Alternative Splicing in Prostate Cancer

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Abstract

Prostate cancer kills one man every 45 minutes. Androgen receptor splice variants (AR-V) appear to play a critical role in the progression of metastatic prostate cancer. AR-Vs are truncated AR isoforms that lack the AR ligand binding domain and remain constitutively active in the absence of androgen, promoting cancer cell proliferation through aberrant activation of AR-mediated cell survival pathways. Consequently, AR-Vs have been proposed to contribute to not only treatment resistance against anti-androgen therapies, but also radio-resistance in patients receiving combination androgen deprivation therapy and radiation by bolstering DNA repair mechanisms. AR-Vs such as androgen receptor variant 7 (AR-V7) have been associated with worse clinical outcomes, however attempts to specifically inhibit or prevent formation of AR-Vs have to date been unsuccessful. Thus, novel therapeutic strategies are desperately needed to address the action of AR-Vs that drive lethal forms of prostate cancer. Disruption of alternative splicing through modulation of the spliceosome is one such potential therapeutic avenue, however our understanding of the inner workings of the spliceosome, and how it contributes to prostate cancer remains incomplete, reflected in the dearth of therapeutic agents able to target the spliceosome. This review outlines our current understanding of the role of the spliceosome in the progression of prostate cancer and explores the therapeutic utility of manipulating this cellular network to improve patient care.

Introduction

Prostate cancer is the second most frequently diagnosed cancer amongst men worldwide [1], with one man dying of prostate cancer every 45 minutes in the United Kingdom [2]. Since the pioneering work of Charles Huggins and Clarence Hodges, who first demonstrated the benefits of androgen deprivation therapy (ADT) in patients with metastatic prostate cancer [3], our understanding of its pathogenesis has increased significantly, particularly with regards to the fundamental importance of the androgen receptor (AR) in all stages of disease from tumorigenesis, to progression and ultimately treatment resistance and death [4].

The androgen receptor and prostate cancer

The AR is a ligand-activated transcription factor that plays a central role in male sexual development. It is a member of the steroid and nuclear hormone receptor super-family and is encoded by the AR gene located on chromosome Xq12 [5], the transcriptional activity of which is modulated by its interactions with more than 200 different transcriptional co-regulators [6]. In prostate cancer, in addition to these regulators, genomic aberrations such as AR copy number gain, mutations and rearrangements are also thought to play a significant role in AR gene expression with AR overexpression in particular being key to the development and progression of castration resistant prostate cancer (CRPC) [7].

The full-length product of AR gene transcription was first reported in 1988 [8] and has a molecular weight of 110 kDa. The AR is comprised of four discrete functional domains (*figure 1*) namely, an N-terminal transcriptional domain (NTD) which is highly variable and inherently disordered [5], a DNA binding domain (DBD) which consists of a highly conserved 66-residue core made up of two zinc-nucleated modules [9], a hinge region and a C-terminal ligand binding domain (LBD) [10]. Of note, while the C-terminal and DBD have been crystalized, the crystal structure of the N-terminus remains elusive, hindering the development of N-terminal targeting agents.

Under normal conditions, the AR is sequestered within the cytoplasm by a complex of heat shock protein (HSP) chaperones [11] and their co-chaperones such as BCL-2-associated-athanogene-1L (BAG-1L). In the presence of androgens, namely dihydrotestosterone (DHT), and to a lesser degree, testosterone, the AR undergoes conformational change [10] and dimerises with other ligand-bound AR to form homodimers. The nuclear localisation of the AR is dependent on the AR bipartite nuclear localisation sequence (NLS), which is highly conserved between many nuclear receptors and contains two clusters of basic amino acids [12]. The NLS is recognised by the transport adaptor proteins, alpha and beta importin, which regulate the shuttling of the AR homodimers into the cell nucleus. In addition, the NLS is also recognised and bound by dynein, a motor protein that interacts with cellular microtubules to enhance AR nuclear translocation via a cytoskeletal transport network [13]. Once in the nucleus, AR binds DNA at specific sites known as androgen-response elements (ARE) through its DBD. In this way, the AR can up- or down-regulate the transcription and activation of various genes, many of which are involved with regulating crucial cellular functions such as growth and proliferation. As a consequence of this ability to regulate cell survival, persistent activation of the AR has been shown to be a pivotal driving force in the carcinogenesis and progression of prostate cancer. Furthermore, inhibition of AR signalling through androgen deprivation remains the standard of care in the treatment of prostate cancer to this day [14]. However, while nearly all patients initially respond to ADT, the duration of response varies from months to years, and ultimately all patients eventually acquire resistance and progress to lethal CRPC [15].

CRPC was long thought of as being an androgen independent entity, however more recently the continuing importance of the AR in the progression of advanced prostate cancer has been better appreciated, culminating in the introduction of abiraterone and enzalutamide into routine clinical practise, which have been shown to provide additional survival benefit in patients with CRPC [16, 17]. Despite the success of these second-generation AR-targeted therapies, treatment resistance continues to be a major challenge, leaving patients with only a limited number of meaningful treatment options following disease progression, namely taxane chemotherapy, which is not without its limitations such as cytopenia and neurotoxicity [18, 19], and targeted therapies that are only efficacious in a subgroup of patients, such as PARP inhibitors or carboplatin in homologous repair DNA repair defective prostate cancers (as yet unapproved) and PD-1 immune checkpoint targeting for mismatch repair defective disease [20]. In addition, with clinical evidence emerging that use of abiraterone at diagnosis of castration sensitive prostate cancer (CSPC) provides improved outcome [21, 22], it is foreseeable that in the future these agents will be used much earlier in the treatment of patients' cancers and potentially result in resistance to anti-androgens occurring at the time of progression from first line therapy rather than as a later event, opening the door to new clinical dilemmas.

The many faces of the androgen receptor
While full-length AR (AR-FL) has been well described in the literature [10, 23], more recently a variety of alternate versions of AR have been shown to exist. Evidence for this first emerged through the work of Dehm and colleagues who identified two truncated AR isoforms lacking the C-terminal domain in 22Rv1 prostate cancer cell lines, encoded by mRNAs with a novel exon 2b at their 3′ end [24]. In addition, they demonstrated that these AR isoforms remained constitutively active, and maintained the proliferation of 22Rv1 cells in the absence of androgen [24]. Since this original work, and with the development of more advanced sequencing techniques, numerous other truncated forms of AR have been reported [23, 25, 26].

