Transcriptome analysis of spliceosome inhibition in human cells

by

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Abstract:

Small-molecule inhibitors of the spliceosome have been shown to have anti-tumour effects, yet their mechanism of cancer inhibition remains unknown. Microarray analysis by our lab identified the activation of the endoplasmic reticulum unfolded protein response (UPR) in cells treated with the splicing inhibitor isoginkgetin (IGG). In this study, we confirmed that IGG activates the UPR, however a second spliceosome inhibitor, pladienolide B (PB) did not show this activation. RNA-seq/gene ontology analysis was also performed on cells treated with IGG, PB, and another splicing inhibitor spliceostatin A, as well as knockdowns of spliceosome components. These data showed support for endoplasmic reticulum activation, but most prominently implicated inflammatory responses. Taken together, spliceosome inhibition appears to elicit a consistent response of inflammation pathway activation, and also implicates endoplasmic reticulum activation, however there are also unique responses to the method of inhibition, suggesting that spliceosome inhibition does not necessarily result in one homogeneous response.

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List of abbreviations:

AP1	Activator protein 1	PAP	Poly A polymerase
ATF4	Activating transcription factor 4	PAS	Polyadenylation signal
ATF6	Activating transcription factor 6	PB	Pladienolide B
BiP	Binding immunoglobulin protein	PERK	Protein kinase R-like ER kinase
BPS	Branch point sequence	PKR	Protein kinase R
CHAC1	Glutathione-specific gamma-glutamylcyclotransferase 1	Pol II	RNA polymerase II
CHX	cycloheximide	PPP1r15A/GADD34	Protein phosphatase 1 regulatory subunit 15A
CPSF	Cleavage and polyadenylation specificity factor	PRPF8	Pre-mRNA-processing-splicing factor 8
CRE	c-AMP response element	RIP1	Receptor-interacting protein 1
DDIT3	DNA damage inducible transcript 3	SF1	Splicing factor 1
DDIT4	DNA damage inducible transcript 4	SF3A3	Splicing factor 3A subunit 3
DE	Differential expression	SF3B1	Splicing factor 3B subunit 1
DOX	Doxycycline	SF3B4	Splicing factor 3B subunit 4
DSE	Downstream sequence element	snRNP200	Small nuclear ribonucleoprotein U5 subunit 200
dsRBD	Double stranded RNA binding domain	SRP	Signal recognition particle
EFTUD2	Elongation factor tu GTP-binding domain-containing 2	SSA	Spliceostatin A
eIF2 α	Eukaryotic translation initiation factor 2 alpha	TFIIB	General transcription factor transcription factor II B
EJC	Exon junction complex	TNFAIP3	TNF alpha induced protein 3
ER	Endoplasmic reticulum	TNFR1	Tumour necrosis factor receptor 1
GADD45	Growth arrest and DNA damage 45	TNFR2	Tumour necrosis factor receptor 2
GCN2	General control non-depressible 2	TNF α	Tumour necrosis factor
HRI	Heme-regulated inhibitor	TPE	TPA-responsive element
IGG	Isoginkgetin	TRADD	TNFR1-associated death domain

IL-17	Interleukin 17	TRAF2	TNF-receptor-associated factor 2
IRE1 α	Inositol requiring enzyme 1	U2AF	U2 associated factor
ISR	Integrated stress response	U2AF1	U2 associated factor subunit 1
I κ B	Inhibitor of nuclear factor kappa B	U2AF2	U2 associated factor subunit 2
JNK	c-Jun N-terminal kinase	uORF	Upstream open reading frame
mRNP	Messenger ribonucleoprotein	UPR	Unfolded protein response
ND	No drug	UTR	Untranslated region
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B-cells	XBP1	X-box binding protein 1
NR4A1	Nuclear receptor subfamily 4 group A member 1		

Introduction:

In eukaryotic cells, protein expression is initiated in the nucleus by transcription of protein coding genes into pre-mRNAs, which are then processed and exported out of the nucleus and into the cytosol where they can be translated into proteins. The process of going from the initiation of transcription to mRNA synthesis to translation of protein involves many complex cellular networks of pre-mRNA processing, mRNA export and localisation, translation, and mRNA degradation.

Transcription is initiated by transcription factors that bind promoter regions in a sequence specific manner, leading to local chromatin remodeling and the recruitment of RNA polymerase II (Pol II), which produces pre-mRNA. In order to recognize the promoter, Pol II works in complex with several general transcription factors, forming a pre-initiation complex. The TATA-box-binding protein, a subunit of the general transcription factor, transcription factor II B (TFIIB), binds DNA upstream of the transcription start site at a specific DNA core promoter sequence, and facilitates the assembly of the other general transcription factors to form the pre-initiation complex (Cramer, 2019). This stage of assembly is sufficient for elongation, however, it is usually not highly active and requires further interaction with trans-acting transcription factors. These transcription factors bind enhancer regions of DNA and are brought into proximity with the pre-initiation complex to increase transcription efficiency (Haberle, 2019).

In the initial stages of elongation, the 5' triphosphate of the pre-mRNA is cleaved by RNA triphosphatase to create a diphosphate terminus, then guanyltransferase adds GMP to the diphosphate, which is then methylated by methyltransferase, resulting in a 7meGppp cap, and

elongation continues (Cowling, 2010). Concurrently with elongation, the non-coding introns are removed by the spliceosome and specific proteins bind the new exon-exon junction to form exon junction complexes (EJCs) where the intron used to be (Hir *et al.*, 2000). After the gene has been transcribed, a poly A tail is added to the 3' end. To do this, the cleavage and polyadenylation specificity factor (CPSF) binds a polyadenylation signal (PAS) in the 3' UTR, and CstF binds a GU-rich downstream sequence element (DSE). Additional proteins complex with these factors, including poly A polymerase (PAP), to catalyse the cleavage of the mRNA between the PAS and the DSE, and the 3' end is then polyadenylated by PAP (McKay, 2014).

In addition to direct modification of the RNA itself, various proteins also bind the pre-mRNA at multiple different sites during transcription and processing to form a messenger ribonucleoprotein (mRNP). These proteins and their functions are not fully understood, but they are known to affect the fate of the mRNA by the various interactions they can facilitate in the cell, including nuclear export, subcellular localization, and degradation. Current evidence shows that a number of mRNP proteins are linked to splicing in some way, such as exon junction complexes or the THO complex, which is a protein thought to be recruited to the site of the first splicing event and interacts with the nuclear pore to facilitate export of the mRNA from the nucleus (Masuda *et al.*, 2005; Moore & Proudfoot, 2009). The fact that these proteins are observed to be a function of splicing also allows them to serve a secondary role as quality controls for the splicing process. Failed recruitment of the THO complex impairs the ability of the mRNA to leave the nucleus thus preventing translation, and failed recruitment of an EJC due to a missed intron can lead to nonsense mediated decay, a process whereby a translating mRNA will be targeted for degradation if the ribosome encounters an EJC fifty or more

nucleotides downstream of a premature stop codon (Rodrigues *et al.*, 2000; Eszter & Nagy, 1998; Moore & Proudfoot, 2009).

Splicing:

Spliceosome assembly:

As stated above, transcription of most eukaryotic protein coding genes results in an RNA product called pre-mRNA that requires further processing before a functional protein can be translated. One of the most notable processing steps is the removal of non-coding nucleotide sequences called introns and the concurrent ligation of the coding nucleotide sequences called exons, in a process known as pre-mRNA splicing. This process is conducted by a ribonucleoprotein called the spliceosome.

The spliceosome is a multi-component ribonucleoprotein complex that undergoes a series of assembly steps and conformational changes in order to remove the introns and ligate the exons during pre-mRNA processing (Figure 1). This is accomplished by two transesterification reactions within the RNA. First, the 2'-OH of a specific adenine located in the branch point sequence (BPS) reacts with the guanine at the intron's 5' splice site forming a lariat structure of the intron. Second, the new terminal 3'-OH of the upstream exon is reacted with the 5' end of the downstream exon, releasing the intron in lariat form and joining the exons in sequence (Ruskin *et al.*, 1984).

Figure 1. Spliceosome assembly and catalysis of exon splicing.

Introns are bound by U1 at the 5' SS, SF1 at the BPS, and U2AF at the 3' SS to form the E complex. Next, U2 is recruited by SF1 and U2AF to form the A complex. The U4/U6.U5 tri-snRNP is then recruited to form the B complex, and U4 and U1 are then ejected to form the B* complex. The B* complex ligates the 5' SS to the BPS, to form the C complex, which then ligates the free 3' end of the upstream exon to the 5' end of the downstream exon, removing the intron and forming the P complex. The spliceosome then releases the pre-mRNA and is disassembled.

These two reactions require numerous protein and ribonucleoproteins working in concert through a series of reactions and conformational changes. The number and nature of all the players in the splicing process is still an ongoing area of study, but it can be broadly described as follows: the process begins with the U1 small nuclear ribonucleoprotein (snRNP) binding the 5' splice site of an intron, splicing factor 1 (SF1) binding the BPS, and U2 associated factor (U2AF) binding the 3' splice site. SF1 and U2AF then recruit the U2 snRNP to the pre-mRNA, displacing SF1 and forming the A complex. A pre-assembled U4/U6.U5 tri-snRNP is then joined in complex with the U1 and U2 snRNPs, forming the precatalytic B complex—the final stage of pre-catalytic assembly. Following the formation of the B complex, the spliceosome undergoes a series of conformational changes resulting in the ejection of the U1 and U4 snRNPs and the formation of the now catalytically active B* complex. The B* complex then conducts the first transesterification reaction of the pre-mRNA, bonding the 5' guanine to the BPS adenine, and causing further conformational changes in the spliceosome, resulting in the C complex. The C complex then catalyzes the second transesterification reaction, ligating the 3' end of the downstream exon to the 5' end of the upstream exon and subsequently forming the P complex. After the second transesterification reaction, the P complex then dissociates from the pre-mRNA, and releases the intron lariat to finish the splicing process (Shi, 2017).

As detailed later, we looked at RNA-seq data from shRNA knockdowns for several RNA-binding splicing components taken from data generated by the Graveley laboratory from a broad-scale knockdown of 276 RNA-binding proteins (Sundararaman, 2016). These are comprised of: SF1, U2 associated factor subunit 1 (U2AF1), U2 associated factor subunit 2 (U2AF2), splicing factor 3A subunit 3 (SF3A3), splicing factor 3B subunit 1 (SF3B1), splicing

factor 3B subunit 4 (SF3B4), pre-mRNA-processing-splicing factor 8 (PRPF8), elongation factor tu GTP-binding domain-containing 2 (EFTUD2), and small nuclear ribonucleoprotein U5 subunit 200 (snRNP200) (Table 1). These nine knockdowns are involved in many of the key steps of pre-mRNA splicing and provide a fairly robust sampling of the overall splicing process, including BPS recognition, 3' SS recognition, U2 recruitment, formation of catalytic spliceosome, catalysis, and snRNA unwinding.

