Identification of New Hit Compounds Using a High-Throughput Phenotypic Screen with SMA Patient iPSC-Derived Motor Neurons

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Introduction

Spinal muscular atrophy (SMA) is an inheritable cause of infant mortality that is characterized by the loss of lower motor neurons and skeletal muscle atrophy. The degeneration of motor neurons is caused by insufficient levels of survival motor neuron (SMN) protein, which is encoded by two nearly identical genes *SMN1* and *SMN2*. Most cases of SMA harbor homozygous deletions of the *SMN1* gene and retain at least one copy of *SMN2*. Hence, a promising treatment strategy is to upregulate levels of the full-length SMN protein originating from the SMN2 gene. Drug discovery screening platforms typically use SMA fibroblasts or lymphocytes, yet the identified molecules often had limited efficacy in SMA mouse models, especially rescuing motor neuron (MN) degeneration. Therefore, MNs from SMA patients should be used early in drug discovery to increase the likelihood of identifying effective small molecule therapeutics. At BrainXell, we established new technologies to rapidly differentiate SMA patient induced pluripotent stem cells (iPSCs) into large quantities of neurons. We also used genome editing to endogenously fuse SMN2 with a nanoluciferase (NLuc) reporter, which enables high-throughput screening (HTS) that monitors the expression levels of SMN after 48 h exposure to each compound. The assay was adapted to meet HTS requirements, including: large batch sizes, 1536-well format, minimal well-to-well variation, short-term culture, plating by automated dispenser, and low reagent volumes. Applying a quantitative HTS approach, we screened the LOPAC, NPC, and MIPE libraries (>6,000 compounds) in a dose dependent manner. After demonstrating feasibility, we expanded the screen to the larger Genesis library (~95,000 compounds) in order to identify novel hit molecules. Compounds that increased SMN2 expression by >20% were considered hits. Analysis of the combined ~100,000 compound qHTS identified 81 hit candidates, which were rescreened in triplicate. Ten compounds increased SMN2 expression by 20% with EC_{50} < 10 µM. We then used an ELISA to validate the increased SMN2 expression after 48 h treatment. This screening paradigm identified and validated at least one new hit compound that has promising efficacy, but the potency will require optimization.

Materials and Methods

Establishment of Reporter iPSC Lines Human iPSCs lines, SMA232 were applied in this study. All iPSCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) as described in the standard protocol (www.wicell.org). We applied CRISPR technology to integrate reporter NLuc into the survival motor neuron 2 (SMN2) locus. CRISPR guide RNA pairs, Cas9-Nickase and Donor plasmid were introduced into iPSCs by electroporation. Neomycin was added in the culture medium to select the resistant cells. The neomycin-resistant iPSC colonies were picked and screened by PCR to detect the integration of the reporter. The PCR products were sequenced to confirm the correct in-frame fusion. In the donor plasmid, we flanked the neomycin resistant cassette with two loxP sites; therefore, it was easily removed by Cre recombination. All the reporter iPSC lines were confirmed without mutation in SMN2 gene or off-target sites.

Neuron Differentiation from Human iPSCs Motor Neuron differentiation from human iPSCs was based on protocols described previously (Du et al. 2015. Nat Commun. 6:6626). Briefly, human iPSCs were treated with small molecules for 1 week to induce neuroepithelial progenitors (NEPs). The NEPs were split and treated in additional patterning molecules for another 1 week to generate subtype-specific neuron progenitors. These progenitors were expanded with the combination of small molecules and frozen in cell freezing medium. To accelerate maturation after thawing and seeding, neurons were cultured in medium supplemented with BrainXell Seeding Supplement for another 1-2 weeks.

Multielectrode Array (MEA) Analysis Neurons were seeded at 40,000 cells per electrode area (16 electrodes in the center of each well) in specialized PDL-coated 48-well MEA plates (Axion BioSystems). From maturation Day 7 to 12, activity was recorded for five minutes from all wells using a Maestro recording chamber (Axion BioSystems).

Quantitative HTS Screening MNPs were thawed and plated with a liquid handling system (MultiDrop) at 1,200-1,500 cells/well in 4 µL into all-white uncoated 1536-well plates (unless otherwise noted). On Day 1 (24 hours after thawing and plating), compounds were added by pin tool. On Day 3 NLuc activity was detected using the Nano-Glo Luciferase Assay kit (Promega). Luminescence signal was measured with a ViewLux system (PerkinElmer). Hits were confirmed using equivalent conditions in a 384-well format.

