Inhibition Of Pre-mRNA Splicing By Small Molecules

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Master's Thesis: 150 word Abstract

To overcome the problems of diseases/mutations due to pre-mRNA splicing errors, current research is being undertaken to investigate RNA- small molecule interactions. In this study RNA- small molecule interactions are investigated through screening various small molecules on nuclear yeast actin pre-mRNA *in vitro*. Ten of thirty-two different small molecules tested have been found to completely inhibit the pre-mRNA splicing mechanism. IC_{50} values were measured for each of the inhibitory small molecules, and neomycin was the strongest inhibitor with an IC_{50} of 80 μM, while cefoperazone was the weakest inhibitor with an IC_{50} of 6.1 mM. Native gel analysis established that each of the ten inhibitors affected spliceosomal complex formation at various steps. These inhibitors will be useful tools for characterizing the splicing complexes that accumulate and map out the path by which splicing complexes assemble. In the long term, these inhibitors may lead to novel therapies for splicing related diseases.

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CHAPTER 1

Introduction

Pre-mRNA splicing is a very complex process which is catalyzed by a large, highly dynamic macromolecular machine called the spliceosome. A significant fraction of all human genetic diseases, including a number of cancers, are believed to result from deviations in the normal pattern of pre-mRNA splicing; yet how and why these deviations occur is not well understood. In order to accumulate sufficient quantities of spliceosomes for biochemical and structural studies small molecule inhibitors could be used to stall the spliceosomes' assembly at distinct intermediates. The term "small molecule" incorporates a broad range of different compounds, which weigh less than 2kDa, ranging from carbohydrates to proteins to nucleic acids. The discoveries of small molecules which modulate pre-mRNA splicing would also present a unique opportunity for the development of a new class of therapeutic agents, in addition to providing a valuable experimental tool for the investigation of spliceosome assembly and function.

1.1 Pre-mRNA Splicing – An Overview

DNA carries the genetic information of a cell and consists of thousands of genes. Each gene serves as a recipe for how to build a protein molecule. The flow of information from the genes determines the protein composition and thereby the functions of the cell. In order to make proteins, the corresponding genes are transcribed into the precursor messenger RNA (pre-mRNA). The pre-mRNA undergoes various processing steps before being transported to the cytoplasm for translation. The first step is removing non-coding intervening sequences, called introns, followed by joining together the remaining

coding sequences, called exons, leaving the final mature mRNA product; the overall process is known as 'mRNA splicing' and occurs only in the nucleus of eukaryotic cells. The resulting mature mRNA may then exit the nucleus and be translated in the cytoplasm. (Lin *et al.,* 1985; Adams *et al.,* 1996)

The importance of mRNA splicing has been shown by the fact that the number of protein-encoding genes in the human genome, at ~25,000, is much smaller than the great diversity of the human proteome (at least 100,000 different proteins). The "missing diversity" in the DNA is made up by the existence of alternative paths of mRNA-splicing; that is, the exons to be spliced together are chosen according to the protein required. In this way, an unspliced mRNA molecule can be used by the cells to produce a variety of spliced mRNA products, and thus a corresponding variety of proteins. This "alternative splicing" is an integral part of the overall process of genetic regulation, and it influences every aspect of the biology of eukaryotes. (Cáceres & Kornblihtt, 2002)

Defects in the regulation of splicing frequently cause or worsen pathological conditions. There is an ever-growing list of diseases attributed to erroneous regulation of splicing, including certain types of cancer and neurodegenerative disorders (Nissim-Rafinia & Kerem, 2002; 2005). The basic features of the structure and function of the spliceosome are already known. In contrast, understanding of the regulation of alternative splicing is only in its early stages. This is due, among other things, to the fact that the selection of exons for splicing is determined by a highly complex interaction between many other proteins. (Varani, 2000; Cáceres & Kornblihtt, 2002)

1.1.1 Yeast as a Model Organism

In eukaryotes, several yeast, particularly *Saccharomyces cerevisiae* ("baker's" or "budding" yeast), have been widely studied, largely because they are quick and easy to grow. The cell cycle in yeast is very similar to the cell cycle in humans, and regulated by homologous proteins. In order to understand the complex mechanism of eukaryotic splicing, simpler eukaryotic splicing mechanisms in yeast, which do not undergo alternative splicing, can be used as a research tool and guide to understand more complex splicing in higher eukaryotes. Genes encoding small nuclear ribonucleoproteins (snRNPs) and other splicing factors are found to be functionally conserved in both vertebrate and insects and are also found in yeasts and slime molds. This indicates that there is some evolutionary preservation of splicing components in a broad range of different eukaryotic species. Thus, study of the simpler splicing mechanism of yeast cells could be applied to the more complex splicing mechanism in human cells and could aid in disease and mutation prevention or inhibition. (Wieben *et al.,* 1983; Lindsey & Garcia-Blanco, 1988)

1.1.2 Mechanism of Pre-mRNA Splicing

Introns are removed from nuclear mRNA precursors via a two-step transesterification reaction. In the first step, the 2'-hydroxyl of an adenosine near the 3' end of the intron attacks the 5' splice site, producing the 5' exon and lariat intron–3' exon intermediates. In the second step the 3'-hydroxyl of the 5' exon intermediate attacks the 3' splice site to give the spliced mRNA and lariat intron products of splicing (Figure 1). (Madhani & Guthrie, 1994)

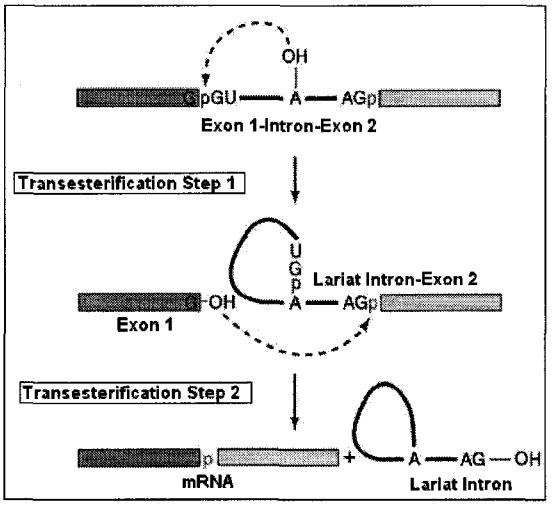


Figure 1: General mechanism of lariat formation from splicing of pre- mRNA.

1.1.3 The Spliceosome

The machinery that catalyzes the splicing event is called the spliceosome which is a complex of five snRNPs and >100 proteins. Each snRNP is composed of a single uridine-rich small nuclear RNA (snRNA) and multiple proteins. The RNAs found in snRNPs are identified as U1, U2, U4, U5 and U6 snRNAs, and participate in several RNA-RNA and RNA-protein interactions. The spliceosome performs the two primary functions of splicing: recognition of the intron/exon boundaries and catalysis of the cut-and-paste reactions that remove introns and join exons. To date, all introns have a 5' GU and a 3' AG identification sequence that the spliceosome recognizes and excises as a lariat (Madhani & Guthrie, 1994).

The snRNAs of the snRNPs play diverse roles in intron recognition and splice site definition and may be intimately involved in spliceosomal catalysis. Splicing involves the step-wise assembly of the spliceosome onto the pre-mRNA. There are four main complexes that are formed denoted spliceosomal complexes E, A, B, and C, respectively.

Their involvement is as follows: Complex E, the commitment complex, is created when U1 joins with the pre-mRNA, attaching at the 5' intron/exon boundary. Complex A is created when U2 binds to the complex at the branch point adenosine. Complex B is created when the triple complex U5•U4/U6 assembles onto the pre-mRNA. Complex C, the active spliceosome, is created when U4 dissociates, allowing U6 to base pair with the snRNA in U2. Splicing occurs, resulting in separation at the 5' exon/intron boundary and formation of the lariat. The joined exons dissociate from the spliceosome/intron complex, leaving the lariat structure behind and the spliceosome dissociates, the snRNPs recycle, and the intron lariat structure is broken into monomers (Figure 2) (Ares & Weiser, 1995).

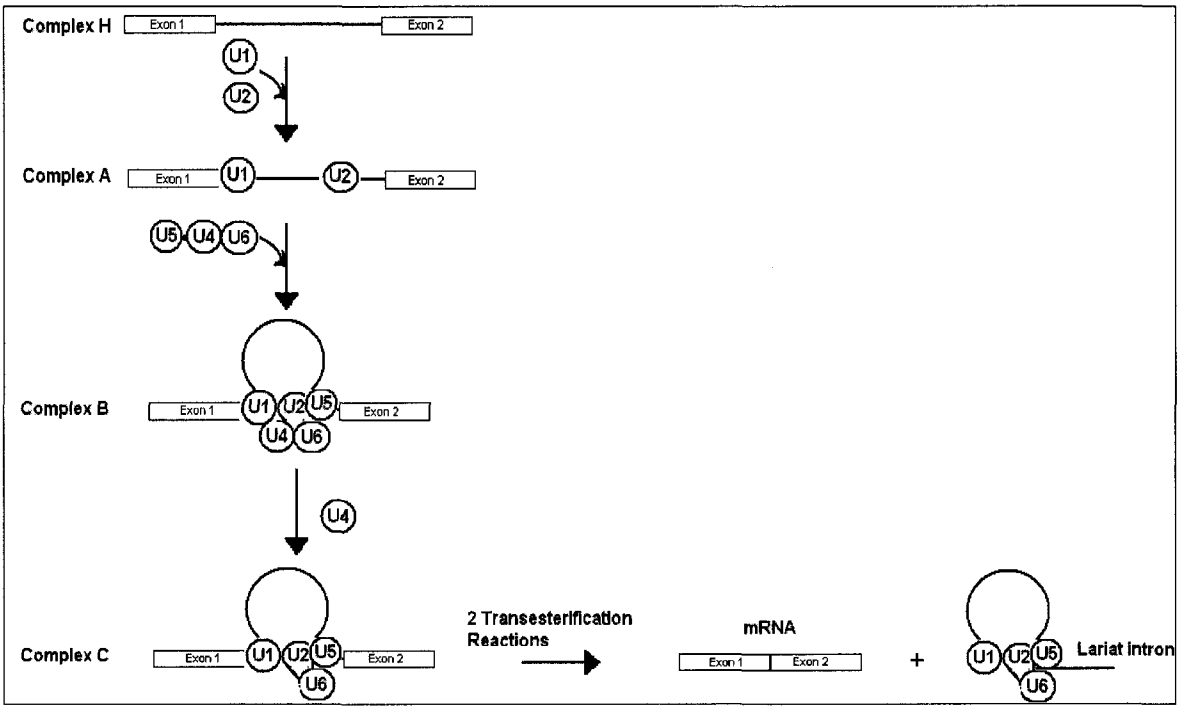


Figure 2: The step wise assembly of snRNPs onto the pre-mRNA leading to the active spliceosome required for splicing.

Spliceosome assembly is highly dynamic, in that complex rearrangement of RNA- RNA, RNA- protein, and protein- protein interactions take place within the spliceosome. Coinciding with these internal rearrangements, both splice sites are recognized multiple times by interactions with different components during the course of spliceosome assembly (Burge *et al.*, 1999; Du & Rosbash 2002). The catalytic component is likely to be U6 snRNP, which joins the spliceosome as a U4/U6 · U5 tri-snRNP (Villa *et al.*, 2002).

1.1.4 Splicing Related Diseases

Approximately 15% of the single base pair mutations that cause human genetic diseases are thought to be linked to pre-mRNA splicing defects. The human mutations database currently contains >3000 entries describing such mutations as cancers caused by aberrant splicing (Levanon & Sorek, 2003). Many of these genetic mutations cause inappropriate exon skipping, which ultimately cause defects in protein expression (Levanon & Sorek, 2003). Other mutations include inclusion or exclusion of more RNA, resulting in longer or shorter exons as well as reduced specificity which could lead to variation in the splice location, addition of one or more amino acids, or more commonly a loss of the reading frame (Levanon & Sorek, 2003). The underlying mechanisms responsible for splicing errors in human disease are poorly understood (Faustino & Cooper, 2003).

Given that the vast majority of human genes contain introns, and that most pre-mRNAs undergo alternative splicing, it is not surprising that disruption of normal splicing patterns can cause human disease (Faustino & Cooper, 2003). A splicing error

that adds or removes even one nucleotide will disrupt the open reading frame of an mRNA; yet exons are correctly spliced from within tens of thousands of intronic nucleotides. This remarkable precision is, in part, built into the mechanism of intron removal because once the spliceosome is assembled the base-paired snRNAs target specific phosphate bonds for cleavage. Mutations in the *cis*- and/or *trans*-acting elements lead to pre-mRNA splicing defects that cause disease (Faustino & Cooper, 2003). *Cis* acting mutations cause disruption in the final pre-mRNA substrate while *trans*-acting mutations cause disruptions in the spliceosomal machinery (Faustino & Cooper 2003). The following section goes over the major types of diseases seen when mutations occur within these *cis*- and *trans*- acting elements.

1.1.4.1 *Cis* - and *Trans* - Acting Mutations

Diseases caused by *cis*-acting mutations disrupt use of alternative splice sites. The following are four examples of such diseases: familial isolated growth hormone deficiency type II, frasier syndrome, frontotemporal dementia and parkinsonism linked to chromosome 17, and Atypical cystic fibrosis. In all cases, mutations in specific genes either cause exon skipping or inclusion (Cartegni and Krainer 2002).

Diseases caused by *trans*-acting mutations disrupt use of spliceosomal and non-spliceosomal components. Two such diseases are caused by mutations that affect the basal splicing machinery: retinitis pigmentosa caused by a mutation in the genes PRP31, HPRP3, and PRPC8 involved in the function of the U4/U6·U5 tri-snRNP (the spliceosome component required for the transition to a catalytically active state) (Zhou *et al.*, 2002) and spinal muscular atrophy caused by a loss of the survivor of motor

neuron gene (*SMN1*) required for the cytoplasmic assembly of the core snRNPs (Cartegni and Krainer 2002).

Due to the complexity of the spliceosome and the components involved, the issue of diseases related to *cis*- and *trans*- acting elements is widely under investigation. Novel therapeutic strategies directed toward correcting or avoiding splicing mutations are now emerging. Approaches include over-expression of proteins that alter splicing of the affected exon (Hofmann *et al.*, 2000; Nissim-Rafinia *et al.*, 2000); use of oligonucleotides to block use of aberrant splice sites and force use of beneficial splice sites (Kalbfuss *et al.*, 2001; Mercatante and Kole 2002); use of compounds that affect phosphorylation of splicing factors (Pilch *et al.*, 2001) or stabilize putative secondary structures (Varani *et al.*, 2000); and high-throughput screens to identify small molecules that influence splicing efficiencies of target pre-mRNAs (Andreassi *et al.*, 2001) to name a few. This thesis is directed toward one of the latter goals; searching for small molecules that influence splicing efficiencies of yeast pre-mRNAs in order to discover tools to control splicing and study the complex nature of spliceosomal components. Such work may lead to the development of therapeutic treatments for genetic diseases.

1.2 Small Molecule Inhibitors of Ribozymes and Mammalian Splicing

The essential role of RNA in many biological processes and in the progression of disease makes the discovery of small RNA-binding molecules an emerging field of interest in drug discovery (Tor *et al.*, 1998; Hertweck *et al.*, 2002; Graveley, 2005). Small molecules that bind to RNAs can be used as a tool for studying the biochemical, genetic, and structural aspects of the many splicing factors involved in pre-mRNA

splicing as they have with ribozymes and the ribosome (Hoch *et al.,* 1998; Park *et al.,* 2000; Bryan & Wong, 2004; Zaman *et al.,* 2003).

1.2.1 Ribozyme Inhibitors

It has been demonstrated that several different types of small molecules act as inhibitors of various biological, RNA-catalyzed, key processes (Sucheck & Wong, 2000; Arya *et al.,* 2001; Bryan *et al.,* 2004; Bakkour *et al.,* 2007), These inhibitors are useful for investigating the pre-mRNA splicing mechanism (Hertweck *et. al.*, 2003; Kaida *et. al.*, 2007).

