKs such as CLK1 and CLK4, or other classes of SR kinases).⁴⁴ We found all of these compounds to be inactive (at $10 \mu\text{M}$) in the MDM2-Luc assay, which eliminated SRPK1, SRPK2, CDK1, CDK2, CDK5 and CDK9 as the *likely* targets that are responsible for the observed activity in our MDM2-Luc cell based assay.

In contrast to the lack of activity of the tool compounds discussed above, the CLK1/2/4 cell-active selective kinase inhibitor KH-CB19 showed modest activity in the MDM2-Luc assay (see Supporting Information).¹⁷ These results, taken together with the recent observations of pronounced modulation of splicing (through inhibition of the CLK mediated phosphorylation of SR proteins¹⁷) by compounds such as Araki compound-2 (see Figure 1),¹⁸ strongly suggested that CLK inhibition was responsible for the alternative splicing effects seen with compounds **1** and **2**; though splicing modulation has not been recognized as an activity of **1** or **2**, to our knowledge. In order to explore the CLKs as possible targets, and to better understand the structure-activity relationships (SAR), additional new structurally related analogs along with the associated CLK biochemical inhibition data were clearly needed so decided to evaluate the most potent of these compounds (compound **1**) for CLK activity. Remarkably, as shown below (see Table 1 and Table 2) we found that compound **1** is a more potent inhibitor of CLK1, CLK2 and CLK 4 than it is as at CDK1, based on biochemical assays.

We therefore prepared a set of analogs starting from the versatile starting material **3** of the general structure **4** containing some of the pharmacophore features of **1** and/or **2** (see Scheme 1 and Table 1) using well-precedented chemistry that has been used to make similar analogs,^{40, 41, 45} in order to explore the nature of the pharmacophore that led to the observed activation of the triple-exon skipping in our assay.³⁸ As can be seen by inspection of Table 1, replacement of the N7 nitrogen with CH completely abrogated all activity at $10 \mu\text{M}$ (compound **11**), as did acetylation of the basic primary amine (compound **12**), and dramatically reduced activity by replacement of the valanol group of **2** with an unsubstituted methyl ether (compound **13**). The absolute and relative stereochemistry of the cyclohexane diamine also affected the activity. By combining potential interaction features from both **1** and **2** into compound **14** and into the acetanilide derivative **15**, we observed strong MDM2-

Luc activity (see Supporting Information). Thus, with this set of compounds we have identified some of the critical geometric and interaction pharmacophore features that lead to activation in the MDM2-Luc assay.

