

## Current status of spinal muscular atrophy research

### Introduction

Over the next century, SMA diagnosis continued to be made based principally on clinical examination and there was little hope for disease-modifying treatment. In 1995, the discovery of the disease-causing gene provided the ability to diagnose SMA with a simple molecular blood test. This has enhanced early diagnosis and resulted in established standards of care for patients. In addition, this discovery triggered a period of rapid advances in understanding the molecular and cellular basis of the disease. This has resulted in the identification of therapeutic targets and the development of SMA animal models, which can be used for preclinical therapeutics investigations. Consequently, therapeutics development in SMA now has strong academic, government, and industry involvement with companies such as Trophos, Repligen Corporation, CA Stem Cell, Inc., ISIS Pharmaceuticals, Inc., Genzyme Corporation, Paratek Pharmaceuticals, Inc., PTC Therapeutics, Novartis Pharmaceuticals, Inc., and Merck & Co., Inc. investing time and money in basic and clinical research. While clinical trials with repurposed drugs have been recently completed in SMA patients, Repligen's recent phase 1 clinical trial approval from the FDA marks the first time a drug developed solely for the treatment of SMA has entered clinical trials, with more likely to come in the next few years. The promise of a treatment for this devastating neuromuscular disease has never been greater.

### Genetics of SMA

The SMA disease-causing gene was mapped in 1990 to a complicated region of chromosome 5q that contains an inverted duplication. Mutations in one of the genes in this area, survival of motor neuron (*SMN1*), cause SMA. These mutations are usually homozygous deletions of exons 6-8; however, frameshift, missense, and nonsense *SMN1* mutations have also been shown to cause SMA. A duplicated and inverted centromeric copy of this gene, *SMN2*, exists in 90-95% of the normal population and in all patients with SMA. The only functional difference between the genes appears to be a C-T transition at position 6 in exon 7 in *SMN2*, which lies within an exonic enhancer sequence and leads to frequent exon 7 skipping during transcription of *SMN2*-derived pre-RNAs. The resulting mRNA transcripts lacking exon 7 (*SMN $\Delta$ 7*) encode a truncated protein product that is rapidly degraded, but a small amount of transcripts arising from the *SMN2* gene are full-length and encode for the full length SMN protein. SMA is thus caused by reduced expression levels of SMN protein. The ability of the *SMN2* gene to correctly encode for functional SMN protein in the absence of *SMN1* gene products plays a key role in modifying phenotypic outcome of SMA patients. There is an inverse relationship between *SMN2* copy number and disease severity with most SMA type I patients having one or two *SMN2* copies, most SMA type II patients having three *SMN2* copies, and most SMA type III patients having 3 or 4 *SMN2* copies. Some individuals who lack *SMN1* have been identified to retain 5 copies of the *SMN2* gene, and do not display SMA symptoms, indicating that adequate *SMN2* activity alone can prevent disease. This observation along with similar observations in mice has focused the majority of effort in SMA therapeutics development on identifying strategies to increase SMN levels.

### Therapeutic Strategies

Because SMN expression levels correlate with disease severity in humans and in mice and postnatal induction of SMN expression in mice is sufficient to prevent disease, a principal focus of therapeutics development in SMA has been to identify strategies to increase SMN protein levels either by activating *SMN2* gene expression, increasing inclusion of exon 7 in *SMN2*-derived transcripts, stabilizing SMN protein, or by replacing the *SMN1* gene. Other efforts have centered on neuroprotection and cell replacement.

### *SMN2* Gene Expression Activation

*Repurposed drugs: histone deacetylase inhibitors (HDACis), hydroxyurea, and prolactin*

HDACis activate gene expression by inhibiting histone deacetylases (HDACs), which deacetylate chromatin histones thereby promoting a tightly coiled, transcriptionally repressed region of chromatin. *SMN2* gene could be activated by a small molecule, the HDACi sodium butyrate. Several HDACis

including valproic acid (VPA), phenylbutyrate, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) are shown to increase *SMN2* expression in patient-derived cell lines and in animal models. This class of drug was also the first to show the ability to substantially improve the phenotype of SMA mice with TSA and SAHA increasing median survival by 40% and 30%, respectively, in different SMA mouse models. As VPA and phenylbutyrate are in clinical use for other indications, they were both taken to clinical trials in SMA patients quickly despite their low potency as HDACis. Recent results from these studies in SMA type II and III patients have show little or no effect. Studies with these compounds are ongoing in SMA type I infants as efficacy may be improved by early delivery. Efforts are also ongoing to identify other more potent, CNS penetrant, and perhaps HDAC enzyme-specific HDACis that may have efficacy in SMA. Identifying such drugs that do not have prohibitive toxicity for chronic use is a challenge for this class of compounds.