RNA molecules that catalyze biological processes are known as ribozymes. Many natural ribozymes catalyze either their own cleavage or the cleavage of other RNAs. Some known ribozymes include RNase P, Group I and Group II introns, hairpin ribozyme, hammerhead ribozyme, hepatitis delta virus ribozyme, and riboswitches. The similarity in the mechanisms of the spliceosome-mediated splicing and these ribozymes, especially the self-splicing introns, has led to the hypothesis that the catalytic core of the spliceosome also functions as an RNA enzyme (Soo-Cheng & Abelson, 1987; Staley & Guthrie, 1998; Nilsen 2003). The following sections detail how different small molecules interact with ribozymes and review current studies of small-molecule inhibitors of human splicing.

1.2.1.1 Self-Cleaving Hammerhead and Hairpin Ribozymes

The hammerhead ribozyme is a small catalytic RNA made up of three base-paired stems and a core of highly conserved, non-complementary nucleotides. These structural features are essential for catalysis of the sequence-specific cleavage of RNA phosphodiester bonds. The hammerhead ribozyme is arguably the best-characterized

ribozyme; its crystal structure has been solved and its kinetic mechanism of cleavage is well established for several different ribozymes. (Pley *et al.*, 1994)

Neomycin was found to be a potent inhibitor of the hammerhead ribozyme cleavage reaction with a kinetic inhibition of 13.5μM. Two hammerhead ribozymes with well-characterized kinetics were used to determine which steps in the reaction mechanism were inhibited by neomycin. The studies found that neomycin interacted preferentially with the enzyme-substrate complex and that this interaction leads to a reduction in the cleavage rate. Although, the site at which neomycin binds the hammerhead ribozyme could not be identified a mode of binding was found. A comparison of neomycin with other aminoglycosides and inhibitors of hammerhead ribozyme cleavage implied that the ammonium ions on neomycin are important for a stronger antibiotic-hammerhead interaction. (Stage *et al.*, 1995)

In comparison, spermine, a positively charged small molecule altered hammerhead ribozyme activity in another manner. The polycation was not found to inhibit hammerhead cleavage but rather reduced the metal ion requirement for the reaction (Dahm & Uhlenbeck, 1991). Thus, it was clear that not every positively charged molecule altered hammerhead cleavage in the same way. It is possible that the different behavior among the cationic molecules resides in how the structure of each adapts to the folded hammerhead. For example, spermine may be able to bind several phosphates along the backbone of either the ribozyme or substrate, and because of its linear and flexible nature, may be able to move with changes in hammerhead ribozyme conformation. In contrast, neomycin, being a more rigid molecule due to its sugar moieties, may only bind a few specifically positioned phosphates in a more structured

region of the hammerhead. This rigidity may prevent of the hammerhead from adopting its active conformation (Stage *et al.,* 1995).

The hairpin ribozyme is also a small catalytic RNA that achieves an active form by docking of its two helical domains in an anti-parallel fashion. A study by Earshaw & Gait (1998) showed that aminoglycoside antibiotics inhibit cleavage of the hairpin ribozyme in the presence of metal ions, with the most effective being 5-epi-sisomicin and neomycin B. In contrast, in the absence of metal ions, a number of aminoglycoside antibiotics at 10mM concentration promote hairpin ribozyme cleavage at a rate of only 13- to 20-fold lower than the magnesium-dependent reaction. These results showed that neomycin B competes with metal ions by ion replacement with the positively charged amino groups of the antibiotic. In addition, the polyamine spermine at 10 mM promoted efficient hairpin ribozyme cleavage with rates similar to the magnesium-dependent reaction.

1.2.1.2 Self-Splicing Group I and II Intron Ribozymes

The Group I and Group II introns are self-splicing introns and are able to splice the lariat product in the absence of any protein factors. A number of small molecules have been found to inhibit the self-splicing of group I and II introns (Bass & Cech, 1986; Yarus, 1988; von Ahsen & Schroeder, 1991; von Ahsen *et al.*, 1991; 1992; Rogers & Davies, 1994; Wank *et al.*, 1994). Inhibitors fall into two classes, those that compete with the substrate guanosine and those that are non-competitive inhibitors. Competitive inhibitors include deoxy- and di-deoxyguanosine (Bass & Cech, 1986), arginine (Yarus, 1988), streptomycin (von Ahsen & Schroeder, 1991), viomycin (Wank *et al.*, 1994) and lysinomicin (Rogers & Davies, 1994). The non-competitive inhibitors are

aminoglycoside antibiotics of the neomycin, gentamicin and kanamycin families (von Ahsen *et al.*, 1991; 1992). The well-studied aminoglycoside, neomycin, has been shown to bind to the internal loop between the stems P4 and P5 and the catalytic core close to the G-binding site of the *td* intron RNA. Splicing inhibition by neomycin was strongly dependent on pH and Mg^{2+} concentration, suggesting electrostatic interactions and competition with Mg^{2+} (Hoch *et al.,* 1993).

1.2.1.3 The Human Hepatitis Delta Virus and HIV-1 Ribozymes

Replication of RNA viruses, such as the human immunodeficiency virus (HIV) and the human hepatitis delta virus (HDV) is dependent upon multiple specific interactions between viral RNAs and viral and cellular proteins. A small molecule that interferes specifically with one or more of these RNA-protein interactions could be an effective antiviral agent (Zapp *et. al.* 1992; Mei & Czarnik, 1995). Zapp *et. al.* (1992) showed that certain aminoglycoside antibiotics, in particular neomycin B and tobramycin, can block binding of the HIV Rev protein to its viral RNA recognition element (RRE). Inhibition appears to be highly selective, resulting from competitive binding of the drug to a small viral RNA region within the Rev-binding site. These results demonstrate that neomycin B and tobramycin can specifically antagonize Rev function *in vitro* and *in vivo* and can inhibit production of HIV. Further work by Zapp *et. al.* (1996) showed that the bulge region of the RRE core element is critical for neomycin B binding as well as Rev binding (Zapp *et al.,* 1996).

Small molecule inhibitors called aromatic heterocyclic compounds in particular a tetra-cationic diphenylfuran (TCD), can block binding of Rev to its high-affinity viral RNA binding site. Inhibition appears to be selective and results from competitive binding

of the drug to a discrete region within the Rev binding site. Interestingly, the molecular basis for the TCD-RNA interaction, as well as the mode of RNA binding differs from previously described aminoglycoside Rev inhibitors. Analysis of a variety of aromatic heterocyclic compounds and their derivatives reveals stereo-specific features required for the inhibition. For example, the alkylamine substituents, which possess some degree of rotational freedom, may be required within the aromatic heterocycle structure to achieve the molecular conformations that block Rev binding. The inhibitory activity of a given cationic aromatic heterocycle may be directly related to its ability to form hydrogen-bond interactions with the RNA. (Zapp *et. al.* 1997)

HDV is a single-stranded RNA virus. A study conducted by Rogers *et al.* (1996) showed that several classes of antibiotics inhibit the self-cleavage reaction of the HDV ribozyme. Of approximately 200 compounds examined, only a small number are active as inhibitors of HDV. Antibiotics of the aminoglycoside, peptide and tetracycline classes all inhibit HDV cleavage at micromolar concentrations. For each antibiotic inhibitor, an antibiotic with a very similar structure, did not inhibit HDV self-cleavage, or inhibited it very poorly. Several antibiotics from other structural classes were tested and found not to inhibit HDV self-cleavage. However, Rogers *et al.* (1996), found that neomycin directly displaces divalent metal ions essential for catalysis in HDV.

1.2.1.4 tRNA Processing RNase P Ribozymes

RNase P is an essential endoribonuclease involved in the processing of tRNA precursors in prokaryotes as well as in eukaryotes. In bacteria, RNase P consists of an RNA subunit and a small basic protein. It has been shown that the catalytic activity of this ribonucleoprotein complex is associated with its RNA subunit (Guerrier-Takada *et*

al., 1983). Mikkelsen *et al.,* (1999) showed that cleavage by RNase P RNA, in the absence as well as in the presence of the RNase P protein, is inhibited by kanamycin, paromomycin and neomycin. Neomycin was found to be the strongest inhibitor with a *K*i value of 35 μM. In addition, neomycin interfered with the binding of divalent metal ions to the RNA, similar to its mechanism in the hammerhead cleavage model. Taken together, these findings suggest that aminoglycosides compete with Mg^{2+} ions for functionally important divalent metal ion binding sites.

1.2.1.5 Riboswitches

Although they are not catalytic RNA, riboswitches are another example of how small molecule interactions could be used as key regulators of RNA-based mechanisms. Riboswitches are genetic control elements that regulate gene expression in a small molecule-dependent way (Davies *et al.*, 1993). Recent studies using specific RNA aptamers to design small molecule- dependent synthetic riboswitches have opened new perspectives in the field of translational control. Hanson *et al.* (2005) identified a tetracycline-binding aptamer capable of controlling translation in *Saccharomyces cerevisiae* by interfering with the formation of the 80S ribosome and preventing it from binding to the cap structure. Weigand *et al.,* (2008) also identified several artificial small molecule-binding riboswitches that respond to the aminoglycoside neomycin. They propose a model composed of a binding pocket of an internal loop as the primary docking site fixing neomycin in a sandwich-like manner. Such binding pockets, characterized by multiple contacts between ligand and RNA, are described for both natural and engineered riboswitches.

These studies have paved the way for the use of small molecules as tools for studying the structural aspects of many ribozymes. These studies also show how small molecules can be used as regulators of different catalytic RNA sequences thus providing insight of how small molecules can be used for studying pre-mRNA splicing and its associated factors. The next section will review studies of small molecules specifically targeting the human splicing system.

1.2.2 Mammalian Splicing Inhibitors

Studies of the dynamic processes involved in mammalian splicing have lead to the discovery of various types of small molecule inhibitors and effectors. The following examples are of different types of small molecule inhibitors which are found for one specific type of mechanism.

1.2.2.1 Peptide Kinase Inhibitors

Small molecule peptides were developed as inhibitors of the interaction between spliceosomal proteins CDC5L and PLRG1 (found in yeast and humans) to determine if they were necessary for the splicing mechanism (Ajuh & Lamond, 2003). The peptides were derived from highly conserved sequences in the interaction domains of both proteins, and were used in *in vitro* splicing experiments as competitors to the cognate sequences in the endogenous proteins.

Mermoud *et al.* (1994) showed that the human protein phosphatase 1 (PP1) prevents pre-spliceosome E complex formation and stable binding of U2 and U4/U6•U5 snRNPs to the pre-mRNA. Thus, splicing catalysis, but not spliceosome assembly, is blocked by inhibiting protein phosphatases and it appears that pre-mRNA splicing, in

common with other biological processes, can be regulated both positively and negatively by reversible protein phosphorylation.

The influenza virus NS1 protein inhibits the nuclear export of mRNAs and Lu *et al.* (1994) demonstrate that the NS1 protein also inhibits pre-mRNA splicing both *in vivo* and *in vitro* where the pre-mRNA forms spliceosomes, but subsequent catalytic steps in splicing are inhibited. The NS1 protein is associated with U6 snRNA in influenza virus-infected cells as well as in splicing extracts from uninfected cells, it is likely that the NS1 protein also inhibits pre-mRNA splicing in infected cells. Surprisingly, the splicing of the viral *nsl* mRNA, the very mRNA that encodes the NS1 protein, was resistant to inhibition by the NS1 protein.

Hu *et al.* (2003) showed that CDK11 complexes promote pre-mRNA splicing *in vivo* and *in vitro*. For instance, $CDK11^{p110}$ complexes were reported to influence transcription as well as interact with the general pre-mRNA-splicing factor RNPS1. Using a two-hybrid interactive screen, the splicing phosphor-protein 9G8 was identified as a partner for $CDK11^{p110}$. They discovered that immunodepletion of $CDK11^{p110}$ reduced splicing and re-addition restored splicing.

1.2.2.2 Synthetic Branched Nucleic Acid Inhibitors

To learn more about the events surrounding branchpoint recognition after the first transesterification step I is complete Carriero & Damha (2003) prepared a series of branched compounds (bRNA and bDNA), and studied the effects of such molecules on the efficiency of mammalian pre-mRNA splicing *in vitro*. They discovered that binding and sequestering of a branch recognition factor by the branched nucleic acids is an early event, which occurs prior to the first chemical step of splicing. In addition, branch

recognition is dependent upon the sequences directly adjacent to the branchpoint nucleotides.

1.2.2.3 Antibiotic Inhibitors

Hertweck *et al.,* (2002) investigated the effects of several antibiotics on *in vitro* splicing of human eukaryotic nuclear pre-mRNA. Of the eight antibiotics studied, erythromycin, Cl-tetracycline and streptomycin were identified as splicing inhibitors in nuclear HeLa cell extract. Cl-tetracycline and the aminoglycoside streptomycin were found to have an indirect effect on splicing by non-specific binding to the pre-mRNA, suggesting that the inhibition was the result of disturbance of the correct folding of the pre-mRNA into the splicing-compatible tertiary structure by the charged groups of these antibiotics. The macrolide, erythromycin, the strongest inhibitor, had only a slight effect on formation of the pre-splicing complexes A and B, but almost completely inhibited formation of the splicing-active C complex by binding to nuclear extract component(s). This results in direct inhibition of the second step of pre-mRNA splicing. This was the first report on specific inhibition of nuclear splicing by antibiotics.

The most recent discovery of a small molecule inhibitor of human pre-mRNA splicing was by Kaida *et al.* (2007). Kaida *et al.* (2007) determined that the methylated derivative of the natural product FR901464, Spliceostatin A inhibited human pre-mRNA splicing by binding to a sub-complex of the U2 snRNP called SF3b. SF3b was isolated and characterized by tagging Spliceostatin A with an affinity protein (Kaida *et al.*, 2007).

Strategies using small molecule to study human pre-mRNA splicing can now be used for testing additional small molecule inhibitors in the more tractable yeast splicing system.

1.3 Potential Inhibitory Splicing Candidates

The ability of small molecules to control expression of specific genes could facilitate studies in many areas of biology and medicine. The objective of this thesis is to find inhibitors of the yeast pre-mRNA splicing system. Finding the right small molecule candidates for targeting RNA structure and mechanistic studies has become a goal for many researchers (Noller, 1991; Wallis *et al.*, 1995; Wang & Rando, 1995; Wang & Tor, 1998; Tor *et al.*, 1998; Sucheck & Wong, 2000; Vicens & Westhof, 2001; Tor, 2003; Zaman *et al.*, 2003). The following categories for small molecules: old and new antibiotics, environmental toxicants and kinase inhibitors are effective candidates for targeting splicing factors in the yeast system and findings will lead into developing tools for studying complex spliceosomal related mechanisms.

1.3.1 Antibiotics

An antibiotic is a chemotherapeutic agent that inhibits or abolishes the growth of micro-organisms, such as bacteria, fungi, or protozoa (Davies *et al.*, 2003). Many antibiotics are relatively small molecules with a molecular weight less than 2kDa because large molecule antibiotics have relative difficulty crossing membranes and traveling systemically throughout the body (Davies *et al.*, 2003). Many antibiotics that are toxic to bacteria are non-toxic to human cells (Davies *et al.*, 2003). In contrast, the basic biochemistries of the fungal cell and the mammalian cell are much more similar (Davies

et al., 2003). This restricts the development and use of therapeutic compounds that attack a fungal cell, while not harming mammalian cells. Similar problems exist in antibiotic treatments of viral diseases. Human viral metabolic biochemistry is very similar to human biochemistry, and the possible targets of antiviral compounds are restricted to very few components unique to a mammalian virus. Targeting RNA is a challenging new approach that is complementary to traditional drug discovery focusing on proteins. One clear benefit of targeting RNA is the potential for the slower development of drug resistance against small molecules. RNA functional domains are more highly conserved and perhaps more accessible than the shapes of enzyme active sites. Thus, it is expected that pathogens will find it difficult to mutate their RNA and develop resistance. (Noller, 1991; Davies *et al.*, 2003; Zaman, 2003)

In order to find more antibiotics such as aminoglycosides that effectively target RNA sequences, antibiotics of different classes should also be tested. As described in section 1.2.3 a few small molecule antibiotics have been shown to inhibit the nuclear splicing mechanism of β-globin pre-mRNA whereas other antibiotics from the same class, showed no effect even though they contained similar functional groups as their inhibitory counterparts (Hertweck *et al.*, 2000). This suggests that inhibition is not entirely specific to compounds found within the same group and no absolute conclusions should be made on an entire class/group based on data obtained from a single compound. The most popular antibiotic leads would be the aminoglycosides, however, there are several different classes of antibiotics yet to be tested, which could be both cost efficient and medically effective.