SMA Patient iPSC-Derived Motor Neurons

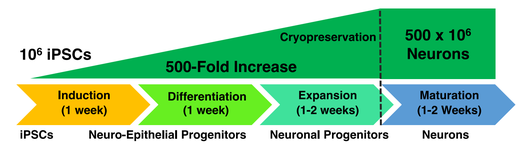

Figure 1. General Protocol for Neuron Production Scheme showing the general production protocol. The time from initiation of iPSC/ESC culture until cryopreservation is 4-6 weeks. Neurons mature 1-2 weeks after plating with BrainXell maturation supplements.

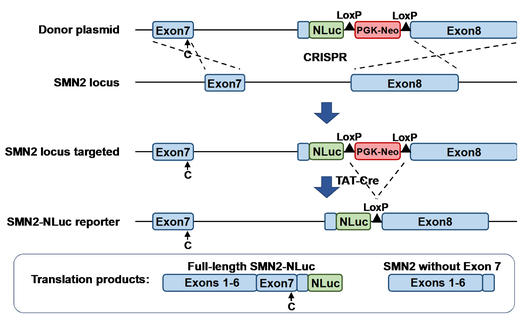

Figure 2. Reporter Construction Schematic showing the strategy to establish the SMN-NLuc reporter line by CRISPR as a single copy at the endogenous locus.

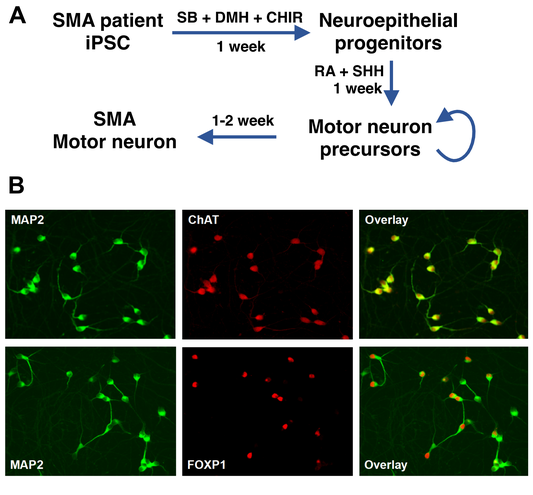

Figure 3. Expression of Motor Neuron Markers *(A)* Motor neurons are differentiated from iPSCs in 3-4 weeks, and can be expanded as precursors prior to maturation. *(B)* Neurons express markers associated with spinal motor neuron identity, including FoxP1, ChAT, and MAP2. In addition, approximately 95% of motor neurons are positive for the mature neuronal marker NeuN.

SMA Assay Development

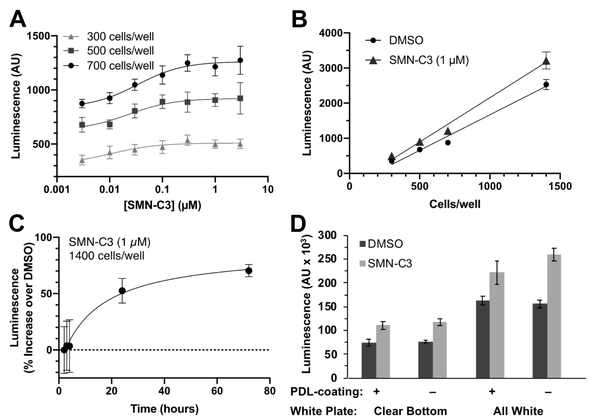

Figure 4. Assay Development. (A) Dose-response curves of SMN-NLuc motor neurons at various cell densities treated for 48 h with positive control compound, SMN-C3 [1]. (B) Linear correlation of cell number versus detected luminescence. At each cell density, SMN-C3 (1 µM) increased luminescence by 30%. (C) Kinetics of SMN expression change after SMN-C3 (1 µM) treatment (compared to DMSO control). (D) Comparison of assay response (luminescence) using plates ± PDL-coating, clear bottom, and all white.

Quantitative HTS (qHTS) Screen

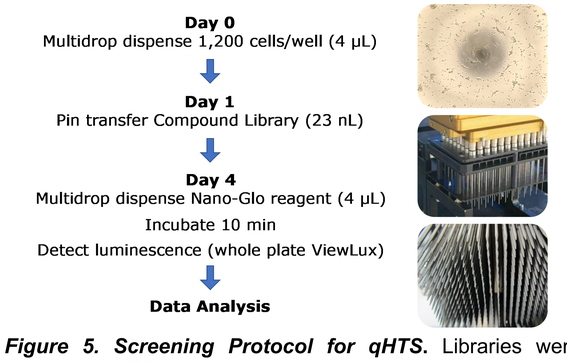

Figure 5. Screening Protocol for qHTS. Libraries were screened in 1536-well format.

