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Cyclic tetrapeptide HDAC inhibitors as potential therapeutics for spinal muscular atrophy: Screening with iPSC-derived neuronal cells

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder that is caused by inactivating mutations in the Survival of motor neuron 1 (SMN1) gene, resulting in decreased SMN protein expression. Humans possess a paralog gene, SMN2, which contains a splicing defect in exon 7 leading to diminished expression of full-length, fully functional SMN protein. Increasing SMN2 expression has been a focus of therapeutic development for SMA. Multiple studies have reported the efficacy of histone deacetylase inhibitors (HDACi) in this regard. However, clinical trials involving HDACi have been unsatisfactory, possibly because previous efforts to identify HDACi to treat SMA have employed non-neuronal cells as the screening platform. To address this issue, we generated an SMA-patient specific, induced pluripotent stem cell (iPSC) derived neuronal cell line that contains homogenous Tuj1 + neurons. We screened a small library of cyclic tetrapeptide HDACi using this SMA neuronal platform and discovered compounds that elevate SMN2 expression by an impressive twofold or higher. These candidates are also capable of forming gems intranuclearly in SMA neurons, demonstrating biological activity. Our study identifies new potential HDACi therapeutics for SMA screened using a disease-relevant SMA neuronal cellular model.

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder that is characterized by cellular death in the anterior horn of the spinal cord in patients.[1] This causes muscle atrophy and progressive paralysis, ultimately resulting in early death in childhood or juvenile stages.[2] SMA is caused by an inactivating mutation in the *Survival of motor neuron 1* (*SMN1*) gene, leading to decreased SMN protein expression.[3] Humans possess a paralog gene, *SMN2*, which contains a splicing defect in exon 7 that reduces expression of full-length, functional SMN protein.[3] SMN plays an important role in spliceosome biogenesis,[4] and loss of SMN results in selective death of motor neurons through mechanisms that are poorly understood.[5] The low level of full-length SMN protein produced by *SMN2* is insufficient to protect against the disease,[3,6] although studies with SMA mouse models have shown that increasing the genomic copy number of *SMN2* to 8 copies is sufficient to overcome the phenotypic defect caused by the absence of functional *SMN1* genes.[7] Clinically, human SMA patients can be stratified by the most widely used ISMAC staging system into stages I to IV, grouping patients according to age of disease onset.[8] The disease stage also correlates with the copy number of *SMN2*.[9] Together with studies in mouse models, there is substantial rationale for increasing the level of *SMN2* mRNA (and therefore increasing amounts of full-length SMN protein) as a potential therapeutic avenue for treating SMA in humans.

A variety of approaches have been employed for therapeutic development to treat SMA.[10] Restoration of SMN protein was able to reverse the pathology and rescue the disease phenotype in a mouse SMA model,[7] paving the rationale for enhancing *SMN2* encoded protein expression as therapeutic approach. Related strategies include transcriptional activation of *SMN2* by histone deacetylase (HDAC) inhibitors,[11,12,47] correction of the *SMN2* mRNA processing defect by antisense oligonucleotides (ASOs)[13] or small molecules,[14] or increasing the half-life of SMN protein with small molecules.[15] Among current investigational therapeutics, ASOs have progressed the furthest in terms of clinical development with

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successful clinical trial results.[16] Interim analysis of a recent clinical trial revealed encouraging results in a subgroup of SMA patients treated with the investigational ASO drug Nusinersen, leading to the first filing of a New Drug Application (NDA) of therapeutics against SMA,[17] and its subsequent approval in 2017.[18] While current progress of ASOs are encouraging, small molecule development for SMA is not without its merits, since ASO based therapeutics in general are still challenged by difficulties of delivery and higher cost per treatment.[19]

Numerous studies in model systems have supported the efficacy of HDAC inhibition in treating SMA,[20–24] although more advanced efforts using HDAC inhibitors in clinical trials have failed.[25,26] It has been pointed out that the aforementioned attempts at developing HDAC inhibitors for SMA have largely focused on pan-HDAC inhibitors.[27] Therefore it would be advantageous to explore the possibility of isoform selective inhibition in SMA therapeutic development. We have previously described the design and characterization of HDAC inhibitors based on cyclic tetrapeptide structures.[28–31] Some of these compounds lack a hydroxamic acid moiety, a zinc binding functional group commonly found in HDAC inhibitors (such as the FDA approved suberoylanilide hydroxamic acid (Vorinostat)). Instead, our cyclic tetrapeptide HDAC inhibitors typically carry an ethylketone or carboxylic acid zinc binding group, which could enable better discrimination between HDAC isoforms and provide greater flexibility in tailored library design for synthesis and screening purposes. Here we describe our approach toward testing a small library of these cyclic tetrapeptides as potential treatments for SMA. We hypothesized that if proven efficacious, this approach could open a new avenue for developing isoform specific HDAC inhibitors for SMA.

In vitro drug screening in SMA patient derived cell lines has previously been carried out using non-neuronal cells such as fibroblasts or lymphoblasts,[20–22] due to the technical difficulties in obtaining neuronal tissue from patients with neurodegenerative disorders such as SMA. With the advent of induced pluripotent stem cell (iPSC) technology,[32] our group optimized a protocol that produces high purity (>90% *Tuj1* positive staining) neuronal cultures that can be used for screening in neurodegenerative disorders. This neuronal screening platform has previously been used for a different neurological disorder (Friedreich's ataxia) to identify a drug candidate that completed a phase 1 clinical trial with promising results.[33] Here, we applied this methodology to generate high purity, SMA patient iPSC-derived neurons for the screening of the aforementioned cyclic tetrapeptide HDACi molecules. From this platform, we have identified prototypical candidates that show promising efficacy in elevating *SMN2* gene expression in patient specific SMA neurons. Our work provides support for further optimization and development in this class of HDAC inhibitors as a potential treatment for SMA.

