Indoprofen Upregulates the Survival Motor Neuron Protein through a Cyclooxygenase-Independent Mechanism

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by rapid degeneration of lower motor neurons in the anterior horn of the spinal cord due to reduced survival motor neuron (SMN) protein. SMA is the leading genetic cause of infant mortality in the United States and Western Europe, with an incidence of 1 in 6000 live births and a carrier frequency of 1 in 40 [1–7]. There is no treatment for this orphan genetic disease. Studies of the underlying molecular pathology of SMA have the potential to reveal essential aspects of motor neuron function, aspects of SMN protein function and regulation, and therapeutic candidates for the disease [8–10].

We developed a high-throughput assay to detect reporter protein production from SMN1- and SMN2-minigene-reporter constructs, denoted SMN1-luc and SMN2-luc. We screened ~47,000 compounds from diverse compound libraries for those that could affect production of the SMN protein [11]. We discovered that two compounds—aclarubicin and 2-[4-(1-oxo-2-isindolinyl)phenyl]propionic acid (indoprofen)—selectively upregulated SMN2-reporter activity. Aclarubicin has been previously shown to modulate the splicing of SMN2 [12]. Here, we describe the SMN regulating activity of indoprofen, a nonsteroidal anti-inflammatory drug.

Results

Indoprofen Selectively Increases Luminescence from an SMN2-Minigene-Reporter Construct

We developed a high-throughput reporter assay designed to detect SMN production. We used C33a cells stably expressing an SMN-minigene with luciferase reporter [13] that consisted of exons 6 through 8 and intervening introns, for either SMN1 or SMN2. Luciferase, the reporter gene’s product, is only produced when proper splicing and translation occurs.

We tested 20,000 compounds from a combinatorial library, 1040 compounds from a National Institute of Neurological Disorders and Stroke (NINDS) library, 2337 compounds from our Annotated Compound Library [14], and 23,685 compounds from our TIC Library, which is a composite of compounds purchased from TimTec, IBS, and ChemBridge that were selected for specific properties, including stereochemical complexity [11]. We discovered that indoprofen significantly increased reporter activity from SMN2-luc cells relative to SMN1-luc cells (Figures 1 and 2). The most effective concentration, 2.8 μg/ml (~10 μM, MW ~ 281.3), resulted in a 3-fold increase in luminescence. The raw data is available in the Supplemental Data of our companion paper in this issue [11] (available online at http://www.chembiol.com/cgi/content/full/11/11/1489/DC1/).

To find related active compounds, we tested those with structures similar to indoprofen, including NSAIDs, and found none that increased luminescence. Com-
Some degree of activity and selectivity, but this may have been due to simple hydrolysis in cells and generation of indoprofen (Figure 1).

Despite the noteworthy increase in SMN2-minigene-reporter activity, real-time RT-PCR using SMA patient fibroblasts failed to show an increase in the ratio of full-length to truncated transcripts, or in the absolute level of transcripts following indoprofen treatment (data not shown). Considering indoprofen’s selective activity for SMN2-luc, it is unlikely that indoprofen is acting post-translationally, as both SMN1-luc and SMN2-luc encode the same protein. We propose that indoprofen has a pre- or cotranslational effect on protein production from SMN2, through a cyclooxygenase-independent mechanism. For example, it is possible that indoprofen, resembling a nucleotide, binds to the SMN2 pre-mRNA and displaces proteins that reduce the rate of translation.

We sought evidence as to whether indoprofen also affects the level of endogenous SMN protein in human cells.

Indoprofen Increases SMN Protein Level in Human Type I SMA Patient Fibroblasts

To assess whether indoprofen treatment affected SMN protein production, we treated type I SMA patient fibroblasts (3813) with indoprofen and assessed protein level by Western blotting (Figure 2). We treated 3813 cells with 5 and 20 \( \mu \text{M} \) indoprofen for 3 days with daily media and compound changes, even though liquid chromatography-mass spectroscopy (LC-MS) revealed that there was no significant degradation of indoprofen over 4 days in cell culture medium (data not shown). Both concentrations of indoprofen treatments yielded similar effects on SMN protein increase, although occasionally higher levels were observed from either treatment concentration (Figure 2). Combining data from both treatments, indoprofen-treated cells resulted in a mean 13% increase in SMN protein production versus untreated cells (independent, one-tailed \( t \) test; \( n = 17 \) [9 treated samples, 8 control samples], \( v = 15, p < 0.014 \)).

