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## Small Molecules in Development for the Treatment of Spinal Muscular Atrophy

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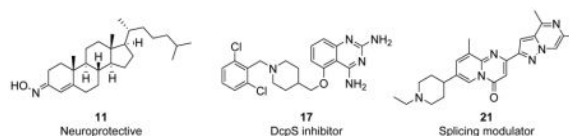
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### Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease resulting from pathologically low levels of survival motor neuron (SMN) protein. The majority of mRNA from the *SMN2* allele undergoes alternative splicing and excludes critical codons, causing an SMN protein deficiency. While there is currently no FDA-approved treatment for SMA, early therapeutic efforts have focused on testing repurposed drugs such as phenylbutyrate (**2**), valproic acid (**3**), riluzole (**6**), hydroxyurea (**7**), and albuterol (**9**), none of which has demonstrated clinical effectiveness. More recently, clinical trials have focused on novel small-molecule compounds identified from high-throughput screening and medicinal chemistry optimization such as olesoxime (**11**), CK-2127107, RG7800, LMI070, and RG3039 (**17**). In this paper, we review both repurposed drugs and small-molecule compounds discovered following medicinal chemistry optimization for the potential treatment of SMA.

### Graphical Abstract



### Keywords

Spinal muscular atrophy; survival motor neuron; small molecule; repurposed drugs; clinical trial

## 1. INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder characterized by progressive muscle wasting and eventual loss of muscle function due to severe motor neuron dysfunction.<sup>1–4</sup> As one of the leading heritable causes of infant mortality worldwide, SMA affects between 1 in 6,000 to 1 in 11,000 live births and has a carrier frequency of approximately 1 in 40 to 50 people.<sup>5–8</sup>

The first cases of SMA were first published in the 1890s by Guido Werdnig and Johan Hoffman, two physicians whose autopsy studies revealed the key pathological feature of the disease: severe loss of  $\alpha$ -motor neurons (MNs) from the anterior horn of the spinal cord.<sup>9</sup> A breakthrough study in 1995 mapped the SMA genes to chromosome 5q13, in which two nearly identical genes encode for the same SMN protein.<sup>10</sup> Initially named telomeric and centromeric SMN, these genes are now more commonly referred to as *SMN1* and *SMN2*, respectively.<sup>11</sup> These two genes are nearly identical and encode the same 294 residue, 38-kDa SMN protein.<sup>10,11</sup> Researchers later determined that SMN is ubiquitously expressed at high levels during embryonic and prenatal development before rapidly decreasing after birth.<sup>12</sup>

*SMN1* and *SMN2* mRNAs are transcribed at similar levels in all cells, but approximately 85% of *SMN2* transcripts result in abnormal SMN protein due to a single nucleotide difference from C to T in the 6<sup>th</sup> nucleotide of exon 7 (encoding residue 840) of the *SMN2* gene.<sup>13,14</sup> The resulting  $\Delta$ 7 *SMN2* mRNA is truncated, lacking the codons for 16 amino acids within exon 7 as well as the translational termination codon.<sup>15,16</sup> The translated  $\Delta$ 7 protein is unstable and quickly degrades in cells.<sup>17–19</sup> The majority of SMA patients lack both copies of the *SMN1* gene altogether, and instead have two or more copies of the *SMN2* gene.<sup>20</sup> In such *SMN1* null patients, *SMN2* expression is the sole source of SMN protein. Accordingly, the *SMN2* gene is unable to fully compensate for the loss of *SMN1* due to its  $\Delta$ 7 mutation and decreased production of normal, full-length SMN protein.<sup>5</sup> About 5% of SMA patients have mutations in the *SMN1* gene that render it dysfunctional.

There are four types of SMA, which are classified by age of onset, age of death, achievements in motor development, and number of *SMN2* copies.<sup>21</sup> Type I (also known as Werdnig-Hoffman disease) patients demonstrate the most severe SMA symptoms and possess only 2 to 3 copies of *SMN2*. Within 6 months of birth, these patients are unable to sit unsupported due to their lack of muscle function.<sup>22,23</sup> Type II SMA (also referred to as Dubowitz syndrome) patients possess 3 to 4 copies of *SMN2* and, notably, are able to sit unsupported.<sup>24</sup> In general, Types III and IV are milder forms of SMA with much later onsets. Type III SMA, referred to as juvenile SMA or Kugelberg-Welander disease, typically manifests after 18 months in patients with 4 copies of *SMN2*. Type IV patients possess 4 to 8 copies of *SMN2* and often demonstrate muscular dystrophy symptoms after adulthood.<sup>21,23,24</sup> Plainly, there is an inverse relationship between the number of *SMN2* copies and the phenotypic severity of this illness.<sup>25,26</sup> A variety of SMA mouse models containing the transgenic human *SMN2* gene were developed to reflect the range of disease severity observed in humans and have been used to evaluate compounds that increase transcription of *SMN2*, the inclusion of exon 7 in the *SMN2* mRNA transcript, and levels of the SMN protein.<sup>27</sup> The  $\Delta$ 7 SMA and the “Taiwanese” or “Li” models are the most commonly used severe mouse models of SMA in preclinical studies.<sup>28</sup> Outcome measures in the severe models include survival (~10–14 days), motor function, neuromuscular junction (NMJ) number and morphology. Less severe mouse models include the 2B<sup>-/-</sup> and *Smn1*<sup>C/C</sup>, which have mutated the single mouse *Smn* allele so that its exon 7 is skipped, as well as the SMN<sup>RT</sup> (SMN read through) and “Burgheron” models with longer survival times (~30–35 days).<sup>29–31</sup>

Currently, there are seven clinical and 11 preclinical agents for SMA treatment, ranging from repurposed drugs, gene therapy, antisense oligonucleotides, and novel small molecules. Gene therapy is an experimental technique that inserts genes into cells via a viral vector. Genes can be delivered systemically by injection of the vectors intravenously or directly into the cerebral spinal fluid (CSF) to infect neurons. AveXis, the first company to test gene therapy in SMA clinical trials, has developed a proprietary intravenous treatment using self-complementary adeno-associated virus (scAAV-9) vectors that carry a codon-optimized *SMN1* sequence and a chimeric intron that reintroduces *SMN1* into MNs resulting in SMN protein overexpression.<sup>32</sup> The effectiveness of the scAAV-9 vector is attributed to its ability to cross the blood-brain barrier (BBB) and to infect neurons by retrograde trafficking from the axon. This modality is in Phase II clinical trials. Another experimental method, antisense oligonucleotide therapy, aims to deliver a synthetic strand of nucleic acids (DNA, RNA, or a chemically modified analogue) that binds a specific regulatory site on pre-mRNA, thereby modifying exon recognition. Ionis (formerly known as Isis) Pharmaceuticals and Biogen have collaborated to develop a chemically modified antisense ribonucleotide, IONIS-SMNRx, that binds to a splice silencer element in intron 7 of *SMN2* and results in its inactivation and the inclusion of exon 7.<sup>33</sup> Although these two treatment options demonstrate the innovative and new strategies being developed over the past few years, from here on, we will focus only on the biology and medicinal chemistry strategies associated with producing small molecule treatments for SMA. We review repurposed drugs as well as five clinical and six preclinical novel small molecules candidate therapies.

## 2. REPURPOSED DRUGS

Finding new uses for existing FDA-approved drugs offers several advantages, including shorter development and approval times, reduced costs, and higher success rates relative to the standard practice of developing new compounds. New drug development can take up to 16 years<sup>34</sup> and incurs significant costs during the drug discovery and preclinical stages.<sup>35</sup> Drug repurposing avoids a number of these expensive processes and takes between three and 12 years for drug approval. Further, the success rates of repurposed drugs are drastically improved; while 10% of new molecular entities from Phase II and 50% from Phase III clinical trials make it to market, the rates for repurposed compounds are 25% and 65%, respectively.<sup>36</sup> With no strong clinical prospects in the SMA drug therapy pipeline, the cost-effective and potentially time-saving testing of repurposed drugs was an attractive source of therapeutic drug discovery.

SMA was granted orphan drug designation by the FDA, which assigns orphan status to drugs and biologics intended for the safe and effective treatment, diagnosis, or prevention of (i) rare diseases and/or disorders affecting fewer than 200,000 people in the U.S. or (ii) diseases and/or disorders affecting more than 200,000 persons, but that are not expected to recover the costs of developing and marketing a treatment drug.<sup>37</sup>

FDA-approved drugs that enhance *SMN2* promoter activity, modulate *SMN2* splicing, and stabilize *SMN2* mRNA or SMN protein, have been explored as potential means of treating SMA (Table 1). Though repurposed drugs examined for the treatment of SMA have resulted

in promising early data, including increased levels of SMN protein, none has successfully made it through Phase III clinical trials.

## 2.1 HISTONE DEACETYLASE INHIBITORS

One group of drugs found to increase *SMN2* promoter activity is histone deacetylase (HDAC) inhibitors. A basic epigenetic mechanism for regulating gene expression is through acetylation and deacetylation of selected lysines within the histones of chromatin. HDAC inhibitors are enzymes that deacetylate chromatin histones and create a tightly coiled, transcriptionally-repressed region of chromatin, and therefore inhibitors of these enzymes activate gene expression. One potential advantage of HDAC inhibitors is the ability to reverse abnormal cell transcription early on, rather than treating downstream translational endophenotypes. It was proposed that HDAC inhibition could induce transcription of *SMN2* and, as a result, could increase the amount of total *SMN2* transcripts, which would lead to more functional SMN protein.<sup>38</sup> To date, several FDA-approved HDAC inhibitors have been investigated for the treatment of SMA. The first HDAC inhibitor to result in increased *SMN2* transcription was sodium butyrate (**1**).<sup>39</sup> Subsequently, phenylbutyrate (**2**), valproic acid (**3**), suberoylanilide hydroxamic acid (**4**), and trichostatin A (**5**) all were shown to increase *SMN2* expression in patient-derived cell lines and animal models.<sup>40–44</sup> Importantly, HDAC inhibition is not specific to *SMN2*, and the expression of multiple genes potentially would be altered, leading to side effects. The results of these approaches are discussed below.