AR protein expression results from the transcription and translation of the AR gene. However, due to the discontinuous nature of eukaryotic genes, with regions of non-coding DNA (introns) interspersed between stretches of coding DNA (exons), when first transcribed the resultant precursor messenger RNA (pre-mRNA) transcript contains both sequences. Therefore prior to translation, nascent pre-mRNA transcripts are edited through the process of splicing, removing unwanted introns and producing mature messenger RNA (mRNA) that can be correctly translated.

Splicing is performed by complex cellular machinery referred to as the spliceosome, the importance of which has recently gained increased recognition with the discovery that through the alternative inclusion and exclusion of exons and introns, termed alternative splicing, a single gene can encode multiple proteins [27], enabling eukaryotic cells to transform a genome that contains only 20,000 genes, into a significantly larger and more diverse proteome of approximately 95,000 proteins [28].

As such, awareness of the role of the spliceosome in numerous diseases, including cancer is growing. However, our understanding of its underlying biological mechanisms remains incomplete, making it an important area of Clinical Research.

The Spliceosome

The spliceosome is a dynamic cellular machine composed of small nuclear ribonucleoproteins (snRNP) and associated protein co-factors. The biosynthesis and assembly of the spliceosome is outlined in **Box 1**.

Importantly, all major steps in spliceosome formation are reversible, suggesting a proof-reading mechanism in operation during splicing, with *in vitro* studies having shown that partially assembled spliceosomes are able to disassemble and reassemble onto alternative splice sites [29], particularly in the early stages of spliceosome assembly, as commitment to splicing increases as spliceosome assembly progresses [29].

Spliceosome regulation
The core constituents of the spliceosome complex are able to define exon-intron boundaries, however, splicing sequences within nascent mRNA precursors often contain too little information to unambiguously define splice sites [30]. In addition, human introns often contain sequences with a high degree of similarity to authentic splice sites. As such, additional *cis-* and *trans*-regulatory factors are required to accurately define exon-intron junctions and maintain fidelity. *cis*-regulatory RNA elements are nucleotide sequences within pre-mRNA transcripts that can modify the splicing of the same pre-mRNA transcript in which they are located. As such, these sequences are referred to as splicing regulatory elements (SREs) and contribute to splicing in a context dependent manner, whereby they can serve as either splicing enhancers or silencers depending on their position within the pre-mRNA transcript [31]. SREs exert their action by recruiting *trans*-acting splicing factors, auxiliary proteins of the spliceosome such as serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonuclear proteins (hnRNP). These proteins interact with core components of the spliceosome, often the snRNPs U1 and U2, to either activate or suppress the splicing reaction by impacting the early steps of spliceosome assembly. In addition, as with SREs, trans-acting splicing factors modify splicing in a context dependent manner. For example, SR proteins can promote splicing when bound to SREs located within exons, but can inhibit splicing when associated with SREs in introns [32].

Other factors contributing to splicing regulation include tissue-restricted protein splicing factors (such as the neuro-oncological ventral antigen (NOVA) [33] and feminizing gene on X (FOX) [34]), the rate of transcription elongation [35], tissue hypoxia [36, 37], heat stress [38, 39], genotoxic stress [40], chromatin structure and nucleosome positioning [41]. Recently this complexity has been furthered with the finding that not only can most splicing factors recognise multiple SREs, but each SRE is often bound by multiple different factors, suggesting the presence of a complex network of protein–RNA interactions working alongside the spliceosome, regulating splicing to not only protect the proteome from error but also to provide cellular plasticity [27].

Alternative splicing
Splicing typically occurs at constitutive splice sites containing a consensus sequence. Splice site selection is reported to crudely depend on the 'strength' of a splice site, with sites that are more adjusted to the consensus sequence producing strong splice sites that are more efficiently recognised by the spliceosome and selected for over weaker sites. However, predominantly through *trans*-acting splicing factors, the spliceosome's regulatory network can modify the strength of these competing sites by silencing stronger splice sites and enhancing weaker ones. In this way, the interplay between these competing spliceosomal 'homing' signals within a nascent pre-mRNA can lead to the preferential selection of non-canonical splice site and result in alternative splicing.

High throughput RNA sequencing studies have shown that alternative splicing is a routine biological process, with 90-95% of human multi-exon gene transcripts demonstrating alternative splicing events, thereby generating protein diversity [42]. Patterns of alternative splicing range from alternative 3' or 5' splice site recognition, to retained introns and mutually exclusive exons, however cassette exon skipping is the most common event in humans [43] (*figure 2*).

Despite the abundance of alternative splicing events, the functional roles of the many spliced isoforms remain uncertain, although we have clear evidence that alternative splicing can play key roles in regulating the functions of many proteins [44-46]. While many speculate alternative splicing is a fundamental factor in biodiversity and evolution [47], it has also been implicated in the pathogenesis of a number of diseases including cancer [43, 48, 49].

The spliceosome in prostate cancer
The role of the spliceosome in prostate cancer is currently a major area of current Clinical Research. While alternatively spliced variants of the AR that remain constitutively active in the absence of androgen are the most well described splicing aberrations in prostate cancer, the spliceosome has been implicated in the pathogenesis of prostate cancer in a number of other ways (*figure 3*).

Mutations of spliceosome regulators
Recurrent somatic mutations in genes encoding splicing factors have been identified in a variety of different cancers such as uveal melanoma [50], pancreatic ductal adenocarcinoma [51], lung adenocarcinoma [52], breast cancer [53] and prostate cancer [54]. Despite this diversity in tumour origin however, most reported spliceosomal mutations occur in one of four genes, namely splicing factor 3B subunit 1 (SF3B1), SR protein splicing factor 2 (SRSF2), U2AF1 and zinc finger RNA-binding motif and serine/arginine-rich 2 (*ZRSR2*) [55]. Of these, mutations of the SF3B1 gene are the most common and have been observed in both haematological and solid malignancies [55], including prostate cancer [56]. Its protein product, SF3B1, is a core spliceosomal protein that binds upstream of the pre-mRNA branch site, and is thought to be required for the recognition of most 3′ splice sites [27]. As such, available evidence suggests that SF3B1 mutations are associated with enhanced recognition of cryptic 3′ splice sites and favour the formation of alternative spliced protein isoforms [57], which are considered an important mechanism of treatment resistance and disease progression in CRPC. However, with a reported incidence in the region of 1% in prostate cancer [54, 56], the contribution of SF3B1 mutations to treatment resistance may prove to be limited.