Indoprofen Increases Number of Nuclear Gems in Human Type I SMA Patient Fibroblasts

The increase in SMN protein production led us to inquire about the effect of indoprofen on the overall number of gems—punctate structures in the nucleus. The number of gems (short for “Gemini of coiled [Cajal] bodies”) directly correlates with SMN protein production [5], and they are found in many adult cell types (especially neurons) and in all fetal tissues. Fibroblasts from normal patients, SMA carriers, and type I SMA patients have \(~80\) gems, \(~40\) gems, and \(~1\) to \(~2\) gems per 100 nuclei, respectively [5].

We treated human type I SMA fibroblasts (2806) with indoprofen (5 and 15 \( \mu \text{M} \)) to observe changes in gem count. Both indoprofen treatments yielded a significant increase in gem count (5 \( \mu \text{M} \): independent, one-tailed \( t \) test; \( n = 17 \) [8 treated, 9 untreated], \( v = 15, p = 6.9 \times 10^{-4} \) and 15 \( \mu \text{M} \): independent, one-tailed \( t \) test; \( n = 16 \) [7 treated, 9 untreated], \( v = 14, p = 1.7 \times 10^{-3} \) (Figure 2). Pooling the two treatments also generated a significant result (independent, one-tailed \( t \) test; \( n = 24 \) [15 treated,
Indoprofen Upregulates SMN

Figure 2. Indoprofen Increases Minigene-Reporter Luminescence, SMN Protein Level, and Number of Nuclear Gems

(A) C33a cells with a minigene-reporter construct containing either SMN1 (red) or SMN2 (blue) treated with indoprofen. A similar result was obtained in a mouse neuronal cell line (NSC34) (data not shown).

(B) Aspirin (acetylsalicylic acid), acetaminophen (4-acetamidophenol), and ibuprofen were tested in C33a cells with the same constructs as in (A).

(C) Type I SMA patient fibroblasts (13) with no SMN1 have less SMN protein than carrier fibroblasts (14) that have 1 copy of SMN1. NSC34, a mouse neuronal cell line, cells (34) with two copies of SMN1 exhibit an effect that is visually indistinguishable from carrier fibroblasts.

(D) Indoprofen-treated cells had a mean 13% increase in SMN protein production over untreated cells (independent, one-tailed t-test; n = 17, p = 0.0139) as determined by densitometric analysis. Indoprofen-treated samples and control sample are noted. An initiation factor (eIF-4e) is shown as a loading control.

(E) Indoprofen treatment of type I SMA patient fibroblasts (2806) results in an increase in gem count when compared to nontreated cells. Blue circles represent samples within each treatment. Red squares represent the mean number of gems per 100 nuclei within each treatment (0 M: 1.3; 5 M: 6.5; and 15 M: 8.3).

9 untreated], [14] n = 22, p = 3.2 x 10^-2]. Additionally, we tested one other type I SMA fibroblast cell line (3813) and one mouse fibroblast cell line (25). These cell lines yielded comparable results to 2806 (see Supplemental Data available online at http://www.chembiol.com/cgi/content/full/11/11/1489/DC1/).

Effect of Indoprofen in a Mouse Model of SMA

In an attempt to find a maximum effective dose, the pharmacokinetics of indoprofen in mice were studied. Pregnant mice were treated with a single dose of 20 mg/kg of indoprofen, using either intraperitoneal (IP) injection or oral gavage, to determine how much indoprofen enters the brain, blood, and embryo after treatment. Consistent with previous rodent studies using 14C-labeled indoprofen [15], liquid chromatography-mass spectrometry (LC-MS) revealed that no indoprofen was present in brain tissue 4 hr after treatment (data not shown). Indoprofen was found in the both the plasma of the pregnant mice and their embryos one hour after treatment. Both routes of administration resulted in ~5.3 µM indoprofen plasma concentration, while the IP method was shown to be more effective than gavage at delivering indoprofen to embryonic tissue. An average concentration of ~3.0 µM indoprofen was found in embryos of IP-treated mice at embryonic day 13 (E13).

