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4

Preventing formation of toxic N-terminal huntingtin fragments through antisense oligonucleotide-mediated protein modification

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4.1. Abstract

Huntington's disease (HD) is a progressive autosomal dominant disorder, caused by a CAG repeat expansion in the HTT gene, which results in expansion of a polyglutamine stretch at the N-terminal end of the huntingtin protein. Several studies have implicated the importance of proteolytic cleavage of mutant huntingtin in HD pathogenesis and it is generally accepted that N-terminal huntingtin fragments are more toxic than full-length protein. Important cleavage sites are encoded by exon 12 of HTT. Here we report proof of concept using antisense oligonucleotides to induce skipping of exon 12 in huntingtin pre-mRNA, thereby preventing the formation of a 586 amino acid N-terminal huntingtin fragment implicated in HD toxicity. *In vitro* studies showed successful exon skipping and appearance of a shorter huntingtin protein. Cleavage assays showed reduced 586 amino acid N-terminal huntingtin fragments in the treated samples. *In vivo* studies revealed exon skipping after a single injection of antisense oligonucleotides in the mouse striatum. Recent advances to inhibit the formation of mutant huntingtin using oligonucleotides seem promising therapeutic strategies for HD. Nevertheless, huntingtin is an essential protein and total removal has been shown to result in progressive neurodegeneration *in vivo*. Our proof of concept shows a completely novel approach to reduce mutant huntingtin toxicity not by reducing its expressing levels, but by modifying the huntingtin protein.

4.2. Introduction

Polyglutamine (polyQ) diseases are a group of autosomal dominant neurodegenerative disorders caused by a CAG triplet repeat expansion in protein coding regions of the genome. The most prevalent polyQ disorder, Huntington's disease (HD), is caused by a CAG repeat expansion in the first exon of the *HTT* gene on chromosome 4p16. The expanded CAG transcript is translated into a mutant huntingtin (htt) protein with an expanded polyQ tract at the N-terminus. Carriers of 40 or more CAG repeats will develop HD, whereas people with 35 to 39 repeats show reduced penetrance (Mcneil *et al.*, 1997). The disease is characterized by motor, psychiatric and cognitive impairments and the typical age of onset lies between 30 and 50 years and is inversely correlated to the number of CAGs (Andrew *et al.*, 1993).

In HD, insoluble protein aggregates are found in the nucleus and cytoplasm of cells, indicating that htt protein misfolding is a common feature (Ross and Tabrizi, 2011). Major neuropathology occurs in the striatum but degeneration is seen throughout the brain when the disease progresses (Vonsattel and Difiglia, 1998). Many cellular processes are affected in HD, as is evident from transcriptional de-regulation, mitochondrial dysfunction, and impaired vesicle transport (Ross and Tabrizi, 2011). Several studies have implicated the importance of apoptosis and specifically proteolytic cleavage of mutant htt in HD pathogenesis (Ehrnhoefer *et al.*, 2011). Exon 1 of the *HTT* gene with an expanded CAG repeat is sufficient to induce symptoms in the R6/2 mouse model of HD (Mangiarini *et al.*, 1996), also shorter htt fragments seems to be more pathogenic than longer htt fragments (Crook and Housman, 2011). Aggregates in brains of HD patients can be stained with antibodies directed at N-terminal epitopes but not C-terminal epitopes of htt (Lunkes *et al.*, 2002), suggesting that aggregates contain truncated N-terminal htt fragments. In apoptotic cells, htt is cleaved by cysteine aspartic acid proteases, called caspases (Goldberg *et al.*, 1996). Mutations in caspase-3 at amino acid positions 513 and 552 and caspase-6 motifs at position 586 in mutant htt resulted in cleavage-resistant neuronal and non-neuronal cells with reduced toxicity and less aggregates *in vitro* (Wellington *et al.*, 2000). *In vivo* it was also shown that mutation of amino acid position 586 in the caspase-6 cleavage motif resulted in reversal of the HD phenotype in a YAC128 mouse model (Graham *et al.*, 2006; Pouladi *et al.*, 2009). These mice express the full human genomic HTT, which is translated into a mutant htt protein with 128 glutamines. Using the same YAC128 model, no improvement of HD phenotype was seen after mutations in the caspase-3 cleavage sites at amino acid positions 513 and 552. This suggests that cleavage at position 586 is an important step in HD neuropathology and results in neuronal dysfunction and neurodegeneration (Graham *et al.*, 2006).