**2.1.1 Sodium Butyrate**—The HDAC inhibitor **1** is a salt of butyric acid with antineoplastic activity.<sup>39</sup> Chang *et al.* were the first to show that the *SMN2* gene could be activated by **1**.<sup>39</sup> To generate lines of immortalized lymphoid cells, the researchers transformed lymphocytes obtained from SMA patients with Epstein-Barr virus. After exposing the lymphocytes to candidate drugs, they performed quantitative reverse transcriptase PCR (qRT-PCR) to determine which drugs resulted in increased production of full-length *SMN2* mRNA. Compound **1** increased both *SMN2* transcription and the inclusion of exon 7 in *SMN2* transcripts, thereby increasing the production of full-length *SMN2* mRNA. A corresponding increase in SMN protein also was seen. Further, there was a 25% extension in the mean survival of Taiwanese SMA mice. The primary disadvantage of **1** is its very short half-life in human serum ( $T_{1/2} = 6$  min), making it unsuitable for therapy.

**2.1.2 Sodium Phenylbutyrate**—In a subsequent study, **2**, an analog of sodium butyrate, was found to increase full-length *SMN2* mRNA and SMN protein in SMA patient-derived fibroblasts.<sup>40</sup> Compound **2** is FDA-approved for urea cycle disorder and has been tested for therapeutic benefit in amyotrophic lateral sclerosis (ALS), cystic fibrosis, and several cancers. Compound **2** is more stable, with a 0.8–1 h half-life in human.<sup>45</sup> There is evidence suggesting that **2** crosses the BBB; **2** was able to lower long-chain fatty acid levels in the brain of mice with x-linked adrenoleukodystrophy<sup>46</sup> and, after IV administration, led to a CSF:plasma ratio of 1:2 in primates.<sup>47</sup>

An uncontrolled, open-label pilot study investigated the use of **2** in Type II SMA patients and resulted in a 2-fold increase in SMN mRNA and improved Hammersmith Motor

Function Scale scores.<sup>48</sup> Two additional phase II/III clinical trials were terminated due to slow enrollment and poor compliance. STOPSMA, an open-label trial for **2**, was conducted to evaluate the compound in pre-symptomatic infants with Types I and II SMA.<sup>49</sup> Although the study was completed in 2013, outcomes have not been reported.

**2.1.3 Valproic Acid**—Compound **3**, an HDAC inhibitor of the aliphatic class, is a carboxylic acid with a pKa of 4.8 that likely is fully ionized at physiological pH.<sup>50</sup> However, **3** can cross the BBB via active transport by medium-chain fatty acid transporters. Compound **3** is used clinically for a number of central nervous system (CNS) diseases, including epilepsy and bipolar disorder, and to prevent migraine headaches.<sup>50</sup>

Compound **3** treatment of SMA patient-derived primary fibroblasts resulted in a 2- to 4-fold increase in SMN protein. This increase coincided with an increase in both total *SMN2* transcripts and full-length exon 7-included *SMN2* transcripts.<sup>41,51</sup> Further analyses revealed increased expression of several splicing factors, each of which had been shown previously to increase *SMN2* exon 7 inclusion. A more recent study testing the efficacy of **3** *in vitro* confirmed an increase in total *SMN2* mRNA, but reported no change in exon 7 inclusion levels.<sup>52</sup> An *in vivo* test of **3** efficacy in homozygous 5058 SMA mice resulted in a 100% increase in median survival, a slight increase in SMN protein in the spinal cord, and improvements in muscle and neuron pathology.<sup>53</sup> However, the authors noted decreases in growth cone size, axon length, motor neuron survival, and increased excitability in axon terminals, which suggested that **3** might be detrimental to motor neuron survival and function.

Five clinical trials using **3** in SMA have been conducted, but none has reported any clinical benefit. A randomized, prospective, placebo-controlled clinical trial designed to test the efficacy and safety of the combined administration of **3** with L-carnitine in ambulatory adults with Type III SMA was performed, but it demonstrated that **3** was not effective in improving strength or function in this population.<sup>54</sup>

**2.1.4 Suberoylanilide Hydroxamic Acid**—Compound **4**, known as Vorinostat, is a potent, hydroxamic acid class HDAC inhibitor that inhibits class I and II HDAC inhibitors at submicromolar concentrations.<sup>42</sup> Compound **4** is FDA-approved for the treatment of cutaneous T-cell lymphoma. *In vitro* experiments showed that **4** increased both SMN protein and mRNA in rat hippocampal neurons, motor neuron cultures, and primary SMA patient fibroblasts.<sup>42</sup> As with other HDAC inhibitors, these increases coincided with an increase in exon 7 inclusion in *SMN2* transcripts. Compound **4** efficacy also was examined in an *in vivo* study using 5024- and 5058-SMA mouse strains.<sup>43</sup> Treatment with **4** increased the lifespan of the SMA mice by 30%, significantly improved motor function abilities, reduced degeneration of motor neurons within the spinal cord, and increased the size of neuromuscular junctions and muscle fibers compared with vehicle-treated SMA mice. SMN RNA and protein levels were elevated significantly in various tissues, including spinal cord and muscle.<sup>43</sup> Compound **4** demonstrated positive *in vivo* effects despite very poor brain access (*e.g.*, brain to plasma ratios between 0.04 and 0.01). Its poor brain penetration likely is due to the fact that **4** is a substrate for the P-glycoprotein (P-gp) and Bcrp1 efflux transporters at the BBB. These transporters are expressed at the blood and spinal cord brain

barriers and restrict a number of drug substances access to the CNS. The current clinical status of **4** is not known by the authors.

**2.1.5 Trichostatin A**—Compound **5** is another HDAC inhibitor of the hydroxamic acid class.<sup>55</sup> Compound **5**, originally developed as an antifungal drug, is a member of a large class of HDAC inhibitors with a broad spectrum of epigenetic activities. A recent study showed that compound **5**-treated SMA 7 mice continued to gain weight, maintained stable motor function, and retained intact neuromuscular junctions long after treatment was discontinued.<sup>55</sup> Compound **5** treatment resulted in a 19% increase in median survival. When additional nutritional supplements were administered, median survival increased by 170%.<sup>55</sup>

A 2014 study by Liu *et al.* found that **5** increased the median lifespan of *SMN2B*<sup>-/-</sup> mice from 20 days to 8 weeks, significantly attenuated weight loss, and improved motor behavior.<sup>56</sup> Interestingly, **5** treatment did not increase the levels of SMN protein in mouse embryonic fibroblasts or myoblasts obtained from *SMN2B*<sup>-/-</sup> mice. In addition, no change in the levels of SMN transcripts or protein in the brain or spinal cord of **5**-treated SMA mice was observed. Furthermore, **5** did not increase SMN protein levels in the hind limb muscle, heart, or liver of *SMN2B*<sup>-/-</sup> mice, suggesting that **5** acts independently of the SMA gene.

**2.1.6 Summary of HDAC Inhibitors**—The hydroxamic acid class of HDAC inhibitors was the first to substantially improve the phenotype of SMA mice, with **5** and **4** increasing median survival by 40% and 30%, respectively.<sup>43,55</sup> However, because **3** and **2** were already in clinical trials, these compounds were studied in SMA patients relatively quickly despite their low potency as HDAC inhibitors. Results from these studies have shown limited but positive effects in open-label studies in SMA Types II and III. The significantly more potent HDAC inhibitors **4** and **5** demonstrate poor HDAC selectivity and are considered to be too toxic for chronic use to potentially treat SMA. The zinc binding hydroxamic acid group is present in all of the HDAC inhibitors advanced into trials for SMA and is generally associated with poor pharmacokinetics and severe toxicity. To fully evaluate the potential of HDAC inhibitors for the treatment of SMA, more potent, HDAC enzyme-specific, non-hydroxamic acid, CNS penetrant compounds are needed.

## 2.2 NEUROPROTECTIVES

**2.2.1 Riluzole**—Riluzole (**6**), a 2-aminobenzothiazole, is FDA-approved for the treatment of ALS.<sup>57</sup> Although **6** was proposed to modulate excitatory neurotransmission mainly through inhibition of glutamate release; the precise neuroprotective mechanisms remain largely speculative. Its pharmacologic properties that may be related to its effect include an inhibitory effect on glutamate release, inactivation of voltage-dependent sodium channels, and the ability to interfere with intracellular events that follow transmitter binding at excitatory amino acid receptors.

Compound **6** exhibits neuroprotective effects in various *in vivo* experimental models of neuronal injury involving excitotoxic mechanisms. In *in vitro* models, **6** protected cultured rat motor neurons from the excitotoxic effects of glutamic acid and prevented the death of cortical neurons induced by anoxia.<sup>57,58</sup> In ALS patients, **6** resulted in increased life

expectancy and reduced rates of neurodegeneration. As a neuroprotective, it did not promote the recovery of lost motor neurons or show an improvement in muscle function. In SMA mouse models, **6** showed stabilization of neuromuscular junctions, but did not prevent the loss of proximal axons.

