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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.

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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 trans-acting 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 IC_{50}s 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

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

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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 analogs[24] 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 Fig. 1). Several groups have independently discovered new diverse small molecule structural classes that effect pre-mRNA splicing, using a range of screening strategies (see Fig. 1). These compounds include TG-003,[16] KH-CB19,[17] Araki Cpd-2,[18] and Madrasin[19] (see Fig. 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 tumorigenesis[27] 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 adenosarcoma,[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 Fig. 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 Fig. 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 4359 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 Fig. 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 μM; this compound inhibits SRPK1 and SRPK2 but does not significantly inhibit other

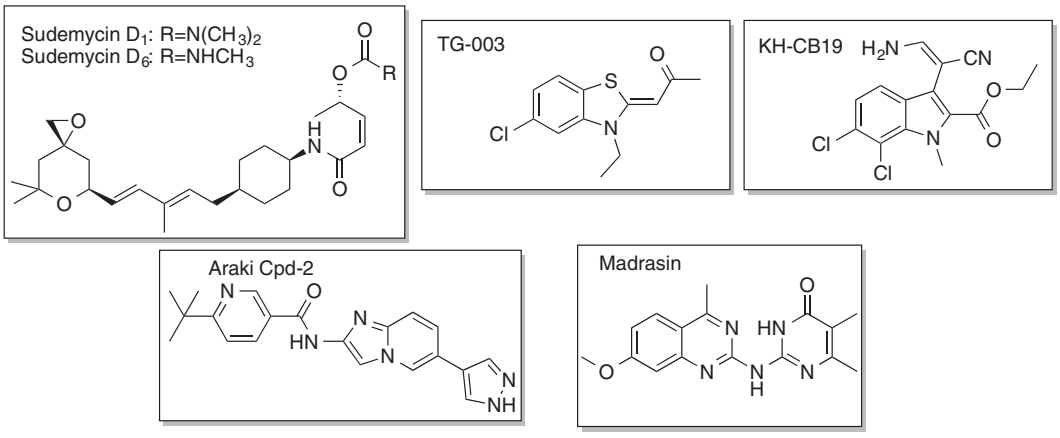

Fig. 1. Some recently reported synthetic alternative splicing modulators.[15–19]

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