Alterations in spliceosome regulator activity
Changes in the activity of splicing factors have been reported to directly impact on tumorigenesis and disease progression in prostate cancer. For example, Src-Associated substrate in Mitosis of 68 kDa (Sam68) is a nuclear splicing factor involved in regulating the splicing of Cyclin D1 (CCND1) [58], which is a central component of cell cycle control. However, Sam68 is activated through extracellular signal-regulated kinase (ERK)-mediated phosphorylation [59], which is dysregulated in approximately a third of human cancers [60] including prostate. As such, Sam68 has been found to be frequently upregulated in prostate cancer [45], and consequently has been associated with the increased expression of a truncated CCND1b isoform, rather than the canonical CCND1a gene product, which promotes the proliferation and survival of prostate cancer cells [45].

Splicing factor upregulation has also been linked with prostatic epithelial-mesenchymal transition (EMT) and disease progression in CRPC. Following androgen deprivation, upregulation of the splicing factor serine/arginine repetitive matrix 4 (SRRM4) has been shown to cause the alternative splicing of RE1-silencing (REST) [61], a neuronal master regulator which normally prevents the expression of neuronal genes such as synaptophysin (SYP) in non-neuronal cells [62]. Consequently, this produces a truncated form of REST that lacks its canonical transcriptional repressor domain and gives rise to a more AR-independent, neuroendocrine (NE) phenotype, which confers a poorer prognosis [63].

As well as directly contributing to disease progression, the upregulation of canonical splicing factors has also been shown to be pivotal in the pathogenesis of other drivers of prostate cancer, such as oncogenes. The proto-oncogene c-MYC is reported to be overexpressed in up to 90% of all primary human prostate cancer lesions [64]. MYC hyperactivation amplifies pre-mRNA production leading to stress on the spliceosome [65]. As such, these cancers are equally dependent on the availability of splicing factors to sustain proliferation and survival as they are on MYC [65], demonstrated by the upregulation of a number of splicing factors such as SRSF1, hnRNPA1 and hnRNPA2 in MYC overexpressing tumours, and the disruption of many vital cell processes which occurs when they are inhibited [65-68].

Alternative splicing of cellular signal transduction pathways
The spliceosome and its associated proteins are involved in the routine operation of a wide range of cellular processes including DNA repair, transcription and nonsense-mediated RNA decay (NMD). For example, through chromatin immunoprecipitation (ChIP) studies, SF3B1 and U2AF1 have been shown to interact with *BRCA1* following DNA damage [69].

Kruppel-like factor 6 (KLF6) is a key tumour suppressor gene that is often mutated in prostate cancer. It encodes a member of the Kruppel-like family of transcription factors which binds DNA and regulates growth-related signal transduction pathways, cell proliferation, apoptosis, and angiogenesis [70]. While wild-type KLF6 has inhibitory effects on cell growth, a common KLF6 germline single nucleotide polymorphism (IVS1−27 G>A/IVSΔA) results in the production of an alternatively spliced isoform, KLF6 splice variant 1 (KLF6 SV1), which enhances cell proliferation, colony formation, and invasion. Furthermore, upregulation of KLF6 SV1 in prostate cancer is associated with worse prognosis [44, 71].

As well as effecting important protein signal transducers, the alternative splicing of cell surface receptors leading to aberrant activation of key survival pathways is an equally important part of the spliceosome's contribution to prostate cancer progression. For example, the fibroblast growth factor-2 receptor (FGFR2) is a tyrosine kinase receptor which when activated by the binding of fibroblast growth factor (FGF), is involved in the regulation of numerous key cellular processes such as proliferation and differentiation which contribute to cell survival [72]. Under normal physiological conditions, the FGFR2 exist in a number of isoforms, which tend to be cell type specific with isoform IIIb expressed in epithelial cells and isoform IIIc expressed in mesenchymal cells. In prostate cancer however, this distribution has been found to change, with isoform IIIc becoming more prevalent [73]. This alters the receptors ligand binding specificity to favour the binding of FGF8b [73], the major FGF isoform expressed in prostate cancer and which is thought to have an important role in disease progression, as evidenced by its association with higher Gleason grade and clinical stage [74].

Splicing therefore impacts on prostate carcinogenesis in a multitude of ways, and while the breadth of these splicing changes suggests the key to endocrine therapy resistance is likely to be multifactorial, currently the most significant role of the spliceosome in the progression of prostate cancer is considered to be its involvement in the generation of alternatively spliced AR receptor isoforms.

Androgen receptor splice variants

To date, a number of AR splice variants (AR-V) have been identified and examined in metastatic CRPC specimens [25, 75, 76] *(figure 1)*, however of these, AR splice variant 7 (AR-V7) is the most well studied and has been associated with both an increased risk of biochemical relapse [77] and poorer overall survival [75, 78-80]. More recently, AR-V9 has been shown to not only be co-expressed with AR-V7, but also shares a common 3' terminal cryptic exon [81]. Furthermore, AR-V9 may also lead to the ligand-independent growth of prostate cancer cells, with high AR-V9 mRNA expression having been reported to be predictive of primary resistance to abiraterone [81], however the clinical significance of this remains uncertain.

AR-V7 is a truncated isoform of the canonical AR-FL protein that lacks the AR LBD but retains both the AR DBD, which mediates AR dimerization and DNA interactions, and the NTD which is responsible for the majority of AR transcriptional activity [82]. Crucially, this conformatory change has been shown to maintain AR-V7 in a constitutively active state in the absence of an androgen ligand, resulting in persistent tumour cell aberrant AR survival signalling [5]. Furthermore, this structural difference may also enable AR-V7 to induce a distinct set of transcriptional programs compared to AR-FL. For example, expression of AR-V7, but not AR-FL, has been positively correlated with the expression of the Ubiquitin Conjugating Enzyme E2 C (UBE2C) gene, which encodes a protein required for the destruction of mitotic cyclins and cell cycle progression in clinical CRPC specimens [83]. However, while this may suggest a shift toward AR-V mediated signalling following anti-androgen therapy in a subset of CRPC tumours, it should be noted that attempts to disentangle the functional role of AR-V7 from that of AR-FL have been challenging and this continues to be an area of active investigation, with further evidence being required before firm conclusions can be drawn on this possibility.

AR-V7 is the most commonly expressed AR-V [25, 82] and its prevalence increases significantly as patients progress to CRPC [26, 84, 85]. This can in part be explained as a consequence of treatment with ADT through two mechanisms. Firstly, AR-V7 expression is intimately linked with AR gene transcription [77] which is increased approximately 10-fold in response to androgen deprivation [82], as such AR-V7 expression is consequently also increased. Secondly, as ligand-dependent AR signalling decreases AR-V7 transcription, inhibition of AR signalling with ADT results in the loss of this negative feedback and upregulates AR-V7 expression [5, 82].