1.3.1.1 Aminoglycosides

Several drugs targeting the ribosomal RNA (rRNA) of bacteria have been in clinical use for over half a century (Moazed & Noller, 1987; Recht *et al.*, 1999). One of these drug classes, the aminoglycoside antibiotics (Figure 3), also target human rRNA, and have been developed as therapeutics for genetic disorders (Wang & Rando. 1995; Wallis *et al.*, 1995; Wang & Tor, 1998; Arya *et al.*, 2001).

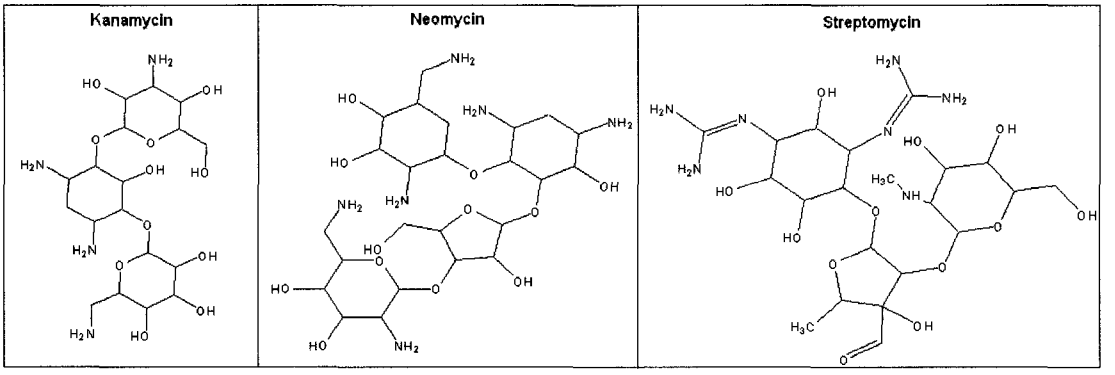


Figure 3: Structures of three aminoglycosides: kanamycin, neomycin, and streptomycin.

The way aminoglycosides bind to ribosomal RNA differs only moderately between prokaryotic 16S and human 18S rRNA (Arya *et al.*, 2001). Nevertheless, aminoglycoside antibiotics only kill bacterial cells. This selective cytotoxicity has been explained by sequence differences and by the occurrence in prokaryotes of transporter proteins that actively take up and concentrated aminoglycosides in the cytoplasm. Cellular uptake mechanisms for aminoglycosides also exist in eukaryotic cells. In the human body, for example, aminoglycosides specifically accumulate in renal tubular epithelial cells and in hair cells of the inner ear, where undesired side-effects are observed. Thus, the expression and activity of cellular uptake mechanisms is an important factor determining the positive and negative biological effects of

aminoglycosides, in addition to the binding to rRNA. (Vicens & Westhof, 2001; Bryan *et al.*, 2004)

A model to explain translational misreading by aminoglycosides has been proposed based on the crystal structure of the 30S ribosomal subunit in complex with a neomycin analogue (Vicens & Westhof, 2001). Critical in this model are two universally conserved adenine residues. In the process of decoding of the mRNA, adenine residues are facing out from the A-site to interact with the codon–anticodon duplex formed between aminoacyl-tRNA and mRNA. Aminoglycoside binding alters the structure of the rRNA so that the two bases are facing out already. This conformational change induced by neomycin analogue reduces the energy required for binding of both cognate (correct) and non-cognate (incorrect) transfer RNAs resulting in an increased error rate of the ribosome. Thus, the ribosome is as important for aminoglycoside action.

Aminoglycosides have played a large role in deciphering the mechanistic and structural aspects of the ribosome by targeting specific sites of the rRNA (Tor *et al.*, 1998; Recht *et al.*1999; Patel & Suri, 2000; Vicens & Westhof, 2001). Aminoglycosides would thus be good candidates for targeting specific splicing factors and/or the pre-mRNA (Varni *et al.*, 2000).

1.3.2 Oxospiro-Compounds from the Manumycin Family

Aminoglycosides are not the only good leads for targeting splicing factors. Other excellent candidates for targeting pre-mRNA sequences would be small molecules that resemble the aromatic heterocyclic compounds discovered by Zapp *et al.* (1997), such as the oxospiro-compounds newly derived from the manumycin family (Figure 6) (Plourde & Fisher, 2002; Plourde *et al.*, 2007).

All manumycins come from micro-organisms isolated from soil samples collected worldwide. The micro-organisms are taxonomically characterized as actinomycetes (genus: Streptomycetes), gram positive, mycelical, and sporulating bacteria. The oxospiro-derivatives are another class of small molecules that have many interesting biological properties *in vitro* and *in vivo* which include antibiotic, antifungal, antiparasitic, anticoccidial, trypanocidal, and insecticidal activities. (Sattler *et al.*, 1998)

Current studies are being undertaken to determine more specific biological relevance of these oxospiro-compounds. Fourteen different types of oxospiro-compounds were made available to be tested in this study. They can be divided into two groups: the precursors and the derivatives. The oxospiro-precursors consist of a core benzene ring with a long alkyl-acid group attachment. The oxospiro-derivatives are made from the oxidative spiroannulation of the precursors, resulting in a spirolactone group attachment to the conjugated ring (Figure 6) (Plourde *et al.,* 1999; Plourde & Fisher, 2002). In addition not all fourteen compounds are in their optically pure form but exist as a mixture of both enantiomers. Enantiomers have identical chemical and physical properties except they behave differently in chiral environments where both enantiomers of a compound are not always biologically active (McMurry, 2000). Single enantiomeric purity increases the number of biologically active molecules in a system (McMurry, 2000).

1.3.3 Environmental Toxicants

A toxicant is a chemical compound that is environmentally hazardous due to their constant stability in the environment (Schuur *et al.,* 1998; Castoldi *et al.,* 2001). Many toxicants are carcinogenic but their mode of action remains unclear (Schuur *et al.,* 1998;

Castoldi *et al.,* 2001). The pre-mRNA splicing system can be utilized to gain more insight into inhibition specificity of lethal small molecules and whether they target RNA.

Two potential well known environmental toxicants are polychlorinated biphenyls (PCBs) and methyl mercury. PCBs are persistent organic pollutants and have entered the environment through both use and disposal. The extent to which PCBs are toxic remains controversial. PCB derivatives have been found to inhibit important biological enzymes such as sulfotransferase isozyme in both human and rat cells (Schuur *et al.,* 1998). PCBs would probably be good candidates for targeting RNA sequences because of their many highly electronegative chlorine groups, which likely interfere with RNA-protein interactions (Hertweck *et al.* 2003).

Methyl mercury is a bioaccumulative environmental neurotoxin and is able to irreversibly inhibit pyruvate dehydrogenase (PDH) in mammalian cells ultimately leading to death (Castoldi *et al.,* 2001). The probable mode of action by methyl mercury would be through its positive ionic charge since it is an organometallic cation where it might displace any catalytic magnesium ions or it may even interact with the negatively charged backbone of the RNA, such as aminoglycosides (Sucheck & Wong, 2000; Arya *et al.,* 2001; Bryan *et al.,* 2004; Bakkour *et al.,* 2007).

1.3.4 Kinase Inhibitors

A kinase is a type of enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to specific target molecules (protein or small molecule); the process is termed phosphorylation. Presence of kinases was tested by kinase peptide inhibitors in the human pre-mRNA splicing system (section 1.2.2). The role of kinases in yeast pre-mRNA splicing, however, remains fairly unclear because ATP is also required

by DExD/H box proteins (ATPase helicases) (Das & Reid, 1999; Dagher & Fu, 2001; Tazi *et al.*, 2005). ATPases hydrolyze ATP at each step of spliceosomal complex assembly, which is thought to show regulation by the ATPases for progression of assembly and splicing (Brow 2002; Silverman *et al.*, 2003; Tazi *et al.*, 2005).

Broad range kinase inhibitors could be used to narrow down what type of kinase groups might be present in the system e.g. cyclin-dependent, Ca^{2+}/calmodulin-dependent, protein C kinases etc.

1.4 Concluding Remarks and Research Objective

In summary, up until now, pharmaceutical industries and research labs have focused on the discovery of compounds that target the protein products of genes and RNA has remained largely unexplored. Small molecules can be used as tools to study the biochemical, genetic, and structural aspects RNA sequences such as they have been used to study the different ribozymes and the ribosome. In addition, many small molecules found to target specific RNA sequences have also become important in the development of therapeutics for genetic disorders (Noller 1999; Varni *et al.*, 2000; Tor 2003).

RNA is an excellent target because it can fold into complex three-dimensional structures which are responsible for the diverse functions of RNA molecules within cells. In this respect, RNA resembles more resembles a protein than DNA, which is less flexible and has a less diverse tertiary structure. The unique shapes in various target RNAs create potential binding sites for small molecules. Targeting at the RNA level is an economical approach to address non-drugable proteins and targets that have failed to

give any sort of leads, as it can build on biological knowledge gathered over years (Arya *et al.*, 2001).

The objective of this study is to investigate the inhibitory effect of small molecules on *in vitro* splicing of a eukaryotic pre-mRNA, actin of yeast, using the above-mentioned candidates. Yeast actin, expressed by the essential gene *ACT1* in *Saccharomyces cerevisiae*, contains only one intron sequence and is an excellent model for study because it can be easily manipulated. In addition, the actin sequence is highly conserved among eukaryotes (section 1.1.1).

Two major assays test for interaction specificity: one with the pre-mRNA, via testing different transcripts; and two with the spliceosomal complexes, via using native gel systems. A study using the yeast pre-mRNA as the target for screening small molecules has no prior precedent; therefore, in order to compare the different small molecules for their effectiveness as inhibitors their concentrations required for 50% splicing inhibition (IC_{50} values) will be determined (methods section).

CHAPTER 2

Identification of Small Molecules that Inhibit Yeast Pre-mRNA Splicing and Accumulate Spliceosomal Complexes *in vitro*

Cellular function is dependent upon the correct expression of genomic information encoded in DNA into functioning products, usually proteins. Prior to protein translation, DNA is transcribed into messenger RNA (mRNA). In eukaryotes, such as yeast or humans, translation generally follows an intermediate step, termed pre-mRNA splicing (Berget *et al.*, 1977; Adams *et al.*, 1996). In pre-mRNA splicing, non-protein-coding regions (introns) are removed from the precursor mRNA, resulting in mature message, which consists of protein-coding regions (exons) (Berget *et al.*, 1977).

Splicing is catalyzed by the spliceosome, a multi-component complex consisting of five different snRNAs and a large number of spliceosomal and non-spliceosomal proteins, which assemble on the pre-mRNA in a stepwise manner before the splicing reaction starts (Cheng & Ableson, 1987; Burge *et al.*, 1999; Du & Rosbash 2002). Native gel analysis has been employed to detect the formation of four distinct spliceosomal complexes, termed H/E, A, B and C, that appear during spliceosomal assembly (Das & Reed, 1999).

Splicing must be carried out with single-nucleotide precision in order to prevent catastrophic changes in the message from occurring (Madhani & Guthrie, 1994; Nissim-Rafinia & Kerem, 2004; 2005). Defects in pre-mRNA splicing are responsible for various human disorders including retinitis pigmentosa, spinal muscular atrophy, myotonic dystrophy, and neoplasia (Faustino & Cooper, 2003). Recent work has focused on small molecules as potential tools for elucidating the role of RNA in a variety of

biochemical processes, anticipating their eventual use as therapeutic agents for a wide range of diseases (Sucheck & Wong, 2000; Tor, 2003; Zaman, 2003).

It has been demonstrated that antibiotics can inhibit various RNA-based processes. The best-known example is the inhibition of prokaryotic protein synthesis (Moazed & Noller, 1987; Recht *et al.*, 1999). For instance, the aminoglycoside streptomycin inhibits bacterial translation by interacting with the 30S ribosomal subunit, which induces misreading of the genetic code (Schroeder *et al.*, 2000). Other aminoglycosides are known to inhibit the catalytic activity of self-splicing group I introns (Hoch *et al.,* 1998), self- cleaving hammerhead ribozymes (Stage *et al.,* 1995), hairpin ribozymes (Earnshaw & Gait, 1998), the hepatitis delta virus ribozyme (Rogers *et al.,* 1996), HIV-1 ribozyme (Mei & Czarnik, 1995), and tRNA processing RNase P RNAs (Mikkelsen *et al.,* 1999).

Previous work has demonstrated that antibiotics can inhibit human splicing *in vitro* (Hertweck *et al.*, 2003), while more recent papers have demonstrated that spliceostatin A, the methylated derivative of anti-tumor compound FR901464, can inhibit human splicing *in vitro* and *in vivo* (Kaida *et al.*, 2007) as well as splicing in the fission yeast *S. pombe* (Lo *et al.*, 2007). Small molecule inhibition of splicing in budding yeast, *S. cerevisiae*, has not yet been demonstrated. Given the powerful genetic and biochemical tools available in *S. cerevisiae*, I sought to determine whether small molecules could inhibit splicing in this highly tractable system. I therefore screened a library of 32 compounds for *in vitro* inhibitory activity, and characterized the positive hits by measuring their IC_{50} and the step of splicing assembly at which they inhibit.

2.1 Materials and Methods

2.1.1 Small Molecules

Table 1 contains a list of all the antibiotics and kinase inhibitors that were purchased from Sigma, with the exception of G1-G14 (enantiomeric mixtures, except G12), which were provided by Dr. Guy Plourde, UNBC, and the environmental toxicants, which were provided by Dr. Laurie Chan, UNBC.

Each small molecule was tested up to a concentration of 10 mM, except the environmental toxicants, which were tested up to 1 mM, the maximum concentration seen for cell toxicity (Hoffman *et al.,* 1996; Olivieri *et al.,* 2000), and the kinase inhibitor roscovitine, which was only tested up to 5 mM because of solubility limitations. The reported inhibitory concentrations were the lowest concentration tested at which there was no detectable in vitro splicing (denoted **LC in** Table 1).

2.1.2 Splicing extract preparation and *in vitro* splicing assays

Splicing extract preparation

Whole-cell extract was prepared from protease deficient yeast strain BJ2168 (Jones, 1991) as described (Ansari & Schwer, 1995) with some modifications: yeast cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30 C to late logarithmic phase (OD 2-2.5) and harvested by spinning at 3000 rpm for 5 min in a Sorvall JA-8.1000 rotor. Cell pellets from 2 L of culture were first washed with 50 ml of cold, sterile double-distilled water and then with 50 ml of AGK buffer (10 mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 200 mM KCI, 0.5 mM DTT and 10% glycerol). The cell pellets were then suspended in 7.5 ml (per 2 L culture) of AGK buffer. The cell suspension was frozen by

drop-wise addition to liquid nitrogen using a syringe with an 18 gauge needle, and stored at -80 C.

Table 1: Candidate inhibitors of *in vitro* splicing.