Recent advances to inhibit the formation of mutant htt using oligonucleotides seem promising therapeutic strategies for HD (Sah and Aronin, 2011). These approaches make use of RNA interference (RNAi), RNAi-like mechanisms using single-stranded RNAs (ssRNAs) or antisense oligonucleotides (AONs) (Sah and Aronin, 2011; Yu *et al.*, 2012). Lowering mutant htt protein levels would prevent all downstream toxic effects, but complete suppression of

htt may not be desirable since wild-type htt has numerous cellular functions. Htt is reported to act as protector of brain cells from apoptotic stimuli (Rigamonti *et al.*, 2000) and is required in adult neurons and testis (Dragatsis *et al.*, 2000). Knock-out of the homologous htt mouse gene was found to be early embryonic lethal (Zeitlin *et al.*, 1995) and htt inactivation in adult mice was shown to result in progressive neurodegeneration (Dragatsis *et al.*, 2000). However, non-allele-specific reduction of both normal and mutant htt transcripts up to 75% was found to be well tolerated in HD rodents and non-human primates (Boudreau *et al.*, 2009; Drouet *et al.*, 2009; McBride *et al.*, 2011; Kordasiewicz *et al.*, 2012) and resulted in phenotypic reversal up to 4 months post treatment (Kordasiewicz *et al.*, 2012). Although studies so far have shown that lowering of htt levels can be well tolerated, safety and specificity of htt transcript lowering drugs after long-term exposure need to be assessed.

Allele-specific reduction would be preferred since this would leave the wild-type htt protein levels unchanged. This was achieved with oligonucleotides directed against single nucleotide polymorphisms (SNPs) unique to the mutant htt transcript (van Bilsen *et al.*, 2008; Lombardi *et al.*, 2009; Pfister *et al.*, 2009; Warby *et al.*, 2009; Zhang *et al.*, 2009; Carroll *et al.*, 2011). Another approach for an allele-specific reduction of mutant htt is targeting the expanded CAG repeat. Oligonucleotides complementary to the CAG repeat were found to result in allele-specific reduction of htt transcript and protein levels in patient derived cells (Krol *et al.*, 2007; Hu *et al.*, 2009; Gagnon *et al.*, 2010; Yu *et al.*, 2012; Chapter 3).

A novel way to alter toxicity of the mutant htt protein is through protein modification. The major advantage of this approach is that htt transcript and protein levels are unchanged. Using AONs it is possible to mask exons from the splicing machinery resulting in exclusion of the targeted exon (Spitali and Aartsma-Rus, 2012). When the reading frame is intact or restored after exon skipping there is subsequent translation of a modified protein. This exon skipping is a promising therapeutic tool that is already in phase II/III clinical trial for Duchenne muscular dystrophy (DMD) (Cirak *et al.*, 2011; van Putten and Aartsma-Rus, 2011).

In this study we use 2'O-methyl modified AONs with a phosphorothioate backbone to induce an in-frame partial exon 12 skip in human htt pre-mRNA. This resulted in a shorter htt protein lacking the 552 caspase-3 and 586 caspase-6 cleavage site, while total htt protein levels were unaltered. Using *in vitro* caspase-6 cleavage assay, AON treated samples showed less 586 N-terminal htt fragments implied in expanded htt toxicity. Injection of a single dose of AONs in the mouse striatum also resulted in removal of the same 552 caspase-3 and 586 caspase-6 cleavage sites, further supporting the concept that proteolytic site removal by exon skipping could be a potential therapeutic approach to prevent formation of toxic N-terminal htt fragments.

4.3. Materials and Methods

Antisense oligonucleotide design

Our AONs were designed following the guidelines described by Aartsma-Rus (Aartsma-Rus, 2012). In short, the AONs were designed to anneal to in silico predictions of potential exonic splicing enhancer sites (ESEs) (Desmet *et al.*, 2009), which have been shown to be an efficient modulator of splicing (Aartsma-Rus *et al.*, 2005; Wilton *et al.*, 2007). Primarily, AON design was based on targeting an open region in the secondary structure of the target exon as predicted by m-fold (Zuker, 2003) and ensuring favorable thermodynamic properties (Aartsma-Rus *et al.*, 2009). Furthermore, cytosine-phosphate-guanine (CpG) dinucleotides were avoided in the AON design, as these potentially activate the Toll-like receptor-9 inflammatory response (Bauer *et al.*, 2001). Finally, sequences were BLAST-verified using megablast general algorithm parameters and short input sequence for the absence of stretches more than 15 homologous nucleotides to the entire genomic sequence of the relevant species.