Hydroxyurea was shown to increase the ratio of full-length to truncated SMN mRNA in SMA patient-derived cell lines perhaps via activation of nitric oxide. Hydroxyurea was also taken to clinical trials in SMA patients as it is FDA-approved for the treatment of sickle cell disease. Despite earlier work documenting modest improvements in manual muscle testing (MMT) scores in Taiwan, a placebo-controlled double-blind study was recently published that contradicted this finding, showing no improvements in the motor function or full-length SMN transcript levels of SMA type II and type III patients after hydroxyurea treatment.

Two groups have recently implicated Stat5 as a regulator of SMN expression. Stat5 was first identified as a downstream target of three compounds able to promote *SMN2* activity, TSA, aclarubicin, and sodium vanadate, and Stat5 knockdown results in decreased SMN expression. More recently, prolactin, a known Stat5 activator, was shown to increase the median life span of severe SMA mice from 14 to 21 days. Recombinant prolactin is FDA-approved for the treatment of prolactin deficiency in women and would be available for investigation in SMA clinical trials. It is unclear, however, whether the doses of recombinant prolactin typically used in clinical practice will be adequate to activate *SMN2* in SMA patients.

#### *Quinazoline derivatives*

Quinazoline derivatives were originally found to increase *SMN2* promoter activity in a screen of over 500,000 compounds in an NSC-34 cell-based screening assay. In a secondary screen, they increased SMN protein levels and gem number in SMA patient-derived fibroblasts. After structure activity relationship studies and lead optimization, a novel 2,4-diaminoquinazoline derivative was identified that showed good CNS penetration and a long half-life following oral dosing in mice. These compounds have been described to be potent inhibitors of DcpS, a scavenger enzyme. This enzyme has nuclear-cytoplasmic shuttling responsibilities and plays a role in first intron pre-mRNA splicing. A lead quinazoline compound was tested *in vivo*, and was shown to have modest behavioral and survival benefits in SMA mice. Phase I clinical trials with this compound have been initiated by the Repligen Corporation. The detailed mechanisms by which DcpS inhibition leads to *SMN2* promoter activation or provides survival and behavioral benefits in SMA mice remains under investigation.

### **Splicing Modulation**

#### *Repurposed drugs: salbutamol*

Salbutamol (albuterol) has been shown to increase full-length SMN transcript levels in SMA patient-derived cell lines. Two pilot clinical studies in SMA type II and III patients suggested modest improvement of motor function over 6-12 month periods. The drug was also well tolerated. Larger randomized placebo-controlled trials are needed to further evaluate the efficacy of this drug in SMA.

#### *Small molecules*

Aclarubicin increases SMN protein levels and gem numbers in SMA patient-derived fibroblasts by increasing exon 7 inclusion. Unfortunately, this compound has prohibitive toxicities for long-term use, as would likely be required for SMA patients. Based on their structural similarity to aclarubicin, tetracycline derivatives from Paratek Pharmaceutical's chemical library were recently screened in a cell-free splicing assay and one derivative, PTK-SMA1, was found to increase the amount of exon 7 inclusion. PTK-SMA1 did not change the splicing patterns of other tested genes, suggesting a specific effect on SMN exon 7 splicing rather than a global effect. PTK-SMA1 also increased exon 7 inclusion when delivered to a mild mouse model of SMA validating target engagement *in vivo*.

PTC Therapeutics, a company focused on developing drugs that target post-transcriptional control of gene expression, has also recently identified compounds that increase exon 7 inclusion thereby increasing SMN protein expression. They have identified three different structural scaffolds that each increases SMN protein levels and extends median survival of severe SMA mice, in one case from 14 to 132 days. These compounds are orally bioavailable and can penetrate the blood-brain barrier, but the mechanism by which they alter SMN splicing has not yet been described.