Ultimately however, the processes determining the expression of AR-V7, as opposed to canonical AR-FL, remain unclear, although there is increasing appreciation for the importance of the spliceosome in this process.

AR-V7 and the spliceosome

The AR-V7 protein product arises from the alternative splicing of AR mRNA at cryptic exon 3 (CE3) as opposed to the canonical AR-FL 3'ss (*figure 1*). While AR gene copy number gain is considered an important determinant of AR-V7 mRNA levels in CRPC metastases [86], this alone does not explain why a proportion of encoded AR mRNA becomes alternatively spliced. For example, in LNCap95 cells, which are not reported to possess this AR copy gain, AR-V7 RNA is still expressed at levels comparable to VCaP cells where AR is amplified [77], whereas the parent cell line LNCaP expresses no AR-V7. Therefore, rather than the alternative splicing of AR mRNA occurring because of random splicing error as a consequence of increased substrate concentration, this instead points towards the existence of a regulatory mechanism responsible for splice site selection.

In preclinical prostate cancer models, Liu and colleagues reported that androgen deprivation increases spliceosomal recruitment to the AR gene, facilitating both AR and AR-V7 splicing [77]. Furthermore, treatment with the anti-androgen Enzalutamide specifically enhances recruitment of a number of splicing factors to the P2 region of the AR mRNA [77] which contains the AR-V7 3' splice site. This group further demonstrated that splicing factors U2AF65 and SRSF1 acted as 'pioneer' factors, directing the recruitment of the spliceosome to the SREs adjacent to the AR-V7 3'ss, and increasing the expression of AR-V7 RNA. Interestingly, while knockdown of these splicing factors resulted in a reduction in AR-V7 RNA levels in both VCAP and LnCap95 cell lines, it did not affect AR-FL levels [77]. Coupling this data with the finding that the expression of U2AF65 is increased in CRPC compared to primary prostate cancer [87], suggests that U2AF65 in particular may play a key role in the progression of CRPC and, more specifically, be important in AR-V7 splicing. HnRNP1 has also been proposed as a regulator of AR-V7 splicing, however the evidence for this is less conclusive. Work by Nadiminty et al. has shown that overexpression of hnRNPA1 significantly up-regulates protein levels of AR-V7, while down-regulation both reduces AR-V7 protein expression and re-sensitises castrate-resistant cell lines to enzalutamide [88]. However, hnRNP1 knock-down has also been shown to reduce AR-FL levels [77], suggesting that hnRNP1 may serve as a general regulator of AR mRNA splicing rather than one specific to AR-V7.

Importantly, and in keeping with the concept of a proof-reading process within the spliceosomal network, AR-V7 splicing appears to be a dynamic and plastic process. For example, the re-introduction of androgen to androgen-deprived cell lines has been shown to repress levels of AR-V7 RNA, with this occurring within 24 hours in VCaP cells. Similarly, in primary cultures from enzalutamide-resistant VCaP xenograft models, both AR and AR-V7 RNA levels significantly decreased when DHT was added [77]. As an interesting aside, it may be the rapidity of this plasticity that contributes to the encouraging efficacy demonstrated by bipolar androgen therapy (BAT) in a recent phase 2 clinical trial. Teply et al observed that 52% of patients with metastatic CRPC that had previously progressed on enzalutamide achieved a 50% reduction in PSA following further treatment with enzalutamide after having received BAT, where patients receive intermittent doses of high-dose testosterone whilst remaining on ADT [89]. While this may suggest that re-sensitisation of treatment resistant prostate cancer to enzalutamide is potentially possible through manipulation of AR-FL and AR-V expression levels by modulating an individual's exposure to testosterone, definitive conclusions regarding this possibility are difficult to elucidate from this cohort given that patient AR-V7 status in this study was determined through analysis of circulating tumour cells (CTC) rather than tissue-based assessment. As such, because over half of the patients included were found to be CTC negative, a large proportion of this cohort could not be assessed for AR-V7 status, and so a number of AR-V7 positive patients could

have been omitted from analyses. Furthermore, pre-clinical evidence in support of this possibility remains inconclusive [90].

Alternative splicing and treatment resistance

Over recent years, appreciation for the role of alternative splicing in the development of treatment resistance against anti-cancer therapies has greatly increased. For example, alternative splicing of Survivin, a member of the inhibitor of apoptosis protein (IAP) family has been reported to confer resistance to taxanes in pre-clinical models of ovarian cancer [91], while the alternative splicing of B-lymphocyte antigen CD19 may promote resistance to immunotherapy with adoptive T cells expressing chimeric antigen receptors (CAR-T) against CD19 in pre-clinical models of B-cell acute lymphoblastic leukaemia [92].

Similarly, even though the development of genome sequencing has heralded the arrival of various new targeted anti-cancer therapies, evidence is emerging that these therapeutic agents are equally vulnerable to the development of resistance as a consequence of alternative splicing. For example, a subset of BRAF mutant melanomas have been reported to acquire resistance to vemurafenib through the expression of a variant BRAF isoform, BRAF(V600E), which lacks exons 4-8, a region that encompasses the RAS-binding domain [93]. Furthermore, and perhaps more pertinently with regards to prostate cancer, alternative splicing has been suggested to promote resistance to PARP inhibition [94].

The PARP inhibitor olaparib utilises the concept of synthetic lethality to exert a therapeutic effect in DNA-repair defective cancers by inhibiting Poly (ADP-ribose) polymerase (PARP), a protein that is important for repairing DNA single-strand breaks. Inhibiting the repair of single strand breaks in this way results in the generation of double strand breaks during cell division, which in tumour cells possessing an inherent inability to repair double strand breaks due to loss or mutation of DNA-repair proteins such as BRCA1 and 2, results in tumour cell death. As such, olaparib has recently been shown to improve overall survival in patients with DNA-repair deficient metastatic Prostate cancer with a response rate of 88% in biomarker positive patients [20], marking a significant step forward in the management of this patient group. PARP inhibition has also demonstrated efficacy in other cancers such as breast [95] and ovarian [96], however of note, evidence is emerging from these cancer types to suggest alternative splicing may contribute to treatment resistance to olaparib. Wang et al. report that a proportion of patients whom possesses PARP-sensitising BRCA1 germline mutations either do not respond to, or eventually develop resistance to, PARP inhibition as a result of frameshift mutations to exon 11, leading to NMD of full-length BRCA1 and the increased expression of an alternatively spliced BRCA1 isoform, BRCA1-Δ11q. In this way, the authors suggest that BRCA1 deficient cancer cells utilise mRNA splicing mechanisms to remove deleterious germline BRCA1 mutations by producing alternatively spliced protein isoforms that retain residual activity and contribute to therapeutic resistance [94]. While it should be noted that BRCA2 mutations are much commoner in prostate cancer than BRCA1 mutations, whether similar patterns and mechanisms of resistance will emerge in prostate cancer will be born out through clinical trials of novel targeted therapies such as these. These examples do however serve to highlight the clinical implications of alternative splicing and add weight to the rationale of harnessing the spliceosome as a novel therapeutic target.