Table 1. Spliceosome components investigated in shRNA knockdown/RNA-seq analysis.

Gene	Associated complex	Function ¹
SF1	BPS	Binds BPS to recruit U2
U2AF1	U2	Interacts with SF1 to recruit U2. Recognizes 3' splice site
U2AF2	U2	Interacts with SF1 to recruit U2. Recognizes 3' splice site
SF3A3	U2	Necessary for U2 binding of pre-mRNA
SF3B1	U2	Aid in recruitment of U2 to mRNA. Prominent splicing inhibitor target
SF3B4	U2	Aid in recruitment of U2 to mRNA
PRPF8	U5	Scaffold protein thought to facilitate many assembly reactions (Grainger & Beggs, 2005)
EFTUD2	U5	GTPase in catalytic core of spliceosome
snRNP200	U5	RNA helicase that unwinds snRNAs.

¹ see text for references

SF1 is responsible for recognition of the BPS and aids in the recruitment of the U2 snRNP (Berglund, 1998). After U2 is incorporated, SF1 dissociates from the pre-mRNA (Rutz & Seraphin, 1999). U2AF1 and U2AF2 are the small and large subunits of U2AF, respectively, which is a protein complex that recognizes the 3' splice site and works with SF1 to recruit the U2 snRNP to the BPS and 3' splice site (Valcarce *et al.*, 1996; Wu *et al.*, 1999). SF3A3, SF3B1 and SF3B4 are components of the U2 snRNP that function in binding of U2 to the pre-mRNA (Behrens *et al.*, 1993; Goals *et al.*, 2003). PRPF8, EFTUD2, and snRNP200 are components of

the U5 snRNP. PRPF8 is thought to function as a scaffold and cofactor for several different components and is essential to the splicing process (Grainger & Beggs, 2005), EFTUD2 is a GTPase that forms part of the catalytic core of the spliceosome, though its exact function is unknown (Agafonov *et al.*, 2016; Frazer *et al.*, 2009), and snRNP200 is a helicase responsible for the unwinding of snRNAs during snRNP remodeling (Absmeier *et al.*, 2016). Together, these nine proteins cover many of the major spliceosome assembly reactions, though it should be noted that several of these components are present throughout the assembly process and the effect of the knockdown of a component may not be representative of the disruption of its canonical role in the spliceosome (e.g. knockdown of the snRNP200 helicase may induce a cellular response unrelated to the loss of helicase activity in the spliceosome).

Splicing inhibition:

The spliceosome as a drug target is a relatively new concept, with a small number of compounds identified to date. Most of this effort has focussed on small molecule inhibitors such as FR901464 derivatives (spliceostatins and sudemycins) and pladienolides that have recently been shown to inhibit spliceosome formation (Leon *et al.*, 2017, Martinez-Montiel *et al.*, 2016). However, these two classes of drugs, like nearly all small molecule inhibitors with known targets, target the SF3B1 subunit of the U2 snRNP. This implies that the range of potential effects of spliceosome inhibition being studied is very limited, considering there are over 200 known spliceosome proteins.

While the effects of spliceosome inhibition are poorly understood and limited largely to the effects of SF3B1 inhibition, there have been some notable observations. One recurring observation is elevated rates of intron retention, which has been shown on an individual gene

level, as well as the transcriptomic level (Effenberger *et al.*, 2016; Yoshimoto *et al.*, 2017). The mechanisms behind this retention have not been elucidated, but one hypothesis is that inhibition of SF3B1 results in a failure to form a functional spliceosome at that intron, and consequently any intron which recruits an inhibitor-bound U2 snRNP will fail to be excised. It is perhaps worth noting that a separate spliceosome forms at each intron, so the inhibition effects may work on an intron by intron basis, rather than affecting splicing for the entire transcript. Another observed effect of splicing inhibition is pre-mRNA leakage; unprocessed or partially processed pre-mRNAs leaking out of the nucleus and into the cytosol (Yoshimoto *et al.*, 2017).

One recurring feature of these small molecule splicing inhibitors that makes them of particular interest is their anti-tumour activity. Spliceostatin, pladienolides, and isoginkgetin (IGG), all examined in this study, have all displayed anti-tumour activity, as have many other classes of splicing inhibitors, and the potential of drugging the spliceosome as a cancer therapy is a rapidly developing area of research (Bonnal *et al.*, 2012, Hsu *et al.*, 2016; Lee & Abdel-Wahab; Lin, 2018; Salton & Misteli, 2017). Small molecule splicing inhibitors have also shown potential in sensitizing cancer cells to other chemotherapeutic drugs (Treiger *et al.*, 2020) and in targeting cancers with spliceosome mutations (Seiler *et al.*, 2018).

Splicing inhibitors:

Spliceostatin A (SSA) is a modified form of a *Pseudomonas* sp. metabolite that was originally identified as an anti-tumour agent (Nakajima *et al.*, 1996). It was later shown to be a splicing inhibitor that targeted SF3B1 (Kaida *et al.*, 2007), and subsequent research into its anti-

cancer mechanisms have tended to focus on its potential to create alternatively spliced genes (Larrayoz *et al.*, 2016; Maguire *et al.*, 2015; Satoh & Kaida, 2016).

PB is a compound originally isolated from *Streptomyces platensis* and, like SSA, it was isolated for its potent anti-tumour activity (Mizui *et al.*, 2004). Again, like SSA, it wasn't until many years later that it was discovered to be a splicing inhibitor, also targeting SF3B1 (Yokoi *et al.*, 2011). There has since been research into the underlying mechanisms of its anti-tumour activity, and again, like SSA, it has been largely focussed on the potential ability of a splicing inhibitor to influence the expression of different splicing isoforms (Hepburn *et al.*, 2018; Sato *et al.*, 2014; Sciarrillo, 2019; Zhang *et al.*, 2020).

IGG is a naturally occurring secondary metabolite of the *Ginkgo biloba* tree that has an inhibitory effect on splicing. While its target is unknown, it has been shown to prevent the stable recruitment of the U4/U6.U5 snRNP trisnRNP, and therefore presumably acts on the spliceosome at a later stage of assembly than PB or SSA (O'Brien *et al.*, 2008). Like SSA and PB, IGG has also been shown to function as an anti-tumour agent for a variety of purported reasons (Heinhaus *et al.*, 2016; Tsalikis *et al.*, 2019; Yoon *et al.*, 2006), yet no conclusive mode of activity for these anti-tumour effects has been established.

All three of these drugs have repeatedly observed instances of spliceosome inhibition and anti-tumour activity across a variety of cell types; however there is relatively little evidence for, or consensus on, the underlying mechanisms (Hepburn *et al.*, 2018; Tsalikis *et al.*, 2019; Zhang *et al.*, 2019). Most studies highlight the change in expression of an alternatively spliced gene or genes in response to spliceosome inhibitor treatment; however, there is still no consensus on which genes or even which pathways are being affected by this alternative

splicing (Endo *et al.*, 2015; Larroyoz *et al.*, 2016; Satoh & Kaida, 2016; Zhang *et al.*, 2020). While discovering alternatively spliced products is undoubtedly important in resolving the increasingly complex picture of spliceosome inhibition, it ignores the more general effects that may be implicated in its anti-tumour activity. For example, it may be that cancerous cells have an increased global level of aberrantly spliced pre-mRNAs that is stressful to the cell, but not lethal; however, spliceosome inhibition may increase the level of aberrantly spliced pre-mRNAs to a point that is lethal to the cancerous cells, yet tolerable to healthy ones. Research directed towards detecting alternatively spliced genes and the activity of their proteins ignores this possibility. The work in our lab has focussed on studying the more general, downstream cellular effects of splicing inhibition, rather than identifying specific alternatively spliced genes.

It should be noted that the presumption that IGG acts at a later stage of the spliceosome's assembly than PB and SSA is of particular importance to this study, since the stage at which a drug acts could theoretically produce different aberrant splice products. For example, SSA and PB target the U2 snRNP, which is responsible for branch point selection, and there is evidence that SSA treatment leads to reduced fidelity of selection site (Corrionero, *et al.*, 2011). In contrast, IGG presumably acts at a later stage of assembly where the BPS has already been selected, presumably with uncompromised BPS selection machinery. This could mean, in theory, that PB and SSA treatment would result in an increase in alternatively spliced products, since the BPS was atypical but the catalysis of splicing was potentially unaffected, whereas IGG treatment would only result in intron retention, since the BPS selection is unaffected but the catalytic form of the spliceosome fails to form.

In pursuing the general effects of spliceosome inhibition, our lab looked at cell cycle arrest in response to both PB and IGG and found that the results were not identical. Microarray analysis of IGG-treated cells showed upregulation of the genes DNA damage inducible transcript 3 (DDIT3), DNA damage inducible transcript 4 (DDIT4), and glutathione-specific gamma-glutamylcyclotransferase 1 (CHAC1), which are upregulated in response to endoplasmic reticulum (ER) stress. From this, we hypothesized that IGG-induced splicing inhibition results in an increase in aberrantly spliced mRNAs, which are then translated into aberrant proteins, which activate an ER-stress pathway called the unfolded protein response (UPR). To further compare the effects of PB and IGG on the general cellular response, this study investigated the activation of the UPR in response to the two drugs.

Unfolded protein response:

While translation is canonically performed in the cytosol, certain proteins, such as those destined for the plasma membrane, organelles, or the secretory pathway, are translated and processed in the ER. The transcripts that are intended to be translated at the ER code for a specific amino acid sequence that is recognized by a signal recognition particle (SRP) that shuttles the actively translating ribosomal complex to the ER where it is bound to the membrane via an SRP receptor (Walter *et al.*, 1981; Gilmore *et al.*, 1982). Translation is continued in the ER with the ribosomal complex translating and releasing its protein product into the lumen of the ER to undergo post-translational modifications and to be exported to its intended destination. During the process of post-translational modification, aberrant proteins can be created due to incorrect folding by chaperones or mistranslated proteins with incorrect

amino acid sequences that cannot be processed correctly (Schwarz, 2016). One of the main ways the ER deals with this issue is the UPR.

The UPR is a stress response to an overabundance of unfolded or misfolded proteins in the ER lumen (Figure 2). The response is initiated by three different ER transmembrane proteins: protein kinase R (PKR)- like ER kinase (PERK), inositol requiring enzyme 1 (IRE1 α), and activating transcription factor 6 (ATF6), each leading to a cellular response designed to reduce stress on the ER. These three sensors are maintained in an inactive state through continuous interaction with the binding immunoglobulin protein (BiP), a multifunctional chaperone protein located in the ER lumen. When the ER becomes overburdened with unfolded or misfolded proteins, ER demand for BiP can increase to the point that it is competed away from its inhibitory signaling role by the misfolded proteins, and the stress response is de-repressed (Saito, 2018).