Compound **6** is able to cross the BBB; however, it is a known substrate for the P-gp efflux transporter. An ALS mouse model showed how the upregulation of P-gp expression correlates with low levels of **6** in the brain. Although it is not known if P-gp is highly expressed in SMA, this concept may explain the inadequate levels of **6** found in *in vivo* studies. The poor pharmacokinetic properties of **6** also may contribute to its poor long-term efficacy.<sup>59,60</sup>

A small Phase I clinical trial enrolling seven compound **6**-treated and three placebo-treated Type I SMA infants showed **6** to be safe. However, no significant differences in survival or motor abilities were observed.<sup>61</sup> Nevertheless, further analysis showed that three patients in the **6** group presented an unusual disease course and were still alive at the age of 30 to 64 months.<sup>62</sup> It is unclear whether **6** is beneficial for SMA patients.

**2.2.2 Hydroxyurea**—Hydroxyurea (**7**), an FDA-approved compound for the treatment of solid tumors and sickle cell anemia, also has been considered for the treatment of SMA.<sup>63</sup> Its ability to increase the expression of fetal hemoglobin suggested it might increase expression of *SMN2*. Compound **7** treatment of SMA-derived lymphocytes stimulated increases in the levels of total and exon 7-included *SMN2* transcripts.<sup>63</sup> A recent study suggested that **7** increases nitric oxide, which, in turn, increases *SMN2* expression.<sup>64</sup> Nevertheless, several clinical trials are underway to evaluate the therapeutic efficacy of **7**. Early uncontrolled trials have shown positive trends, although none was statistically significant. A subsequent controlled trial reported no benefit of **7** in SMA treatment.<sup>65</sup>

Compound **7** has poor brain penetration in rat (*e.g.*, brain to plasma ratio of 0.12–0.25), although it is not considered to be a P-gp substrate.<sup>66</sup> However, it has low lipophilicity (*e.g.*, cLogP = –1.8 and hydrogen-bond donors (HBD) = 4). These physicochemical properties likely combine to limit passive transport across the BBB.

## 2.3 ANTIBIOTICS

**2.3.1 Ceftriaxone**—Despite having physicochemical properties outside of those normally considered necessary for CNS penetration (*e.g.*, MW = 554 amu, PSA = 208 Å<sup>2</sup>, hydrogen-bond acceptors (HBA) = 15), the  $\beta$ -lactam antibiotic ceftriaxone (**8**) crosses the BBB through active transport.<sup>67</sup> Though **8** was demonstrated to increase glutamate reuptake and improved the phenotype of ALS mice,<sup>68</sup> a clinical trial of **8** in ALS was unsuccessful. Compound **8** was evaluated in severe SMA mice and showed a modest extended survival.<sup>69</sup> Treated animals demonstrated a modestly improved neuromuscular phenotype and increased survival. The neuroprotective effect appears to be mediated by increased levels of the glutamate transporter Glt1, the transcription factor Nrf2, and SMN protein. This study provided the first evidence of a potential positive effect of  $\beta$ -lactam antibiotics as a treatment for SMA, but further studies are needed.

**2.3.2 Albuterol**—Another FDA-approved compound repurposed for SMA is albuterol (**9**).<sup>70</sup> Compound **9** is a  $\beta_2$  adrenergic receptor agonist used to relieve the symptoms of asthma and chronic obstructive pulmonary disease and increase skeletal muscle strength.<sup>70</sup> Compound **9** has been shown to increase full-length SMN transcript levels in severe SMA patient-derived cell lines. In SMA-derived fibroblasts, **9** stimulated *SMN2* mRNA and SMN protein production.<sup>71</sup>

Compound **9** has poor brain penetration in rat (*e.g.*, brain to plasma ratio of 1:20). The very low lipophilicity (*e.g.*, cLogP <1) and the 4 H-bond donors likely hinder passive diffusion across the BBB.<sup>72</sup>

A small, uncontrolled pilot study was performed to determine the clinical benefit of **9** in Type II and Type III SMA patients, and demonstrated a statistically significant increase in muscle function.<sup>73</sup> An additional open-label trial using daily **9** treatments in Type II SMA patients reported possible functional improvement on the Hammersmith Motor Functional Scale. However, the data for this study should be cautiously interpreted because of the relatively small sample size and possible placebo effect.<sup>74</sup> A sufficiently powered, randomized, double-blinded, placebo-controlled trial needs to be performed using **9** in SMA patients as none has been published to date.

**2.3.3 Aclarubicin**—Aclarubicin (**10**) is a member of the anthracycline drug class approved for the treatment of acute non-lymphocytic leukemia that was identified in a cell-based screen for compounds that correct *SMN2* splicing.<sup>75</sup> Compound **10** was shown to increase both total and exon 7-included *SMN2* transcripts as well as SMN protein in primary SMA-derived fibroblasts. Compound **10** interacts with and antagonizes the activity of Type II DNA topoisomerases.<sup>5</sup> Compound **10** can also intercalate into DNA and inhibit DNA replication, DNA repair, and RNA protein synthesis.

Despite observations of its activity on SMN expression by two independent groups,<sup>75,76</sup> the use of **10** is restricted due to intrinsic toxicity. However, other related anthracycline derivatives did not affect *SMN2* splicing, suggesting that the mechanism underlying compound **10**-modulated splicing is different from other “anthracycline-like” activities. If the mechanism can be identified, it may be possible to design new analogs with less toxic effects.

### 3. SMALL MOLECULES IN CLINICAL TRIALS

#### 3.1 NON-SPECIFIC TREATMENTS FOR SMA

There are five small molecules currently being tested in Phase I to Phase III clinical trials for SMA. Two of these five compounds, neuroprotective olesoxime (**11**)<sup>77</sup> and muscle protective CK-2127107 (structure not disclosed),<sup>78</sup> are non-specific to SMA, meaning that the mechanism targets the CNS and muscles in general, and neither molecule directly affects the pathway of SMA. Each of these non-specific SMA small molecules will be discussed in terms of identification, rationale, mechanism of action, progression to date, and medicinal chemistry properties.



**3.1.1 Olesoxime**—Compound **11** is a novel neuroprotective compound that is non-specific to the disease mechanism of SMA (Table 2). This compound was identified in a cell-based screen as an agent protective against motor neuron death induced by neurotrophic factor withdrawal.<sup>79,80</sup> This agent acts to preserve the integrity of mitochondria in neurons under cellular stress by preventing mitochondrial permeability transition pore openings. There is evidence that **11** binds to a translocator protein (18 kDa) and voltage-dependent anion channel, two proteins located on the outer mitochondrial membrane.<sup>80</sup> Compound **11** is closely related to cholesterol and exists as a stable mixture of *syn*- and *anti*- isomers at the 3-position. Compound **11** is highly lipophilic (cLogP = 10) and is able to cross the BBB, although it has poor aqueous solubility at pH 7.4 and must be dosed orally in an oily excipient.<sup>77</sup>

Compound **11** was developed as a neuroprotective therapy for ALS and SMA. *In vitro* neuronal cell death studies demonstrated a dose-dependent increase in cell survival with the use of **11** in trophic factor deprivation assays. When dosed at 3 mg/kg in SOD1G93A transgenic mouse models of ALS, **11** resulted in the prevention of weight loss, a delay in severe muscle function decline, and a significant increase in lifespan. In another study, **11** was subcutaneously administered (30 mg/kg) daily to 21-day old SMNF7/F7 SMA mice and resulted in a significant increase in life span. Only 15% of vehicle-treated mice survived longer than 40 days, whereas 45% of compound **11**-treated mice lived beyond 40 days.<sup>77</sup>

Compound **11** is brain penetrant and is not a P-gp substrate. In mice, daily doses of **11** (3 mg/kg) resulted in a brain to plasma ratio of 0.35:1 after 7 days of treatment, and 0.51:1 after 42 days of treatment. Indeed, in a mouse nerve crush study, repeated dosing demonstrated drug accumulation in brain tissue over time, while the drug concentrations in plasma remained constant.<sup>77</sup>

Compound **11** did not provide significant treatment benefits during a Phase III clinical trial in patients with ALS, and it did not receive FDA approval. Although the compound showed no major effect on the survival of ALS patients, **11** was known to be safe in healthy individuals and was advanced directly into Phase II clinical trial studies for SMA.<sup>80,81</sup> A randomized, double-blind Phase II clinical trial study was conducted to assess the efficacy and safety of **11** in SMA Type II and Type III non-ambulant patients ranging in age from 3 to 25 years old. Although no results were reported, a second Phase II clinical trial is underway. This trial, which is currently in the recruitment process, will focus on the nature, frequency, and severity of adverse events, as well as the effects on laboratory values, vital signs, and electrocardiography parameters.