Since the pharmacophores present in **1** and **2** do not match the previously reported consensus pharmacophore for agents that target the SF3B1 splicing protein,^{15, 20, 24} it appeared unlikely that **1** or **2** target the sudemycin D6 binding site on SF3B1. However, since it is known that CLK inhibition can lead to modulation of pre-mRNA splicing,¹⁸ and since we observed that the weakly cell-active, but selective, CLK inhibitor KH-CB19 shows activity in the MDM2-Luc reporter assay, we decided to explore the activity of **1** (and our active analogs) in regard to their biochemical inhibition of CLK 1-4 (see Table 2). The results summarized in Table 2 clearly show that CGP-74514A (**1**), which has been considered a selective CDK inhibitor⁴⁶ actually also shows significant inhibition of CLK 1, 2, and 4 and so can be considered a dual CLK/CDK inhibitor. Additionally some of our analogs of **1** and **2** were more active for three of the four CLK subtypes and the potency trend of CLK inhibition across the subtypes is similar to the trend that was observed in the MDM2-Luc reporter assay, in that more potent CLK inhibitors (compounds **9**, **10**, **14**, **15**) showed a strong luciferase response (see Supporting Information). Interestingly compound **8** shows some modest (~5 fold) selectivity for CLK2 over CLK1 and CLK4 without significant CDK1,4,6 activity and compounds **9**, **10**, **14** and **15** that showed the strongest Luc activation also show potent CLK inhibition for the 1, 2 and 4 subtypes. We also screened compounds that were inactive in the triple-exon skipping assay, which were the closest analogs to the active compounds (**11**, **12** and **13**) for CLK1 activity (as described in the Experimental Section below) Analogs **11** and **12** have IC₅₀s for CLK1 >> 1 μM (data not shown) and thus serve as negative controls that further strengthen the connection between the CLK activity in our compounds and the activity observed in the triple-exon skipping screen. Compound **13** is active in the CLK assay, but has very poor solubility (data not shown), which may explain its inactivity in the reporter assay. The weakly cell active tool compounds Madrasin¹⁹ and TG-003¹⁶ were also screened for activity in the MDM2-Luc assay at a concentration of 10 μM, but neither compound showed significant activity in our hands in this screen (data not shown). This is in line with the weak activity reported for Madrasin with an EC₅₀ of ~ 20 μM.¹⁹ In regard to TG-003 it should be noted that the other CLK inhibitor tool compound that showed activity (KH-CB19, also reported to be weakly active in cells at 10 μM)¹⁷ barely passes the minimal hit criteria in our MDM2-Luc screen. This suggests that TG-003 may be even less 'drug like' in terms of its cell penetration. Given all of this information we selected the active compounds **8**, **9**, and **14** for kinase profiling against 25 kinases⁴² at a concentration of 1 μM in duplicate for each analog (see Figure 4 below and the Supporting Information). This kinase profiling screen showed <50% inhibition against the entire 22 diverse kinase panel for compound **8**, with some significant off-target activity for **9** only against Flt3, Src(1-530) and off-target activity for **14** against Flt3, Plk1, and Src(1-530) (see Supporting Information for complete screening results).

Given these promising results we decided to prepare a follow-up set of optically pure analogs **16–25** (see Table 3) that holds the R₁ group constant. This set of analogs includes different ring constraints and derivatives with a single stereocenter (compounds **22–25**). This

set of compounds was then subjected to the same kinase inhibition assays that had been performed on the compounds shown in Tables 1 and 2. This data is presented in Table 4. Many of these analogs showed potent activity at CLK1, CLK2 and CLK4 but exhibited some consistent variation depending on the stereochemistry of the diamine group and the nature of the diamine constraint. Compound **16** (with the 1*R*,2*S* configuration of the cyclohexyldiamine) showed the most potent inhibition of CLK2 (6 nM) of any compound evaluated. Compound **17**, the enantiomer of **16**, showed similar activity at CLK1 and CLK4 but was significantly less active at CLK2 (26 nM). A similar trend is seen with the enantiomers **18** and **19**. An even more pronounced stereochemistry driven structure-activity difference is seen between the 1*S*,2*S* cyclohexyldiamine analog **20** and its enantiomer **21**, with a potency difference of >10 fold for CLK2.

We also considered the published co-crystal structures CLK1 with the CLK selective inhibitor KH-CB19,¹⁷ and the promiscuous debromohymenialdisine,⁴⁷ which may be helpful in developing a pharmacophore hypothesis (see Figure 5) as part of future work. As shown in Figure 5 a potential a hypothetical binding mode can be proposed as a conceptual starting point. This possible pharmacophore is based on our initial SAR data, and the published CLK1/debromohymenialdisine structure together suggest a potentially important charge-charge hydrogen-bond interaction between CLK1 Asp-325 and the basic primary amine in the active selective compounds such as **8**, **9**, **14**, and **15**. Future work can test this hypothetical pharmacophore.