Antibiotic	Class	LC
Kanamycin	Aminoglycoside	2.5mM
Neomycin B	Aminoglycoside	250μM
Streptomycin	Aminoglycoside	5mM
Cefoperazone	Cephalosporin	10mM
Erythromycin	Macrolide	NI
Tetracycline	Aminocyclitol	NI
Ampicillin	Penicillin	NI
Ciprofloxacin	Quinolone	NI
Bacitracin	Polypeptide	NI
Sulfamethizole	Sulfonamide	NI
Chloramphenicol	Phenicol	NI
G5	Oxospiro-Compound	5mM
G6	Oxospiro- Compound	5mM
G11	Oxospiro- Compound	5mM
G12	Oxospiro- Compound	1mM
G14	Oxospiro- Compound	5mM
G1 – G4	Oxospiro- Compound	NI
G7 – G10	Oxospiro- Compound	NI
G13	Oxospiro- Compound	NI
Kinase Inhibitor		
Staurosporine	Broad Range Protein Kinase Inhibitor	3mM
Roscovitine	Broad Range Cyclin-dependent Kinase Inhibitor	NI**
Environmental Toxins		
PCB mixture A1254	Biphenyl	NI*
PCB – # 126	Biphenyl	NI*
PCB – # 99	Biphenyl	NI*
PCB – # 77	Biphenyl	NI*
Methyl Mercury Salt	Transition metal	NI*

NI non-inhibitor at 10mM; * except toxicants at 1mM (the maximum concentration seen for cell toxicity (Hoffman *et al.*, 1996; Olivieri *et al.*, 2000)

** Roscovitine was only tested up to 5mM because of solubility limitations

LC is the lowest concentration tested at which there is no detectable nuclear *in vitro* splicing

The frozen cell pellets were homogenized to a very fine powder using a mortar and pestle. The powder was allowed to thaw slowly at 4 C, was stirred for 30 min, and then centrifuged at 18 000 rpm for 30 min in a Sorvall JLA-25.5 rotor. The supernatant from this spin was centrifuged at 37 000 rpm for 1 h in a Beckman Ultracentrifuge 70.1Ti rotor. After the spin, the pale yellow aqueous phase was carefully removed and dialyzed twice against 2 L of buffer D (20 mM HEPES-KOH pH 7.9, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT and 20% glycerol) for 1.5 h each.

Template and radioactively labeled pre-mRNA *in vitro* transcript preparation

BJPS149 template (truncated ACT1, 590ntds, Staley & Mayas, 2002) was linearized with HindIII and the precursor RNA was synthesized by run-off transcription using T7 RNA polymerase (Roche). Templates, YOL047C (163 nucleotides) and UBC4 (290 nucleotides), for *in vitro* transcription were amplified by PCR from yeast genomic DNA using primers listed in table 2.

Table 2: Primers used for constructing YOL047C and UBC4 templates from yeast genomic DNA

YOL047C	Forward	oSDR339	5'AATTAATACGACTCACTATAGGGAACATGTCTTCTTC TAAACGTATTGCTAAAGAACTAAGTGATCTAGAAAG3'
	Reverse	oSDR340	5'GATATAGATCATCGCCGACTGGACCGGCTGAACATGA AGTAGGTGGATCTC3'
UBC4	Forward	oSDR345	5'AATTAATACGACTCACTATAGGGTTTGGAAAGACCTA GAGTCGTCGCAC3'
	Reverse	oSDR346	5'AGGAAAAATAGATGCAAATAATCCGAGTTTCCC3'

Preparation of pre-mRNA transcripts for in vitro splicing assays was synthesized in 50uL reactions. A 10 μL reaction containing 1 mM Roche 10x T7 RNA polymerase buffer, 0.5 mM NTPs (CTP, UTP, ATP), 1 mM GTP, 50μg/ml template, 0.5 μL of

1000units/ml Ribonuclease Inhibitor (RNAsin) (Promega), 2.5 μL of 125 μCi/ml α-^{32}P-labelled GTP, 0.5 μL of 400units/mL T7 RNA polymerase (Roche) was incubated for 1.5 hours at 37 C, followed by addition of 40 uL 1xTE buffer (10 mM Tris, 1mM EDTA).

The 50 uL reactions were purified from unincorporated nucleotides by using a G25 spin column. 4 fmol of the precursor were used per 10 uL splicing assay.

In Vitro Pre-mRNA Splicing Assays

Standard splicing of the Actin pre-mRNA in BJ2168 nuclear extract was performed at room temperature for 30 minutes. Splicing reactions were performed in 10μL reactions (Schwer & Guthrie, 1991) using the following standard conditions: 2.5 mM $MgCl_2$, 0.1 M KPO_4 (pH 7.0), and 30% PEG 3000, 40% (v/v) BJ2168 extract, 2 mM ATP, and 1uL 4fmol internally ^{32}P-GTP labeled actin pre-mRNA *in vitro* transcript, and 1 μL of small molecules at various concentrations. The reactions were stored on ice before and after incubation and were stopped by the addition of stop solution (3 M NaOAc, 500 mM EDTA, 10% SDS, 10 mg/mL *E. coli*tRNA).

Splicing reactions were extracted with phenol/chloroform/isoamyl alcohol (39: 59: 2, v/v), and back extracted with chloroform. The aqueous phase was ethanol precipitated. Following a wash with 70% ethanol, the pellets were resuspended in 7 M urea formamide gel loading dye, and the products separated on denaturing 6% polyacrylamide 7 M urea gels at 400 V for 1 hour. The gels were dried under vacuum for 10-15 minutes at 80 C. Once dry, they were exposed to a PhosphoImager screen overnight. The resulting autoradiogram was visualized and the bands quantitated with the Molecular Dynamics PhosphorImager and associated software. Splicing efficiency was

defined as the percent of final product bands (mRNA and lariat) divided by the sum of all five bands (pre-mRNA, lariat-3'exon, 5'exon, excised lariat, and ligated exons).

2.1.3 Spliceosome Assembly Gels

Spliceosomal complex assembly was analyzed as described (Reed & Das, 1999). Aliquots of splicing reaction containing ^{32}P- labeled transcript were taken at the indicated 8 time points (0, 1, 2, 5, 10, 15, 20, 25 minutes) and, prior to loading on to the gel, 4.44 uL of heparin mixture (4 mg/mL heparin, 50% glycerol and trace bromophenol blue for visualization) was added to each 10 uL reaction. The samples were run on non-denaturing 1.5% agarose gels in tris/glycine buffer to separate individual spliceosomal complexes at 70 V for 3.5 hours. The gels were fixed with 10% acetic acid and 10% methanol for 30 minutes, and dried under vacuum for 40 minutes at 80 C. The dried gels were exposed to a PhosphorImager screen for visualization overnight.

2.1.4 IC_{50} Determination

Percent splicing of the actin pre-mRNA reporter in the presence of inhibitor was determined for a range of inhibitor concentrations up to complete inhibition for each small molecule (LC values Table 1) (splicing efficiency was around 60% in the absence of inhibitor).

Percent maximum splicing versus concentration was plotted for each inhibitory small molecule and the concentration required to achieve 50% splicing, IC_{50}, was estimated from these plots. The maximum splicing efficiency in the absence of inhibitor

was normalized to 100% in order to look for the concentration that reduces maximum splicing by 50%.

Each titration assay was performed in triplicates and exhibited less than 5% standard error deviations from the average (Table 3). Error bars were calculated for each small molecule IC_{50}, using excel's standard error calculation, which is as follows: STDEV(values calculated for IC_{50} from each triplicate), divide by, SQRT (COUNT(values calculated for IC_{50} from each triplicate)) (Table 3).

Table 3: IC_{50} standard error calculations using excel

Inhibitory Small Molecule	**IC_{50} gel 1**	**IC_{50} gel 2**	**IC_{50} gel 3**	**Average**	**Standard Deviation**	**Square Root**	**Standard Error**	**Estimated IC_{50}**
Cefoperazone	6.40	6.20	5.80	6.10	0.06	1.73	0.033	6.10 ± 0.033 mM
Kanamycin	0.71	0.90	0.65	0.75	0.13	1.73	0.075	0.75 ± 0.075 mM
Neomycin	0.08	0.09	0.07	0.08	0.01	1.73	0.005	0.08 ± 0.005 mM
Streptomycin	2.10	1.90	1.85	1.95	0.13	1.73	0.076	1.95 ± 0.076 mM
Staurosporine	1.94	2.00	1.89	1.94	0.06	1.73	0.032	1.94 ± 0.032 mM
G5	0.75	0.80	0.60	0.72	0.10	1.73	0.060	0.72 ± 0.060 mM
G6	0.66	0.70	0.90	0.75	0.13	1.73	0.074	0.75 ± 0.074 mM
G11	1.80	1.70	1.80	1.77	0.06	1.73	0.033	1.77 ± 0.033 mM
G12	0.59	0.75	0.55	0.63	0.11	1.73	0.061	0.63 ± 0.061 mM
G14	2.61	2.70	2.80	2.70	0.10	1.73	0.055	2.70 ± 0.055 mM

All values are in mM

2.2 Results

2.2.1 Ten Small Molecules Found to Inhibit Nuclear Splicing *In Vitro*

To search for molecules that inhibit splicing at specific steps in spliceosome assembly, the effects of thirty-two different small molecules on actin pre-mRNA splicing in yeast nuclear extract were investigated (Table 1). Twenty-five of the compounds tested were antibiotics. In order to study how different structural properties of a compound could affect splicing, compounds that represent different classes of antibiotics were investigated. The first antibiotics (erythromycin, streptomycin, tetracycline, and neomycin) were chosen because of their ability to bind to RNA sequences non-specifically (Mei & Czarnik, 1995; Mikkelsen *et al.,* 1999; Schroeder *et al.,* 2000; Hertweck *et al.,* 2002;). Fourteen of our small molecules were newly synthesized oxospiro-compounds from the manumycin family. In addition, a variety of common environmental toxins as well as kinase inhibitors were also tested.

The small molecules were added to *in vitro* splicing reaction mixtures at a starting concentration of 10 mM. Of the thirty-two different compounds tested, ten were found to inhibit splicing, as indicated by a large reduction in mature ligated mRNA and excised lariat RNA relative to the amount of unspliced pre-mRNA (Figure 4). The rest of the twenty-two small molecules showed no significant inhibitory effect on pre-mRNA splicing even at 10 mM (Roscovitine was only tested up to 5mM because of solubility limitations).

Of the ten inhibitory molecules, three were the aminoglycosides kanamycin, streptomycin and neomycin (Figure 4). From the remaining eight different classes of antibiotics tested, it was found that cefoperazone, a third generation cephalosporin,

inhibited splicing (Figure 4). Three different types of polychlorinated biphenyls (A1254, 77, 99 and 126) and methyl-mercury were also tested; however none of these environmental toxicants inhibited pre-mRNA splicing (Figure 5). To determine whether yeast splicing requires kinase function *in vitro,* the effect of a broad range inhibitor of nuclear protein kinases, staurosporine, as well as a broad range inhibitor of cyclin-dependent kinases, roscovitine were tested. A 5mM concentration of roscovitine did not exhibit any effect on splicing (Figure 5) while staurosporine at 3mM showed complete inhibition (Figure 4).

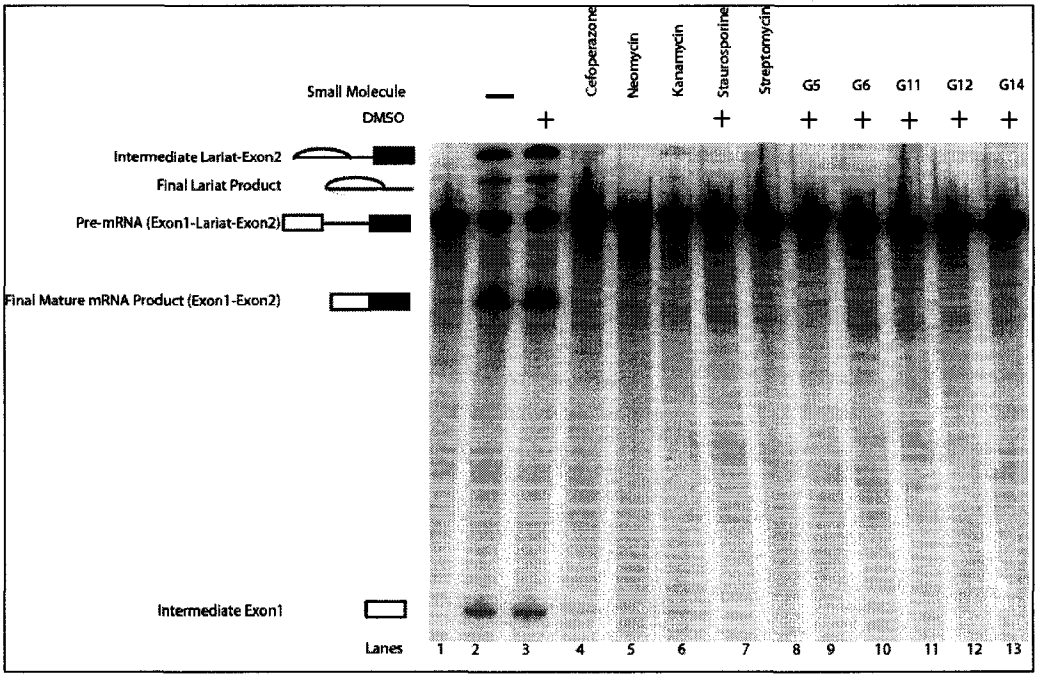


Figure 4: Splicing inhibition by ten small molecules. Actin pre-mRNA splicing reactions analyzed on a 6% denaturing polyacrylamide gel and visualized by autoradiography. Locations of pre-mRNA and product mRNA bands are indicated at left. Splicing reactions are shown in the absence of inhibitor at 0 minutes (lane 1) and 30 minutes (lane 2), and with a DMSO control at 30 minutes (lane 3). Lanes 4-13 are reactions containing compounds at the concentrations given in Table 1.