**3.1.2 Compound CK-2127107**—In collaboration with Astellas Pharma, Cytokinetics has developed CK-2127107, which is believed to be a 2-aminoalkyl-5-*N*-heteroarylpyrimidine derivative that slows calcium release from fast skeletal muscle troponin and sensitizes the sarcomere to calcium (Table 2). The sarcomere is the contractile unit of skeletal muscle in the body. Within the sarcomere, calcium ions (Ca<sup>2+</sup>) are the key regulatory ions in the signaling process. The binding of Ca<sup>2+</sup> to troponin C in the troponin complex of the muscle allows tropomyosin movement, actin-myosin interaction, and muscle contraction.<sup>78</sup> As the magnitude of Ca<sup>2+</sup> release is proportional to the level of neuromuscular transmission and the

force of muscle contraction, control of this ion provides a therapeutic target of interest to treat SMA and other skeletal muscle-related diseases.<sup>82</sup> With this mechanism of action, CK-2127107 helps increase skeletal muscle contractility and enhances physical performance in patients with neuromuscular diseases.<sup>78</sup>

Cytokinetics also developed an earlier small-molecule fast skeletal troponin activator, Tirasemtiv (**12**), and reported the first positive results from this mechanism of action by increasing skeletal muscle force and exercise performance in healthy and neuromuscular-diseased rats.<sup>78</sup> Compound **12** is structurally distinct from CK-2127107, but it demonstrates efficacy in improving muscle strength and produces a significantly higher force output at submaximal levels of motor nerve stimulation (Figure 2). Compound **12** is currently in Phase III clinical trials for ALS treatment and provides proof-of-concept for this mechanism of action. Through the same mechanism, CK-2127107 has displayed multiple areas of effectiveness, improving exercise tolerance *in vivo* in a rat model of heart failure-mediated skeletal myopathy, increasing sarcomere Ca<sup>2+</sup> sensitivity in left anterior descending coronary artery heart failure (LAD-HF) skeletal fibers *in vitro*, increasing force output at submaximal stimulation frequencies in the diaphragm and hind-limb muscles, and improving performance in LAD-HF rats.<sup>78</sup> Importantly, CK-2127107 also has been shown to selectively activate fast skeletal myofibrils (EC<sub>50</sub> = 3.4 μmol/L and maximal activation = 3.6-fold increase) with no effect on slow skeletal or cardiac myofibrils. Although this study showed the effects of CK-2127107 in a heart failure rat model, there is a correlation between the effects in this mouse model and those of SMA mouse models, as both display significant muscle atrophy. CK-2127107 may decrease the severity of symptoms and allow for an increase in patient muscle function in SMA.<sup>78</sup> The unique mechanism of CK-2127107 may allow combination therapy with agents that specifically increase levels of the SMN protein.

A number of patents on the use of amino-pyridazines for the treatment of neuromuscular disorders (*e.g.*, Markush **13**, Figure 2) have published and the 2-aminopyridine (**14**) is shown as a representative analog.

Phase I clinical trials tested the safety, pharmacokinetics, and pharmacodynamics of CK-2127107 in healthy individuals using three randomized, placebo-controlled studies. Single and repeated doses of the compound were well-tolerated and increased the response of muscle to nerve activation.<sup>85</sup> With positive safety results in the first phase of clinical trials, the drug is now moving into Phase II studies. Cytokinetics has opened enrollment for this double-blind, randomized, placebo-controlled clinical trial to test the pharmacodynamics effect of CK-2127107 on skeletal muscle function and fatigability in teens and adults with SMA Types II, III, and IV.

### 3.2 SPECIFIC TREATMENT FOR SMA

Treatment concepts shifted from non-specific to specifically targeting the *SMN2* gene after this gene was discovered to produce normal SMN protein. The *SMN2*-derived SMN protein can be directly or indirectly upregulated through (i) increased transcription of *SMN2*, (ii) increased inclusion of exon 7 *SMN2* (iii) stabilization of full-length exon 7-included *SMN2* mRNA, and (iv) stabilization of the SMN protein. In this section, the identification,

rationale, mechanism of action, progression to date, and medicinal chemistry properties of each small molecule specific to SMA-targeted therapy in clinical trials will be discussed.

**3.2.1 Compound RG3039 (17)**—To identify compounds that increase the transcription of *SMN2*, a high-throughput, cell-based screen was performed by Aurora Biosciences (which was subsequently acquired by Vertex Pharmaceuticals) under the sponsorship of Families of SMA.<sup>86</sup> The screen identified a class of 2,4-diaminoquinazolines (DAQ) (Figure 3; **15** and **16**).<sup>86,87</sup> The DAQ series of compounds was shown to increase total *SMN2* mRNA with a proportional increase in exon 7-included *SMN2* mRNA transcripts and SMN protein in SMA patient-derived primary fibroblasts. Medicinal chemistry optimization of the DAQ series focused on modifications to the 5-position and ultimately led to the discovery of the O-linked 2,6-dichlorobenzyl piperidine analog **17** (Table 2).<sup>88</sup>

Following a single oral dose (10 mg/kg) in adult mice, **17** resulted in low plasma exposure (*e.g.*, AUC<sub>last</sub> (plasma) = 157 ng.h/mL), but it displayed significant partitioning into the brain (AUC<sub>last</sub> (brain) = 6762 ng.h/mL) and consequently resulted in a very high brain to plasma ratio of 16:1.

Next, **17** was tested in three SMA mouse models. In the Taiwanese mouse model of SMA, in which the transgenic mice only have 2–4 copies of human *SMN2* and no murine SMN (generated to be similar in survival to the SMA  $\gamma$  by Li *et al.*), Compound **17**-treated mice had improved motor abilities and a 38% increase in lifespan compared to vehicle-treated SMA mice.<sup>90</sup> When tested in the intermediate 2B/– SMA mouse model (average survival 18.5 to 21 days), **17** extended survival by 100% relative to vehicle. When the study ended on day 112, 27% of compound **17**-treated mice were still alive. In addition to improved lifespan, the muscle function and weight gain of the compound **17**-treated mice improved as well.<sup>88</sup>

In order to identify the binding target of the DAQ series, a suitably active <sup>125</sup>I radiolabeled compound was identified (Figure 3; **18**) and used to identify potential binders in human protein microarrays.<sup>89</sup> Compound **18** was found to bind the scavenger decapping enzyme (DcpS), and binding was confirmed using X-ray crystal structure determination.<sup>89,91–93</sup> DcpS participates in 3′-5′ mRNA degradation, in which the protein targets the 5′ cap structure following 3′ end mRNA decay to release m<sup>7</sup>Gp and ppN products. In a separate pathway, DcpS also was shown to be a cofactor for Xrn1, a 5′-3′ exoribonuclease, and to regulate RNA stability through a transcript-specific mode of action. Thus, small molecules in the quazoline series, such as **17**, appear to stabilize RNA levels through inhibition of DcpS.<sup>94</sup>

In the SMA  $\gamma$  mouse model, **17** was administered intraperitoneally at 10 mg/kg for 10 days and resulted in 90% DcpS inhibition within 2 h of the last dose. DcpS levels remained 80% inhibited 72 h after dosing. To examine the effects of **17** on survival in the SMA  $\gamma$  mouse model, mice were treated with daily doses (10 mg/kg) until death. Drug-treated mice showed a 26% increase in median survival in comparison to vehicle-treated mice and displayed an overall increase in weight gain and motor function. A modest increase in full-length SMN transcript levels was detected; however, no increase of the SMN protein in the CNS was

detected.<sup>95,96</sup> Notably, **17** improved survival, muscle function, and weight gain in all three mouse models, which varied in SMA severity.

A Phase I clinical trial to evaluate the safety profile of **17** in healthy volunteers began in 2012. Repligen, Inc. has released several reports about the success of this trial, detailing the effectiveness of **17** and its minor side effects. Pfizer Inc. acquired rights to this compound but subsequently terminated the project and further development of **17** as a treatment for SMA is questionable.

**3.2.2 Compound RG7800**—PTC Therapeutics aimed to identify compounds that selectively modulate *SMN2* inclusion of exon 7. Through the use of a HEK293 human embryonic kidney cell line harboring an *SMN2* mini-gene reporter assay, a library of proprietary small molecules was screened, and several chemical classes of compounds that induced exon 7 inclusion were identified. Deep sequencing of all mRNAs revealed that altered exon processing was highly specific to *SMN2* transcripts. Following medicinal chemistry optimization in a collaborative project with Roche, three orally available compounds (*i.e.*, the coumarins SMN-C1 (**19**) and SMN-C2 (**20**) and the pyrido[1,2-*a*]pyrimidin-4-one SMN-C3 (**21**)) with nanomolar potency were identified.<sup>97</sup> These compounds increased full-length *SMN2* mRNA levels and concomitantly reduced *SMN7* mRNA levels in SMA Type I patient fibroblasts after 24 h of treatment.<sup>97</sup>

In RNA sequence studies, **21** was determined to be specific to SMA, displaying affinity for *SMN2* exon 5 and 7 splice junctions. When dosed orally in the mild-type SMA mouse model, **21** increased *SMN2* mRNA and SMN protein in the brain and muscle tissues by 90% and 50–70%, respectively. Compound **21** also showed an excellent PK profile; after oral administration (10 mg/kg), adult wild-type mice demonstrated a 3:1 brain penetration ratio and a plasma concentration peak of compound at approximately 2 to 2.5 h. In support of these findings, intraperitoneal injection of **21** in the severe SMN7 mouse model resulted in increased SMN protein levels in the brain and muscle tissue by up to 150% and 90%, respectively. Dosing the animals daily with **21** (0.3 mg/kg) allowed 50% of the mice to survive past 40 days. Another study dosed *SMN7* mice daily (1 or 3 mg/kg) and resulted in 90% survival until the end of the study or 65 days.<sup>97</sup>

PTC Therapeutics, in partnership with Roche, advanced RG7800 (structure not disclosed) as its clinical candidate and began a Phase I single-ascending dose study.<sup>98</sup> The study revealed that the compound was safe and well-tolerated in healthy volunteers. Following this success, adult and pediatric SMA patients began to enroll in a randomized, placebo-controlled Phase Ib/IIa clinical trial. However, PTC/Roche suspended this clinical trial over concerns regarding an eye condition that developed in long-term animal studies. A Phase I trial began recruiting healthy volunteers in January 2016 to evaluate the safety profile and pharmacokinetic tolerability of an alternative candidate, RG7916 (structure not disclosed). Dosing regimens in the study will include a combination of RG7916 and itraconazole, an antifungal, as well as a placebo.