Overall, however, notwithstanding this growing body of literature, currently with regards to CRPC, AR-Vs represent the most well-established, and clinically important, mechanism through which alternative splicing is thought to contribute to treatment resistance.

AR splice variants and treatment resistance

AR-Vs have been proposed as a biologically credible mechanism of treatment resistance through the restoration of AR signalling. Preclinical studies have shown that inhibition of AR-V7 can re-sensitise enzalutamide-resistant prostate cancer cell lines to anti-androgen treatment [97-99]. Furthermore, AR-Vs have also been implicated in treatment failure in patients receiving combined ADT and radiotherapy, with AR-V aberrant signalling bolstering the DNA damage response and increasing the clonogenic survival of prostate cancer cells after irradiation [100].

However, the evidence to support AR-Vs role in resistance remains inconclusive. Despite the advantageous characteristics conferred by their structural properties, which allow AR-Vs to remain constitutively active in the absence of androgen, in practise only a minority of AR-Vs have demonstrated this ability in AR transactivation reporter assays [4], raising questions regarding the clinical significance of the majority of AR-Vs. A proposed explanation for this observation is that most AR-Vs are truncated after exon 3 and lack a complete NLS, therefore are expected to be predominantly sequestered within the cytoplasm [101]. AR-V7 is however the exception to this rule, and despite having an incomplete NLS, has been shown to have a significant nuclear residence time [5].

AR-Vs have therefore been counter-proposed as being a consequence of the physiological response to androgen deprivation. In support of this is the rapidity of their increase following ADT. In xenograft models, protein levels of both AR-FL and AR-V7 have been shown to increase in just two days following castration and reach peak levels at two weeks, with AR-V7 mRNA being only a fraction of total AR-FL levels [101]. In addition, the re-introduction of androgen in these models returns these levels to baseline in only eight days [101]. Thus, if AR-Vs were to cause treatment resistance, one would expect this to occur much sooner than seen clinically [16, 17]. In support of this argument, while a number of clinical studies corroborate reports that AR-V7 expression confers a worse prognosis and contributes to treatment resistance, some groups have failed to validate this relationship. For example, in a study by Watson et al. overexpression of AR-V7 in LNCaP cell lines, which do not innately express AR-V7, did not confer resistance to enzalutamide both *in vitro* and *in vivo* [101]. Furthermore, retrospective analyses of patient records by Bernemann et al. identified six out of 21 AR-V7 positive patients who experienced a beneficial response to treatment with abiraterone or enzalutamide, suggesting a subgroup of AR-V7 positive patients may obtain benefit from novel anti-androgen therapy despite detection of AR-V7 splice variants in their circulating tumour cells [102]. Similarly, a prospective study by To el al. found no significant difference in PSA response nor median PSA progression free survival between AR-V7 and AR-V9 positive and negative patients treated with novel anti-androgen therapy, concluding that AR-V expression did not predict outcome in metastatic CRPC patients receiving abiraterone or enzalutamide [103].

However, it is important to recognise that nearly all studies reported to-date rely on the identification of AR-V7 status from CTCs. Therefore, both positive and negative associations of AR-V7 expression with clinical outcomes in CRPC have to be interpreted with careful consideration of the validity of AR-V7 assays utilised, with multiple lines of evidence clearly indicating limitations to these binary assays [75, 79-82, 102, 103]. Firstly, the ability of each assay to only determine AR-V7 status (whether mRNA or protein) in patients with CTCs needs to be considered; CTC positive AR-V7 negative patients are not the same as CTC negative patients in which AR-V7 status cannot be determined, though CTC negative patients were recently shown to have the best prognosis after treatment with abiraterone and

enzalumaide [104]. Secondly, although assays measuring AR-V7 protein expression overcome concerns with regard AR-V7 mRNA stability, they remain susceptible to antibody off-target liabilities with the Abcam/Epitomics antibody previously described in the EPIC AR-V7 assay having this major limitation [105]. Moreover, consideration needs to be given to the possibility that in any one patient there may be large numbers of AR-V7 negative cells despite AR-V7 positivity which may mean that these patients will still benefit from abiraterone or enzalutamide. Finally, these molecular association studies will need to be supported by further understanding of AR-V7 biology and the development of novel therapies that abrogate AR-V7 signalling, and induce robust responses in patients with CRPC. Only then will the biological and clinical significance of AR-V7 be truly confirmed; this remains a priority of for the field and an unmet urgent clinical need.

Utilising the spliceosome to overcome treatment resistance
There are a number of strategies currently under investigation to therapeutically utilise the spliceosome as summarised in ***table 1***.

Targeting the core spliceosome complex
Through large-scale drug screens, a number of bacterial fermentation products have been identified which demonstrate potent anticancer activity through modulation of the core spliceosome complex, and can be broadly categorised into three drug classes, namely pladienolides, herboxidienes and spliceostatins *(table 1)*. While these compounds are structurally distinct, they share a common mechanism of action whereby they all bind SF3B1 [106]. Under normal conditions, SF3B1 interacts with U2AF65 to recruit U2 to the intron 3'ss. However, by binding to SF3B1, these compounds interfere with these early stages of spliceosome assembly and destabilise the interaction between U2 and its pre-mRNA target, modifying splice site selection [107]. This perturbation of U2 also causes an accumulation on unspliced pre-mRNA in the cell nucleus, of which a small proportion has been shown to 'leak out' into the cytoplasm and undergo translation, generating aberrant proteins products which themselves can be cytotoxic [108, 109]. In addition, a number of these compounds have also been shown to decrease levels of vascular endothelial growth factor (VEGF), inhibiting tumour angiogenesis in vivo [110].