We tested the effect of indoprofen on the viability of SMA model mice, which lack murine Smn but contain
Table 1. Numbers of Non-SMA and SMA Litters and Embryos from Untreated and Indoprofen-Treated Mother Mice

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<thead>
<tr>
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<th>Non-SMA</th>
<th>SMA</th>
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<tr>
<td>Untreated</td>
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</tr>
<tr>
<td>Indoprofen treated</td>
<td>4</td>
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<thead>
<tr>
<th></th>
<th>Non-SMA</th>
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<tr>
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<tr>
<td>Indoprofen treated</td>
<td>39</td>
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a human SMN2 transgene (i.e., Smn-/-; TgSMN2+/+) [16]. To generate litters that contained 25% Smn-/-; TgSMN2+/+ mice, we mated Smn-/-; TgSMN2+/+ mice with Smn+/+; TgSMN2+/+ mice. Our SMA model mice were derived from the published transgenic model created in C57BL/6 and crossed with FVB (Taconic Labs) [16]. These mice were backcrossed once with a wild-type FVB mouse to generate our C57BL/6/FVB mice. We found that in this strain of Smn-/-; TgSMN2+/+, embryos die at approximately embryonic day 11 (E11).

To determine whether indoprofen could increase the viability of these SMA model embryos, we treated such pregnant mice twice daily for the first 14 days of pregnancy by IP injection with 5 mg/kg indoprofen in phosphate buffered saline, the maximum dose that exhibited no toxicity within the 14 days. On embryonic day 14 (E14), we genotyped the embryos and ascertained the number of Smn-/-; TgSMN2+/+ (SMA genotype) embryos (Table 1). At E14, none of the 7 untreated litters harbored any SMA genotype embryos, whereas 3 of 7 indoprofen-treated litters harbored SMA genotype embryos (Fisher Exact test, p = 0.096). Thus, there was a trend in which indoprofen increased the viability of SMA model mice. As expected, indoprofen treatment significantly increased the mean litter size from 5.7 embryos to 6.9 embryos (independent, one-tailed t test; n = 14, p = 0.040). There was also a trend in which indoprofen increased the number of SMA embryos (Fisher Exact test, p = 0.073).

Discussion

Most SMA patients have a homozygous deletion of SMN1 and are consequently left with an insufficient level of SMN protein to prevent disease. This protein has been further implicated in many cellular processes including snRNP biogenesis [17–21], mRNA splicing [22–26], apoptosis [27–30], and axonal transport [31–33].

We have discovered a nonsteroidal anti-inflammatory drug (indoprofen) that produces an increase in luminescence in SMN2-luc cells compared to SMN1-luc and untreated cells. This SMN2-selective activity indicates that indoprofen is not simply acting on the construct’s promoter. Indoprofen treatment of type I SMA patient fibroblasts results in a small increase in SMN protein level, and in the number of nuclear gems. Finally, indoprofen treatment caused a trend in which embryonic viability in SMA model mice was increased. Other candidate drugs for treating SMA patients have shortcomings, such as severe side effects [34], extremely short half-life [35, 36] or nonselectivity of mechanism [37–39]. Indoprofen is an NSAID with minimal side effects [40, 41] that may be worthy of study as a therapeutic agent for SMA. As indoprofen is not modifying splicing, possible additive effects may result from combination treatment with other drugs. In addition, it may serve as a chemical probe to identify proteins that regulate the production of SMN protein.

Significance

Chemical genetic screening approaches can be used to discover both novel therapeutic agents and chemical tools for genetic diseases. We describe such an approach for the pediatric genetic disease spinal muscular atrophy (SMA). We developed a high-throughput assay that detects production of an SMN-luciferase reporter protein, and we used this assay to identify compounds that increase production of SMN protein in cells. In a screen of ~47,000 compounds, we discovered that a nonsteroidal anti-inflammatory drug (indoprofen) increases endogenous SMN protein level in type I SMA patient fibroblasts. Additionally, we found that indoprofen caused a 5- to 6-fold increase in the number of nuclear gems, an indicator of greater intracellular SMN protein concentration. No other NSAIDs displayed this ability to upregulate SMN protein level. This suggests that SMN upregulation is not caused by inhibiting COX activity (the known target of NSAIDs). Finally, we found that indoprofen caused a trend toward increased viability of SMA model mice. Indoprofen may prove to be a treatment for SMA, and it may serve as a chemical tool to identify novel proteins regulating SMN protein production.

Supplemental Data

Detailed experimental procedures are available as Supplemental Data at http://www.chembiol.com/cgi/content/full/11/11/1489/DC1/.

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References

4. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P.,
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