**3.2.3 Compound LMI070**—Novartis performed a high-throughput screen of over 1.4 million compounds using a minor modification of the *SMN2* splicing reporter construct

described by Cherry *et al.*<sup>99</sup> In this screening assay, the reporter was introduced into the NSC-34 mouse neuroblastoma/spinal cord hybrid cell line.<sup>100</sup> With a hit rate of <1%, a pyridazine class of orally active small molecules was identified and shown to elevate levels of the full-length SMN protein via the inclusion of exon 7. After substantial lead optimization and drug discovery efforts, two compounds from this series, NVS-SM1 (**22**) and NVS-SM2 (**23**), showed increased exon 7 inclusion and upregulated SMN protein expression in both SMA patient fibroblasts and SMN<sup>-/-</sup> 5025-mouse myoblasts (Figure 5).<sup>100</sup> The related analog NVS-SM3 (**24**) was inactive, indicating that the un-substituted pyrrole common to **22** and **23** is important for activity.<sup>100</sup>

Short-term dosing of **22** (1 mg/kg and 3 mg/kg) in the severe SMN<sup>-/-</sup> mouse model demonstrated an increase in both SMN protein levels in the brain and survival of the animals by 50% and 62% relative to the doses. In a long-term SMN<sup>-/-</sup> mouse model study, one cohort received daily doses of **22** (1 mg/kg) for an additional two weeks until day 49, and another received vehicle between days 36 and 49. Body weight and survival measurements between cohorts were nearly identical, supporting the duration effectiveness of **22** and suggesting that termination of treatment after 28 days allows sustained rescue from SMA phenotypic symptoms.<sup>100</sup>

Unexpected effects of alternative splicing prompted RNA sequencing studies to determine if **22** changed global gene expression or splice site selection. Following **22** treatment, 175 genes were altered by >2-fold with little difference in splicing factors or RNA binding proteins. Splice site analysis detected 39 **22** specific events in 35 genes, indicating a very selective modulation of splicing. The mechanism of **22** exon 7 recognition was investigated with a series of chimeric splicing cassettes. This analysis revealed that the 21 nucleotides surrounding the 5' splice site in intron 7 of *SMN2* were enough to promote **22** specific changes in exon 7 inclusion. Surface plasmon resonance established changes in the disassociation kinetics between the *SMN2* mRNA and the U1 snRNP in the presence of **22**, suggesting that it interacts with the 5' splice site of *SMN2* intron 7 and stabilizes its interaction with the U1 snRNP complex. Combined, these results provide the first mechanistic insights for a sequence-specific, small molecule splicing modulator.<sup>100</sup>

The clinical lead compound in this series, LMI070 (structure not disclosed),<sup>101</sup> has begun Phase II clinical trials to evaluate safety, tolerability, and efficacy in SMA patients. An open-label, multi-part, first-in-human clinical trial is now recruiting in Europe. During the trial, LMI070 will be orally administered to Type I SMA infants to determine the maximum tolerated dose and optimal dosing regimen.

#### 4. PRECLINICAL SMALL MOLECULES

There are six small molecules currently in preclinical development for the treatment of SMA. All of these compounds are reported to be specific to the SMA-disease pathway, although not all of the companies and academic laboratories have published data to support these claims. Without published data, many of these compounds cannot be assessed for medicinal chemistry properties and their progress remains unknown.

#### 4.1 Indiana University and the Laboratory for Drug Discovery in Neurodegeneration

The Androphy group at Indiana University and the Laboratory for Drug Discovery in Neurodegeneration (LDDN) at Brigham and Women's Hospital combined the SMN promoter with exons 1–6 and an exon 7 splicing cassette in a single construct with a luciferase reporter to identify compounds that increase SMN transcription, increase exon 7 inclusion, or potentially stabilize the SMN mRNA or protein. Stable clonal HEK293 cell lines that expressed *SMN1* or *SMN2* reporters were isolated to reduce variation due to factors such as plasmid number. Using this *SMN2* reporter assay, over 115,000 compounds were screened at the LDDN, and two hit compounds (LDN-75654 (**25**) and LDN-76070 (**28**)) were discovered to increase SMN expression from the *SMN2* gene (Table 3).<sup>99</sup>

Compound **25** produced a >240% or 3.4-fold increase in the luciferase activity of the assay with an EC<sub>50</sub> of 2 μM (Figure 6). Compound **25** is a 5-isopropyl isoxazole bearing a 3-chloro-4-fluorophenyl carboxamide at the 3-position. Compound **25** is compliant with Lipinski's rules for drug-like molecules, has physicochemical properties that are considered suitable for BBB penetration<sup>102</sup> (MW = 283 amu, cLogP = 3.4, PSA = 51 Å<sup>2</sup>, HBD = 1, HBA = 3), and promotes increases in SMN expression in SMA-derived fibroblasts. However, **25** was inactive for SMN protein induction *in vivo*. Administration to SMN 7 mice revealed that **25** did not consistently elevate levels of SMN protein in the brain or spinal tissue of the animals. Compound **25** had poor aqueous solubility (6 μM at pH 7.4) and a short microsomal half-life in mouse microsomes (T<sub>1/2</sub> = 5 min). The lack of metabolic stability and poor solubility presumably accounts for its poor pharmacokinetics and disappointing performance *in vivo*. Modifications made to the aryl ring of **25** revealed that the 3-chloro-4-fluorophenyl could be replaced by a phenyl ring (Figure 6; LDN-5021 (**26**)) with a significant improvement in both potency (EC<sub>50</sub> = 0.28 μM) and solubility (59 μM at pH 7.4), although the mouse microsomal stability was still poor (T<sub>1/2</sub> = 5 min).<sup>103,104</sup> The introduction of the 2-pyridyl group (Figure 6; LDN-5065 (**27**)) to replace the phenyl group gave a compound of similar potency and solubility to **26** but with significantly improved stability in mouse microsomes.

The mechanism of action of **25** was studied in *SMN2*-luciferase reporter cell lines using qRT-PCR. No change was detected in the mRNA levels, but concentration-dependent increases were observed in luciferase activity, suggesting that **25** and its analogs function through a post-transcriptional mechanism.<sup>99</sup>

Another distinct hit, compound **28**, produced a >180% or 2.8-fold increase in the luciferase activity of the assay, with an average EC<sub>50</sub> of 8.3 μM (Figure 7).<sup>99</sup> Compound **28** is a 3,4-dihydroquinolinone and structurally distinct from the first lead, **25**. Further, **28** follows Lipinski's rules and had acceptable physicochemical properties for potential brain penetration (MW = 410 amu, cLogP = 3.3, PSA = 57 Å<sup>2</sup>, HBD = 1, HBA = 5). Using qRT-PCR, **28** was found to increase SMN transcription in the *SMN2*-luciferase reporter cell line.<sup>99</sup>

Additional *in vitro* studies showed **28** had good stability in mouse liver microsomes (T<sub>1/2</sub> = 40 min), but had poor aqueous solubility (4 μM at pH 7.4). In a preliminary efficacy study, the un-optimized lead **28** was found to be active *in vivo*. Daily treatment of SMN 7 mice

with **28** (20 mg/kg) increased SMN protein levels and survival. Compound **28**-treated mice had a mean increase of SMN protein levels in the spinal cord up to 6-fold greater than untreated or vehicle-treated animals. Additionally, median survival increased from 7 days (vehicle-treated) to 16 days (compound **28**-treated), a 2.4-fold increase in lifespan.<sup>99</sup>

## 4.2 NIH Chemical Genomics Center

Using the cell-based *SMN2*-luciferase reporter assay previously described by Cherry *et al.*, the NIH Chemical Genomics Center (NCGC) screened a library of 210,386 compounds to identify those that increased expression of full-length SMN transcripts.<sup>105</sup> Following hit validation and prioritization, 1-(4-(4-bromophenyl)thiazol-2-yl)piperidine-4-carboxamide (**29**) was identified as a promising hit that increased SMN protein production in a dose-dependent manner (Figure 7).<sup>105</sup>

The hit, **29** ( $EC_{50} = 1.22 \mu\text{M}$ , 268% increase in *SMN2*) underwent medicinal chemistry optimization, including piperidine modification, thiazole replacement, and phenyl substitutions. Switching the location of the aryl substituent from the 4- to the 5-thiazole position and replacing the 4-bromo phenyl with the electron-donating 4-thiomethyl phenyl (*e.g.*, ML200 (**30**),  $EC_{50} = 31 \text{ nM}$ , 576% increase in *SMN2*) conferred increased potency and efficacy (Figure 7).<sup>105</sup> Unfortunately, **30** had a very short half-life in mouse liver microsomes ( $T_{1/2} = 2 \text{ min}$ ) and low permeability in Caco-2 cells ( $P_{\text{app}} \text{ A to B} = 1.6 \times 10^{-6} \text{ cms}^{-1}$  and  $P_{\text{app}} \text{ B to A} = 0.2 \times 10^{-6} \text{ cms}^{-1}$ ) with a poor efflux ratio of 0.125.

Replacement of a potential metabolic liability, the 4-thiomethyl phenyl group, by 3,4-dihydro-2H-benzo[b][1,4]dioxepin-7-yl and replacement of the amide by carboxylic acid produced ML372 (**31**) ( $EC_{50} = 12 \text{ nM}$ , 325% increase in *SMN2*).<sup>105</sup> Compound **31** possessed a more stable half-life in mouse liver microsomes ( $T_{1/2} > 60 \text{ min}$ ) and high permeability in Caco-2 cells ( $P_{\text{app}} \text{ A to B} = 33.8 \times 10^{-6} \text{ cms}^{-1}$  and  $P_{\text{app}} \text{ B to A} = 44.1 \times 10^{-6} \text{ cms}^{-1}$ ) with an efflux ratio close to unity (*i.e.*, 1.3).