Figure 2. Unfolded protein response

The unfolded protein response consists of three pathways initiated by three distinct receptors, ATF6, PERK, and IRE1 α , which are kept in an inactive, monomeric state through interactions with the chaperone protein BiP. Accumulation of misfolded protein draws BiP away from the three receptors, allowing them to oligomerize into an active form. Upon oligomerization, ATF6 is secreted from the ER membrane and processed into a transcription factor by the Golgi apparatus, PERK phosphorylates eIF2 α to inhibit translation (not shown) and activate ATF4, and IRE1 α splices inactive XBP1 mRNA into its active form, which is translated into a transcription factor. Together these three responses reduce protein load and upregulate a host of genes involved in stress responses and protein folding to alleviate the stress cause by the accumulation of misfolded proteins.

PERK:

PERK is an ER transmembrane protein that is kept in an inactive, monomeric state under normal cellular conditions through its interactions with BiP. BiP also binds tightly to misfolded proteins so as misfolded proteins accumulate in the ER, the amount of BiP available to interact with the luminal domain of PERK decreases. When available BiP levels are insufficient to keep PERK in a monomeric state, PERK will oligomerize into a dimer or tetramer state and become active. Activated (derepressed) PERK has kinase activity and will phosphorylate the eukaryotic translation initiation factor 2 alpha (eIF2 α), resulting in a global decrease in cap-dependent translation and reducing the protein load on the stressed ER (McQuiston & Diehl, 2017).

In addition to the reduced protein load, phosphorylation of eIF2 α also leads to increased expression of activating transcription factor 4 (ATF4). ATF4 is a basic leucine zipper transcription factor that recognizes C/EBP-ATF response elements, causing upregulation or repression of a wide variety of stress-responsive proteins involved in, among other pathways, antioxidant response, apoptosis, and autophagy (Kasai *et al.*, 2019). ATF4 also activates the pro-apoptotic ATF4-ATF3-DDIT3 pathway, which contains three of the genes upregulated in our lab's unpublished microarray analysis—DDIT3, DDIT4, and CHAC1 (Mungrue *et al.*, 2009; Whitney *et al.*, 2009). ATF4 mRNA contains two upstream open reading frames (uORFs) in its 5' UTR which serve to control expression at the translational level. The 5'-proximal uORF (uORF1) is only three codons long and serves as the translation initiation site. The downstream uORF (uORF2) overlaps with the ATF4 coding region but in a different frame, so translation will ultimately be terminated if this uORF is used (Vattem & Wek, 2004). Under normal cellular conditions, translation will be initiated at uORF1, terminated after the three codons have been

translated, and another ternary complex will form on the scanning 40S ribosome before it reaches uORF2. This will result in the translation of the out-of-frame ATF4 and subsequent termination of translation. However, if the levels of phosphorylated eIF2 α are such that the scanning 40S ribosomal subunit cannot assemble a functional ribosome in time to re-initiate translation at the second uORF, but can assemble one before the start of the ATF4 coding region, it will then translate the functional ATF4 protein (Young & Wek, 2016). In this way, ATF4 is a translationally controlled stress response transcription factor that is specifically upregulated in response to global translation inhibition via eIF2 α deactivation.

IRE1 α :

IRE1 α is negatively regulated by BiP similarly to PERK. IRE1 α is a transmembrane protein with a luminal N terminal, a cytosolic C terminal, a Ser/Thr kinase domain, and an endoribonuclease domain (Sidrauski & Walter, 1997). Through interaction with BiP, IRE1 α is kept in an inactive, monomer state. Without this interaction, IRE1 α will dimerize and auto-phosphorylate into an enzymatically active form, which in turn activates the transcription factor X-box binding protein 1 (XBP1) (Amin-Wetzel *et al.*, 2017). XBP1 is a gene that encodes an mRNA called XBP1u that does not encode the functional XBP1 transcription factor under normal cellular conditions. However, after IRE1 α undergoes dimerization and auto-phosphorylation, its endonuclease activity will remove a 26-nucleotide intron from the cytosolic XBP1u mRNA, causing a frameshift in the transcript which then codes for an active transcription factor, in a peculiar example of non-spliceosome-mediated, cytosolic splicing (Sidrauski & Walter, 1997; Wu *et al.*, 2015). Translation of this transcription factor then leads to

upregulation of a host of genes associated with ER protein production, folding, and degradation (Wu *et al.*, 2015).

ATF6:

ATF6 is also held in an inactive state by BiP interactions. ATF6 contains a luminal C-terminus, a cytosolic N-terminal leucine zipper, and a golgi localization signal that is bound by BiP under normal cellular conditions (Haze *et al.*, 1999; Shen *et al.*, 2002). In the case of ER stress where BiP levels are insufficient for proper ATF6 interaction, the golgi localization signal becomes accessible and ATF6 is exported out of the ER membrane (Chen *et al.*, 2002; Shen *et al.*, 2002). ATF6 is then taken to the Golgi apparatus where site-1 protease and site-2 protease cleave the cytosolic N-terminus into an active transcription factor, leading to upregulation of genes containing ER stress response elements, which include a number of chaperone proteins (Ye *et al.*, 2000; Shen *et al.*, 2002; Wang *et al.*, 2000; Yamamoto *et al.*, 2007).

Generally, the UPR is a three-branched adaptive stress response that is activated in response to increased need for ER-associated chaperone activity. It is generally an adaptive response that works to reduce ER-protein load by inhibiting the production of further proteins and upregulating chaperone proteins to aid in protein folding to ease cell stress. However, this stress pathway can also lead to apoptosis if the stress signals are not resolved.

UPR in cancer

The rapid and constant growth of cancerous cells is metabolically demanding and requires high levels of activity from translation machinery, including those located in the ER. Coupled with other factors such as the hypoxic environment and genomic aberrations of cancerous cells, ER stress is a common feature in cancer. These cells need to take advantage of

the ER-stress-relieving elements of the UPR, as well as avoiding its apoptotic ones, in order to proliferate. This suggests the UPR has potential as a target for cancer treatment (Oakes, 2020).

While ER stress and UPR activation are frequently observed characteristics of cancerous cells, the modulation of the UPR as a method of cancer inhibition has produced varying results.

PERK-ATF4 induction has been shown to cause apoptosis in B-chronic lymphocytic leukemia cancer cells; however it has also been shown that activation of this pathway leads to upregulation of genes that increase tolerance to hypoxia, promote angiogenesis, increase autophagy, and downregulate apoptosis, all of which help support tumour survival.

IRE1 α activation has been shown to slow the progression of tumour growth and increase the survival of mice with colorectal tumours. Conversely, its activation in human breast cancers have been shown to have an oncogenic effect by facilitating adaptation to hypoxia. ATF6 has received much less research, but seems to confer a cytoprotective effect, and current evidence suggests that its activation exhibits no anti-tumour effect (Walczak *et al.*, 2019).

These varying results have provided some evidence that the UPR may prove useful as a target in cancer treatment; however they also imply that the mechanisms are quite complex and will require much further research to properly understand the effects of targeting the UPR.

Hypothesis and rationale:

Spliceosome inhibition is a novel and rapidly advancing area of chemotherapeutic research, but the mechanisms behind its anti-tumour activity are not understood. This study sought to investigate the general cellular response to spliceosome inhibition, particularly in terms of gene expression. Microarray analysis suggested that DDIT3, DDIT4, and CHAC1 were upregulated in response to the spliceosome inhibitor isoginkgetin, which are all

transcriptionally upregulated by ATF4, a transcription factor that plays a prominent role in the unfolded protein response. Therefore, we hypothesized that spliceosome inhibition triggers the unfolded protein response. This stress response may affect the outcome of spliceosome inhibition in cancer cells because they are known to exploit the UPR for cell survival, and an increase in this already active stress response could preferentially induce apoptosis in cancer cells, providing a possible explanation for the well-established anti-tumour potential of splicing inhibitors.

Materials and methods:

Cell culture:

NF-hTERT and HeLa tet-off d2EGFP -UTR cells were grown in Dulbecco's Modification Eagle's Medium (Wisent) supplemented with 12% fetal bovine serum (Gibco) and 1.25% penicillin-streptomycin (Hyclone). HCT116 and HCT116 XBP1-mNG (neon green) cells were grown in McCoy's 5A medium (Hyclone), supplemented with 9% newborn calf serum (Gibco), 3% fetal bovine serum (Gibco), and 1.25% penicillin-streptomycin (Hyclone). Cells were seeded at a density of 2.5×10^5 cells per 6 cm dish for flow cytometric and qRT-PCR analyses before being treated with 3 mL of medium containing either DMSO (Sigma Canada), IGG (EMD Millipore), PB (EMD Millipore), or thapsigargin (Tg) as a positive control. Tg is a well-established inducer of ER stress that inhibits the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase. This causes an efflux of Ca^{2+} from the endoplasmic reticulum, preventing its use as a cofactor for calcium-dependent chaperone proteins, which results in impaired protein folding and subsequent ER stress (Oslowski & Urano, 2013).

qRT-PCR:

After drug treatment, HCT116 cells, HeLa Tet-off d2EGFP cells (for d2EGFP only), and NF-hTERT cells (for BiP, CALR, and PDIA6 only) were rinsed with PBS and RNA was extracted using either the RNeasy Mini Kit (Qiagen) (CHAC1, DDIT3 and DDIT4 analysis) or the EZ-10 DNAaway RNA Mini-Preps kit (Biobasic) (BiP, CALR, PDIA6) following the manufacturer's recommendations. Total RNA was quantified by spectrophotometry and reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with sample RNA concentrations normalized to the lowest sample. The expression of CHAC1, DDIT3, DDIT4, d2EGFP and GAPDH mRNA was determined using Bioline SensiFAST Probe HI-ROX Mix with TaqMan® Gene Expression Assay primers Hs00225520_m1, Hs00358796_g1, Hs01111686_g1, Mr04329676_mr Enhance, Hs02758991_g1, respectively (ThermoFisher Scientific). Expression of CALR, HSPA5, PDIA6, and GAPDH was measured using the SensiFAST SYBR Hi-ROX Kit (Applied Biosystems) using the following primers:

Table 2: Primers used in SYBR green qRT-PCR experiments

	Forward	Reverse
CALR	5'- CCTGCCGTCTACTTCAAGGAG -3'	5'- GAACTTGCCGGAAGTGAAGAC -3'
BiP	5'- CATCACGCCGTCCTATGTCG -3'	5'- CGTCAAAGACCGTGTTCTCG -3'
PDIA6	5'- GGACACTGCAAAAACCTAGAGC -3'	5'- CCAGAACCTGATTGACTGTAGCA -3'
GAPDH	5'- GGAGCGAGATCCCTCCAAAAT -3'	5'- GGCTGTCATACTTCTCATGG -3'

Flow cytometry:

For translation shutdown analysis, HeLa Tet-off d2EGFP cells with a constitutively active trans-gene were treated with 30 μ M IGG or 25 nm PB for 24 hours then washed with PBS and collected via trypsinization. Cellular d2EGFP fluorescence was quantified via flow cytometry and normalized to the no drug control.