#### *Antisense oligonucleotides*

Another strategy that has been pursued to increase exon 7 inclusion is the use of antisense oligonucleotides (ASOs), modified nucleotides that bind specific mRNA sequences. This binding can mark a specific mRNA for degradation or in the case of SMA therapeutics, binding to specific cis-acting splicing regulatory motifs can promote exon 7 inclusion. Different ASOs utilizing various chemistries as well as a transsplicing molecule have been studied in cell and animal models. ISIS Pharmaceuticals has developed a 2'-O-2-methoxyethyl-modified ASO (ASO-10-27 or ISIS SMNRx), which they have shown in collaboration with Adrian Krainer's laboratory and Genzyme is able to correct ear and tail necrosis in a mild mouse model of SMA and extend median survival in severe SMA mice from 16 to 26 days after ICV injection. There is also proof-of-principle evidence for the clinical relevance of this drug documenting putatively therapeutic ASO levels in the spinal cords of cynomolgus monkeys after intrathecal delivery. Furthermore, recent data show an even more striking benefit when ASO 10-27 is given systemically in severe SMA mice, with some mice surviving for more than 1 year. The enhanced benefit with systemic delivery suggests the requirement to restore SMN in other tissues aside from the CNS. Whether this is relevant to the human disease has yet to be determined. ISIS Pharmaceuticals plans to submit an IND (investigational new drug) in the coming months, with the goal of starting a phase 1 trial in SMA patients this winter using a single intrathecal ISIS SMNRx injection as a starting dose

### **Protein Modulation**

#### *Repurposed drugs: aminoglycosides and proteasome inhibition*

Aminoglycosides are known for their ability to induce translational readthrough of stop codons. In the case of SMN2 transcripts, it was postulated that readthrough of the initial stop codon would extend the length of the C-terminal of the truncated SMN protein thereby improving its stability. Aminoglycosides increased gem number and SMN protein levels in SMA patient-derived fibroblasts and geneticin (an aminoglycoside) improved motor behavior, but not survival in SMA mice with some evidence of toxicity. In contrast, a novel aminoglycoside, TC007, provided a ~30% increase in median survival when given to severe SMA mice by ICV injection.

Recently has showed that the FDA-approved ubiquitin proteasome inhibitor, Bortezomib, increased SMN protein levels in muscle and other peripheral tissues, but not in the CNS when delivered to severe SMA mice by intraperitoneal injection. When combined with TSA, which is a CNS penetrant drug, there was an improvement in survival of SMA mice that was better than that seen with TSA alone. These data provide proof of principle that inhibition of SMN protein degradation can reduce SMA disease severity. Nonetheless, both aminoglycosides and proteasome inhibitors will have to overcome challenges of drug toxicity before they can be legitimate treatments for SMA patients.

#### *SMA Project*

In 2003, the National Institute of Neurological Disorders and Strokes (NINDS) founded the SMA Project, a research initiative based at and directed by the NIH. This marked the first time that the NIH embarked on a mission to develop therapeutics in-house for a particular disease, with a goal of filing an IND in 5 years. SMA Project currently has two sets of compound series in development. The first, indoprofen analogs, are based on the observation that indoprofen increased SMN levels and nuclear gem counts in SMA patient-derived fibroblasts and provided a modest survival benefit to SMA mouse embryos. The second set of compounds, approximately 200 benzimidazoles, was independently generated from a high throughput screen and lead candidates are being generated from these hits.

### **Gene Therapy**

Gene therapy provides the opportunity to restore a normal form of the *SMN1* gene to SMA patients; however, effective delivery to a difficult-to-access cell such as a motor neuron has been considered an

almost impossible challenge until very recently. Several groups have accomplished this using self-complementary adeno-associated virus vectors. scAAV9 in particular was shown to have tropism for MNs in neonatal animals when injected intravascularly. Both *SMN1* scAAV9 and scAAV8 were demonstrated to remarkably improve the SMA phenotype after early postnatal delivery in severe SMA mice. The first two papers outlining this work were published weeks apart and employed two different methods. Both studies resulted in increased median survival of severe SMA pups compared to vehicle-treated counterparts, but interestingly delivery of the gene intravenously provided a greater survival benefit when compared to ICV injections, with 6 of 7 severe SMA mice living past 250 days compared to a median survival of 157 days in the ICV-injected mice. Other studies have also shown dramatic extensions of median lifespan using IV scAAV9 delivery, one study showing an increase from 14 to 69 days and another showing an increase from 27 to 199 days in SMA mice. Recently a cynomolgus macaque was injected with scAAV9-GFP on postnatal day 1 and saw robust green fluorescent protein (GFP) expression in dorsal root ganglia and MNs 25 days later, providing evidence that IV scAAV9 injections are able to efficiently transfect the CNS in a large nonhuman primate. Preclinical studies are ongoing to address the potential challenges of toxicity, delivery, and manufacturing for human clinical trials.