Pharmacokinetic studies in mice (oral dosing at 30 mg/kg) demonstrated that **31** reached high plasma levels (plasma  $C_{\text{max}} = 44 \mu\text{M}$ ) with a long plasma half-life ( $T_{1/2} > 11 \text{ h}$ ). However, there was significant resistance to brain penetration (brain  $C_{\text{max}} = 1.2 \mu\text{M}$ ), and half-life in the brain was  $> 13.7 \text{ h}$ . In addition, **31** demonstrated high plasma protein binding (94.9%). Although **31** showed good oral absorption, its poor CNS penetration and high plasma protein binding indicated that free levels of **31** in brain were likely to be low following higher oral dosing (30 mg/kg).<sup>105</sup>

To determine their mechanism of action, **30** and **31** were studied using qRT-PCR. RNA expression and exon 7 inclusion levels were measured within a SMA reporter cell line, in which there was no increase in exon 7 mRNA. These compounds may, therefore, act post-transcriptionally to increase the stability of SMN protein by inhibition of its degradation.<sup>105</sup>

## 4.3 Kozikowski and Rubin Laboratories

An image-based screen of annotated chemical libraries revealed that small molecule inhibitors of the glycogen synthase kinase (GSK-3 $\beta$ ) (*e.g.*, alsterpaullone (**32**) and AR-A014418 (**33**)) could elevate SMN levels (Figure 8).<sup>106</sup> In general, GSK-3 $\beta$  is known to

regulate protein stability. More specific to SMA, there is a consensus GSK-3 $\beta$  phosphorylation site on serine 4 of the SMN protein.<sup>106</sup> When this residue becomes phosphorylated, SMN protein degrades at a faster rate. Compound **32** is a known GSK-3 $\beta$  inhibitor, and treatment of mouse motor neurons with **32** resulted in increased amounts of SMN protein levels.<sup>106,107</sup> This evidence suggests that the inhibition of GSK-3 $\beta$  is a potential therapeutic approach to treat SMA.

Kozikowski and Rubin recently described a potent ATP-competitive GSK-3 $\beta$  inhibitor, BIP-135 (**34**) (GSK-3 $\beta$  IC<sub>50</sub> = 21 nM).<sup>108</sup> In SMA patient fibroblasts, **34** (25  $\mu$ M, 72 h exposure) stimulated a 7-fold increase in SMN protein levels and displayed neuroprotective properties in an SMA-related, cell-based model of oxidative stress-induced neurodegeneration. Subsequently, **34** was administered daily by intraperitoneal injection to SMN<sup>-7</sup> neonatal mice in multiple doses (25, 75, and 125 mg/kg) in 100% DMSO. Compound **34** did not increase motor function or levels of SMN mRNA or protein, but it did improve median survival in the SMN<sup>-7</sup> mouse model at the 75 mg/kg dose, increasing the lifespan by 2 days in comparison to untreated mice.<sup>108</sup>

Pharmacokinetic data (*e.g.*, brain levels) were not reported for **34**, and the choice of 100% DMSO as the vehicle for the *in vivo* study suggests poor compound solubility. Nevertheless, the modest extension in survival demonstrates potential for GSK-3 $\beta$  inhibitors as therapeutics; however, more potent and selective inhibitors with better drug-like properties and pharmacokinetics are required.

#### 4.4 Paratek Pharmaceuticals

As previously mentioned, **10** was identified in a cell-based screen specifically designed to detect SMN protein upregulation via increased inclusion of exon 7 in *SMN2*. Although the precise mechanism in which **10** increases SMN expression is unclear, it was demonstrated to change the splicing ratio between exon 7-included and exon 7-excluded *SMN2* transcripts.<sup>75</sup> More importantly, **10** was found to be toxic in SMA Type I fibroblast 3061 cells dosed at concentrations >10 nM. Although toxic, the distinct mechanism of **10** was unlike any other tetracycline derivatives. Therefore, it is predicted that structure-activity relationship (SAR) studies can provide similarly structured compounds without the toxic effects.

Paratek Pharmaceuticals reported that a lead compound from its novel series of tetracycline-like analogs, PTK-SMA1 (**35**) (Table 3), alters the splicing pattern of exon 7 and has shown progress in *in vitro* and *in vivo* studies.<sup>109</sup> A dose of **35** (10  $\mu$ M) increased the levels of SMN protein in SMA patient fibroblasts by about 40%, relative to vehicle-treated cells. Compound **35** (MW = 605 amu, cLogP = 1.3, PSA = 194  $\text{\AA}^2$ ) is a tetracycline derivative, modified at the C7 position of the tetracycline backbone.<sup>109</sup> Due to its large molecular size and polarity, the compound does not demonstrate good CNS drug-like properties and is hindered in its ability to penetrate the brain tissue *in vivo*. When **35** was administered via intraperitoneal injection (25 and 50 mg/kg) in a mild SMA Type III mouse model over 6 days, a 1.5-fold increase of exon 7 inclusion within liver cells was found. Only data from liver was collected because the compound is unable to cross the BBB.<sup>109</sup> Additional SAR studies must focus on BBB penetration to increase SMN expression in the CNS. Otherwise,



the novel treatment may not be an effective therapy. No further information has been published on the progress of this preclinical phase project.

#### 4.5 BioBlast Pharma

BBrm2 is the repurposed FDA-approved drug, azithromycin, redesigned in a proprietary intrathecal formulation. Azithromycin is an antibiotic approved to treat acute bacterial exacerbation of chronic obstructive pulmonary disease, acute bacterial sinusitis, and pneumonia, among other uses. Read-through agents cause skipping or read-through of the translational termination codon at the end of exon 7 and result in a protein that has an extended C-terminus.<sup>110</sup> Aminoglycoside antibiotics (from a class of FDA-approved drugs including tobramycin, geneticin, amikacin, and TC-007 (**36**)),<sup>111</sup> were found to induce higher levels of functional SMN protein after administration at the stop codon of exon 7 in the SMN 7 protein; however, toxicity limited any attempts to translate these agents into therapeutic candidates. With a safe profile, BBrm2 was shown to increase SMN protein expression and function in SMA patient cell lines, as well as increase SMN expression of the spinal cord, body weight, motor function, and survival in a SMN<sup>RT</sup> transgenic mouse model dosed via intracerebroventricular (ICV) administration. BioBlast Pharma reported that testing of the small molecule has been extended to additional studies in the Burgheron mouse model, where significant increases in tail length, body weight, and tissue SMN levels were observed.<sup>112</sup>

## 5. DISCUSSION

Repurposed drugs offer significant savings in discovery and development time and costs. However, many repurposed drugs were not originally developed for diseases that required brain penetration, limiting their utility in CNS diseases. While many of the compounds described in the preceding section demonstrated promising *in vitro* activity (e.g., increased SMN protein levels or neuroprotection), often there was no evidence of adequate brain penetration or target engagement.<sup>102,113,114</sup> Indeed, a number of the compounds studied *in vitro* and *in vivo* for SMA have very poor brain penetration. When repurposing an approved drug for a CNS disease, there is a risk that negative information about a particular target or disease mechanism may be generated in the rush to achieve Phase II efficacy studies. For example, although a potent, HDAC-enzyme-specific, brain-penetrant molecule may have utility in the treatment of SMA, the non-specific mechanism through which HDAC inhibitors such as **2** and **3** act means these drugs are likely to cause side effects with long term usage, decreasing interest for developing this class of compound. Furthermore, many of the clinical trials of repurposed drugs in SMA were open label and ineffectively powered in trial design, so that it is difficult to know whether the therapeutic strategy or target had validity. Nonetheless, much was learned on how to develop a clinical trial and evaluate outcome measures. For instance, transport of a severely ill SMA Type 1 patient to a distant medical center could itself have deleterious effects that complicate trial result interpretations. There are also ethical issues in withholding or limiting palliative treatments during trials. Although repurposing small molecules can expedite clinical testing, opportunities were missed to develop new SAR and discovery molecules with suitable brain penetration, level of effect on the SMA target, off-target toxicity and specificity.

Clinical trials with agents specifically designed to target SMA and *SMN2* have been initiated. Evidence shows that increasing the amount of SMN protein has the potential to decrease disease severity and symptoms. Because the *SMN2* gene was discovered to produce some SMN protein, it has become a specific therapeutic target for SMA even though the threshold level of SMN protein required to decrease SMA symptoms remains undetermined.<sup>115</sup> Depending on their mechanism of action, small molecules can be administered independently or in combination to help decrease SMA symptoms. Cherry *et al.* demonstrated the use and success of small molecule combination therapy targeted to SMA. The study used **25** (a small molecule that functions at the post-transcriptional level) and **28** (a small molecule that affects the transcription of the *SMN2* gene) individually and in combination with the pan-HDAC inhibitor **4** in the *SMN2*-luciferase reporter assay.<sup>99</sup> The combination of **25** with either **28** or **4** produced a greater-than-additive stimulation of the *SMN2*-luciferase reporter. Not only does this complementation confirm the separate and distinct mechanisms in which the two compounds work, but the results also suggest a synergistic relationship. On the other hand, **4**, when combined with **28**, appeared to overwhelm the transcriptional machinery and obscure the effect of **28**. These results suggest that the compounds stimulate *SMN2*-luciferase expression similarly.<sup>99</sup> Therapeutic approaches that combine modalities acting through different pathways may maximally increase SMN protein production and be of greatest clinical benefit.