For IRE1 α activity analysis, HCT116 XBP1-mNG cells were treated with 30 μ M IGG or 25 nM PB for 24 hours then washed with PBS and collected via trypsinization. Cellular mNG fluorescence was quantified via flow cytometry and normalized to the no drug control.

RNA-seq analysis and gene ontology:

Raw RNA-seq reads of nine different spliceosome-component shRNA knockdowns in both K562 and HepG2 cells were obtained from the ENCODE project database (Davis *et al.*, 2018; ENCODE project consortium, 2012; Sundararaman *et al.*, 2016). These included SF1 (ENCSR562CCA, ENCSR644AIM), U2AF1 (ENCSR342EDG, ENCSR372UWV), U2AF2 (ENCSR904CJQ, ENCSR426UUG), SF3A3 (ENCSR454KYR, ENCSR374NMJ), SF3B1 (ENCSR047QHX, ENCSR896CFV), SF3B4 (ENCSR081XRA, ENCSR148MQK), PRPF8 (ENCSR137HKS, ENCSR998MZP), snRNP200 (ENCSR943LIB, ENCSR003LSA), and EFTUD2 (ENCSR117WLY, ENCSR620OKS). RNA-seq reads for HeLa cells treated with igg and DMSO were obtained from the European Bioinformatic Institute, study accession PRJNA292827 (Tseng *et al.*, 2015). RNA-seq reads for HeLa cells treated with SSA and DMSO were obtained from the European Bioinformatic Institute, study accession PRJEB23117 (Yoshimoto *et al.*, 2016). RNA-seq reads for TOV21G ovarian cells treated with Pb and DMSO were obtained from the European Bioinformatic Institute, study accession PRJNA280861 (Wan *et al.*, 2015). Alignment of all reads from the five different studies was performed using HiSat 2 v2.1.0 (Kim *et al.*, 2015). Raw sequence alignments were aligned to the ENSEMBL GRCh38 human genome reference assembly. The alignments were sorted using samtools and then the reads were counted and annotated using ht-seq v0.10.0 (Anders *et al.*, 2014) with the ensemble GRCh38 human annotated gene list. Differential expression of the counts was calculated using deseq2 v3.8 (Love *et al.* 2014),

comparing all replicates of each test condition to their respective controls. Gene ontology analysis of all genes calculated to have $\geq$ two-fold expression increase was performed using g:Profiler (Reimand *et al.*, 2007). In cases where the number of candidate genes was too large for ontological analysis, the top 1000 genes with lowest p value amongst those with a $\geq$ two-fold change were chosen. Ontology results were also trimmed for any biological process or pathway that had more than 1000 genes to avoid overly general results.

Results:

PERK activation:

In previous unpublished work in the laboratory, HCT116 cells were treated with IGG for 8 hours and oligonucleotide microarray analysis was performed to identify differentially expressed mRNAs. DDIT3, CHAC1, and DDIT4 were among the few IGG-induced transcripts suggesting that the PERK branch of the UPR was activated by IGG. We confirmed that IGG significantly increased the expression of these mRNAs within 8 hours using qRT-PCR (Figure 3, left side). Therefore, IGG does indeed appear to increase the expression of these ATF4 target genes.

We had previously found that IGG and PB have subtle differences in cell cycle regulation (Vanzyl et al, 2018), so we sought to determine whether PB led to similar changes in gene expression. While IGG treatment led to significant upregulation of DDIT3, CHAC1, and DDIT4 at 8 hours, PB did not and we only detected significant increases in the expression of DDIT3 at later times (Figure 3, right side). Taken together, IGG appeared to induce ATF4-regulated transcripts but PB does not consistently do so.

Figure 3. Isoginkgetin, but not pladienolide b, upregulates ATF4-regulated mRNAs.

HCT116 cells were treated with either no drug (ND) 15 μ M or 30 μ M IGG with volume matched DMSO and 10nM or 25nM PB for 8 hours or 24 hours. RNA was collected, reversed transcribed, quantified using qRT-PCR, and normalized to GAPDH expression. Error bars represent SEM from at least three independent experiments. Data was analyzed by one-way ANOVA and Tukey test, and significance is reported relative to each time point's respective ND control (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). IGG data was provided by Erin Vanzyl.

We next measured global translation levels in response to spliceosome inhibition, as PERK activation leads to a global shutdown of cap-dependent translation via phosphorylation of eIF2 α . To do this, HeLa cells containing a constitutively expressed and short-lived d2EGFP transgene with a doxycycline-regulated promoter (Figure 4A) were treated with PB or IGG, with cycloheximide and doxycycline serving as positive controls, and fluorescence per cell was measured after 24 hours via flow cytometry. The relative fluorescence per cell is expressed relative to untreated controls and expressed as a percentage. Our results indicate that IGG led to a significant decrease in fluorescence, while PB did not (Figure 4B). Positive controls for d2EGFP transcription (DOX) and translation (CHX) decreased fluorescence, as expected. To ensure that the splicing inhibitors did not block d2EGFP mRNA expression, despite the absence of introns in the reporter construct, qRT-PCR was used to determine the effect of these drugs on d2EGFP mRNA levels. While shutting off transcription of the reporter gene with DOX effectively reduced transgene mRNA expression, IGG did not (Figure 4C). Therefore, IGG appears to inhibit d2EGFP at the protein level but not at the mRNA level, consistent with decreased translation of the reporter mRNA. Curiously, PB consistently increased reporter mRNA expression (Figure 4C). Taken together, IGG and PB again appear to elicit different effects, despite the fact they are both splicing inhibitors.

Figure 4. Isoginkgetin, but not pladienolide b, decreases translation levels of a constitutively transcribed d2EGFP reporter gene, suggesting global translation inhibition.

HeLa Tet-off d2EGFP cells were treated with either 30 μ M IGG, 25 nM PB, or no drug for 24 hours, with a cycloheximide translation inhibition control and doxycycline transcription repression control. A) HeLa d2EGFP construct for a d2EGFP transgene, consisting of a tetracycline (or doxycycline) -regulated, minimal promoter, a coding region, a PAS polyadenylation signal sequence, and a PEST sequence (not shown) that targets the protein for degradation, allowing for earlier detection of translation shutdown. B) Cells were collected via trypsinization and cellular fluorescence was measured using flow cytometry, and cellular fluorescence was normalized to the ND control. Error bars represent SEM from at least three independent experiments. Data was analyzed by a one-way ANOVA and Tukey test, and significance is reported relative to the ND control (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Data includes contributions from Erin Vanzyl. C) mRNA was collected, reverse transcribed, and the d2EGFP gene was quantified by qRT-PCR analysis. Error bars represent SEM from at least three independent experiments. Data was analyzed by a one-way ANOVA and Tukey test, and significance is reported relative to the ND control (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Data provided by Erin Vanzyl.

IRE1 α & ATF6 activation:

After gathering evidence on the downstream results of PERK activation, we next looked upstream. Since activation of PERK and indeed all three branches of the UPR canonically occurs through the same mechanism (BiP depletion), we then decided to test if the other two components of the UPR (IRE1 α & ATF6) were activated by either drug.

To assess the activation of IRE1 α we used an XBP1 reporter gene and measured fluorescence in response to spliceosome inhibition. As mentioned earlier, IRE1 α , once activated, performs the unique function of splicing out a 26nt sequence from XBP1, which changes the sequence to then code for an ER-stress transcription factor. The reporter construct outlined in Figure 5B contains a GFP gene downstream of and out of frame with a segment of the XBP1 gene (Nougarede *et al.*, 2018). Upon removal of the 26nt sequence of the XBP1 gene fragment the GFP reading frame is restored, allowing for the expression of the GFP reporter and effectively providing a fluorescent indicator of IRE1 α activity. Our results show that both IGG and PB led to increased cellular fluorescence, with PB eliciting the stronger response, suggesting that both spliceosome inhibitors are activating the IRE1 α branch of the UPR.

To assess activation of ATF6, HCT116 cells were treated with PB or IGG for 8 hours, the mRNA was collected, and expression levels of the ATF6-regulated genes BiP, CALR, and PDIA6 were quantified via qRT-PCR. None of these ATF6-regulated transcripts increased in response to IGG, PB or our positive control, Tg. Therefore, a non-transformed human fibroblast cell line (NF-hTERT cells) was also tested. Here, Tg was able to increase ATF6 target gene expression but IGG and PB did not. Taken together, IGG and PB do not appear to activate the ATF6 branch of the UPR and HCT116 cells may have a defect in this branch of the UPR.

In summary, there is evidence that IGG activates PERK and IRE1 α , but not ATF6, as indicated by the inhibition of global translation and increased expression of ATF4-regulated genes, and the increased fluorescence observed in our XBP1/GFP-reporter cell line, but no upregulation of any of the ATF6-regulated genes. There is evidence that PB is only able to activate the XBP1 branch of the UPR, as it increased fluorescence in our XBP1/GFP reporter assay, but did not inhibit translation, it did not upregulate any of our selected ATF4 targets apart from a delayed DDIT3 response, and it did not upregulate any of our selected ATF6 targets.

Figure 5. IGG and PB activate IRE1α but not ATF6

A) XBP1 vector for reporting IRE1α activity. An mNeonGreen GFP reporter gene is downstream of nucleotides 410-633 of the XBP1 gene, out of reading frame. Upon activation of IRE1α, 26nt's are removed from the XBP1 region, correcting the GFP reading frame and allowing for its expression. The vector also contains a nuclear localization downstream of GFP which aids in signal detection. Figure taken from Nougarede *et al.*, 2018. B) HCT116 XBP1-mNG cells were treated with either no drug (ND), 30 μM IGG, IGG-volume-matched DMSO, or 25 nM PB, with a Tg positive control. Cells were collected via trypsinization and cellular fluorescence was measure via flow cytometry. Error bars represent SEM from at least three independent experiments. Data was analyzed by a one-way ANOVA and Tukey test, and significance is reported relative to the ND control (* = p < 0.05, ** = p < 0.01, *** = p < 0.001). Data provided by Erin Vanzyl. C) HCT116 and NF-hTERT cells were treated with either no drug (ND), 30 μM IGG, IGG-volume-matched DMSO, or 25nM PB for 8 hours, with a Tg positive control. RNA was collected, reversed transcribed, quantified using qRT-PCR, and normalized to GAPDH expression. Error bars represent SEM from at least three independent experiments. Data was analyzed by a one-way ANOVA and Tukey test, and significance is reported relative to the ND control (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

RNA-seq analysis of IGG, PB, and SSA treatment:

Although our original microarray data suggested that IGG-induced spliceosome inhibition led to activation of the UPR, further analysis with both IGG and a second inhibitor, PB, gave conflicting results. Since IGG presumably targets the spliceosome at a different step, we then questioned whether spliceosome inhibition led to one general response, or if the response varied depending on what component of the spliceosome was being targeted. During the course of this study, RNA-seq data from cells treated with PB, IGG, and a third inhibitor SSA, which also targets SF3B1, became publicly available but were not analyzed in this context. We analyzed these data to compare the transcriptomic responses of different splicing inhibitors to determine if there is a common, general response to small molecule spliceosome inhibition, or if each drug has its own unique transcriptomic response. To perform this analysis, we collected raw RNA-seq data for TOV21G human ovarian cells treated with PB and HeLa cells treated with SSA and IGG from Wan *et al.* (2015), Yoshimoto *et al.* (2016), and Tseng *et al.* (2015), respectively. We calculated the differential expression of these data in response to their drug treatments and then compared the number of common genes that showed a significant differential expression greater than or equal to two-fold (Figure 6).