We reprogrammed human SMA fibroblasts (GM03813) with the standard four transcription factor cocktail (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) developed by Yamanaka and colleagues to yield induced pluripotent stem cells (iPSCs).[34] Primary fibroblasts were obtained from a 3-year-old male SMA patient who was homozygous for deletion of exons 7 and 8 in the *SMN1* gene, but had three copies of the *SMN2* gene (line GM03813). The iPSCs were characterized by immunostaining and qRT-PCR of pluripotency markers to confirm pluripotency (Fig. 1a and b). These cells express reduced levels of SMN2 mRNA and SMN protein relative to unaffected cells (Fig. 1c).

The iPSCs were then subsequently differentiated into neuronal cells using a recently developed protocol.[33] We optimized our neuronal differentiation protocol to achieve a highly homogenous population of neuronal cells (Fig. 2a, b), which present with >90% Tuj1 positive cellular staining, measured by FACS (Fig. 2c).[33] This ensured that our drug screening would be carried out using a homogeneous background with minimal complicating responses from non-neuronal cells in the population.

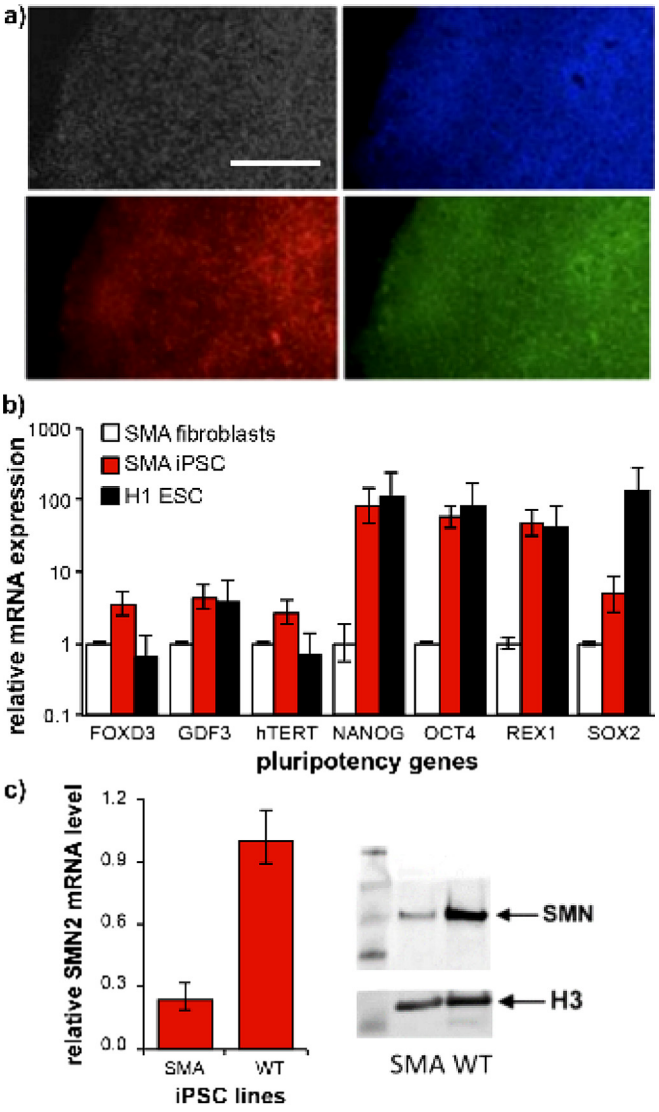

Fig. 1. Generation of SMA patient specific iPS cells. (a) Induced pluripotent stem cells (iPS) derived from GM03813 SMA fibroblasts were immunostained for pluripotency markers. Top left, phase contrast (uncolored); top right, DAPI staining for nuclei (blue); bottom left, Oct4 transcriptional regulator (red); bottom right, stage-specific embryonic antigen (SSEA3, green). Scale bar = 250 μm. (b) Pluripotency marker genes are highly expressed in SMA iPSCs at similar levels to the authentic ES cells. For comparison, the mRNA levels for these genes in the parent GM03813 fibroblasts are far lower than in either the iPSCs or H1 ES cells (note the log scale). (c) Importantly, full-length SMN mRNA (left) and protein (right) levels are lower in the SMA iPSCs than in the iPSCs from an unaffected individual (GM08333).

We previously developed a class of HDAC inhibitor compounds using the natural product apicidin as an inspiration for design. Apicidin was chosen due to its high HDAC inhibition potency and its relatively weak Zn^{2+}-coordinating capability (compared to hydroxamic acids) that could afford improved HDAC isoform selectivity. We developed scaffolds containing one or two *beta*-amino acids in the backbone, resulting in 13- or 14-membered-ring derivatives (Fig. 3).[28–31] High-resolution NMR structures were determined for many of the compounds, allowing us to construct a rudimentary three-dimensional pharmacophore model for HDAC inhibition.[28] In summary, our work yielded conformationally well-defined derivatives that are low-nanomolar HDAC inhibitors with moderate isoform selectivity, comparable to or better than apicidin.[28–31]