Combination therapies can be applied to lead compounds when the mechanism of action is known and distinct for each molecule. Compound **11** and CK-2127107 might be paired with any SMA-specific treatment because the neuroprotective and muscular protective mechanisms provide them with a unique therapeutic opportunity compared to other treatments in the pipeline. Analyzing the mechanisms of more specific treatments, **17** has been shown to bind DcpS, an enzyme that participates in mRNA degradation.<sup>94</sup> In comparison, LMI070 was determined to be a selective splicing module that works to increase the stabilization between U1 snRNP binding and the 5' splice site.<sup>100</sup> Compound **17** and LMI070 work to stabilize *SMN2* mRNA levels and increase exon 7 inclusion of *SMN2* mRNA, effectively working through two distinctive *SMN2*-targeted pathways. Importantly, some evidence suggests that RG7800 works through a similar mechanism as LMI070. The exact mechanism of RG7800 has not been reported, but affinity studies demonstrated that the compound shows moderate specificity for *SMN2* exon 5 and 7 splice junctions.<sup>97</sup> Based on their structural similarities, LMI070 and RG7800 may bind to the same protein or RNA structure in *SMN2* intron 7. Expanding to other treatment types, the combination of IONIS-SMNRx, which increases exon 7 inclusion, with a drug that stabilizes the half-life of the SMN protein may be a more effective in clinical trials. With multiple therapeutic targets in the SMA disease pathway, there is the potential to manage the symptoms of SMA with a variety of treatment options and combinations.

## 6. CONCLUSION

Despite the lack of FDA-approved treatments for SMA, the last fifteen years have seen dramatic progress in the development of therapeutic strategies. These advances have widened our understanding of the disease process and helped researchers explore new options for treatment. Progress in the field of SMA therapy is attributed to understanding

SMN function, *SMN2* exon 7 splicing regulation, and the therapeutic opportunities that stem from SMN production defects. Thus far, the current use of repurposed drugs has not provided a short cut to disease treatment, due in large part to non-specific mechanistic pathways and lack of CNS-penetration. However, neuroprotective compounds, such as **11**, and muscle targeting agents, such as CK-2127107, continue to progress through clinical trials due to their ability to target general atrophy symptoms. Very novel approaches that appear to specifically enhance the inclusion of *SMN2* exon represent strong evidence that drug-like molecules can target RNA splicing. The field of SMA continues to advance upon the construction of extensive biology findings, sophisticated drug screening technologies and medicinal chemistry efforts that have translated to numerous therapeutic opportunities for SMA.

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## Abbreviations

<b>SMA</b>	spinal muscular atrophy
<b>SMN</b>	survival motor neuron
<b>MNs</b>	motor neurons
<b>CSF</b>	cerebral spinal fluid
<b>NMJ</b>	neuromuscular junction
<b>SMN<sup>RT</sup></b>	SMN read through
<b>scAAV-9</b>	self-complementary adeno-associated virus
<b>BBB</b>	blood-brain barrier
<b>HDAC</b>	histone deacetylase
<b>qRT-PCR</b>	quantitative reverse transcriptase PCR
<b>ALS</b>	amyotrophic lateral sclerosis
<b>CNS</b>	central nervous system
<b>P-gp</b>	P-glycoprotein
<b>HBD</b>	hydrogen-bond donor
<b>HBA</b>	hydrogen-bond acceptor
<b>MW</b>	molecular weight
<b>cLogP</b>	calculated LogP

<b>PSA</b>	polar surface area
<b>LAD-HF</b>	left anterior descending coronary artery heart failure
<b>DAQ</b>	diaminoquinazoline
<b>DepS</b>	scavenger decapping enzyme
<b>LDDN</b>	Laboratory for Drug Discovery in Neurodegeneration
<b>NCGC</b>	NIH Chemical Genomics Center
<b>GSK</b>	glycogen synthase kinase
<b>SAR</b>	structure-activity relationship

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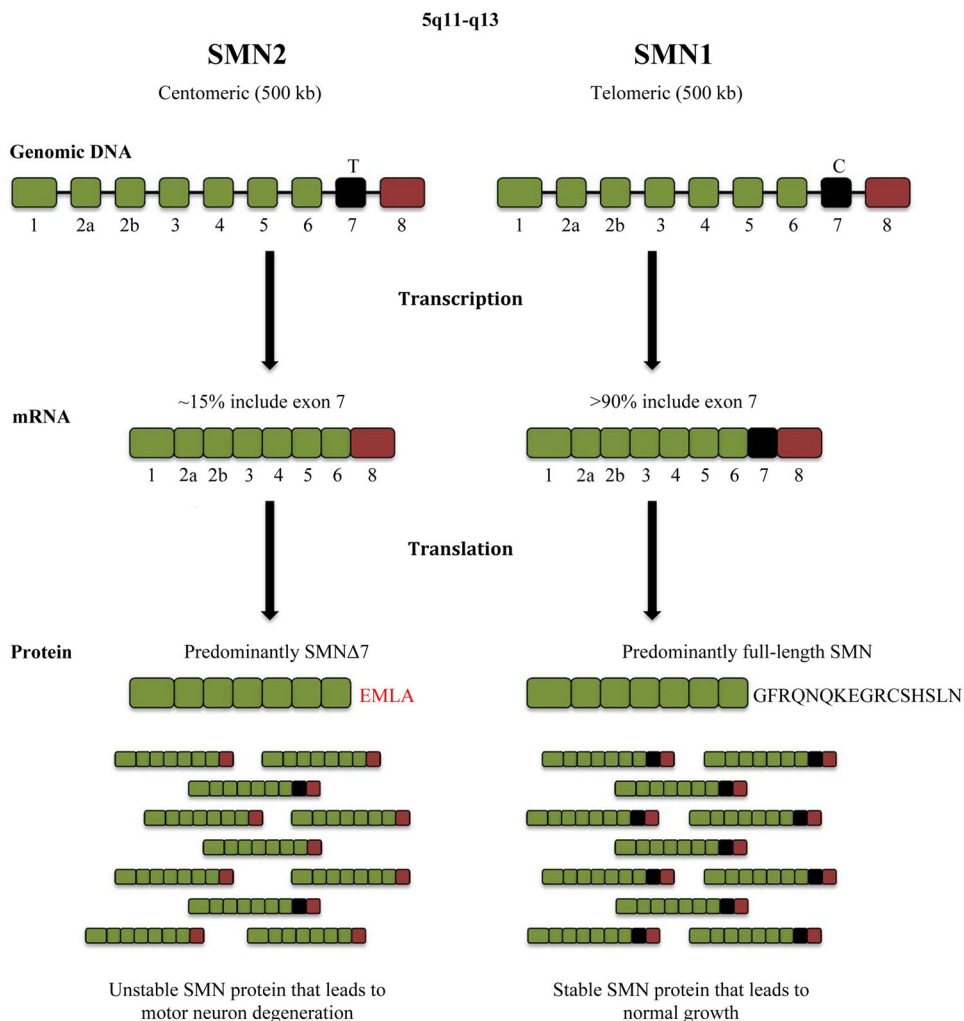
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## Biographies

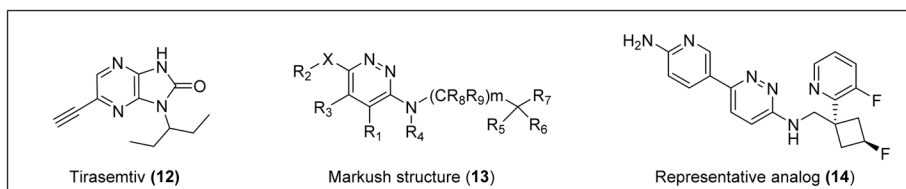
**Alyssa N. Calder** received her B.S. (summa cum laude) degree in Chemistry from Northeastern University in 2015. She performed undergraduate research at Northeastern University under Professor Graham Jones on the development of fluoro-pharmaceuticals and PET ligands utilizing continuous flow micro-reactor technology as well as at Genzyme Corporation, working on peptide chemistry for immune mediated disease discovery. Alyssa joined the LDDN in 2014, where she has focused her efforts on synthesizing and optimizing small molecules for the SMA project as the laboratory closes in on identifying a developmental candidate.

**Elliot Androphy**, M.D. is the Kampen-Norins Chair of the Department of Dermatology at Indiana University School of Medicine. After a post-doctoral fellowship at the National Cancer Institute/NIH, he began his SMA research at Tufts Medical Center, where his lab identified the critical difference between *SMN1* and *SMN2* exon 7. He served as Vice Chair for Research of the Department of Medicine and Director of the MD/PhD Program at the University of Massachusetts Medical School, where his research team focused on the mechanisms of exon 7 recognition and developed the first cell-based high-throughput screens to identify drug-like compounds that increase levels of the SMN protein. His lab continues to develop novel therapeutics and investigates the functions of the SMN protein in neurons.

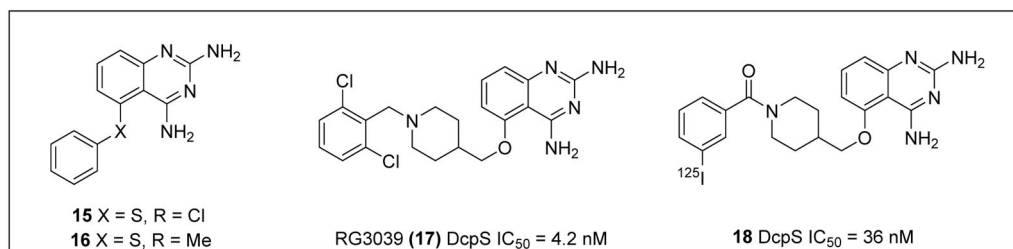
**Kevin Hodgetts**, Ph.D. is a medicinal chemist, Director of the LDDN and Assistant Professor of Neurology at Brigham and Women's Hospital and Harvard Medical School. He works in collaboration with researchers to translate discoveries in basic biology into opportunities for drug discovery.