CONCLUSION

We have used our triple-exon skipping assay to screen a small molecule library (composed of 830 known drugs and 4,359 bioactive compounds³⁹, see Supporting Information) and have identified initial hits. Using directed chemistry, which included a range of optically pure analogs, we also identified new more potent and selective CLK inhibitors and associated structure-activity relationships. The fact that our screen identified CDK inhibitors that also show off-target effects against the highly homologous CLK subfamily is not surprising, given the close structural relationship between these two branches of the kinase family tree and the reported partial selectivity of many clinical kinase inhibitors.⁴⁹ We used dose response in our MDM2-Luc exon-skipping assay and the orthogonal PCR MDM2 alternative splicing assay in Rh-18 cells (see Supporting Information) to validate the active hits and analogs and as a guide in the development of SAR. Some of the new analogs showed significant activity in the MDM2-Luc assay and are potent biochemical inhibitors of CLK subtypes 1, 2 and 4. Several of these compounds show reasonable overall kinase selectivity, based on data from a total of 29 kinases (including CLK1-4; CDK 1, 4, and 6; and a screen against a panel of 22 oncogenic kinases, see SI). It is important to note that the well-studied tool compound CGP-74514A (**1**) shows significantly more biochemical activity against CLK 1, 2, and 4 than either CDK 1 or CDK 2, under the assay conditions that we used (see Experimental section below). Additionally, we found that CLK inhibition generally correlated with MDM2-Luc activity in this series of compounds. The lack of a close correlation between the MDM2 triple exon skipping and CLK inhibition and biochemical activity may be due to the fact that inhibitors of CLKs do not efficiently inhibit this unusual type of alternative splicing modulation, which has only been previously

reported for SF3B1 modulators.^{22, 24, 38} The use of the RT PCR assay as a confirmatory counterscreen for MDM2 exon skipping in Rh-18 cells validates this splicing effect in full length MDM2 pre-mRNA in a different cell background.²² Using this screening approach we have identified compounds that are dual CLK2/CDK1 inhibitors (such as **1**) that we optimized to yield more specific CLK inhibitors (such as SRI-29329, **8**). This is a notable result since CLK2⁵⁰ and CDK1⁵¹ have been independently identified as potential targets for breast cancer. Therefore these active analogs, SAR results and our MDM2-Luc assay may facilitate the development of new probe and lead molecules with potential application in breast cancer, and other diseases. Additionally the MDM2-Luc assay has proven its utility in the identification of compounds with splicing modulatory activity at targets other than SF3B1. The utility of the triple-exon skipping splicing modulatory screen is remarkably selective. None of the 830 FDA approved drugs showed activity at 10 μ M in our assay, indicating that this assay may be useful in selectively identifying compounds that act through other unique mechanisms involving modulation of pre-mRNA splicing that may be relevant to innovative drug discovery. Additionally our observations of overlapping pharmacophores for the CLK and CDK (and potentially structurally related CDK inhibitors) should be considered when interpreting biological results that are based on the cellular or in vivo responses to compounds such as CGP-74514A (**1**).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CLK	cdc2-like kinase
MDM2	mouse double minute 2 homolog
Luc	luciferase
TEA	triethylamine
DIPEA	diisopropylethylamine
EtOAc	ethyl acetate

References

1. Kramer A. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annual review of biochemistry. 1996; 65:367–409.
2. Wahl MC, Will CL, Luhrmann R. The spliceosome: design principles of a dynamic RNP machine. Cell. 2009; 136(4):701–718. [PubMed: 19239890]