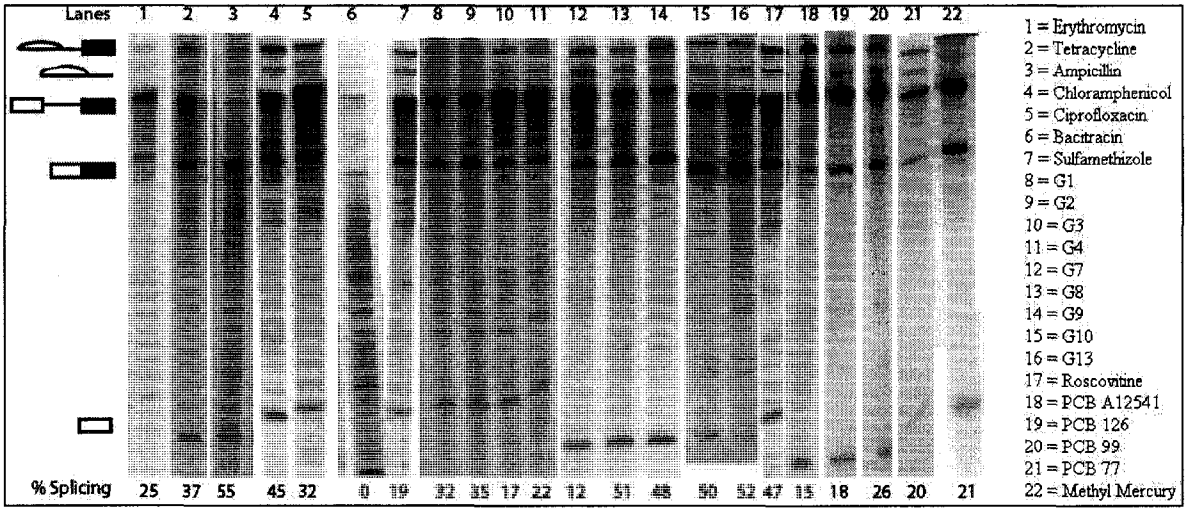
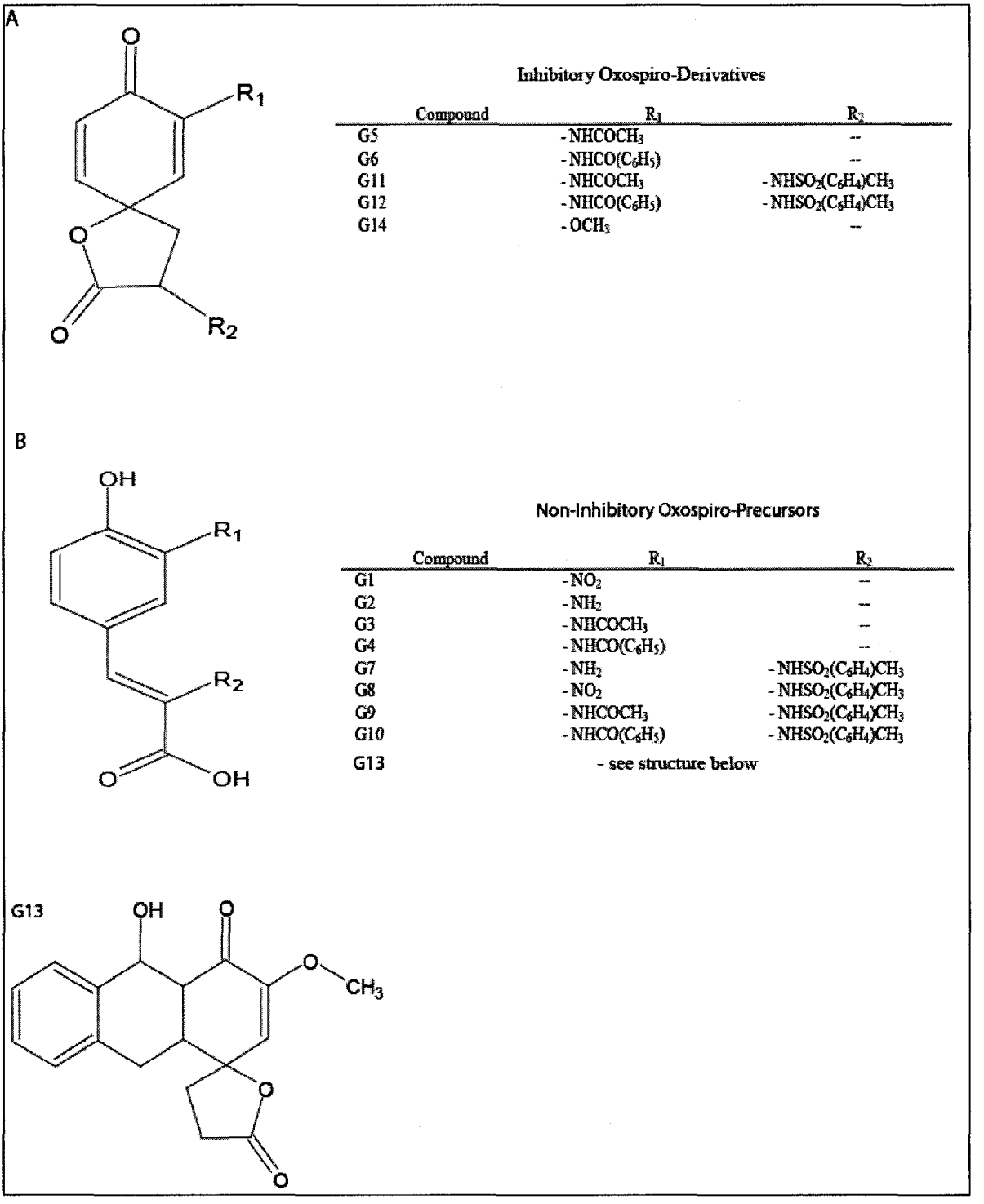


Figure 5: 22 Small molecules did not inhibit actin pre-mRNA splicing *in vitro*. Actin pre-mRNA splicing reactions analyzed on a 6% denaturing polyacrylamide gel and visualized by autoradiography. Locations of pre-mRNA and product mRNA bands are indicated at left. Non-inhibitory small molecules are listed to the right with their respective lane numbers found on the gel. Splicing efficiency for each splicing reaction is given at the bottom of the gel.

Novel oxospiro compounds of the biologically active manumycin family were obtained (a generous gift of Guy Plourde; Plourde *et al.*, 2007). To determine whether they exert their biological effects in part through inhibition of pre-mRNA splicing, these compounds (denoted G1-G14) were tested in the splicing assay. The results showed five of the 14 oxospiro-compounds tested (Figure 6) completely inhibit splicing (Figure 4). The 5 inhibitory oxospiro-compounds shared a common core structure (Figure 6A) in which there is a lactone ring attached to a conjugated ring, suggesting that the groups R1 and R2 are not involved in binding to the targets. Although G13 has a similar core structure to the inhibitors (Figure 6B), it did not inhibit splicing (Figure 5). It is the only oxospiro-compound which contains an extra cyclohexane ring fused to the core.



Inhibitory Oxospiro-Derivatives

Compound	R_1	R_2
G5	$-NHCOCH_3$	--
G6	$-NHCO(C_6H_5)$	--
G11	$-NHCOCH_3$	$-NHSO_2(C_6H_4)CH_3$
G12	$-NHCO(C_6H_5)$	$-NHSO_2(C_6H_4)CH_3$
G14	$-OCH_3$	--

Non-Inhibitory Oxospiro-Precursors

Compound	R_1	R_2
G1	$-NO_2$	--
G2	$-NH_2$	--
G3	$-NHCOCH_3$	--
G4	$-NHCO(C_6H_5)$	--
G7	$-NH_2$	$-NHSO_2(C_6H_4)CH_3$
G8	$-NO_2$	$-NHSO_2(C_6H_4)CH_3$
G9	$-NHCOCH_3$	$-NHSO_2(C_6H_4)CH_3$
G10	$-NHCO(C_6H_5)$	$-NHSO_2(C_6H_4)CH_3$
G13	- see structure below	

Figure 6: Oxospiro-compound inhibitors share a common core structure. (A) Oxospiro derivative inhibitors and (B) non-inhibiting precursors (Plourde *et al.*, 2002; 2007). The identity of the R groups is tabulated on the right.

2.2.2 Effectiveness of Inhibitory Small Molecules (IC_{50} values)

To compare the potency of the inhibitors, the concentration of inhibitor required for 50% inhibition of pre-mRNA splicing relative to a no inhibitor control (the apparent IC_{50}) was measured (Figures 7, 8 & 9). Percent splicing of the actin pre-mRNA reporter was measured in the presence of each small molecule for a range of concentrations up to the LC concentrations listed in Table 1. Figure 7 is an example of Cefoperazone titration gel used to determine its IC_{50}. It can clearly be seen from the gel that as the concentration of cefoperazone increases the percent splicing decreases (Figure 7). Splicing in the control reactions without cefoperazone was approximately 60% of total pre-mRNA (Figure 7: lanes 2 and 3) and as the concentration increased to 10 mM (Figure 7: lane 15) splicing decreased to undetectable levels. Two to three replicates of the titration assay were reproduced for each inhibitor at each concentration, which showed very low, if any, standard deviations of the replicated averages (<5%) (Table 3).

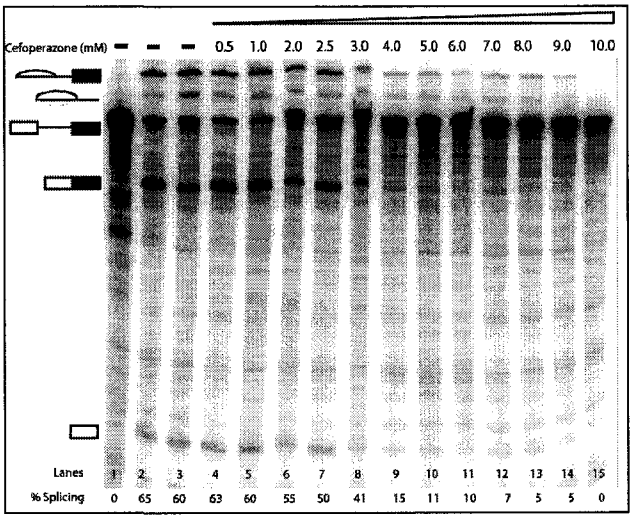
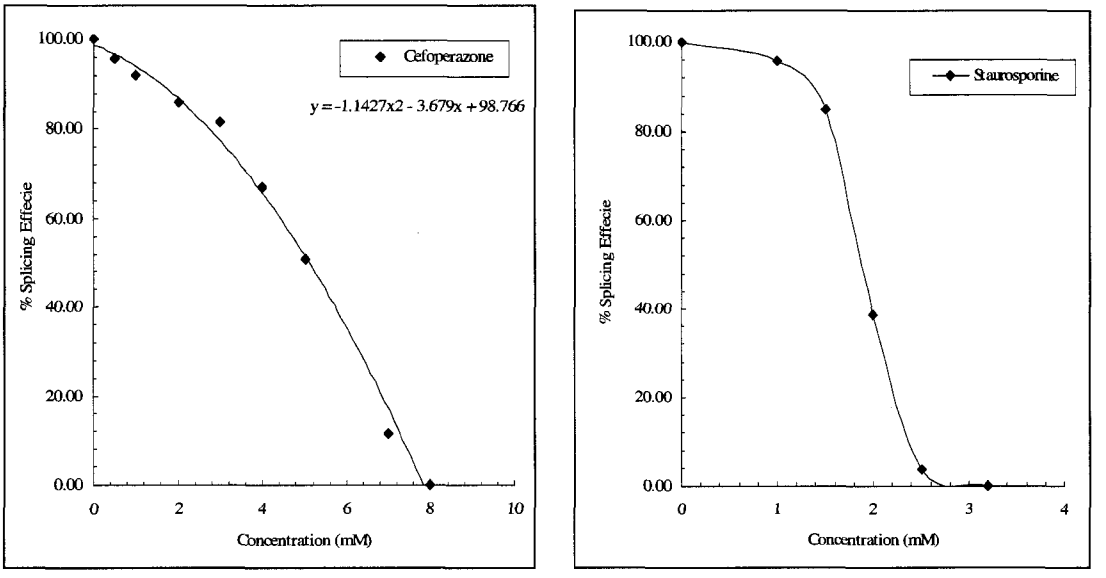


Figure 7: Cefoperazone inhibits pre-mRNA splicing with an apparent IC_{50} of 6.1mM. Denaturing polyacrylamide gel analysis of splicing with increasing concentrations of Cefoperazone (indicated at top of gel). Locations of pre-mRNA and product mRNA bands are indicated at right, and fraction of RNA spliced in each lane is indicated below the gel.

To determine the IC_{50}, splicing efficiency was plotted as a function of inhibitor concentration for each compound tested (Figure 8). Best fit equations were achieved for all inhibitors, in which kanamycin, G5 and G6 required a log scale to obtain a better fit equation (Figure 8). Cefoperazone and staurosporine were the only two inhibitors with non-linear regression (Figure 8). The IC_{50} values were determined from the midpoint of these graphs (Figure 9). As expected from initial experiments used to determine inhibitory activity, neomycin had the lowest IC_{50} of 0.08 mM and cefoperazone the highest IC_{50} of 6.10 mM, while the remainder had IC_{50} values in the low millimolar range (Figure 9). These IC_{50} values can be used to compare the effectiveness of the small molecules as inhibitors.



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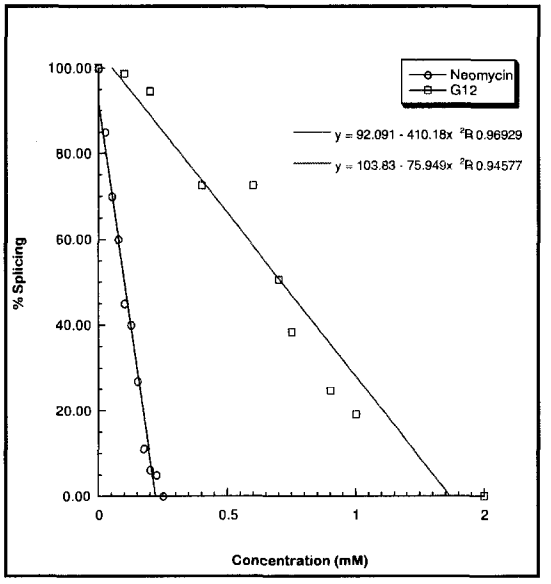

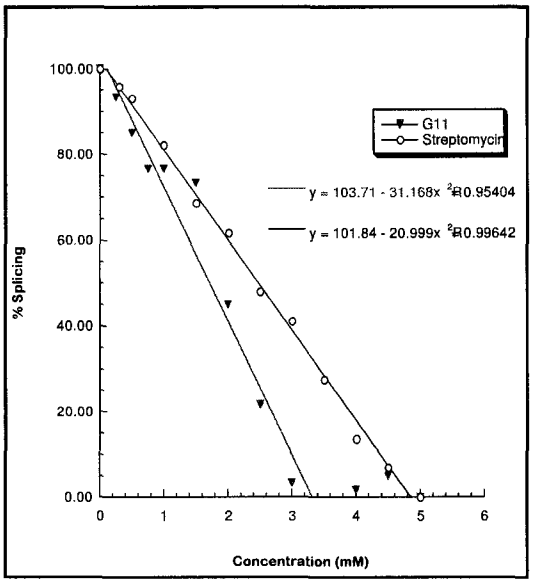

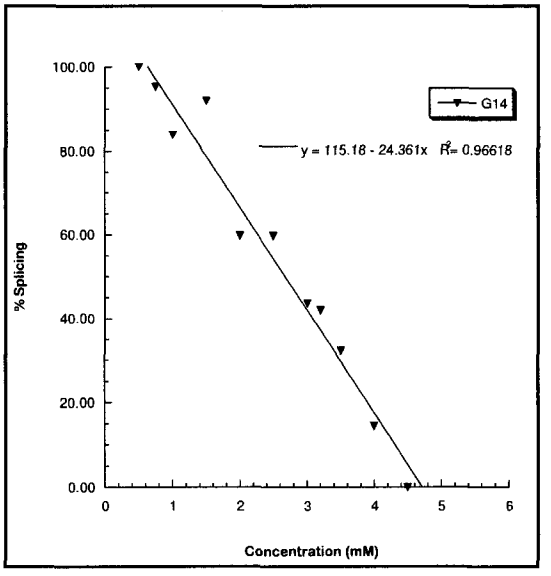

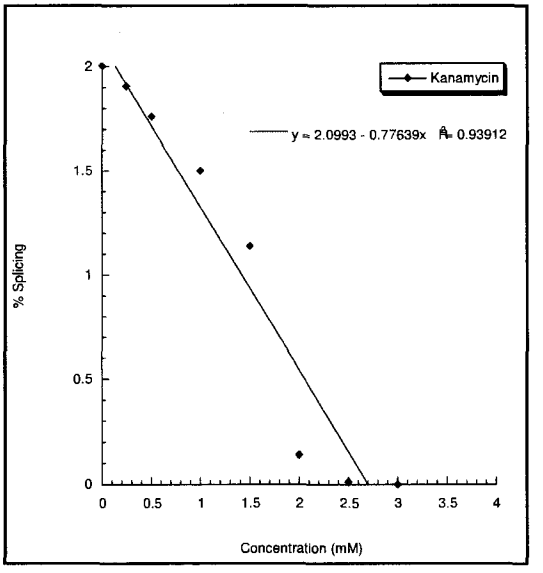

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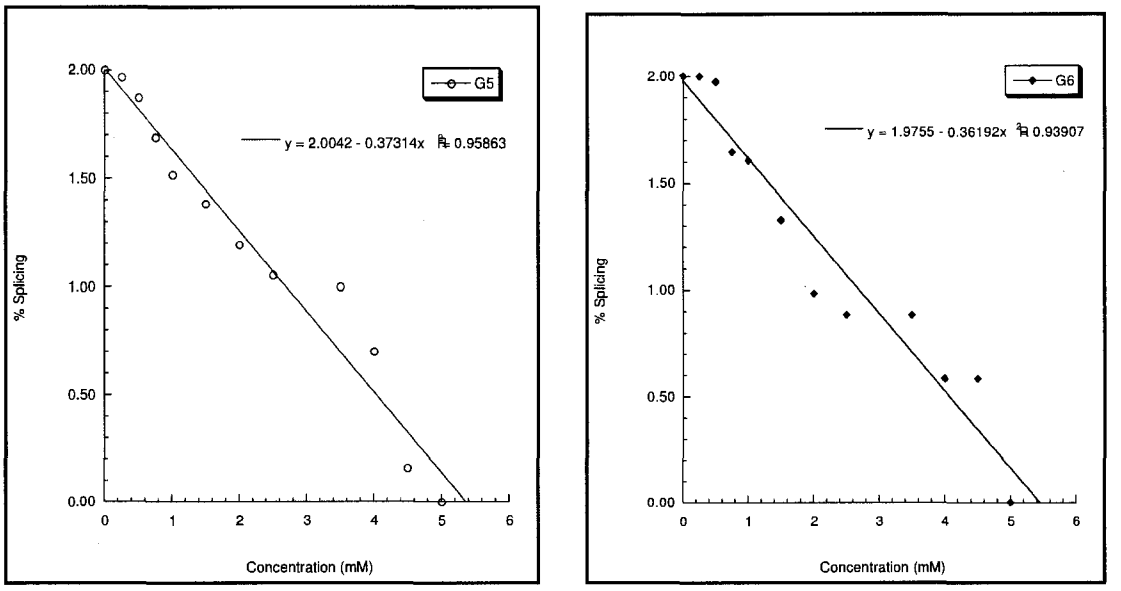


Figure 8: Neomycin has an IC_{50} of 0.08 mM. Splicing efficiency was plotted versus inhibitor concentration to determine the IC_{50}. The plots' of kanamycin, G5 and G6 were plotted on a log scale using a linear regression.