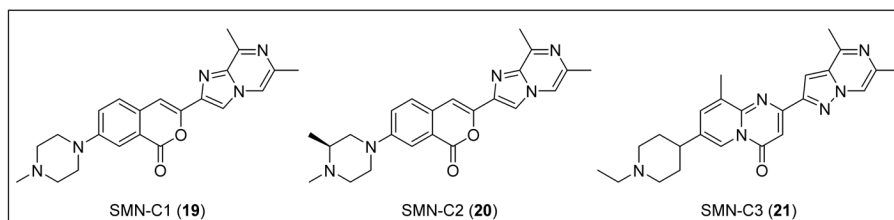
**Figure 1.** SMA is caused by a loss of *SMN1*. The two SMA genes, *SMN1* and *SMN2*, are found on chromosome 5q11-13 and encode for SMN protein. In most patients with SMA, complete lack of an *SMN1* gene results in all SMN protein being derived from *SMN2* expression. Although all patients retain one or more copies of the *SMN2* gene, it is unable to fully compensate for loss of *SMN1*. Only about 15% of *SMN2* transcripts encode for normal, full-length SMN protein.<sup>5</sup>



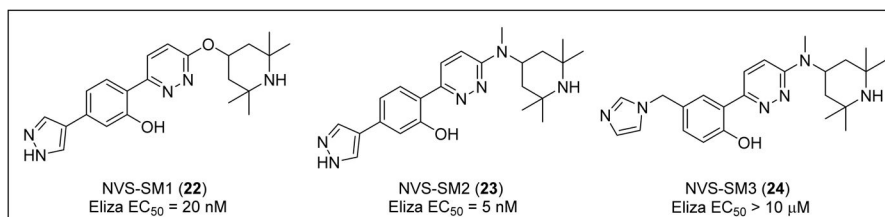
**Figure 2.**  
Examples of Cytokinetics small molecules.<sup>83,84</sup>



**Figure 3.**  
Discovery of clinical compound **17** and target ID probe compound **18**.<sup>89</sup>

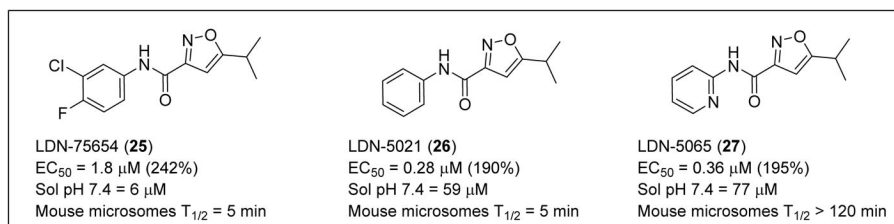


**Figure 4.**  
Examples of PTC/Roche small molecules.

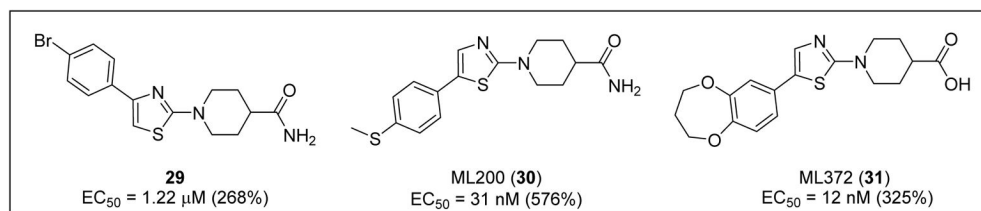


**Figure 5.**  
Examples of active and inactive pyridazines reported by Novartis.

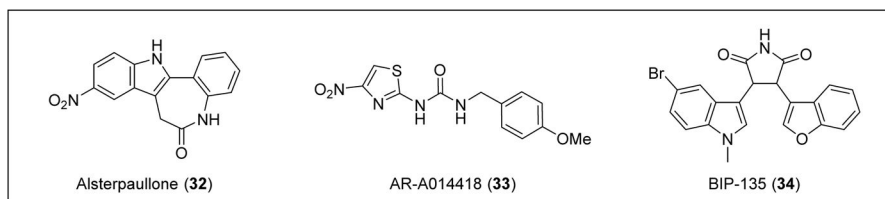




**Figure 6.**  
Preliminary optimization of **25**.



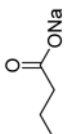

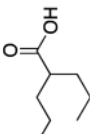

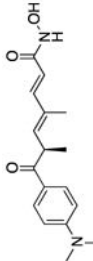
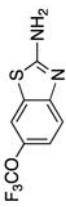
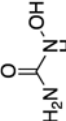
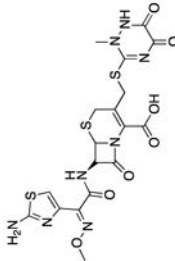
**Figure 7.**  
Discovery of **30** and **31**.

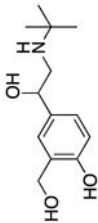
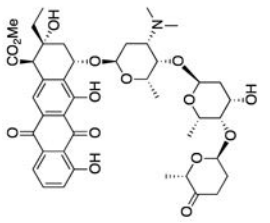


**Figure 8.**  
GSK-3 $\beta$  inhibitors: **32**, **33**, and **34**.

Table 1

Small molecules that were repurposed for the treatment of SMA.

Name	Structure	Mode of Action	MW <sup>a</sup>	cLogP <sup>b</sup>	PSA <sup>c</sup>	HBA <sup>d</sup>	HBD <sup>e</sup>	Ref
Sodium butyrate (1)		Weak class I HDAC inhibitor	110	0.6	26	2	1	39
Sodium phenylbutyrate (2)		Weak class I HDAC inhibitor	186	2.0	26	2	1	40,45–49
Valproic Acid (3)		Weak class I HDAC inhibitor	144	2.8	37	2	1	41,50–54
Suberoylamide hydroxamic acid (4)		Potent HDAC class I and II	264	1.0	78	5	3	42,43
Trichostatin A (5)		Potent HDAC class I and IIb	302	1.9	70	5	2	44,55,56
Riluzole (6)		Neuro-protective	234	3.2	48	4	2	57–62
Hydroxyurea (7)		Increases Nitric Oxide	76	-1.8	75	4	4	63–66
Ceftriaxone (8)		Unknown	554	0.02	209	18	5	67–69

Name	Structure	Mode of Action	MW <sup>a</sup>	cLogP <sup>b</sup>	PSA <sup>c</sup>	HBA <sup>d</sup>	HBD <sup>e</sup>	Ref
Albuterol (9)		β2 Adrenergic Receptor Agonist	239	0.06	73	4	4	70-74
Aclarubicin (10)		Antibiotic	812	3.4	217	16	4	75, 76

<sup>a</sup> MW, molecular weight;

<sup>b</sup> cLogP, calculated LogP;

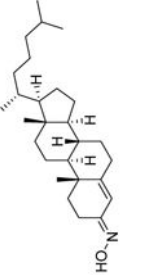
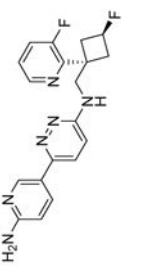
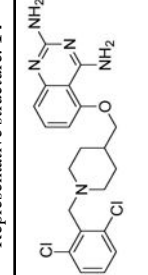
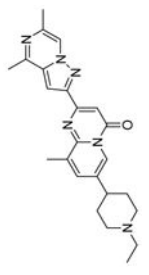
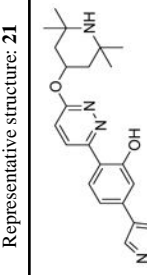
<sup>c</sup> PSA, polar surface area;

<sup>d</sup> HBA, hydrogen-bond acceptor;

<sup>e</sup> HBD, hydrogen-bond donor.

Table 2

Chemical properties of small molecules in clinical development for SMA.

Name	Structure	Mode of Action	MW <sup>a</sup>	cLogP <sup>b</sup>	PSA <sup>c</sup>	HBA <sup>d</sup>	HBD <sup>e</sup>	Ref <sup>f</sup>
Olesoxime ( <b>11</b> )		Neuro-protective	400	10	33	2	1	77, 79–81
CK-2127107	 Representative structure: <b>14</b>	Fast skeletal troponin activator	368	2.2	88	6	3	78, 82–85
<b>17</b>	 Representative structure: <b>21</b>	DcpS inhibitor	432	5.3	89	6	4	86–96
RG7800	 Representative structure: <b>21</b>	Sequence-specific splicing modulator	416	3.7	64	7	0	97, 98
LM1070	 Representative structure: <b>22</b>	Sequence-specific splicing modulator	393	3.2	91	3	1	100, 101

<sup>a</sup>MW, molecular weight;<sup>b</sup>cLogP, calculated LogP;<sup>c</sup>PSA, polar surface area;

HBA, hydrogen-bond acceptor;  
HBD, hydrogen-bond donor;  
 $f_j$  Ref. reference number

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Table 3

Preclinical small molecules in development for SMA.

#	Structure	Mode of Action	MW <sup>a</sup>	cLogP <sup>b</sup>	PSA <sup>c</sup>	HBA <sup>d</sup>	HBD <sup>e</sup>	Ref <sup>f</sup>
25		Unknown (Post-transcriptional)	282	3.5	51	4	1	99, 103
28		Unknown (Transcriptional)	410	3.2	57	5	1	99
31		Unknown (Post-transcriptional)	360	2.7	71	7	1	105
34		Glycogen synthase kinase 3-β inhibitor	422	3.9	59	5	1	108
35		Unknown (Transcriptional)	605	1.3	194	12	7	109
36		Read-through inducing	483	-7.9	288	15	16	111



$^a$ MW, molecular weight;

$^b$ cLogP, calculated LogP;

$^c$ PSA, polar surface area;

$^d$ HBAs, hydrogen-bond acceptor;

$^e$ HBs, hydrogen-bond donor;

$^f$ Ref, reference number

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