## **Neuroprotection**

### *Repurposed drugs: riluzole and ceftriaxone*

Riluzole is an FDA-approved drug for the treatment of amyotrophic lateral sclerosis (ALS) and there is interest to evaluate it for efficacy in SMA. A phase I clinical trial showed Riluzole to be safe in SMA type I patients and a recent study indicated that this compound has similar pharmacokinetic properties in SMA patients to those seen in ALS patients.

The beta-lactam antibiotic ceftriaxone was demonstrated to increase glutamate reuptake and improve the phenotype of ALS mice. Consequently, a clinical trial of ceftriaxone is ongoing in ALS patients. This drug was evaluated in severe SMA mice and shown to modestly extend survival.

### *Olesoxime*

A novel neuroprotective compound currently being studied for the treatment of neurodegenerative diseases including ALS and SMA is Olesoxime (TRO19622), a cholesterol-like compound being developed by Trophos. This drug was identified in a cell-based screen for compounds that protected rat primary motor neurons from degeneration induced by trophic factor withdrawal. Although the drug mechanisms are not entirely understood, the drug has been shown to bind TSPO and VDAC, proteins located on the outer mitochondrial membrane, suggesting a role of mitochondrial signaling in the mechanism of drug action. Recruitment for an ongoing clinical trial of Olesoxime in SMA patients in Europe was recently completed.

## **Cell Replacement**

Another treatment strategy that is being actively investigated in SMA is cell replacement. Embryonic stem (ES) cells can be differentiated into neural stem cells and then functional MNs under stringent growth and differentiation conditions usually involving retinoic acid, sonic hedgehog, and neurotrophic factors. One group reported the *in vivo* benefit of neural stem cell injections in severe SMA mice using intrathecal injections after differentiating neural stem cells from mouse spinal cord neurospheres. SMA mice treated with these neural stem cells displayed increased survival and motor behavioral benefits, as well as increased MN number and size in the spinal cord when compared to SMA littermates.

Work by California Stem Cell Inc. has recently resulted in the development of new methods to create high purity human neuronal progenitor cells. The ability to do this on a large scale is instrumental in the development of these cells as potential therapeutics. This group has also demonstrated phenotypic benefits in animal models of ALS, SMA, and spinal cord injury, further validating the potential of this therapeutic.

## **Ongoing Screens**

There are many ongoing efforts to identify other novel drugs for SMA. These include high throughput screening campaigns by Novartis and by Merck in collaboration with Gideon Dreyfuss. In the latter effort, native SMN protein-inducing compounds have been identified during a high throughput screen of

over one million small molecules in SMA patient-derived cells. Hits are currently being optimized and their mechanisms of action are being characterized. Two additional recent studies highlight the utility of novel screening assays. Lee Rubin's group at the Harvard Stem Cell Institute uses a new image-based method that identifies increases in gem number, gem intensity, and SMN levels independent of cellular location. In addition, by screening compounds at varying concentrations and those with known targets and pathways instead of diverse chemical libraries, the investigators were able to provide mechanistic evidence that implicates GSK-3 kinase as an SMN regulator. Elliot Androphy's group created a new luciferase-based screening assay that allows detection based on increases in *SMN2* promoter activity, increases in exon 7 inclusion, and/or stabilization of the SMN protein. In a primary screening of over 200,000 compounds, over 6,000 compounds were initially identified. After elimination of many compounds postulated to interfere with the assay and those that also nonspecifically increased activity of an *SMN1*-luciferase construct, 21 compounds were tested in a secondary assay. From these compounds, one lead series underwent structure and relationship studies that led to the identification of 2 compounds that showed good oral bioavailability and CNS penetrance in mice. These compounds are aryl piperidines whose mechanism of action has yet to be described.

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