3. Hang J, Wan R, Yan C, Shi Y. Structural basis of pre-mRNA splicing. *Science*. 2015
4. Webb TR, Joyner AS, Potter PM. The development and application of small molecule modulators of SF3b as therapeutic agents for cancer. *Drug Discovery Today*. 2013; 18(1–2):43–49. [PubMed: 22885522]
5. Bonnal S, Vigevani L, Valcarcel J. The spliceosome as a target of novel antitumour drugs. *Nat Rev Drug Discov*. 2012; 11(11):847–859. [PubMed: 23123942]
6. Kaida D, Motoyoshi H, Tashiro E, et al. Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. *Nature chemical biology*. 2007; 3(9):576–583. [PubMed: 17643111]
7. Kotake Y, Sagane K, Owa T, et al. Splicing factor SF3b as a target of the antitumor natural product pladienolide. *Nature chemical biology*. 2007; 3(9):570–575. [PubMed: 17643112]
8. Hasegawa M, Miura T, Kuzuya K, et al. Identification of SAP155 as the target of GEX1A (Herboxidiene), an antitumor natural product. *ACS chemical biology*. 2011; 6(3):229–233. [PubMed: 21138297]
9. Liu X, Biswas S, Berg MG, et al. Genomics-Guided Discovery of Thailandstatins A, B, and C As Pre-mRNA Splicing Inhibitors and Antiproliferative Agents from *Burkholderia thailandensis* MSMB43. *Journal of Natural Products*. 2013; 76(4):685–693. [PubMed: 23517093]
10. Nakajima H, Sato B, Fujita T, Takase S, Terano H, Okuhara M. New antitumor substances, FR901463, FR901464 and FR901465. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *The Journal of antibiotics*. 1996; 49(12):1196–1203. [PubMed: 9031664]
11. Nakajima H, Hori Y, Terano H, et al. New antitumor substances, FR901463, FR901464 and FR901465. II. Activities against experimental tumors in mice and mechanism of action. *The Journal of antibiotics*. 1996; 49(12):1204–1211. [PubMed: 9031665]
12. Mizui Y, Sakai T, Iwata M, et al. Pladienolides, new substances from culture of *Streptomyces platensis* Mer-11107. III. In vitro and in vivo antitumor activities. *The Journal of antibiotics*. 2004; 57(3):188–196. [PubMed: 15152804]
13. Eskens FA, Ramos FJ, Burger H, et al. Phase I pharmacokinetic and pharmacodynamic study of the first-in-class spliceosome inhibitor E7107 in patients with advanced solid tumors. *Clin Cancer Res*. 2013; 19(22):6296–6304. [PubMed: 23983259]
14. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011; 478(7367):64–69. [PubMed: 21909114]
15. Lagisetti C, Palacios G, Goronga T, Freeman B, Caufield W, Webb TR. Optimization of antitumor modulators of pre-mRNA splicing. *Journal of medicinal chemistry*. 2013; 56(24):10033–10044. [PubMed: 24325474]
16. Muraki M, Ohkawara B, Hosoya T, et al. Manipulation of alternative splicing by a newly developed inhibitor of CLKs. *The Journal of biological chemistry*. 2004; 279(23):24246–24254. [PubMed: 15010457]
17. Fedorov O, Huber K, Eisenreich A, et al. Specific CLK inhibitors from a novel chemotype for regulation of alternative splicing. *Chemistry & biology*. 2011; 18(1):67–76. [PubMed: 21276940]
18. Araki S, Dairiki R, Nakayama Y, et al. Inhibitors of CLK protein kinases suppress cell growth and induce apoptosis by modulating pre-mRNA splicing. *PloS one*. 2015; 10(1):e0116929. [PubMed: 25581376]
19. Pawellek A, McElroy S, Samatov T, et al. Identification of small molecule inhibitors of pre-mRNA splicing. *The Journal of biological chemistry*. 2014; 289(50):34683–34698. [PubMed: 25281741]
20. Lagisetti C, Pourpak A, Jiang Q, et al. Antitumor compounds based on a natural product consensus pharmacophore. *Journal of medicinal chemistry*. 2008; 51(19):6220–6224. [PubMed: 18788726]
21. Lagisetti C, Pourpak A, Goronga T, et al. Synthetic mRNA splicing modulator compounds with in vivo antitumor activity. *Journal of medicinal chemistry*. 2009; 52(22):6979–6990. [PubMed: 19877647]
22. Fan L, Lagisetti C, Edwards CC, Webb TR, Potter PM. Sudemycins, novel small molecule analogues of FR901464, induce alternative gene splicing. *ACS chemical biology*. 2011; 6(6):582–589. [PubMed: 21344922]