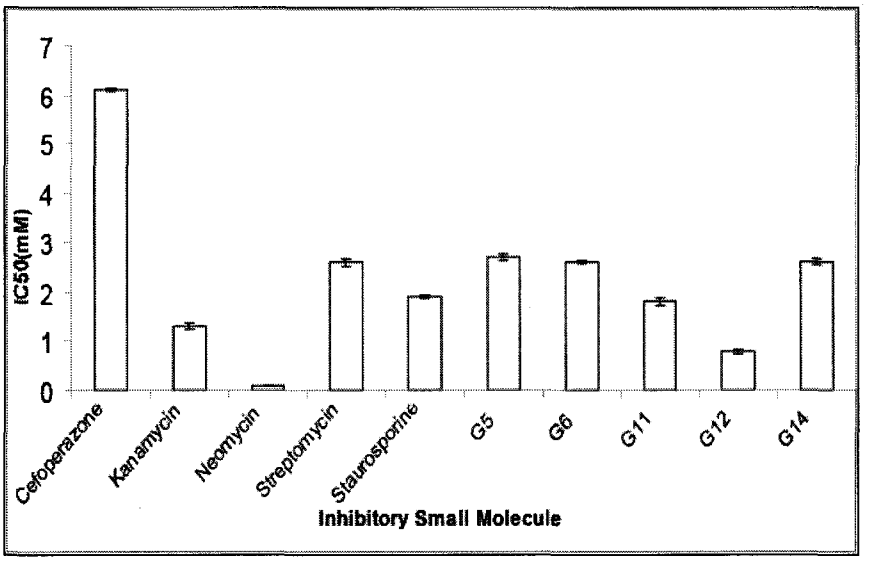


Figure 9: IC_{50} for all splicing inhibitors. Standard deviations are calculated from triplicate measurements of inhibition (Table 3).

2.2.3 Inhibitors Function at Different Steps of Splicing

To determine the step of splicing at which each inhibitor acts, spliceosomal complex accumulation was determined in the presence of each of the ten small molecules (Figure 10). The different spliceosomal complexes H, A, B and C were identified in a time course splicing assay using native agarose gel separation (Das & Reed, 1999).

In the absence of inhibitors, four complexes containing pre-mRNA can be distinguished on native agarose gels by their different mobilities (Figure 10A). At 0 minutes, complex H predominates, consisting of numerous heterogeneous nuclear RNP (hnRNP) proteins.(Das & Reed 1999; Jurica & Moore 2002). Complex A consists of the U2 snRNP bound to the pre-mRNA. Complex B consists of the pre-catalytic assembly in which the U4/U6 hybrid and the U5 snRNP have joined the complex, , while complex C is the catalytically active stage formed by U4 dissociation and U2 base pairing to U6. The identity of the each complex was determined by Das and Reed (1999) through isolation of the complexes and Northern analysis of their snRNA composition.

The inhibitors fall into several classes based on the step at which they appear to block spliceosome assembly. Aminoglycosides streptomycin and neomycin, kinase inhibitor staurosporine, and oxospiro-compound G12 cause accumulation of complex H (Figure 10A). The native gels of G12 contain sharper complex H bands than the more smeared bands of neomycin, streptomycin, staurosporine, and the control (-) inhibitor lane. Sharp bands may indicate less heterogeneity in the accumulating complexes. In contrast, the aminoglycoside kanamycin was the only one that showed an apparent accumulation of complex A (Figure 10B). Unlike streptomycin and neomycin,

kanamycin also showed more distinct complex A accumulating as time progresses from 10-25 minutes.

The oxospiro-compounds G6, G11, and G14 cause accumulation of the B complex (Figure 10C). All three native gels show that as complex B accumulates there is also a build-up of the other two complexes H and A. There is also more complex B with both G11 and G14 compared to G6. Interestingly, there is more complex A than B for G6, and almost equal amounts of complexes A and B for G11. There is less complex A than complex B for G14. This suggests that each compound inhibits splicing assembly in a different way.

Oxospiro-compound G5 and the cephalosporin cefoperazone both caused an accumulation of complex C (Figure 10D) and no formation of mature product. Accumulation of complex C also results in accumulation of complex B, but not of complexes H and A (in contrast to the block at complex B in figure 10C). Additionally, in the presence of cefoperazone complex C accumulates more than complex B, whereas with G5 complexes B and C accumulate to similar levels (Figure 10D).

2.2.4 Transcript Specificity of Inhibition

To determine whether the inhibitors are specific for actin pre-mRNA, or whether they exhibit general inhibition of pre-mRNA splicing, the effect of the inhibitors on two other transcripts was tested. The results showed that in addition to inhibiting actin pre-mRNA, these ten small molecules also completely inhibit splicing of two other pre-mRNA substrates: YOL047C and UBC4 (Figure 11).

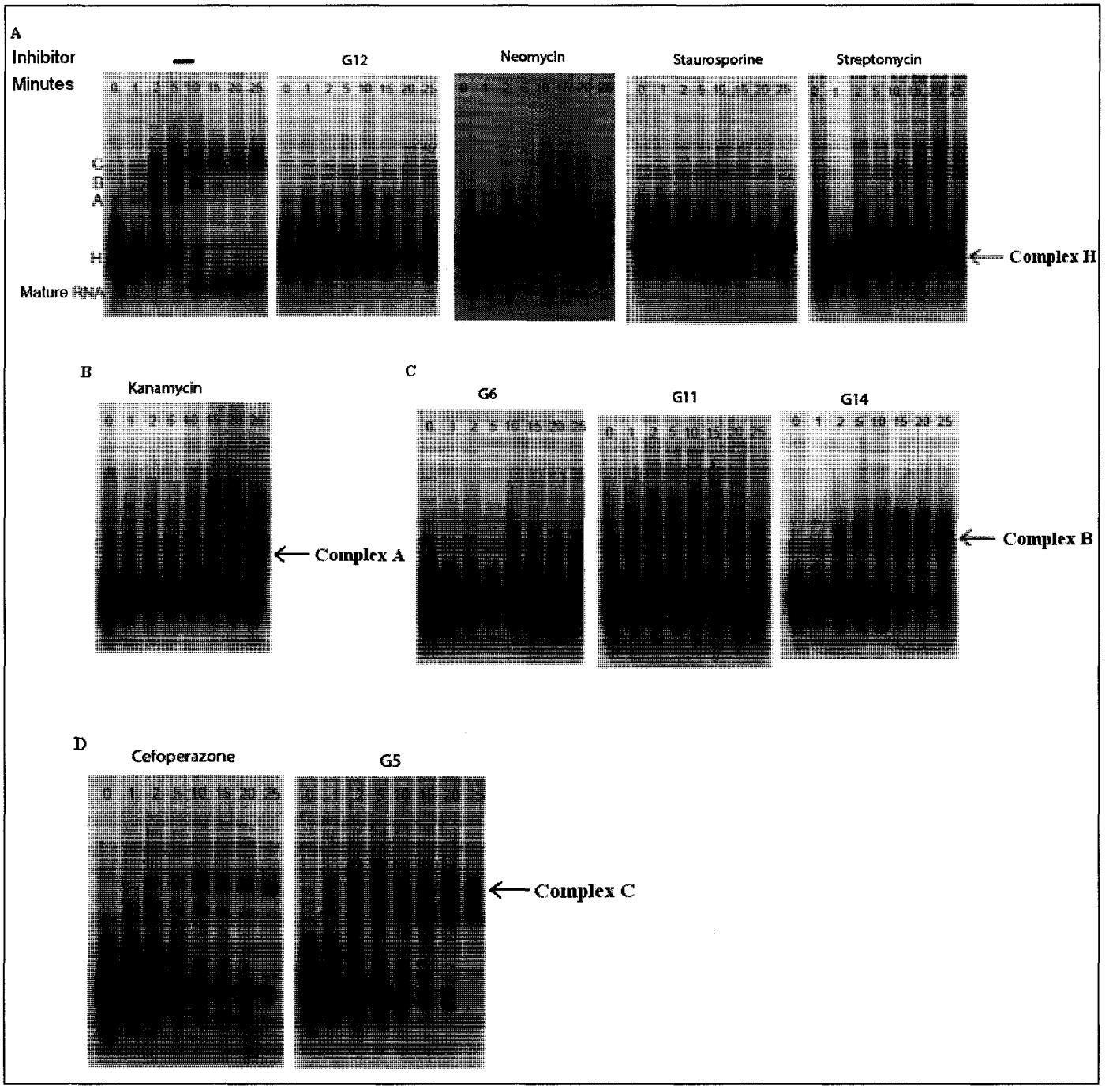


Figure 10: Splicing inhibitors block spliceosome assembly at distinct steps.
Time-dependent formation of the four different spliceosomal complexes H, A, B, and C. Splicing reactions in the absence of inhibitor were stopped at the indicated times and analyzed by mini-native agarose gels (-) = no inhibitor. (A) G12, Staurosporine, Streptomycin, and Neomycin respectively show no spliceosomal assembly; (B) Kanamycin shows a A complex; (C) G6, G11, G14 show a B complex; and (D) G5 and Cefoperazone show a C complex. Complexes were separated on a non-denaturing 1.5% agarose gel run in Tris-glycine buffer.

The transcript used for the splicing assay in Figure 11A was partially degraded. Nevertheless, it is clear that there is a dramatic reduction of product mRNA in the presence of inhibitors compared to the control reaction. Quantitation revealed that splicing of all three transcripts was inhibited at 95% at the inhibitor concentrations listed in Table 1. These results confirm that the ten small molecules do not require a conserved pre-mRNA sequence in order to exert their inhibitory effects, and suggest that these small molecules either inhibit splicing by binding RNA non-specifically or via interactions with the splicing machinery.

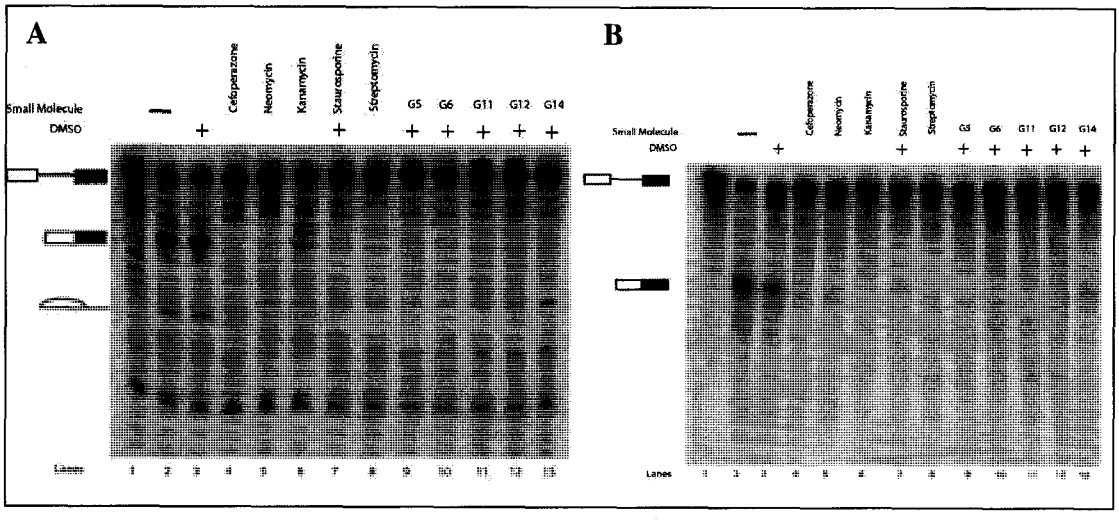


Figure 11: Splicing inhibitors are not transcript specific. (A) YOL047C and (B) UBC4 transcripts were spliced in the presence of each inhibitor, analyzed on a 6% denaturing polyacrylamide gel, and visualized by autoradiography. Locations of pre-mRNA and product mRNA bands are indicated at left. Splicing reactions are shown in the absence of inhibitor at 0 minutes (lane 1) and 30 minutes (lane 2), and with a DMSO control at 30 minutes (lane 3). Lanes 4-13 are reactions containing compounds at the concentrations given in Table 1.

2.3 Summary and Interpretation of Results

In a search for small molecules with which to study splicing, the sensitivity of a yeast *in vitro* splicing reaction to inhibition by a variety of chemical compounds has been investigated. Ten of the thirty-two small molecules tested were found to inhibit splicing of actin pre-mRNA prior to the first transesterification step. The 10 small molecules were also found to inhibit splicing of two other pre-mRNA substrates, YOL047c and UBC4, demonstrating that the inhibitors are not specific for actin. The IC_{50} value of all of the inhibitors was determined to assess their effectiveness. Of the compounds tested, neomycin was found to be the strongest inhibitor with an IC_{50} of 0.08 mM, while the remaining IC_{50}s were in the range of 1-6 mM (Figure 9).

To learn more about the inhibitory mechanism of each of these compounds, the arrested splicing step was determined by native gel analysis. Each inhibitor resulted in accumulation of one or more complexes in the yeast spliceosome assembly pathway (Figure 10). Four of the ten inhibitors showed a complete block in spliceosome assembly, one accumulated spliceosomal complex A, two blocked assembly after formation of complex B, and three resulted in accumulation of complex C (Figure 10).

Of the ten inhibitors, neomycin, kanamycin, and streptomycin are aminoglycosides known for their non-specific interactions with RNA. Cefoperazone is a third generation cephalosporin chosen to determine whether other classes of antibiotics inhibit splicing. Staurosporine is a broad range protein kinase inhibitor chosen to determine if yeast splicing is regulated by kinases, which has not been shown biochemically, but only suggested from gene expression analysis (Schena *et al.,* 1996; Dagher & Fu, 2001). The remaining five (G5, G6, G11, G12, G14) are new compounds

known as oxospiro-derivatives of the manumycin family, derived from actinomycetes bacteria, that have not been characterized previously.