However, while the clinical utility of these agents has been well demonstrated in pre-clinical studies, for example the dose-dependent growth inhibition seen in prostate cancer xenografts following treatment with pladienolide B [111], early phase clinical trial results have been more mixed. Two phase 1, open-label, single-arm, dose-escalation studies have assessed the pladienolide E7107 in patients with locally advanced or metastatic solid tumours, which although showed that E7107 was generally well tolerated and produced both dose-dependent and reversible inhibition of pre-mRNA processing in target genes *in vivo*[112], both were suspended as a result of unexpected incidences of bilateral optic neuritis [112, 113].

H3B-8800, a small molecule modulator of SF3B1 [114] has also entered a phase 1 clinical trial (NCT02841540) to determine the maximum tolerated dose and recommended Phase 2 dose in patients with Myelodysplastic Syndromes (MDS), Acute Myeloid Leukaemia (AML), or Chronic Myelomonocytic Leukaemia (CMML) where recurrent heterozygous mutations of SF3B1 are though to play a pathological role. If found to be efficacious in subsequent phase 2 and 3 trials, H3B-8800 could provide proof of principle that targeting the spliceosome is a genuine treatment strategy, and opens the door to a variety of new therapeutic avenues. However, the toxicity and tolerability of these agents will equally prove to be important factors as to whether or not these agents make their way into routine clinical use.

Targeting spliceosomal regulatory proteins

Rather than target the core spliceosome, an alternative approach to modulating splicing is to target its protein regulators. For example, a variety of compounds have been identified that can inhibit SR protein phosphorylation, which has been shown in lab models to inhibit splicing [115]. TG-003, a benzothiazole, is one such agent and functions as an inhibitor of CLK1, CLK2 and CLK4, members of the CDC2-like (or LAMMER) family of dual specificity protein kinases. These kinases are typically involved in the phosphorylation of SR proteins in the cell nucleus [116], inhibition of which results in the inhibition of splicing and dissociation of spliceosomal nuclear speckles [116].

More recently, bromodomain and extra-terminal (BET) inhibition, a promising therapeutic approach currently undergoing clinical evaluation in CRPC (NCT03150056, NCT02711956), has also been shown to effect alternative splicing by modulating spliceosomal regulators [117]. In a study by Asangani et al. the BET inhibitor JQ1 was found to decrease the expression of AR-V7 in pre-clinical models of CRPC by down-regulating the activity of splicing factors SRSF1 and U2AF65, and in doing so re-sensitised resistant prostate cancer cells to AR targeted therapy [118]. However, as with therapeutic agents targeting the core spliceosomal complex, the long-term success of BET inhibition as a clinically useful therapeutic modality will hinge on the toxicity profile BET inhibitors demonstrate in ongoing clinical trials.

Other small molecule inhibitors of the spliceosome

A number of other small molecules have also been identified as being capable of modulating the spliceosome, some of which have been reported to have efficacy in cancer. However, these studies have generally been limited by their use of cell-free and non-mammalian models [119], as such, currently the therapeutic application of these agents is considered limited. Despite this, some interesting results have been seen with a number of these agents. For example, NB-506, a glycosylated indolocarbazole derivative that inhibits the capacity of topoisomerase I to phosphorylate SRFS1, has been shown *in vitro* to disrupts early spliceosome assembly and produces a cytotoxic effect in murine P388 leukaemia cells [120]. In addition, anti-tumour activity has also been shown pre-clinically with the biflavonoid natural plant product isoginkgetin, at least in part through its ability to interfere with the recruitment of the snRNP U4/U5/U6 and inhibiting splicing by precluding the transition from spliceosomal complex A to B [121].

Targeting the spliceosome in oncogene-driven cancers

As described previously, MYC overexpression places considerable oncogenic stress on the spliceosome resulting in cells becoming equally dependent on the spliceosome for survival, as they are on MYC. This has led to the hypothesis that in these tumours, inhibition of the spliceosome may produce an anticancer effect. In support of this view recently it has been reported that in xenograft models of MYC dependent breast cancer, spliceosome dysregulation through the inhibition of SF3B1 with sudemycin D increases survival and limits metastases [65]. Ultimately however, while intriguing, whether this is a principle will be applicable to other similarly important genomic aberrations, or if the clinical utility of this approach will be limited to MYC dependent cancers in a subset of tumour types remains to be seen.

Targeting alternatively spliced variants

When devising therapeutic strategies to target pathological alternatively spliced variants, in addition to considering those generated through the action of the spliceosome as discussed previously, it is equally important to take into account protein variants resulting from alternative means such as genomic fusions or rearrangements which have been described in many cancers to impact key proteins including AR and PD-L1 [86]. As such, while targeting the spliceosome remains a key consideration in this process, given the multiple routes through which alternatively spliced variants can arise, the concept of directly targeting these protein variants, rather than their mechanism of origin seems logical.

Efforts to target alternatively spliced proteins remain attractive but doing so directly with small molecule inhibitors has to date proved challenging, often due to the inherent nature of these alternatively spliced variants. For example, as truncated alternatively spliced AR variants lack the AR LBD, alternative target sites are required to facilitate their inhibition. However, the disordered nature of the AR NTD, renders a consistent target site difficult to ascertain and has hindered drug development along this avenue, requiring the development of novel therapeutic strategies. One such approach that has been proposed is the use of monoclonal antibodies such as GP369, which specifically blocks the IIIb splice variant of FGFR2 [122]. GP369 showed efficacy in inhibiting tumour growth in pre-clinical studies of human cancer cell lines and tumour xenografts driven by activated FGFR2 signalling [123], however while a phase I trial in patients with advanced stage solid tumours known to express FGFR2 was opened (NCT02368951), the trial was terminated early. Despite this setback, the ability to target alternatively spliced protein isoforms using monoclonal antibodies may yet help circumvent the difficulties associated with directly inhibiting splice variants which have hampering drug discovery efforts in this regard to date.

Oligonucleotide therapy
Oligonucleotide-based therapies utilise engineered oligonucleotides designed to hybridize with RNA sequences known to be responsible for specific splicing events, to prevent their alternate splicing and the production of pathological erroneous protein products. The potential of these therapeutics has so far been best realised in neurodegenerative conditions where late-stage clinical trials are underway in Duchenne muscular dystrophy [124] and spinal muscular atrophy [125]. However, while the question remains as to whether oligonucleotide therapy is a viable treatment approach in cancer, particularly where these splicing events are more diverse, evidence in support of this approach stems from work by Smith et al. who have developed a novel RNA splice-switching oligonucleotide designed to induce skipping of exon 11 of the BRCA1 gene, which is key to the function of BRCA1 in DNA damage repair [126]. In doing so the authors report to have successfully rendered wild-type BRCA1 expressing cell lines more susceptible to PARP inhibitor treatment [126]. However, while this provides a fascinating potential therapeutic strategy for targeting BRCA1-functional cancers, the challenge in this setting would be to maintain BRCA1 functionality in non-cancer cells to minimise potentially widespread toxicity.