All raw RNA-seq reads mapped to the human GRCh38 genome with alignment rate greater than 90%. DeSeq2 calculation of differential expression is as follows: Pb had 9629 genes with significant differences in expression, 823 of which had greater than two-fold increase, SSA had 4974 genes with significant differences in expression, 544 of which had greater than two-fold increase, and IGG had 16369 genes with significant differences in expression, 6953 of which had greater than two-fold increase (Figure 6). Of these genes that

had greater than two-fold increase, 52 were present in all three treatments, 51 were present in only PB and SSA, 294 were present in only PB and IGG, 108 were present in only IGG and SSA, and 496 were unique to PB, 333 were unique to SSA, and 6499 were unique to IGG.

These data show a couple notable trends. First, IGG upregulated a strikingly high number of genes compared to SSA and PB. Second, there was a proportionally greater overlap in the genes upregulated by PB and SSA compared to those upregulated by IGG, which is to be expected given that they both target the same protein. However, there were also many genes that were uniquely upregulated by PB or SSA. They also had a considerable overlap with IGG, although this represents a relatively small fraction of the large number of differently expressed transcripts. In total, these results would suggest that the answer to our question is more complicated, and that splicing inhibition does seem to have some shared response, but also a prominent response that is unique to the method of inhibition.

Figure 6. Venn diagram comparing the number of significantly upregulated genes in response to three splicing inhibitor drugs: isoginkgetin, pladienolide b, and spliceostatin a.

Differential expression (DE) of the transcriptome was calculated and all significantly upregulated genes ($P < 0.05$, $\log_2 \text{DE} \geq 1$) were tallied and compared between the three treatments.

Next, a gene ontology analysis of these significantly differentially expressed transcripts was conducted for each drug treatment and the significantly enriched pathways were compared among the three treatments. The g:Profiler software that was used reports several different categories, but only the “KEGG Pathway” and “Biological Process” categories were included in this study as they were deemed to be the most informative for our purposes. KEGG is a cellular pathway database founded by researchers at Kyoto University (<https://www.genome.jp/kegg/>) and Biological Process is taken from the GO database (<http://geneontology.org/>).

The ontology results show that the three drugs share 4 pathways in common, IL-17 (interleukin 17) signalling, TNF α (tumour necrosis factor alpha) signalling, response to lipopolysaccharide, and response to molecule of bacterial origin, all related to inflammatory response (Figure 7). IGG and PB shared no pathways that they did not share with SSA, IGG and SSA shared two pathways that they did not share with PB, (MAPK signalling and response to antibiotic), and SSA and PB shared seven pathways that they did not share with IGG, which include several additional inflammation-related pathways (Supplemental table 2). All three drugs again influenced quite a few pathways that were unique: IGG having 16, PB having 23, and SSA having 40 (Supplemental table 2).

All three drugs show TNF α signaling and IL-17 signaling as their two lowest p-value KEGG pathways (Table 3). Biological processes were less consistent for their two lowest p-value results, but all included some biological process related to inflammation. Notably, endoplasmic reticulum processes were significantly enriched in cells treated with IGG (Supplemental table 2) and SSA (Table 3), but not PB. This is consistent with our previous findings that suggest that IGG

affects the endoplasmic reticulum more than PB does. It is also worth noting that the significant ontology categories that were shared by two drugs but not all three were still largely all related to inflammation, however this is not the case for the pathways that were upregulated by only one drug, which were much more variable (Supplemental table 2), reaffirming our previous finding that there is both a shared response to spliceosome inhibition as well as unique responses to each drug.

Figure 7. Venn diagram comparing the significantly enriched pathways in ontology analysis
RNA-seq data was accessed from the European Bioinformatics Institute for HeLa cells treated with IGG and SSA and TOV-21G cells treated with PB. Ontology analysis was performed and the significantly enriched pathways were compared between the three treatments.

Table 3. Top two lowest p value gene ontology results from RNA-seq data collected from HeLa cells treated with pladienolide b, isoginkgetin, and spliceostatin A.

	GO: Biological Process	KEGG pathway
PB	<ul style="list-style-type: none"> • detoxification of inorganic compound • stress response to metal ion 	<ul style="list-style-type: none"> • IL-17 signaling pathway • TNFα signaling pathway
IGG	<ul style="list-style-type: none"> • response to lipopolysaccharide • neuron death 	<ul style="list-style-type: none"> • TNFα signaling pathway • IL-17 signaling pathway
SSA	<ul style="list-style-type: none"> • protein localization to endoplasmic reticulum • SRP-dependent cotranslational protein targeting to membrane 	<ul style="list-style-type: none"> • TNFα signaling pathway • IL-17 signaling pathway

RNA-seq analysis of shRNA knockdown of spliceosome components:

In addition to the RNA-seq analysis of the small molecule splicing inhibitors, we also performed an RNA-seq analysis of nine different spliceosome component shRNA knockdowns using publicly available, raw RNA-seq data from the Brenton Gravelly lab at Yale university, accessed through the ENCODE project website. Their lab conducted a broad scale experiment that identified hundreds of RNA-binding proteins and subsequently performed shRNA knockdown on ~300 of these proteins followed by RNA-seq analysis in two unrelated cell lines. From this data set, we identified nine knockdowns that were key elements of the spliceosome: SF1, U2AF1, U2AF2, SF3A3, SF3B1, SF3B4, PRPF8, EFTUD2, and snRNP200. Unlike the similarity of most small molecule inhibitors of the spliceosome, these proteins include components of different snRNP complexes so they likely interfere with splicing at a variety of points. Our analysis of this data was performed to provide a drug-independent second analysis of the transcriptomic effects of spliceosome inhibition without the potential off-target effects of the compounds examined.

All raw RNA-seq reads mapped to the human GRCh38 genome with alignment rate greater than 85%. DeSeq2 calculation of differential expression is as follows:

Table 4. RNA-seq gene counts for spliceosome component shRNA knockdowns

P < 0.05 represents all genes that were found to be significantly differentially expressed.

Log2 DE ≥ represents all significant genes that were upregulated more than two-fold

	K562		HepG2	
	p < 0.05	Log 2 DE ≥ 1	p < 0.05	Log2 DE ≥ 1
SF1	4059	536	2188	597
U2AF1	2874	77	3665	3665
U2AF2	4689	256	4796	1638
SF3A3	5015	288	7540	1025
SF3B1	4121	487	4829	710
SF3B4	4526	249	2393	439
PRPF8	6161	825	2133	2
EFTUD2	4552	358	2739	65
snRNP200	3684	3684	1873	538

Ontology analysis confirmed that spliceosome inhibition results in significant upregulation of elements of the inflammation pathway as well (Table 5). Amongst the most common KEGG pathway upregulations were cytokine-cytokine interaction, TNF α signaling, and inflammation (data not shown). Inflammation pathway results were present in at least one cell line in seven of our nine knockdowns, only being absent in the U2AF1 and SF3B4 knockdowns, which returned overall poor results and perhaps were not significantly knocked down. The endoplasmic reticulum pathway was significantly enriched in the SF1, SF3B1, and snRNP200 knockdowns as well, again supporting our previous findings of an ER response to splicing inhibition via isoginkgetin. Taken together with the small molecule splicing inhibitor ontologies, our results seem to suggest that the inflammation pathway is significantly upregulated in response to splicing inhibition, particularly the TNF α and/or IL-17 pathways, however the ER is often implicated as well.

Table 5. Top two gene ontology results from RNA-seq data collected from K562 and HepG2 cells with shRNA knockdown of various spliceosome components.

Green boxes indicate that an inflammation-related process or pathway was significantly enriched in the ontology results, red boxes indicate they were not (not all data shown). An asterisk indicates that an ER-related process or pathway was significantly enriched in the ontology results. NA indicates that no significant results were returned.

shRNA target gene	K562		HepG2	
	Biological Process	KEGG pathway	Biological Process	KEGG pathway
SF1*	<ul style="list-style-type: none"> regulated exocytosis regulation of cell growth 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator anatomical structure development 	<ul style="list-style-type: none"> NA NA
U2AF1	<ul style="list-style-type: none"> fibroblast migration response to cAMP 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA
U2AF2	<ul style="list-style-type: none"> cytokine production cytokine-mediated signaling pathway 	<ul style="list-style-type: none"> Cytokine-cytokine receptor interaction Hematopoietic cell lineage 	<ul style="list-style-type: none"> regulation of cell adhesion leukocyte differentiation 	<ul style="list-style-type: none"> TNFα signaling pathway MAPK signaling pathway
SF3A3	<ul style="list-style-type: none"> extracellular matrix organization inflammatory response 	<ul style="list-style-type: none"> ECM-receptor interaction Transcriptional misregulation in cancer 	<ul style="list-style-type: none"> negative regulation of interleukin-8 production mucosal immune response 	<ul style="list-style-type: none"> p53 signaling pathway Viral carcinogenesis
SF3B1*	<ul style="list-style-type: none"> response to cAMP regulation of leukocyte mediated immunity 	<ul style="list-style-type: none"> Cytokine-cytokine receptor interaction PI3K-Akt signaling pathway 	<ul style="list-style-type: none"> lipid transport cellular hormone metabolic process 	<ul style="list-style-type: none"> Complement and coagulation cascades Steroid hormone biosynthesis
SF3B4	<ul style="list-style-type: none"> cellular response to biotic stimulus positive regulation of miRNA metabolic process 	<ul style="list-style-type: none"> Transcriptional misregulation in cancer NA 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA
PRPF8	<ul style="list-style-type: none"> cytokine production regulation of cell adhesion 	<ul style="list-style-type: none"> Cytosolic DNA-sensing pathway IL-17 signaling pathway 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA
EFTUD2	<ul style="list-style-type: none"> cytokine production cytokine-mediated signaling pathway 	<ul style="list-style-type: none"> Cytokine-cytokine receptor interaction Hematopoietic cell lineage 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA
snRNP200*	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> NA NA 	<ul style="list-style-type: none"> cell-substrate adhesion anion transport 	<ul style="list-style-type: none"> TNFα signaling pathway NA

Discussion:

In this study we sought to assess the cellular response to spliceosome inhibition, looking particularly at the implication of the unfolded protein response, following on the evidence of our microarray analysis. We found that the splicing inhibitors IGG and PB produce different results in their effects on the UPR. As hypothesized, treatment of cells with IGG led to decreased global translation levels and increased transcription of several ATF4-regulated transcripts including DDIT3, DDIT4, and CHAC1 within 8 hours, providing some support for the activation of PERK. We also found that IGG activated IRE1 α but not ATF6. In contrast, PB did not inhibit translation of our reporter construct and it did not induce transcription of CHAC1, or DDIT4, and DDIT3 was only significantly induced at 24 hours, suggesting there is no activation of PERK. Further analysis of the UPR showed that PB did appear to activate IRE1 α but not ATF6. Subsequent RNA-seq and ontology analysis indicated the most significantly enriched pathway in response to IGG, PB, and SSA to be inflammation, particularly TNF α signalling. This was also confirmed with shRNA knockdowns of various splicing components which showed seven of the nine knockdowns were significantly enriched for inflammation-related pathways.