23. Gundluru MK, Pourpak A, Cui X, Morris SW, Webb TR. Design, synthesis and initial biological evaluation of a novel pladienolide analog scaffold. *Medchemcomm*. 2011; 2(9):904–908. [PubMed: 21927710]
24. Lagisetty C, Yermolina MV, Sharma LK, Palacios G, Prigaro BJ, Webb TR. Pre-mRNA splicing-modulatory pharmacophores: the total synthesis of herboxidiene, a pladienolide-herboxidiene hybrid analog and related derivatives. *ACS chemical biology*. 2014; 9(3):643–648. [PubMed: 24377313]
25. Effenberger KA, Urabe VK, Prichard BE, Ghosh AK, Jurica MS. Interchangeable SF3B1 inhibitors interfere with pre-mRNA splicing at multiple stages. *Rna*. 2016; 22(3):350–359. [PubMed: 26742993]
26. Convertini P, Shen M, Potter PM, et al. Sudemycin E influences alternative splicing and changes chromatin modifications. *Nucleic Acids Res*. 2014; 42(8):4947–4961. [PubMed: 24623796]
27. David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes & development*. 2010; 24(21):2343–2364. [PubMed: 21041405]
28. Ogawa S. Splicing factor mutations in myelodysplasia. *International journal of hematology*. 2012; 96(4):438–442. [PubMed: 23054646]
29. Damm F, Nguyen-Khac F, Fontenay M, Bernard OA. Spliceosome and other novel mutations in chronic lymphocytic leukemia and myeloid malignancies. *Leukemia*. 2012; 26(9):2027–2031. [PubMed: 22484420]
30. Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D. Myeloid malignancies: mutations, models and management. *BMC cancer*. 2012; 12:304. [PubMed: 22823977]
31. Cancer Genome Atlas Research N. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *The New England journal of medicine*. 2013; 368(22):2059–2074. [PubMed: 23634996]
32. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012; 490(7418):61–70. [PubMed: 23000897]
33. Maguire SL, Leonidou A, Wai P, et al. SF3B1 mutations constitute a novel therapeutic target in breast cancer. *The Journal of pathology*. 2015; 235(4):571–580. [PubMed: 25424858]
34. Imielinski M, Berger AH, Hammerman PS, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell*. 2012; 150(6):1107–1120. [PubMed: 22980975]
35. Harbour JW. Genomic, prognostic, and cell-signaling advances in uveal melanoma. *American Society of Clinical Oncology educational book/ASCO American Society of Clinical Oncology Meeting*. 2013:388–391.
36. Hubert CG, Bradley RK, Ding Y, et al. Genome-wide RNAi screens in human brain tumor isolates reveal a novel viability requirement for PHF5A. *Genes & development*. 2013; 27(9):1032–1045. [PubMed: 23651857]
37. Hsu TY, Simon LM, Neill NJ, et al. The spliceosome is a therapeutic vulnerability in MYC-driven cancer. *Nature*. 2015
38. Shi Y, Joyner AS, Shadrick W, et al. Pharmacodynamic assays to facilitate preclinical and clinical development of pre-mRNA splicing modulatory drug candidates. *Pharmacology research & perspectives*. 2015; 3(4):e00158. [PubMed: 26171237]
39. Walters BJ, Lin W, Diao S, et al. High-throughput screening reveals alsterpaullone, 2-cyanoethyl as a potent p27Kip1 transcriptional inhibitor. *PloS one*. 2014; 9(3):e91173. [PubMed: 24646893]
40. Imbach P, Capraro HG, Furet P, Mett H, Meyer T, Zimmermann J. 2,6,9-trisubstituted purines: optimization towards highly potent and selective CDK1 inhibitors. *Bioorganic & medicinal chemistry letters*. 1999; 9(1):91–96. [PubMed: 9990463]
41. Chang YT, Gray NS, Rosania GR, et al. Synthesis and application of functionally diverse 2,6,9-trisubstituted purine libraries as CDK inhibitors. *Chemistry & biology*. 1999; 6(6):361–375. [PubMed: 10375538]
42. Uitdehaag JC, Verkaar F, Alwan H, de Man J, Buijsman RC, Zaman GJ. A guide to picking the most selective kinase inhibitor tool compounds for pharmacological validation of drug targets. *British journal of pharmacology*. 2012; 166(3):858–876. [PubMed: 22250956]