Conclusion
Splicing events represent a plausible mechanism of treatment resistance and disease progression in CRPC and have been proposed as a potential therapeutic target. Drug discovery efforts to date have however been challenging and utilising the spliceosome as a therapeutic tool seems attractive. However, as yet no spliceosome inhibitors have made an impact in prostate cancer clinical practise, largely due to the complexity of the spliceosome, and a lack of understanding of its biology.

Further research is therefore required to discover the mechanisms underpinning the splicing abnormalities thought to contribute to the progression of CRPC, as well as the consequences of inhibiting these factors, before the true utility of these therapies can be realised.

Competing Interests

GVR has served as a consultant to Pfizer, Astellas, Sanofi and Bayer and receives funding from Bayer in addition to having ownership interest in EtiraRx, GaudiumRx and C-diagnostics. JL has served as a paid consultant/advisor for Sun Pharma, Janssen, and Sanofi; has received research funding to his institution from Orion, Astellas, Sanofi, Constellation, and Gilead; and is the lead inventor of a technology that has been licensed to A&G, Tokai, and Qiagen.AP, AS, JW, AN and JSdB are employees of The Institute of Cancer Research, a not for profit research organization and independent college of The University of London, that has a commercial interest in abiraterone. AP, AS, JW, AN and JSDB have no personal fiscal interest in abiraterone. JSdB has served as an advisory board member for Astellas, AstraZeneca, Bayer, Genmab, Genentech, GlaxoSmithKline, Janssen, Medivation, Merck-Serono, MSD, Menarini, Orion Pharma, Pfizer and Sanofi-Aventis.
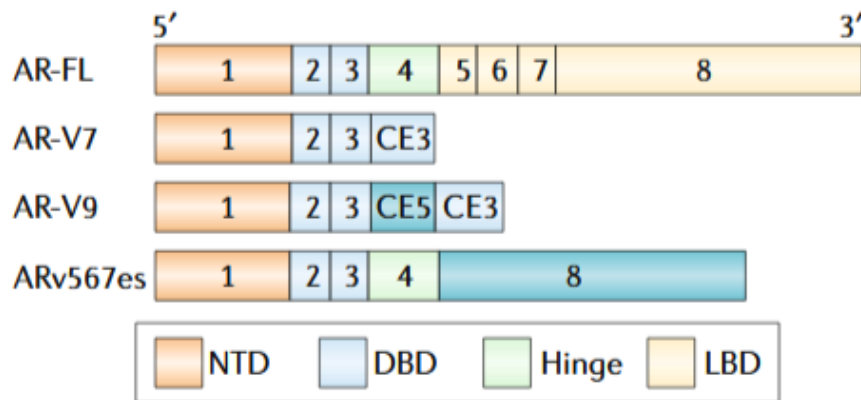
Fig. 1 |AR splice variants. A schematic diagram depicting the full- length androgen receptor (AR- FL) alongside a selection of its truncated protein isoforms, the androgen receptor (AR) splice variants (AR- Vs) AR- V7 , AR- V9, and ARv567es. These proteins share identical amino- terminal domains (NTDs) and DNA- binding domains (DBDs) but have unique carboxy- terminal extensions. AR- V7 and AR- V9 have a common 3′-terminal cryptic exon (CE), while ARv567es has a complete hinge region and nuclear localization signal, similar to that of the full-length protein, but lacks a ligand- binding domain (LBD).
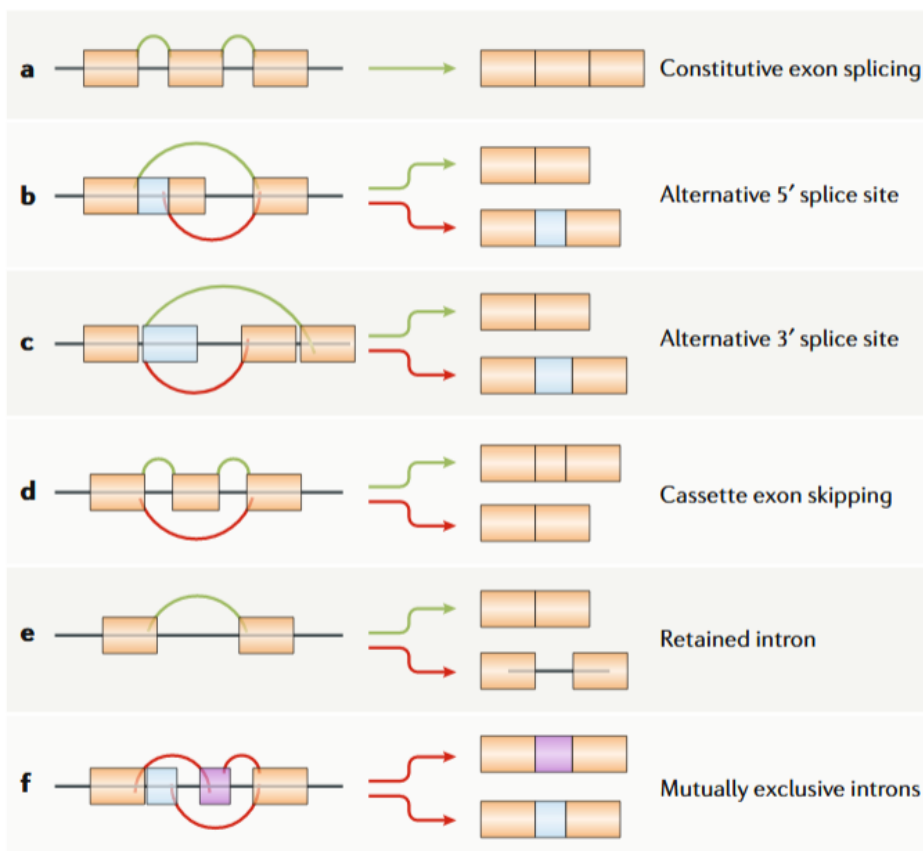