Integrated stress response:

While the data from our previous microarray study suggested that IGG may activate the PERK branch of the UPR, the UPR gene expression results of this current study suggest this may not be exclusively the case. Since PERK is activated by the same general process as ATF6 and IRE1 (BiP depletion), it would be expected that if IGG was causing an accumulation of misfolded proteins that draw BiP away from the UPR sensors, then all three would be activated, not just

PERK. In this study, IGG was shown to inhibit global translation and activate a selection of ATF4's downstream target genes, which are both hallmarks of PERK activation. IGG was also able to increase expression of an XBP1-reporter gene, suggesting that IGG led to increase endoribonuclease activity of IRE1. However, we did not detect activation of ATF6-regulated transcripts in response to IGG in either cell line tested. However, this does not conclusively refute the activation of PERK by IGG, as it may be possible that there is repression of the ATF6 pathway through crosstalk with other pathways, and further studies testing the direct activation of PERK will be necessary, particularly measuring the levels of phosphorylated PERK and examining IGG responses in PERK null or knockdown cells. The PERK-dependent UPR is also a component of the integrated stress response (ISR), the activation of which could produce similar results to those observed in our study.

The ISR is an umbrella term for several different stress pathways, including the UPR, which ultimately result in the phosphorylation of eIF2 α and the subsequent shutdown of cap-dependent translation. There are four known pathways in the integrated stress response, each using a different eIF2 α kinase in response to different cellular stresses. These are: protein kinase R (PKR), in response to viral infection; general control non-depressible 2 (GCN2), in response to amino acid deprivation; heme-regulated inhibitor (HRI), in response to oxidative stress; and PERK, previously described in this paper (Pakos-Zebrucka *et al.*, 2016). In this study we indirectly tested for activation of PERK by testing for translation shutdown, with positive results for IGG, however activation of any number of these other branches should, in theory, produce similar results; namely, decreased levels of translation and preferential translation of

the active form of ATF4, and subsequent upregulation of its downstream targets ATF3, DDIT3, and DDIT4, among others.

PKR:

PKR is a constitutively expressed serine/threonine kinase with two N-terminal double stranded RNA binding domains (dsRBDs). Binding of a dsRNA to the dsRBDs facilitates dimerization and activation of the protein through a conformational change that exposes the kinase domain and allows it to phosphorylate eIF2 α and consequently inhibit cap-dependent translation, activating ATF4 (Meurs *et al.*, 1990). While PKR primarily functions as a mediator of anti-viral response, it can be activated in response to many cellular pathways, including heat shock proteins, oxygen radicals, and, importantly for our purposes, inflammation and ER stress (Gal-Ben-Ari *et al.*, 2018). PKR may be activated in response to splicing inhibition as well, since inhibition of the spliceosome has been shown to cause great accumulation of unprocessed pre-mRNAs in the nucleus (Yoshimoto *et al.*, 2017), which may be recognized by PKR. This notion is largely speculative, but PKR is active in the nucleus (Jeffrey *et al.*, 1995), and pre-mRNAs are known to form secondary structures (Buratti & Baralle, 2004, Jin *et al.*, 2011), and there is even some evidence of direct PKR activation through pre-mRNA binding (Namer *et al.*, 2017).

Activated PKR can lead to apoptosis directly, through the FADD/caspase-8/caspase-3 and caspase-9 pathways, (Roretz & Gallouzi, 2010; Gil *et al.*, 20012) as well as indirectly through ATF4, and may be a player in the anti-tumour activity observed in response to splicing inhibition. PKR also activates the apoptotic TNF α pathway (Goh *et al.*, 2000; Takada *et al.*, 2007, which was shown to be activated by spliceosome inhibition in our data (Table 3, Table5), though TNF α also activates PKR, so if these two pathways are in fact implicated in spliceosome

inhibition, the nature of their respective activations will need to be investigated (Sharma *et al.*, 2011).

GCN2:

GCN2, under normal conditions, is an inactive serine/threonine kinase homodimer consisting of an N-terminal domain, a pseudokinase domain, a kinase domain, a HisRS-like domain, and a C-terminal domain (Masson, 2019). The inactivated conformation of this protein prevents the catalytic kinase domain from binding its substrate; however, under low amino acid conditions where there is an overabundance of deacylated tRNAs, binding of the HisRS-like domain of GCN2 to deacylated tRNA causes a conformation change that exposes the catalytic pocket and allows for phosphorylation of eIF2 α (Lageix *et al.*, 2015; Wek *et al.*, 1995). In this way, GCN2 functions as a sensor for low amino acid conditions and shuts down translation in response, which in turn upregulates ATF4. Splicing inhibition could potentially affect this pathway through global metabolism alterations as a result of global splicing alterations that shift cellular homeostasis towards a state of amino acid deprivation (e.g. impaired amino acid synthesis pathways), and there is some existing evidence that splicing disruption can sensitize cells to amino acid deprivation (Dalton *et al.*, 2019).

HRI:

HRI functions as a heme sensor in red blood cells and is unlikely to be implicated in our findings. While there is some interest in its potential as a therapeutic target outside red blood cells (Burwick & Aktas, 2017), it is again unlikely to be implicated in the current state of understanding of spliceosome inhibition barring a specific splicing aberration that leads to activation of HRI.

Inflammation:

The most significant result from the RNA-seq analysis of this study is the activation of the inflammatory pathway, as evidenced by its implication in seven of the nine shRNA knockdowns, as well as the four overlapping significantly enriched pathways of the drugs all being related to inflammation (TNF, IL-17, response to lipopolysaccharide, response to molecule of bacterial origin). The two most significantly upregulated pathways in response to SSA, PB, and IGG were IL-17 signalling and TNF α signalling. Since IL-17 is an activator of TNF α and all of the upregulated genes detected in this study that belong to the IL-17 pathway also belong to the TNF α pathway, (i.e. no elements of the IL-17 pathway were significantly upregulated except those in the TNF α pathway), they will be discussed in terms of only TNF α signalling.

TNF α signalling:

TNF α is a cytokine that initiates a pro-inflammatory signal through the binding of two types of receptors, TNFR1 and TNFR2 (Figure 8) (Baud & Karin, 2001). In TNFR1 binding, TNFR1-associated death domain (TRADD) is recruited to the cytosolic end of the TNFR1 protein which subsequently recruits receptor-interacting protein 1 (RIP1) and TNF-receptor-associated factor 2 (TRAF2) (Hsu *et al.*, 1995; Hsu *et al.*, 1996; Stanger *et al.*, 1995). TNFR2 binding activates TRAF2 directly rather than through the recruitment of TRADD (Rothe *et al.*, 1995). Recruitment of TRAF2 leads to the activation of two major pathways: NF- κ B and MAPK (Lowenthal *et al.*, 1989; Natoli *et al.*, 1997). Our data shows activation of several elements of MAPK and NF- κ B signalling (Table 6), however, it does not show significant upregulation of the genes leading from the activation of TNFR1/2 through to the activation of MAPK signalling and NF- κ B,

suggesting that the small molecule splicing inhibitors are activating many downstream elements of the TNF α signalling pathway, but likely due to cross talk with another pathway further upstream of the MAPKs and NF- κ B.

Figure 8. Tumour necrosis factor signalling pathway

The TNF α cytokine activates the TNFR1 receptor, triggering the formation of the TRADD-TRAF2-RIP1 complex which activates NF- κ B and MAPK pathways. NF- κ B is activated when IKK β is post-translationally modified to allow it to ubiquitinate I κ B α , removing it from NF- κ B, allowing it to migrate to the nucleus and activate many pro-survival genes. MAPK is activated when TRAF2 activates an MKKK, which activates MKK3/MKK6, which activate p38, and MKK4/MKK7, which activate JNK. Together JNK and p38 activate subunits of the AP1 transcription factor, which up or down regulates a wide array of genes, depending on the cellular context. JNK and p38 also facilitate the migration of NR4A1 from the nucleus and to the mitochondria to complex with bcl-2 and trigger apoptosis.

MAPK signalling:

MAPKs are protein kinases that function to transduce a variety of cellular signals through a three-tiered phosphorylation cascade, from MKKKs to MKKs to MAPKs. The major MAPKs implicated in TNF α signalling are c-Jun N-terminal kinase (JNK), activated by phosphorylation by MKK4 or MKK7 (Derrijard *et al.*, 1995; Moriguchi *et al.*, 1997), and p38, activated by phosphorylation by MKK3 or MKK6 (Enslen *et al.*, 1998). Both JNK and p38 are capable of producing a wide variety of cellular responses, including activation of transcription factors, leading to even further complexity in their response, though they are often categorized into cell growth, proliferation, cycle arrest, and apoptosis (Cuadrado & Nebreda, 2010; Dhanasekaran & Reddy, 2008). In this study JNK appears to be the most obviously activated as evidenced by the strong suggestion of activator protein 1 (AP1) activation, described later (Table 6).

JNK was originally described as a kinase that phosphorylates the c-JUN transcription factor that dimerizes with Fos or ATFs to form the AP1 transcription factor, however, it has since been shown to perform a variety of different functions (Dhanasekaran & Reddy, 2008). Cellular response to JNK is multi-faceted and often context-specific, with the potential for both anti- and pro- apoptotic responses depending on the cell type, the post-translational modifications in the network, and cross-talk with other signalling pathways (Dhanasekaran & Reddy, 2017). Of particular importance to this study is the potential of JNK to induce apoptosis, which may help explain the anti-tumour properties of the splicing inhibitors

There are a variety of proposed methods through which JNK can induce apoptosis. As stated above, JNK activation by the MKKs allows it to phosphorylate and activate c-Jun, leading

to the formation of the AP1 transcription factor (Hibi, *et al.*, 1993). AP1 has a wide variety of targets including a number of pro-apoptotic genes (Dhanasekaran & Reddy, 2017) and mutation of the c-Jun gene has been shown to confer resistance to apoptosis (Bjorkblom, *et al.*, 2008). JNK is also known to phosphorylate p53 and p73, both tumour suppressor proteins, potentially stabilizing its protein expression by inhibiting degradation (Fuchs *et al.*, 1998; Jones *et al.*, 2007).