43. Parry D, Guzi T, Shanahan F, et al. Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. *Molecular cancer therapeutics*. 2010; 9(8):2344–2353. [PubMed: 20663931]
44. Fukuhara T, Hosoya T, Shimizu S, et al. Utilization of host SR protein kinases and RNA-splicing machinery during viral replication. *Proc Natl Acad Sci U S A*. 2006; 103(30):11329–11333. [PubMed: 16840555]
45. Oumata N, Bettayeb K, Ferandin Y, et al. Roscovitine-derived, dual-specificity inhibitors of cyclin-dependent kinases and casein kinases 1. *Journal of medicinal chemistry*. 2008; 51(17):5229–5242. [PubMed: 18698753]
46. Dai Y, Dent P, Grant S. Induction of apoptosis in human leukemia cells by the CDK1 inhibitor CGP74514A. *Cell cycle*. 2002; 1(2):143–152. [PubMed: 12429924]
47. Bullock AN, Das S, Debreczeni JE, et al. Kinase domain insertions define distinct roles of CLK kinases in SR protein phosphorylation. *Structure*. 2009; 17(3):352–362. [PubMed: 19278650]
48. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of computational chemistry*. 2004; 25(13):1605–1612. [PubMed: 15264254]
49. Fabian MA, Biggs WH 3rd, Treiber DK, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nature biotechnology*. 2005; 23(3):329–336.
50. Yoshida T, Kim JH, Carver K, et al. CLK2 Is an Oncogenic Kinase and Splicing Regulator in Breast Cancer. *Cancer Res*. 2015; 75(7):1516–1526. [PubMed: 25670169]
51. Kang J, Sergio CM, Sutherland RL, Musgrove EA. Targeting cyclin-dependent kinase 1 (CDK1) but not CDK4/6 or CDK2 is selectively lethal to MYC-dependent human breast cancer cells. *BMC cancer*. 2014; 14:32. [PubMed: 24444383]

Figure 1.

Some recently reported synthetic alternative splicing modulators.^{15, 16, 17, 18, 19}

Figure 3.
The most active PCR validated hits from a run of the MDM2-Luc exon-skipping screen.

Figure 4.
Active CLK inhibitors that were profiled against 22 oncogenic kinases (see Supporting Information).

Figure 5.

Left Panel A representation of the structure of debromohymenialdisine (K0010) bound to CLK1 using the published coordinates,⁴⁷ highlighting the hydrogen bonds to the ligand (as blue lines). This figure was prepared using UCSF Chimera.⁴⁸ This perspective is similar to that shown in Figure 5. **Right Panel:** A hypothetical 2D overlay of the hydrogen bond pharmacophore features from K0010 (blue) over the structure of CLK inhibitor **14** (shown in grey) from Table 1 above. The overlay is intentionally offset so that similar features are visible in both structures. Abbreviations: HB-Don= hydrogen-bond donor; HB-Acc= hydrogen-bond acceptor. The notation 'Asp-325?' refers to a hypothetical interaction with this residue in CLK1, assuming the overlay is correct.

Scheme 1. Synthesis of Substituted Diaminopurine and Diaminopyrrolo[2,3-*d*]pyrimidine Analogs

Reagents and conditions:

(a) EtBr or *i*-PrI, K₂CO₃, DMSO; (b) ArNH₂, TEA, BuOH, 100 °C, 16 h; (c) R₂NH₂, TEA, NMP, 160 °C, 16 h; (d) Boc-R₂NH₂, DIPEA, 110 °C; (e) TFA, CH₂Cl₂, rt, 1 h.