The high success rate in finding splicing inhibitors (10 of 32 compounds tested) may reflect the complexity of the splicing process, which provides over 100 individual molecular targets for potential inhibitors, and a much larger number of molecular interactions. With the exception of neomycin, however, these inhibitors are weak, so it is also possible that they are simply non-specific inhibitors of splicing. A comparison of inhibitors to non-inhibitors argues against this interpretation, as similar compounds have different effects, both on splicing generally and on the specific assembly step that is affected.

2.3.1 Inhibitory small molecules: structure and mechanism

The following sections describe the ten inhibitory small molecules and their association with various proteins and RNAs, which have been reported by other research groups. The last section then describes two models which have been proposed to explain the spliceosomal complex accumulation seen in the presence of the small molecules.

Aminoglycosides

In order to look for compounds that inhibit yeast pre-mRNA splicing, aminoglycoside antibiotics were tested as they are known to inhibit other RNA-based processes. Aminoglycosides are multiply charged compounds of high flexibility that have been found to bind RNA non-specifically through electrostatic interactions between the positively charged nitrogen groups and the negatively charged backbone (Stage *et al.*,

1995; Zapp *et al.,* 1996; 1997; Tor *et al.,* 1998;). Another explanation suggests that aminoglycosides compete with Mg^{2+} ions for functionally important divalent metal ion binding sites in catalytic RNAs (Hoch *et al.,* 1993; Earshaw & Gait 1998; Rogers *et al.* 1996; Mikkelsen *et al.* 1999). All three aminoglycosides tested - neomycin, kanamycin, and streptomycin - inhibited pre-mRNA spicing. This observation, taken together with the established mechanisms of aminoglycoside function, suggests that these inhibitors interact directly with RNA to block splicing. Whether, however, the hypothesized interaction occurs with the pre-mRNA or with the snRNAs that constitute part of the splicing machinery was not determined.

Oxospiro-derivatives

Of the fourteen oxospiro compounds tested, five inhibited the splicing mechanism. Notably, the five inhibitors share a common core structure containing a spirolactone ring. The non-inhibitory oxospiro compounds are in the open carboxylate form, with the exception of G13, which has a bulky double-ring system on the side that is conserved in the inhibitory molecules. The five inhibitory compounds differ only at the two R groups, which vary considerably in size, from small linear chains to large benzyl derivatives. This strongly suggests that the other, conserved portions of the molecule are responsible for interaction with molecular targets. Given the conservation of chemical structure among these five inhibitors, it is surprising that they have such different effects on spliceosome assembly. Manumycin analogues have been found to inhibit a wide range of enzymes in a wide range of organisms, including farnesyltransferase in plants (Pei *et al.,* 1998) and yeast (IC_{50} 5 – 13 μM), and the human polymorphonuclear elastase, (IC_{50} of

4.0 μM) (Tanaka *et al.*, 1996). These studies suggest that manumycin analogues exert their inhibitory effects by interacting with specific protein enzymes, therefore spliceosomal proteins may be potential targets of the oxospiro-derivatives tested. As there are over 100 proteins associated with the spliceosome, it will be important to identify which one is targeted by these inhibitors.

Cephalosporin: Cefoperazone

The antibiotic cefoperazone is a third generation cephalosporin. It was tested because non-aminoglycosides, the aminocyclitol Cl-tetracycline, and the macrolide erythromycin, were found to inhibit human pre-mRNA splicing *in vitro* (Hertweck *et al.*, 2002). To determine if more classes of antibiotics inhibited splicing *in vitro*, one compound from each of eight different classes of antibiotic was tested. Interestingly, however, the two non-aminoglycosides found to inhibit human pre-mRNA splicing did not inhibit yeast pre-mRNA splicing in contrast to cefoperazone. This could mean that erythromycin and Cl-teteracycline are inhibiting human splicing factors which do not exist in the much simpler yeast system.

Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls by competitively inhibiting the transpeptidases involved, making the particularly effective against gram-negative bacteria (Greenwood & Whitley 2002). The observation that Cefoperazone exerts its inhibitory effects by interacting with specific protein enzymes suggests that spliceosomal proteins may be potential targets.

Kinase Inhibitors: Staurosporine

Several studies have suggested a role for kinases in human splicing (Prasad *et al.*, 1999; Du *et al.*, 2000; Dickinson *et al.*, 2002; Hu *et al.*, 2003). In order to address whether kinases also function in yeast splicing, the effect of kinase inhibitors was tested in the splicing assay. While the cyclin-dependent kinase inhibitor roscovitine had no effect on splicing at any concentrations tested, the broad range protein kinase inhibitor staurosporine was found to inhibit actin pre-mRNA splicing with an IC_{50} of 1.9 mM. Staurosporine has been found to inhibit a variety of kinases including PKA, PKG, MLCK, PKC, CaMK, tyrosine kinases, and phosphorylase kinase. Inhibition is *via* interaction with the ATP binding site and it induces PKC translocation (Sigma-Aldrich product information S3939).

Observation of splicing inhibition by staurosporine provides tantalizing evidence for a possible role for kinases in yeast splicing. Kinases frequently mediate signal transduction and consequently regulation of biochemical processes by environmental signals. Historically, yeast splicing was not thought to be regulated, but recent papers suggest otherwise (Pleiss *et al.*, 2007). Splicing inhibition by staurosporine is therefore consistent with the possibility that regulation of yeast splicing is mediated by protein kinases.

Further support for a role for kinases in yeast splicing was provided by studies done by Parker *et al.* (1997), which found four peptide inhibitors of protein kinases PKA and PKC that were effective inhibitors of both yeast spliceosomal complex C assembly and yeast splicing. The four peptide kinase inhibitors target their kinase with the following affinities: GS peptide Ki = 7.5 μM (Pearson *et al.*, 1985), CBD peptide IC_{50} =

24 μM (Payne *et al.,* 1988), CaMK II inhibitor K_i = 3.5 μM (Smith *et al.,* 1992) and CaMK II substrate K_i = 135 μM (Yamagata *et al.,* 1991). In this case, the IC_{50} measured for staurosporine inhibition of yeast splicing is 1-3 orders of magnitude weaker than that of peptide inhibitors, and 5-7 orders of magnitude weaker than its inhibition of known kinases. This raises questions about whether it is actually inhibiting a kinase. Further investigations are required to determine exactly how staurosporine exerts its inhibitory effects.

Toxicants

Environmental toxicants were also tested to investigate the effects they might have on eukaryotic pre-mRNA splicing. Both PCBs and methyl mercury were tested. None of the PCBs tested, nor methyl mercury, inhibited splicing at higher concentrations than what is thought to enter into mammalian systems from environmental pollutants (Schuur *et al.,* 1998; Castoldi *et al.,* 2001). This suggests that these environmental toxins do not exert their effects via splicing inhibition.

Potential Usefulness

The main focus of this work was to identify small molecule inhibitors of splicing that could be used for further molecular dissection of the splicing mechanism. For example, in humans the recent demonstration of splicing inhibition by spliceostatin A allowed the authors to block the splicing reaction at a specific step and to demonstrate the presence of the SF3b complex at that step (Kaida *et al.*, 2007). The work presented in this study, demonstrates for the first time that oxospiro compounds derived from the manumycin

family can act as effective splicing inhibitors. As this is a novel class of splicing inhibitor, it is reasonable to hope that the oxospiro compounds will trap spliceosomes at a previously uncharacterized step of assembly. Ultrastructural studies, particularly crystallography and electron microscopy, stand to benefit greatly from effective inhibitors of spliceosome assembly, as sample heterogeneity is probably the greatest current challenge to those techniques. The ability to trap specific complexes efficiently would therefore lead to major advances in determining the structure of the spliceosome. Beyond their potential utility as probes of splicing mechanism, it is hoped that further refinements of these inhibitors will lead to useful therapeutic agents.

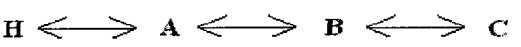
2.3.2 A Framework for Understanding Complex Accumulation in Spliceosome Assembly

Active spliceosome complex formation can be observed by native gels in three distinct stages: A (early), B (middle) and C (late). Each complex corresponds to different snRNPs joining or leaving the pre-mRNA. In this study, the results showed that splicing was inhibited prior to the first transesterification step by all ten inhibitors, however each small molecule resulted in blocking or stalling of specific spliceosomal complexes (A, B and/or C) (Figure 10 and Table 4).

It might be expected, *a priori*, that inhibition of a particular step in splicing would lead to accumulation of the complex immediately preceding that step. This is not what is generally observed, however, as many of the inhibitors tested here led to accumulation of two or more complexes.

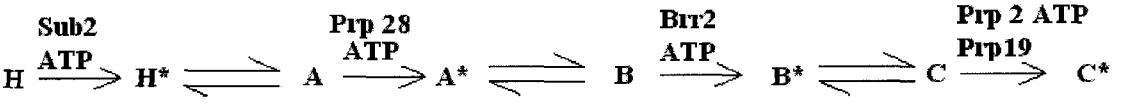
In this section I examine two models to explain this unanticipated result.

One model is a simple system of linked equilibria, in which the block of one step results in accumulation of the previous steps,



This is a thermodynamic model, in which the relative free energies of each complex along the pathway determines the extent to which each complex accumulates. In this model, the ratio of two complexes, say H to A, should not change with the blocked step. In other words, whether the block occurs between A and B or between B and C should not alter the ratio of H to A, if they are in equilibrium. The observation of very different ratios of H to B and B to C ratios with cefoperazone and G5 argue against this model.

The second model is a system of binding equilibria separated by commitment steps, in which the block could be either in the binding step or in the commitment step (asterisk) for a particular complex. Many researchers have proposed that the commitment steps correspond to ATP hydrolysis carried out by associated ATPase proteins (Tazi *et al.*, 2005; Silverman *et al.*, 2003; Brow, 2002). Some of the candidate ATPases and the steps at which they are proposed to function are listed in Table 4.



In this model, inhibition of a binding step would result in accumulation of committed complex immediately preceding it (e.g. if binding of triple-snRNP is blocked, the A* complex would accumulate). Conversely, inhibition of a commitment step would result in accumulation of both species in the preceding equilibrium, in a ratio determined by

their relative energies. This model therefore predicts that complex accumulation is dictated in part by energetics and in part by kinetics, as well as by the specific inhibitor target.

Table 1: Possible DExD/H box (ATPase) protein targets of splicing inhibitors

Small molecule	**Accumulation of spliceosomal complex**	**ATP- dependent helicase DExD/H box proteins**	**The function of ATPases prior the splicing mechansim**
Staurosporine, Strepomycin, Neomycin, G12	H	Sub 2	required by the U2 snRNP for addition to the branchpoint site and formation of the branchpoint-dependent commitment complex (Rutz and Seraphin, 1999)
Kanamycin	A, H	Prp 28	required for the activation of the triple snRNP U4/U6•U5 (Stevens *et al.*, 2002)
G6, G11, G14	B, A, H	Brr2	required for the unwinding of the U4/U6 duplex (Lauber *et al.*, 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996; Kim and Rossi, 1999; Stevens *et al.*, 2002)
Cefoperazone, G5	C, B (small amounts of H)	Prp 2	required after binding of U2 to the pre-mRNA and prior to formation of the functional spliceosome (Roy *et al.*, 1995)
		NTC (Prp19) (not a DExD/H)	associated with the spliceosome after binding of U2 to the pre-mRNA and prior to formation of the functional spliceosome (Tarn *et al.*, 1993)

ATPases may provide a system of regulated control for spliceosome assembly on to the pre-mRNA. For example, the Brr2 ATPase is proposed to facilitate the transition between complex B and complex C, and as a result, provides an opportunity for rejection of substrates that do not efficiently proceed to complex C. This modulation of transition to the next commitment step, with its opportunity for discard, is likely repeated at multiple points in both assembly and post-catalytic phases (Konarska & Query, 2005).

All 5 of the inhibitory oxospiro-derivatives contain the same core structure, yet they all block at different spliceosomal assembly steps. It could be that each oxospiro

derivative actually inhibits a specific ATPase, since all the ATPases are also very similar. So each oxospiro inhibitor may be specific for a particular ATPase.

A final possibility is that all of the inhibitors change the kinetics of assembly without creating a thermodynamic block. In this case, the effectiveness of the inhibitor should correlate with the step at which assembly is blocked, that is that strong inhibitors would block early, leading to accumulation of H complex, whereas weaker inhibitors would allow more assembly and therefore apparent accumulation of later complexes. This is consistent with the observation that the strongest inhibitor, neomycin, causes accumulation of complex H, whereas the weakest, cefoperazone, results in accumulation of C complex. The range of IC_{50} values is too small, however, to allow a rigorous test of the correlation. If inhibition is simply due to an overall slowing of the reaction, it should be possible to detect formation of mature product by allowing the reactions to proceed longer.

Chapter 3

General Discussion

3.1 Future Work

In order to gain insight into how these ten small molecules exert their inhibitory effects and what their targets may be, the techniques of crosslinking and biotinylation could be utilized. In addition, since the ten inhibitory small molecules provide a new means for stalling and accumulating spliceosomal complexes, they could further be isolated for biochemical and structural studies using the techniques of fractionation and affinity purification. These avenues of investigation, for finding the targets of the small molecules, as well as purifying the spliceosomal complexes, are discussed in this section.

3.1.1 Determination of Inhibitory Small Molecule Targets Through Crosslinking and Biotinylation

Future studies aimed at identifying the targets of these ten small molecules, whether they are pre-mRNA or spliceosomal components, should be a priority. Such investigations could be done through photo-crosslinking studies in which the inhibitory small molecule is covalently attached to a photoreactive group like nitroguaiacol. Photoactivation of the nitroguaiacol leads to covalent bond formation with the inhibitor's binding partner, allowing purification and identification of the partner. This would aid in determining if the pre-mRNA is the target. Abad & Amils (1990) synthesized such photoreactive derivatives of streptomycin while maintaining its mode of action in the bacterial ribosome. This method also requires some means of labeling the inhibitor, for

example through incorporation of radioisotopes, so that the crosslinked target can be detected on protein or RNA gels.

An alternative is biotinylation of the inhibitor, which involves covalently attaching a biotin tag to the small molecule without affecting its function, and using the biotin tag for pulling out the complex (pre-mRNA or spliceosomal proteins) it may be interacting with. Kaida *et al.* (2007) performed this experiment by biotinylating spliceostatin A and pulling out a sub-complex of the U2 snRNP called SF3b. Purification is achieved through affinity chromatography with a column that has avidin (a natural binding partner for biotin) bound to it, and detection is possible through avidin-tagged detectors like the fluorescent dye HABA (2-(4-hydroxyazobenzene), in which HABA dye is bound to avidin and yields a characteristic absorbance. When a biotinylated inhibitory small molecule is introduced it would displace the dye, resulting in a change in absorbance at 500 nm. The absorbance change is directly proportional to the level of biotin in the sample. Biotinylation has mostly been reported with antibodies, nucleic acids, or proteins, however if a suitable biotin derivative can be made for each of the small molecules here the method would be quite useful in a direct pull-down of the target compound (Kotake *et al.,* 2007).

3.1.2 Spliceosomal Complex Isolation and Purification

In order to study the biochemical and structural aspects of the spliceosome, another priority would be to purify each spliceosomal sub-complex and the components involved in each assembly step. Other strategies have been devised for accumulating specific splicing complexes (Silvia *et al.,* 1990; Jurica & Moore, 2002), however, the

conditions are different and in most cases are not as easy and simple as addition of a readily available small molecule. The most common methods employed to date for isolating spliceosomes combine size fractionation with affinity purification (Konarska & Sharp 1986; Jurica *et al.,* 2002). Most common is glycerol gradient fractionation and treatment with heparin (a polyanion that disrupts nonspecific or loose protein: nucleic acid interactions). Heparin shifts the pre-mRNA peaks from 40S to 15S, 25S, and 35S, which likely corresponds to E/H, A, and B/C complexes, respectively (Grabowski & Sharp 1986). These much smaller fractions could be isolated from the gradients for further biochemical analysis or purification to obtain a more homogeneous sample (Lindsey & Garcia-Blanco, 1999). Gel filtration is another size fractionation method for purifying large amounts of spliceosomes (Garcia-Blanco *et al.,* 1989). On a Sephacryl S-500 column, label originating from pre-mRNA in splicing reactions elutes in three main peaks. The earliest corresponds to a mixture of A, B, and C complexes, the second to E/H complex, and the third to substrate degraded by the many RNases present in nuclear extract.