NF-κB:

NF-κB is a transcription factor that is an early response to many cellular stressors and is capable of inducing a wide array of cellular responses, often broadly categorized into pro-inflammatory responses and cell proliferation and growth responses (Brenner *et al.*, 2015; Zhang *et al.*, 2017). As such, it is widely implicated in a variety of cancers (Annibaldi & Meier, 2018; DiDonato *et al.*, 2012).

In unperturbed cells, the NF-κB protein is kept inactive through binding of the inhibitor of nuclear factor kappa B (IκB) proteins, which perform the dual action of interacting with the DNA binding domain of NF-κB, rendering it transcriptionally inactive, as well as localizing it to the cytosol where transcription cannot be performed (Baeuerle & Baltimore, 1988). Canonical activation of NF-κB occurs through activation of IKK proteins which target IκB proteins for degradation, thus freeing the DNA-binding domain of NF-κB and allowing it to be shuttled to the nucleus for transcription of its target genes (Mercurio *et al.*, 1997).

NF-κB serves as one of the central regulators of inflammation and has a very complex interaction network. During tumorigenesis, cells undergo a variety of stressors as metabolic aberrancies accumulate, and NF-κB is often activated in response to these stressors. Its targets

are often context specific due to the complexity of its network and the complexity of the controls exerted on the members of its network, but the general consensus is that NF- κ B often serves as an oncogene that is exploited by cancerous cells to cope with the metabolic stresses that accompany tumorigenesis (Annibaldi & Meier, 2018; Brenner *et al.*, 2015; DiDonato *et al.*, 2012).

Most upregulated inflammatory genes:

To provide a more specific view of the RNA-seq analysis, we've included a selection of inflammation-related genes that were among the 50 most upregulated genes in response to IGG, PB, or SSA treatment (Table 6). The nature of this signalling pathway precludes conclusive interpretation simply from mRNA expression, but these genes may serve as useful clues for future analysis of splicing inhibition.

AP1:

c-Jun, c-Fos, and FosB were all among the 50 most upregulated genes in response to either IGG, PB, or SSA treatment, though not always upregulated by all three. These genes are transcription factors that dimerize to form the AP1 transcription factor. As mentioned above, AP1 regulates numerous genes and can produce a wide variety of effects based on the context in which it is active. AP1 is a dimeric protein complex that can be composed of JUN family proteins (c-JUN, JUNB, JUND), FOS family proteins (c-FOS, FOSB, FRA-1, FRA-2), ATF family proteins (ATF1-6, BATF, BATF-2, BATF-3, JDP2), and MAF family proteins (c-MAF, MAFA, MAFB, MAFF, MAFK, MAFG), with probably more as yet undiscovered (Garces *et al.*, 2018). With so many different possible arrangements, as well as post translational modifications, it is difficult to say

anything conclusively just from RNA-seq data, but some general observations about AP1 could prove useful going forward.

The composition of the AP1 proteins has been shown to affect what genes get up- or down-regulated, though information is sparse on the majority of dimer compositions (Hess *et al.*, 2004). JUN, ATF, and MAF proteins can homodimerize, whereas FOS proteins cannot, and only forms heterodimers with one of the other members of AP1 (Gazon, *et al.*, 2018; Halazonetis *et al.*, 1988). The most commonly reported dimer, and the most commonly studied, is c-JUN-c-FOS. This heterodimer recognizes DNA regions called TPA-responsive elements (TPE) and is usually what is being referred to when the term “AP1” is used (Angel *et al.*, 1987). ATF proteins recognize DNA regions called c-AMP response elements (CRE), which are also weakly recognized by JUN and FOS (Chinenov & Kerppola, 2001). MAF proteins bind MAREI and MAREII sequences, which are extensions of TPE and CRE (Kataoka *et al.*, 1994; Kerppola & Curran, 1994). These various transcription factors and their binding sites help illustrate how many compositions of AP1 can lead to a wide variety of cellular responses, but again, these remain poorly understood

Regarding the role of AP1 in our data specifically, a few observations may be noteworthy. First, c-Fos is upregulated by all three drugs by roughly the same degree, however the alternative FosB gene is upregulated by almost twice as much by all three drugs. Both FosB and c-Fos dysregulation is implicated in osteblastomas (Fittall *et al.*, 2018), and both appear to be involved in breast cancer invasion (Milde-Langosch *et al.*, 2004). FosB specifically was shown to be necessary for prostate cancer invasion in response to transforming growth factor beta (Barrett *et al.*, 2017), however FosB was also shown to be involved in fibroblast-growth-factor-

2-mediated S-phase delay (Vitorino *et al.*, 2018), suggesting that it cannot be easily classified as an oncogene or tumour suppressor.

The other prominent result in our data in regard to AP1 is the very high upregulation of c-Jun by IGG (and SSA to a lesser extent), compared to the downregulation of c-Jun by PB (Table 6). PB does however upregulate another Jun family protein, JunB, as do IGG and SSA. Like Fos, c-Jun and JunB also evade classification as either oncogenes or tumour suppressors, though the narrative tends to be that c-Jun is oncogenic and JunB is tumour suppressive. Towards this narrative, c-Jun promotes tumourigenesis in squamous cell carcinoma (Jin *et al.*, 2011) and in response to oncogenic Ras (Schutte *et al.*, 1989), whereas JunB inhibited tumourigenesis in both cases. JunB has also been shown to bind to c-Jun to inhibit its activity (Deng & Karin, 1993), However another study found that all Jun family proteins were positively correlated with proliferation of diffuse large B-cell lymphoma. Further examples of the pro- and ant- tumour effects of c-Jun and JunB have been reviewed here (Piechaczyk & Farras, 2008; Shaulian, 2010), further highlighting that the role of c-Jun and JunB in tumour cell proliferation is complicated and unpredictable with our limited data. A supplemental table of the differential expression of all AP1 components has been included (Supplemental table 3).

IER3:

IER3 is transcriptionally regulated by AP1, p53, and NF- κ B, among other transcription factors, and though its exact function is not known, it has been shown to be a stress-response protein with both pro- and anti- apoptotic effects. IER3 upregulation has been observed to induce apoptosis in a variety of cells and in response to a variety of signals, including anti-cancer drugs, TNF α signalling, and radiation (Arlt & Schafer, 2011). Importantly for our data,

IER3 has been shown to cause apoptosis through NF- κ B inhibition (Muerkoster *et al.*, 2008), which is concordant with our strong upregulation of other NF- κ B inhibitors, and the apoptotic response of TNF α signalling has been shown to be greatly increased if the NF- κ B arm of the pathway is inhibited (Baichwal & Baeuerle, 1997; Bergmann *et al.*, 2001; Diessenbacher *et al.*, 2008; Yang *et al.*, 2004). It should also be noted that IER3 upregulation was far stronger in response to IGG than PB or SSA, suggesting that perhaps the drugs are activating TNF α signalling in different ways.

IL24:

IL24 is a cytokine that has been shown to have anti-cancer effects across a broad range of cancers and was only found to be upregulated in response to IGG in our study. IL24 has been implicated in a variety of apoptosis pathways as well, including p38, PKR, and ER stress (Sarkar *et al.*, 2002; Pataer *et al.*, 2002; Menezes *et al.*, 2019). It has been shown to activate the p38 pathway, and inhibition of this pathway inhibited apoptosis (Sarkar *et al.*, 2002). It has also been shown to post-transcriptionally activate BATF-2 (which was significantly downregulated by PB in our data (Supplemental table 3)), which binds to c-Jun to form a version of AP1, and its activation led to increased levels of apoptosis which were also reversed when p38 signalling was inhibited (Dash *et al.*, 2015). Notably, adenoviral overexpression of IL24 has been shown to induce apoptosis in a PKR-dependent manner, where both inhibition of PKR protein and deletion of its gene prevented apoptosis in response to IL24 overexpression (Pataer *et al.*, 2002). As discussed above, PKR is part of the ISR which shuts down translation in response to various cellular stresses, which may help explain our observation that IGG, but not PB, shuts down translation, as IGG is also the only drug that upregulated IL24. Of final note, IL24 has also

been shown to interact with BiP, the protein responsible for keeping the UPR receptors in an inactive form. IL24 resulted in activation of p38 and apoptosis in cancer cells, both of which were abrogated when the BiP binding region of IL24 was disrupted, providing evidence for some amount of cross talk between the UPR activation seen in our microarray and the p38 activation suggested by our RNA-seq analysis (Gupta *et al.*, 2006).

PPP1r15A:

Protein phosphatase 1 regulatory subunit 15A (PPP1r15A), also known as GADD34, was found to be upregulated in response to all three drugs. This gene is noteworthy as it encodes the protein responsible for dephosphorylation of eIF2 α and restoring translation (Novoa *et al.*, 2001). PPP1r15a is upregulated by DDIT3 and is thought to serve as a negative feedback loop to maintain transiency of the stress response, as well as allow for the translation of apoptotic genes if the stress response is transduced too long (Novoa *et al.*, 2001). The specific upregulation of PPP1r15a in response to PB could also help explain the surprising, slightly increased levels of d2EGFP translation in our d2EGFP shutdown experiment if this gene is being upregulated in the absence of a translational shutdown.

GADD45:

Growth arrest and DNA damage 45 (GADD45)A and GADD45B, hereafter referred to as GADD45, were both strongly upregulated in response to all drugs. GADD45 is a stress response molecule activated in response to a variety of cellular stresses. Most pertinent to our study, it is strongly associated with apoptotic response, though the exact method is not fully understood (Salvador *et al.*, 2013). It is upregulated by c-JUN in response to p38 and JNK activation and in return activates p38 and JNK in a positive feedback loop (Salvador *et al.*, 2013). The apoptotic

response in this signalling cascade is thought to be in part by GADD45 binding MTK1, which then phosphorylates JNK and p38 to induce a strong apoptotic response (Takekawa & Saito, 1998).

NR4A1:

Nuclear receptor subfamily 4 group A member 1 (NR4A1) functions primarily as a transcription factor, however it has also been reported to migrate from the nucleus to the mitochondria where it associates with bcl-2 to induce mitochondria-mediated apoptosis (Pawlak *et al.*, 2015). One recent study showed that the plant-derived small molecule apaensin induced NR4A1-mediated apoptosis via activation of JNK and p38. JNK was shown to be necessary for nuclear export of NR4A1, whereas p38 was necessary for its interaction of bcl-2 and subsequent induction of apoptosis (Zhou *et al.*, 2014). This is of particular relevance to our study, since p38 and JNK activation are widely implicated in our RNA-seq analysis, and NR4A1 was highly upregulated, especially by PB (Table 6).