Table 1
 Analogs of **1** and **2** and their activity in the MDM2-Luc reporter assay, prepared as shown in Scheme 1^a

Compound	Ar	X	R ₁	R ₂ -NH	MDM2/Luc	Compound	Ar	X	R ₁	R ₂ -NH	MDM2/Luc
CGP-74514A (1)	3-Cl-phenyl	N	Et		Active	10	3-Cl-phenyl	N	i-Pr		Active
5	3-Cl-phenyl	N	Et		Active	11	3-Cl-phenyl	CH	i-Pr		NA
6	3-Cl-phenyl	N	Et		Active	12	3-Cl-phenyl	N	Et		NA
7	3-Cl-phenyl	N	i-Pr		Active	13	3-Cl-phenyl	N	i-Pr		NA

Compound	Ar	X	R ₁	R ₂ -NH	MDM2/Luc	Compound	Ar	X	R ₁	R ₂ -NH	MDM2/Luc
8	3-Cl-phenyl	N	<i>i</i> -Pr		Active	14	3-Cl-5-NH ₂ -phenyl	N	<i>i</i> -Pr		Active
9	3-Cl-phenyl	N	<i>i</i> -Pr		Active	15	3-Cl-5-AcNH phenyl	N	<i>i</i> -Pr		Active

^a Active means a significant MDM2-Luc reporter dose response (greater than approximately 3 fold over DMSO control, where DMSO is set as Luc-Fold = 1.0) in luciferase activation based on the average of three experiments. Sudenycin D6 serves a positive control.

Abbreviations: NA, not active. See Experimental Section and Supporting Information for more details.

Table 2

The biochemical inhibition results for the active compounds from Table 1^a

Compound	CLK1 IC ₅₀ (nM)	CLK2 IC ₅₀ (nM)	CLK3 IC ₅₀ (nM)	CLK4 IC ₅₀ (nM)	CDK1 IC ₅₀ (nM)	CDK4 IC ₅₀ (nM)	CDK6 IC ₅₀ (nM)
CGP-74514A (1)	148	111	>1000	104	382	573	>1000
5	868	498	>1000	>1000	546	>1000	>1000
6	371	190	>1000	498	>1000	>1000	>1000
7	400	120	>1000	634	>1000	>1000	>1000
SRI-29329 (8)	78	16	>1000	86	>1000	>1000	>1000
9	38	11	>1000	50	674	541	>1000
10	74	26	>1000	70	>1000	>1000	>1000
14	18	16	>1000	54	973	>1000	>1000
15	19	13	>1000	64	>1000	930	>1000

Table 3Second-generation analogs of **1** and **2**, prepared as shown in Scheme 1.^a

Compound	Ar	R ₂ -NH	Compound	Ar	R ₂ -NH
16	3-Cl-5-NH ₂ -phenyl		21	3-Cl-5-NH ₂ -phenyl	
17	3-Cl-5-NH ₂ -phenyl		22	3-Cl-phenyl	
18	3-Cl-5-AcNH-phenyl		23	3-Cl-phenyl	
19	3-Cl-5-AcNH-phenyl		24	3-Cl-phenyl	
20	3-Cl-5-NH ₂ -phenyl		25	3-Cl-phenyl	

Table 4

The biochemical inhibition results for the active compounds from Table 3^a

Compound	CLK1 IC ₅₀ (nM)	CLK2 IC ₅₀ (nM)	CLK3 IC ₅₀ (nM)	CLK4 IC ₅₀ (nM)	CDK1 IC ₅₀ (nM)	CDK4 IC ₅₀ (nM)	CDK6 IC ₅₀ (nM)
16	17	6	>1000	25	532	>1000	>1000
17	21	26	>1000	28	>1000	>1000	>1000
18	19	7	>1000	30	>1000	611	>1000
19	26	17	>1000	31	>1000	>1000	>1000
20	56	14	>1000	124	>1000	>1000	>1000
21	274	163	>1000	648	>1000	>1000	>1000
22	31	26	>1000	61	>1000	>1000	>1000
23	66	34	>1000	102	>1000	>1000	>1000
24	71	28	>1000	77	>1000	>1000	>1000
25	118	92	>1000	299	>1000	>1000	>1000

^a Enzyme inhibition of CLK subtypes 1–4 and CDK 1, 4, and 6.