Affinity purification of splicing complexes is most often mediated by modifying the pre-mRNA substrate. One method is to randomly incorporate biotinylated nucleotides during *in vitro* transcription. Spliceosomes assembled on such substrates will bind tightly to streptavidin resin under native conditions (Grabowski & Sharp, 1986; Gozani *et al.,* 1994; Neubauer *et al.,* 1998). Alternatively, spliceosomes assembled on unmodified pre-mRNAs can be captured on streptavidin resin with biotinylated antisense oligonucleotides (Ryder *et al.,* 1991). However, elution of biotin/streptavidin conjugates

requires denaturation, limiting the applicability in cases where the goal is to analyze the function of the purified complexes or 3D structure determination.

Isolation of splicing complexes for subsequent functional or structural studies requires a means for elution under native conditions. Theoretically, RNA aptamers selected to bind a stationary ligand would be ideal for this purpose, and many such aptamers have been described (Wang & Rando, 1995; Bachler *et al.,* 1999; Patel & Suri, 2000; Berens *et. at.*, 2001; Srisawat & Engelke, 2001). For instance, the Luhrmann lab purified splicing complexes assembled on pre-mRNAs containing the tobramycin aptamer (Luhrmann *et al.*, 2004). Reed *et al.,* (2000) describe an affinity purification system for purifying mammalian splicing complexes. This method consists of incorporating binding sites for the MS2 coat protein into the substrate pre-mRNA and using an MS2 coat protein:maltose binding protein (MS2:MBP) fusion as an affinity tag. The fusion protein can be eluted from the amylose resin under native conditions with free maltose. In yeast, the TAP tag is a similar equivalent to the MS2:MBP tag developed by Seraphin and co-workers (Puig *et al.,* 2001) and has been used to purify low-abundance, endogenous complexes containing splicing factors. Subsequent studies have employed mass spectroscopy to identify a plethora of associated proteins (Jurica & Moore, 2002; Ohi *et al.,* 2002; Stevens *et al.*, 2002; Gavin *et al.,* 2006).

3.2 Concluding Remarks

The search for small molecules as potent and selective RNA binders comes from the desire to control cell function at the RNA level, which depends on how strongly the inhibitors interact with their targets. In order to investigate which functional groups on

the small molecules are responsible for the inhibitory response, different functional group substitutions can be made. Ultimately, functional group substitutions may lead to lower IC_{50} values. Lower IC_{50} values represent a more potent interaction with the target, allowing easier isolation of the small molecule – RNA or protein complexes for purification assays. The following section discusses where such functional groups substitutions on the small molecules could be possible.

All five oxospiro-derivative inhibitors of yeast pre-mRNA splicing contained the same core structure (Figure 6A) except with different R1 and R2 groups. This means that it may be possible to replace these R groups by other substituents without destroying the inhibitory mode of action. A family of compounds of this sort could be generated using combinatorial chemistry techniques. Combinatorial chemistry is one of the important new methodologies developed by researchers in the pharmaceutical industry to reduce the time and costs associated with producing effective and competitive new drugs (Newman 2007). Synthesis of molecules in a combinatorial fashion can quickly lead to large numbers of molecules. For example, a molecule with two points of diversity (R1 and R2) can generate $N_{R1} \times N_{R2}$ possible structures, where N_{R1} and N_{R2} are the number of different substituents utilized. For these reasons oxospiro-compounds with the common core would be ideal for synthesizing a family of small molecules where the R1 and R2 groups are varied.

Specific R1 and R2 groups discriminate between the splicing factors they target. For instance, of the five oxospiro-compounds: one showed a complete spliceosomal assembly block, three showed a block of complex B, and one showed a block of complex C, in which two compounds which have the same R1 groups but different R2 group also

showed different effects e.g. G5 and G11. Since all three steps involve their own set of various splicing factors, each compound must be targeting a specific splicing factor in order to exert its inhibitory effects. This makes them ideal tools for further studying the biochemical and structural aspects of each step during splicing.

G5 is of particular interest since it was the only oxospiro-compound that blocked spliceosomal complex C assembly in native systems. In most cases it has been implied that once the assembly of the spliceosome has reached this point it should be activated to begin the splicing mechanism. However, since no mature product was observed, in contrast to the (-)-inhibitor control, and splicing was completely inhibited, the C complex is indeed being stalled for 2 – 25 minutes. This means the G5-complex C interaction is quite stable and could potentially be purified for further studies.

The strongest inhibitor of the five oxospiro-derivatives was G12 and it was also the only oxospiro-compound present in its enantiomerically pure from. Having enantiomeric purity could thus be very important in discovering a more potent inhibitor with a lower IC_{50} value. The lower IC_{50} values may result because only one enantiomer (of the mixture of enantiomers) is biologically active; therefore less of the active enantiomer is present in comparison to the- only- enantiomerically pure form.

For a very long time now the mode of inhibition of various RNA reactions by aminoglycosides have been under investigation where one of the widely applied binding models is said to be due to 'surface electrostatic complementarity' (Tor 2003). Surface electrostatic complementarity arises when the three-dimensional projection of positively charged ammonium groups toward the negatively charged RNA surface is employed. The high charge density of the aminoglycosides, together with their unique

structural features (namely, conformationally fixed six-membered rings that can rotate around flexible glycosidic bonds) and the geometrical degeneracy of ammonium groups, allow these compounds to favorably model themselves to match the electrostatic requirements of the RNA surface (Wang & Tor 1998). So the number and position of the ammonium and hydroxyl groups control the effectiveness of the aminoglycosides as potent inhibitors for their targets. In this case the IC_{50} values of the three aminoglycosides did increase according to the order of relative strength of inhibitors with the most amino groups, neomycin (the strongest inhibitor of yeast pre-mRNA splicing) to kanamycin and then streptomycin.

Cefoperazone, a cephalosporin, disrupts the synthesis of the peptidoglycan layer of bacterial cell walls by competitively inhibiting the transpeptidases involved (Greenwood & Whitley 2002). Cefoperazone contains a β-lactam nucleus in its molecular structure (Figure 12).Other known small molecules with this same core structure should also be tested to see if they exhibit the same inhibitory effects on pre-mRNA splicing. If positive inhibitor results are found then the two R1 and R2 groups on the β-lactam nucleus can be modified to obtain different properties and make various therapeutic analogs of cefoperazone and develop even more potent inhibitors.

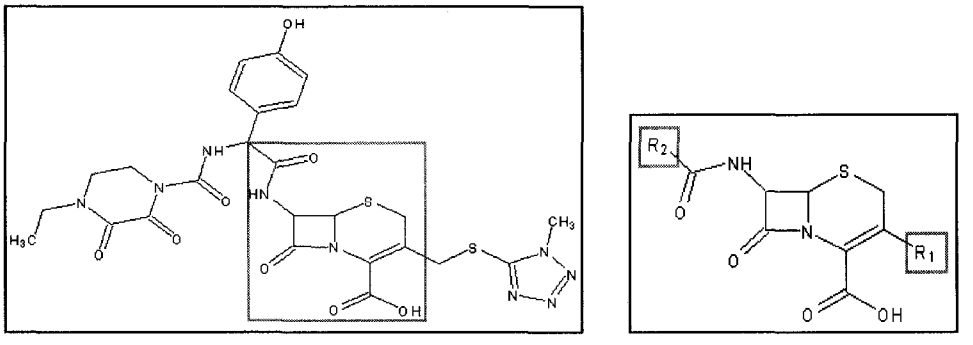


Figure 12: Cefoperazone (left) and β-lactam core structure (right).

Staurosporine is a natural product originally isolated in 1977 from bacterium *Streptomyces staurosporeus* and was the first of over 50 alkaloids to be isolated with this type of bis-indole chemical structure (Figure 13). Staurosporine was discovered to have biological activities ranging from anti-fungal to anti-hypertensive and led to investigation for potential in anti-cancer activity. The ability of staurosporine to stall the first spliceosomal complex assembly step may be due to the stronger affinity of staurosporine to the ATP-binding site on a particular protein kinase. More specific protein kinase inhibitors would have to be tested to narrow down the targets.

In order to determine if the structure of staurosporine is the cause of inhibition then other small molecules with the same bis-indole core structure should also be tested. The antibiotic rebeccamycin (Figure 14) has a similar bis-indole core but does not inhibit protein kinases, and would be an excellent candidate (Shinoda *et al.*, 2007). Therefore, if rebeccamycin also inhibits yeast pre-mRNA splicing it can be concluded that inhibition by staurosporine is not due to inhibition of protein kinases.

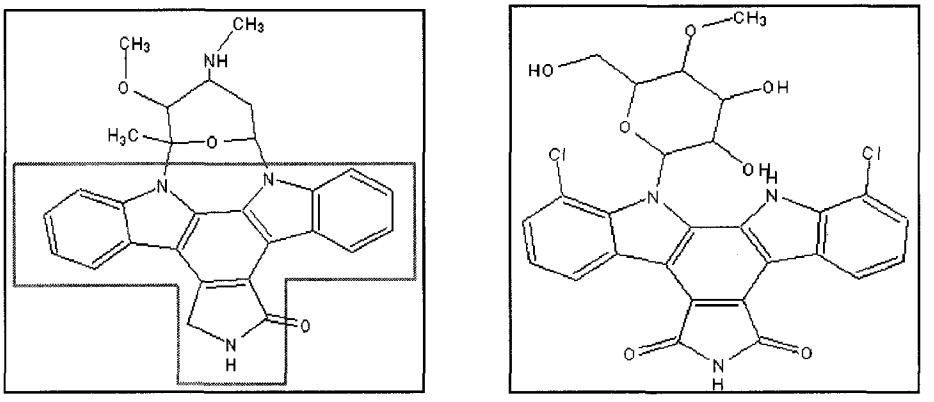


Figure 13: Staurosporine (left) with bis-indole core structure (box) and derivative rebeccamycin (right).

This study provides the first demonstration that five oxospiro-derivatives , in addition to five non-oxospiro compounds, are inhibitors of yeast pre-mRNA splicing and result in stalling of the spliceosomal sub-complexes H, A, B, and C. To define the molecular and structural entities that mediate small molecule-RNA recognition will facilitate the future design of small molecule therapeutics, especially in the case of the five oxospiro-compounds in which its different R groups could be replaced by other substituents without destroying its inhibitory mode of action. The structure-activity profile derived from the initial studies of oxospiro-derivatives and the data found here will be useful for the future design of more potent oxospiro-derivative inhibitors.

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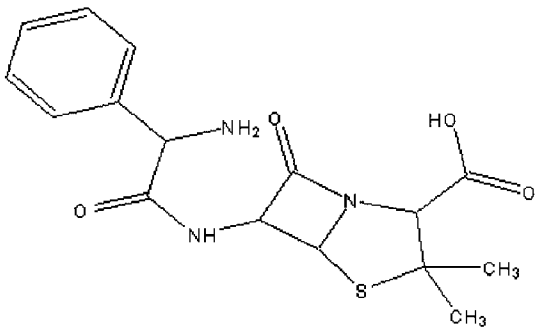
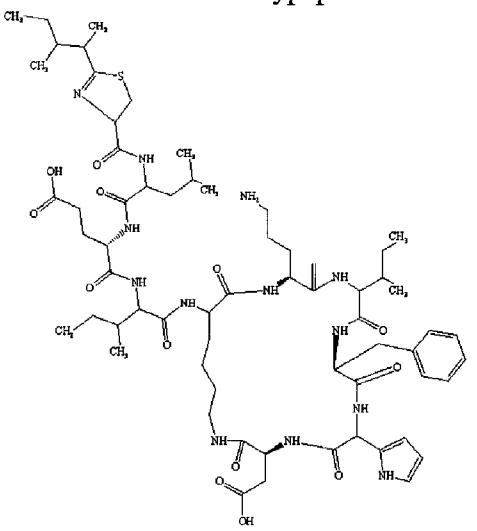
Appendix – Chemical Structures

Structures of non-inhibitory small molecules of nuclear yeast pre-mRNA splicing

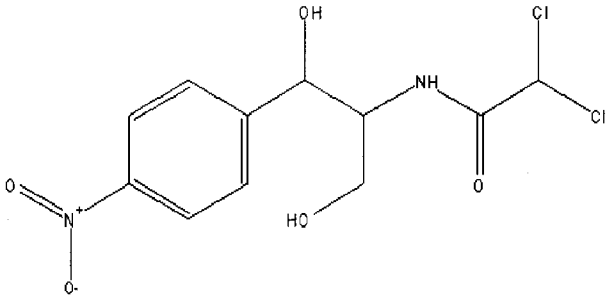
Ampicillin-Penicillin



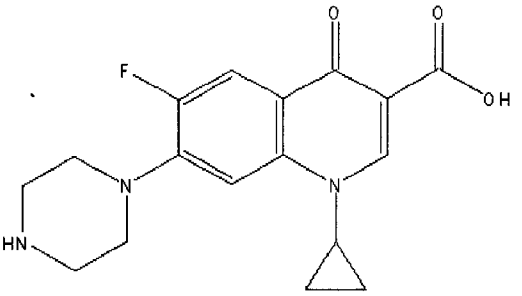
Bacitracin-Polypeptide

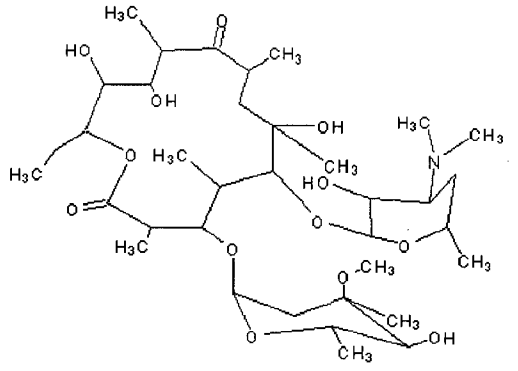
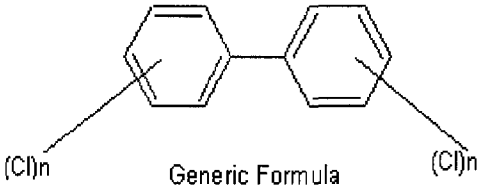
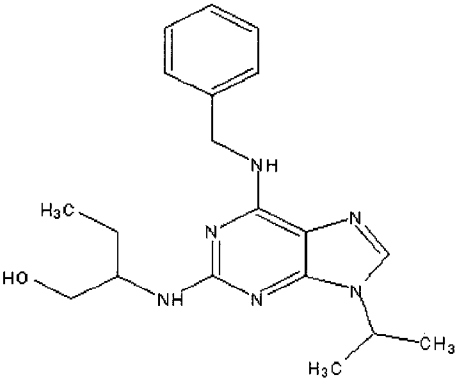
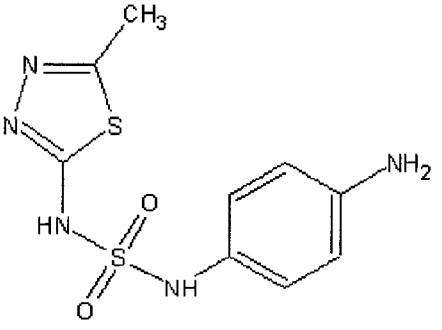


Chloramphenicol-Phenicol



Ciprofloxacin-Quinolone



Erythromycin-Macrolide 	Methyl Mercury- Environmental Toxicant 
PCBs-Environmental Toxicant  Generic Formula n= 126, n= 99, n= 77, A1254= mixture of all 3	Roscovitine Kinase Inhibitor-Ckds 
Sulfamethizole-Sulfonamide 	Tetracycline-Aminocyclitol