I κ B α and TNFAIP3:

I κ B α and TNF alpha induced protein 3 (TNFAIP3) are both involved in suppressing the NF- κ B response, though through different mechanisms. I κ B α is the predominant I κ B protein, referred to above, responsible for binding NF- κ B, blocking its DNA-binding domain, and localizing it to the cytosol (Baeuerle & Baltimore, 1988; Gupta *et al.*, 2010). TNFAIP3 acts further upstream in the NF- κ B activation pathway, removing ubiquitin chains from RIP1 and NEMO, which are necessary for their activation of NF- κ B via ubiquitination and degradation of the I κ B inhibitor proteins (Wertz, *et al.*, 2004; Das *et al.*, 2018). The downregulation of NF- κ B is of particular importance since, as mentioned above, TNF-pathway-mediated apoptosis is

greatly increased if NF- κ B is inhibited as well, and inhibition of the NF- κ B pathway has seen success as an anti-tumour strategy (Gupta *et al.*, 2010; Paul *et al.*, 2018).

Table 6. A selection of inflammation-related genes from the top 50 most increased protein coding genes in RNA-seq analysis of cells treated with IGG, PB, or SSA.

“Not sig.” indicates DeSeq2 did not return a p value < 0.05, likely indicating there were insufficient reads in the sequencing.

Gene	Function	Fold increase		
		IGG	PB	SSA
c-Jun	Subunit of AP1 transcription factor	164.8	0.6	6.0
IER3	General signalling responder to a variety of cell stressors	116.3	2.7	10.1
TNF	Pro-inflammatory cytokine that activates MAPK and NF-KB signalling	97.5	28.7	25.1
FosB	Subunit of AP1 transcription factor	45.4	54.4	31.9
IL24	Apoptotic cytokine	44.0	Not sig.	Not sig.
PPP1R15A	Dephosphorylates eIF2 α	39.4	19.5	47.2
c-Fos	Subunit of AP1 transcription factor	27.3	25.7	21.1
GADD45B	Pro-apoptotic signalling protein	13.9	6.8	81.0
NR4A1	Transcription factor/induces apoptosis by binding bcl-2	9.5	35.4	10.1
GADD45A	Pro-apoptotic signalling protein	7.8	15.3	14.2
TNFAIP3	Inhibitor of NF-KB	7.7	37.7	127.1
IκBα	Inhibitor of NF-KB	4.3	6.1	16.3

Future directions:

Much of the data generated in this study is preliminary and will likely require substantial further study before any conclusive statement can be made about the downstream effects of splicing inhibition. Specifically in regard to advancing on the findings of this study, the most important next steps would be resolving the role of the UPR and ISR in response to splicing inhibition and a more thorough investigation into the individual effects of splicing inhibition on the TNF α pathway in light of the RNA-seq data.

For future UPR and ISR research, the most crucial next step will be to determine the mechanism behind the translation shutdown observed in response to IGG. This will involve measuring the levels of phosphorylated eIF2 α to determine if that is the mechanism behind the translation shutdown and if so, then determining the enzyme responsible for its phosphorylation. Once determined, the downstream mechanisms of apoptosis can then be investigated, as well as confirmation that the upstream source of activation is in fact splicing inhibition and not an off-target effect of the drug or a non-canonical role of its target protein.

Future TNF α research will be more general, as the only lead we have is mRNA expression. Initial investigation could start by investigating p38 and JNK activation via the MAPK cascade by measuring phosphorylation levels. AP1 activity could be investigated as well, as it is highly implicated in our seq data, although its effects are very broad range and context specific. The study of the AP1 response to spliceosome inhibition would perhaps best be focussed on the regulation of some of its key targets, as well as determining the upstream activators. Lastly, the effects of the genes listed in table 6 could be investigated as well, as more information than simply transcriptional upregulation will be needed to determine if they are contributing to the apoptotic and anti-tumour effects of splicing inhibition. This includes protein expression, cellular localization, post translational modifications, and genetic interactions. It will also be important to assess these traits temporally, since TNF α signalling is a very complicated pathway that can produce completely opposing responses depending on cellular context, which will likely be shifting over time during spliceosome inhibition.

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Supplemental material:

Supplementary Table 1. Significantly upregulated genes in response to IGG, PB, and SSA treatment.

Genes in this table correspond to the 52 shared genes in Figure 6.

Ensembl ID	Gene name	Ensembl ID	Gene name
ENSG00000008517	IL32	ENSG00000212163	SNORD91A
ENSG00000081041	CXCL2	ENSG00000213782	DDX47
ENSG00000087074	PPP1R15A	ENSG00000213859	KCTD11
ENSG00000100906	NFKBIA	ENSG00000224959	None
ENSG00000112096	SOD2	ENSG00000225091	SNORA71A
ENSG00000116717	GADD45A	ENSG00000235354	RPS29P16
ENSG00000118503	TNFAIP3	ENSG00000237927	AL078604.2
ENSG00000119508	NR4A3	ENSG00000240106	RN7SL146P
ENSG00000119725	ZNF410	ENSG00000241112	RPL29P14
ENSG00000120129	DUSP1	ENSG00000249661	TNRC18P1
ENSG00000122035	RASL11A	ENSG00000252759	AC005037.2
ENSG00000122877	EGR2	ENSG00000259158	ADAM20P1
ENSG00000123358	NR4A1	ENSG00000259771	AC092756.1
ENSG00000125148	MT2A	ENSG00000260144	AC100827.2
ENSG00000134107	BHLHE40	ENSG00000260190	AL807752.5
ENSG00000136244	IL6	ENSG00000260708	AL118516.1
ENSG00000142871	CCN1	ENSG00000270640	AC104695.4
ENSG00000149798	CDC42EP2	ENSG00000272688	AP005329.3
ENSG00000163660	CCNL1	ENSG00000275216	AL161431.1
ENSG00000175213	ZNF408	ENSG00000276107	AC037198.1
ENSG00000175592	FOSL1	ENSG00000276980	AC008760.2
ENSG00000182774	RPS17	ENSG00000278709	NKILA
ENSG00000184047	DIABLO	ENSG00000279520	AC093525.8
ENSG00000201264	SNORD73B	ENSG00000279901	AC092117.2
ENSG00000206754	SNORD101	ENSG00000282057	AC092807.4
ENSG00000207181	SNORA14B	ENSG00000284138	ATP6V0CP4

Supplementary Table 2. Significantly enriched ontology pathways in response to IGG, PB, or SSA.

Information here is represented graphically in figure 7.

PB only	IGG only	SSA only	IGG & SSA	PB & SSA	Pb & IGG & SSA
AGE-RAGE signaling pathway in diabetic complications	Alcoholism	Epstein-Barr virus infection	MAPK signaling pathway	Hepatitis B	IL-17 signaling pathway
Malaria	Systemic lupus erythematosus	Human T-cell leukemia virus 1 infection	response to antibiotic	Legionellosis	TNF signaling pathway
Mineral absorption	embryonic organ development	Kaposi sarcoma-associated herpesvirus infection		NF-kappa B signaling pathway	response to lipopoly-saccharide
cellular response to cadmium ion	fat cell differentiation	Pertussis		NOD-like receptor signaling pathway	response to molecule of bacterial origin
cellular response to chemokine	intrinsic apoptotic signaling pathway	RNA catabolic process		cell chemotaxis	
cellular response to copper ion	myeloid cell differentiation	RNA splicing		cellular response to interleukin-1	
cellular response to zinc ion	negative regulation of immune system process	Ribosome		response to interleukin-1	
cellular transition metal ion homeostasis	negative regulation of neuron death	SRP-dependent cotranslational protein targeting to membrane			
cellular zinc ion homeostasis	neuron apoptotic process	Salmonella infection			
chemokine-mediated signaling pathway	neuron death	cellular response to biotic stimulus			
detoxification	regionalization	cellular response to corticotropin-releasing hormone stimulus			
detoxification of copper ion	regulation of hemopoiesis	cellular response to fibroblast growth factor stimulus			
detoxification of inorganic compound	regulation of myeloid cell differentiation	cotranslational protein targeting to membrane			

leukocyte chemotaxis	regulation of neuron death	cytoplasmic translation			
regulation of smooth muscle cell proliferation	response to mechanical stimulus	establishment of protein localization to endoplasmic reticulum			
response to cadmium ion	response to muscle stretch	leukocyte cell-cell adhesion			
response to chemokine		mRNA catabolic process			
response to copper ion		muscle tissue development			
response to zinc ion		myeloid leukocyte migration			
smooth muscle cell proliferation		negative regulation of MAPK cascade			
stress response to copper ion		negative regulation of hemopoiesis			
stress response to metal ion		negative regulation of phosphorylation			
zinc ion homeostasis		negative regulation of protein phosphorylation			
		negative regulation of response to external stimulus			
		negative regulation of transferase activity			
		nuclear-transcribed mRNA catabolic process			
		nuclear-transcribed mRNA catabolic process, nonsense-mediated decay			
		p38MAPK cascade			
		protein deubiquitination			
		protein localization to endoplasmic reticulum			
		protein targeting to ER			
		protein targeting to membrane			
		regulation of DNA-binding transcription factor activity			
		regulation of inflammatory response			
		response to corticosteroid			
		response to corticotropin-releasing hormone			
		response to fibroblast growth factor			
		response to steroid hormone			
		skeletal muscle cell differentiation			
		translational initiation			

Supplementary table 3. Fold change of AP1 subunits in response to IGG, PB, or SSA.

All numbers represent fold change. NA indicates that DeSeq2 did not return a significant result, likely due to low read depth. Green indicates a fold change greater than two, red indicates a fold decrease of greater than two.

AP-1 subunit	IGG	PB	SSA
JUN	164.7544663	0.561384798	5.963078348
JUNB	29.36036491	6.209675813	4.566118659
JUND	6.275450168	0.755343055	1.127815304
FOS	27.33561514	25.65695643	21.08530402
FOSB	45.38963135	54.40180113	31.89351417
FOSL1	3.615753204	5.305082845	25.70958027
FOSL2	0.577772586	0.27537406	0.31165175
ATF1	0.468545054	0.286865329	0.49197883
ATF2	0.505246082	0.332531036	0.634924407
ATF3	5.663818012	11.50595959	15.08844942
ATF4	1.573002822	0.309360464	1.33272352
ATF5	0.796522595	NA	1.561524225
ATF6	0.881855011	1.466224164	NA
BATF	NA	NA	NA
BATF-2	NA	0.085493371	NA
BATF-3	2.584797706	1.291073188	0.636153369
JDP2	0.441172001	0.369795128	0.271745132
c-MAF	NA	NA	NA
MAFA	19.56500792	1.118490811	8.44137009
MAFB	33.04405093	1.45512316	8.012025999
MAFF	29.18399742	1.889439612	30.62844054
MAFG	0.311562152	0.821744923	3.918975759
MAFK	0.348989651	0.811640045	9.158445765