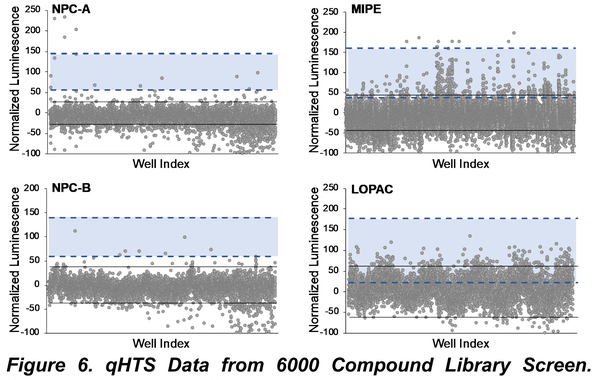

Figure 6. qHTS Data from 6000 Compound Library Screen. Black lines indicate ± 2 standard deviations from the mean of the DMSO control. Blue dashed lines indicate ± 2 standard deviations from the mean of the positive control compound, SMN-C3 (1 µM).

Quantitative HTS (cont'd)

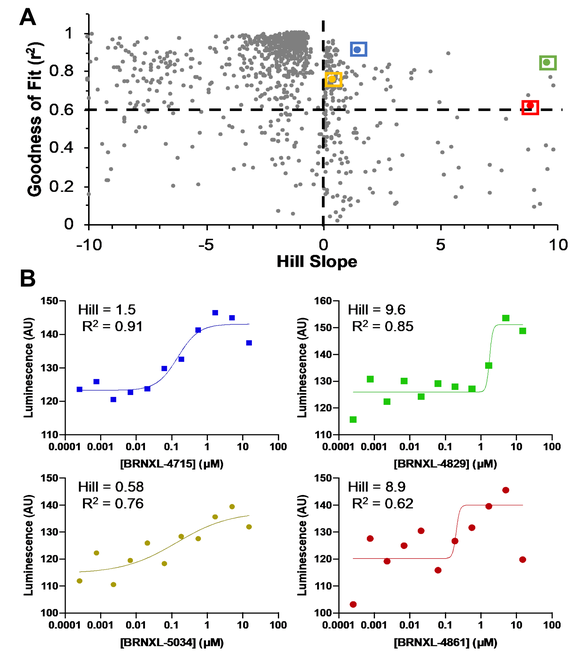

Figure 7. qHTS Data from Rescreen of 431 Hit Compounds from the Genesis Library. (A) Scatter plot of curve fit features of all dose-response curves. Each compound was tested in triplicate. Compounds with at least two dose-response curves with positive hill slope and goodness of fit (r^2) >0.6 were considered hits. (B) Example data from A. Colors correspond to respective data point.

Table 1. Summary of qHTS from Each Library Screen.

Library	Number of Compounds	CV	Response Window	Initial Hits	Verified Hits
LOPAC	1,280	8%	24%	5	–
NPC-A	1,408	6%	37%	3	1
NPC-B	1,408	8%	38%	2	–
MIPE	1,920	12%	36%	29	9
Genesis	95,000			42	6

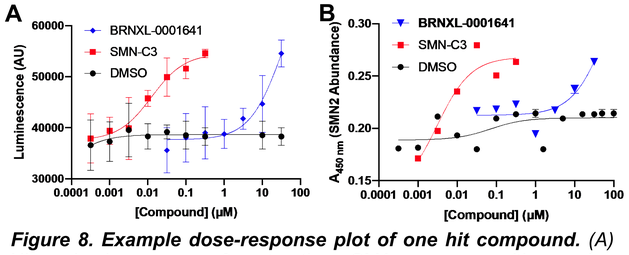

Figure 8. Example dose-response plot of one hit compound. *(A)* NLuc luminescence after treating SMA neurons with varying concentrations of BRNXL-1641, positive control compound SMN-C3, or equivalent volume of DMSO as negative control. Error bars represent one standard deviation among four replicates. *(B)* Preliminary results of an SMN2 ELISA using the same conditions as in panel A.

BRNXL-1641 increases SMN2 expression to equivalent levels as control compound, SMN-C3, but with 100-fold less potency.

References (1) Naryshkin, N. A., et al. (2014). "Motor neuron disease. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy." Science **345**(6197): 688-693.