Fig. 2 |Summary of constitutive and alternative splicing events.a| Graphic depiction of constitutive splicing where introns are removed and sequential exons are ligated to produce mature mRNA. b,c| Alternative splicing, in which

changes in 5' and 3' splice site selection can result in the generation of alternatively spliced protein variants such as androgen receptor (AR) splice variant 7 (AR- V7), which possesses a 3'-terminal cryptic exon. d| Exon skipping, in which a cassette exon is spliced out of the nascent mRNA transcript altogether, along with its adjacent introns. e| Intron retention; an intron that does not form part of the canonical mRNA transcript is not removed and remains within the mature mRNA. f| Splicing, in which complex events that give rise to mutually exclusive alternative splicing events, in which only one of a set of two or more exons in a gene is included in the final transcript can also occur. Orange exons indicate those that are part of the canonical mRNA sequence; blue or purple exons indicate alternative sequences that might or might not be included in the mature mRNA. Black lines indicate introns, green lines indicate constitutive splicing patterns, and red lines indicate alternative splicing events.
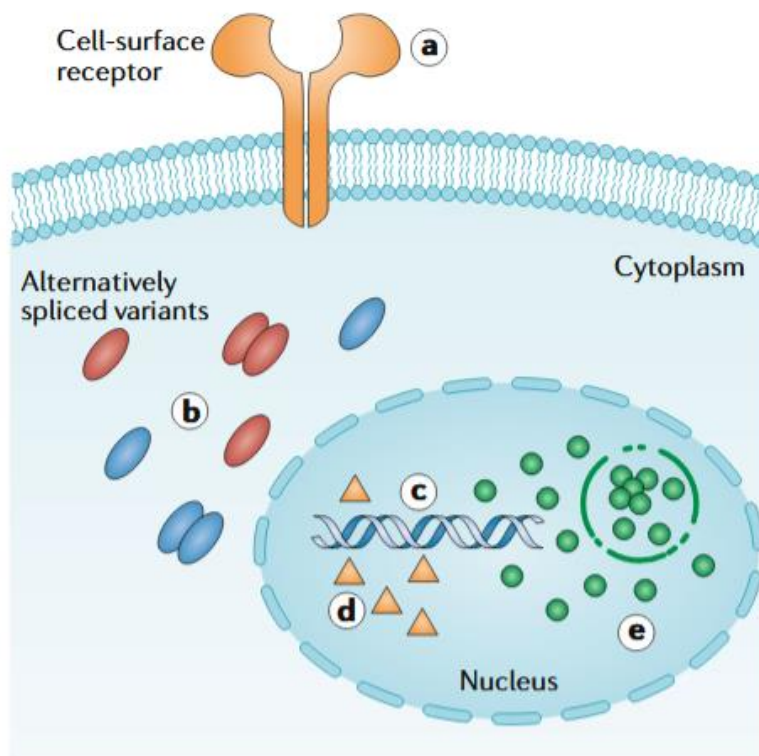

Fig. 3 |Mechanisms through which the spliceosome contributes to tumorigenesis and disease progression in prostate cancer.a| Alternative splicing of cell- surface receptors such as the FGFR have been reported to cause aberrant activation of key survival pathways in the absence of circulating androgens. b| Constitutively active splice variants of intracellular transcription factors such as the androgen receptor (AR; red ovals) have been linked with disease progression in patients with castration- resistant prostate cancer and are correlated with inferior overall survival outcomes. c| Gain- of-function mutations in cis- regulatory elements have been proposed to increase AR transcription in the absence of circulating androgens. d| Alternative splicing of key cellular regulatory proteins (orange triangles) such as G1–S- specific cyclin D1 (CCND1), a central component of cell cycle control, can promote the proliferation and survival of prostate cancer cells. e| Upregulation, as well as alternative splicing, of nuclear splicing factors (green circles) such as Kruppel-like factor 6 (KLF6) is able to increase cellular proliferation, colony formation, and invasion, as well as epithelial–mesenchymal transition, which contributes to AR-independent treatment resistance.

	Targeting the core spliceosome complex				Targeting spliceosomal regulatory proteins				Other small molecule inhibitors	
Agent	*Pladienolides A–G [127, 128]* *E7107 [111]*	*Herboxidiene (GEX1A) [129]*	*FR901463, FR901464 and FR901465 [130]* *Meayamycin B [131]* *Spliceostatin A [108]*	*H3B-8800 [114]*	*TG003 [116]*	*SRPIN340 [132]*	*Cpd-1, Cpd-2 and Cpd-3 [133]*	*GSK525762 [134]* *ZEN003694 [135]* *OTX105/MK-8628 [118]*	*Isoginkgetin [121]*	*NB‑506 [120]*
Actions	*Bind to and inhibit SF3B1 to destabilise recruitment of snRNP U2* *Decrease levels of VEGF* *Cell cycle arrest in G1 and G2/M* *Disrupts spliceosome assembly* *Generate truncated form of cell cycle inhibitor p27 which is still functional but more robust* *Reduce number of nuclear speckles* *Reduced tumour angiogenesis*			*Small molecule modulator of SF3B1* *Preferential lethality toward spliceosome-mutant cancer cells due to retention of short, GC-rich introns* *Currently in Phase I clinical trial (NCT02841540)*	*Competitive antagonist of CLK binding of ATP* *Inhibition of CLK enzymatic phosphorylation and activation of splicing factors e.g. SR proteins* *Dissociation of nuclear speckles*	*Competitive antagonist of SRPK1 and SRPK2 binding of ATP* *Nicotinamide inhibitor* *Inhibits SRPK phosphorylation and activation of splicing factors e.g. SR proteins* *Modulates splicing of VEGF*	*Inhibition of both CLKs and SRPKs, components of the splicing machinery that are crucial for exon selection* *CLK1, CLK2, SRPK1 and SRPK2* *Reduced phosphorylation of SR proteins* *Causes enlargement of nuclear speckles* *Causes widespread splicing alterations*	*Inhibitors of bromodomain and extra-terminal (BET) proteins BRD2, BRD3, BRD4 and BRDT* *Downregulate expression of splicing factors* *Decrease alternative splicing events in pre-clinical models* *Currently ongoing clinical evaluation (NCT03150056, NCT02711956)*	*Biflavonoid natural plant product that interferes with the recruitment of the snRNP U4/U5/U6* *Prevents transition from spliceosomal complex A to B*	*Inhibits the SRFS1 phosphorylation by topoisomerase I* *In vitro disrupts early spliceosome assembly and produces a cytotoxic effect*

Table 1: Small molecules reported to have effect on splicing. snRNP = small nuclear ribonuclearprotein, CLK = CDC2-like kinase; SRPK = serine and arginine protein kinase; SRPIN340 = N-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl; VEGF = vascular endothelial growth factor.

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