Table 1. Antisense oligonucleotides sequences used for transfection and injection.

AON Name	Sequence (5' - 3')
AON12.1	GUCCCAUCAUUCAGGUCCAU
Control AON	UCCUUUCAUCUCUGGGCUC
mAON12.1	GGCUCAAGAUGUCCUCCUCAUCC
mAON12.2	UUUCAGAACUGUCCGAAGGAGUC
mAON13	GGCUGUCCUAUCUGCAUG
Scrambled AON	CUGAACUGGUCUACAGCUC

Cell culture and transfection

Patient derived fibroblasts from HD patients (GM04022, purchased from Coriell Cell Repositories, Camden, USA) and controls (FLB73, a kind gift from Dr. M.P.G. Vreeswijk, LUMC) were cultured at 37°C and 5% CO2 in Minimal Essential Medium (MEM) (Gibco Invitrogen, Carlsbad, USA) with 15% heat inactivated fetal bovine serum (FBS) (Clontech, Palo Alto USA), 1% Glutamax (Gibco) and 100 U/ml penicillin/streptomycin (Gibco).

AON transfection was performed in a 6-wells plate with 3 µl of Lipofectamine 2000 (Life Technologies, Paisley, UK) per well. AON and Lipofectamine 2000 were diluted in MEM to a total volume of 500 µl and mixtures were prepared according to the manufacturer's instruction. Four different transfection variables were used: 1) transfection with 1-200 nM AON12.1, 2) transfection with h40AON2 directed against exon 40 of the *DMD* gene (Control AON) (Aartsma-Rus *et al.*, 2002), 3) transfection without AON (Mock). For AON sequences, see **Table 1**. Mixtures were added to a total volume of 1 ml of MEM. Four hours after transfection, medium was replaced with fresh medium containing 5% FBS. A control AON with a 5' fluorescein label was used to ascertain optimal transfection efficiencies by counting the number of fluorescent nuclei (in general, over 80% of all nuclei). All AONs consist of 2'-O-methyl RNA and contain a full-length phosphorothioate backbone (Eurogentec, Liege, Belgium).

RNA analysis

Twenty four hours after the first transfection total RNA was isolated from cells using the Aurum Total RNA Mini Kit (BioRad, Hercules, USA), with an on-column DNase treatment for 30 minutes. RNA was eluted in 40 µl elution buffer and cDNA was synthesized from 500ng total RNA using the Transcriptor First Strand cDNA Synthesis Kit with Random Hexamer primers at 65°C (Roche, Mannheim, Germany).

PCR was performed using 2 µl cDNA, 10x PCR buffer with 1.5 mM $MgCl_2$ (Roche), 0.25 mM dNTPs, 10 pmol of both forward and reverse primer (Eurogentec), 1U FastStart Taq DNA Polymerase (Roche), and PCR grade water to a final volume of 20 µl. PCR was performed with primers flanking exon 9 to 16 of the human sequence (see **Table 2**). The PCR program started with a 4 min initial denaturation at 95°C, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 59°C, 70 sec elongation at 72°C, after which a final elongation step was performed at 72°C for 7 min.

Lab-on-a-Chip was performed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), using the Agilent DNA 1000 Kit.

The qPCR was performed on RNA extracted from striatal tissue isolated from mouse brain, using 2 µl of 5x diluted cDNA, 20x EvaGreen-qPCR dye (Biotium, Hayward, USA), 10x PCR buffer with 1 mM $MgCl_2$ (Roche), 0.25 mM dNTPs (Roche), 2.5 pmol forward primer, 2.5 pmol reverse primer, 0.35U FastStart Taq DNA Polymerase (Roche), and PCR grade water to a total volume of 10 µl. Primer pairs located in various exons of htt were selected for qPCR using Primer3 software (Rozen and Skaletsky, 2000) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) and ribosomal protein L22 (Rpl22) were used as reference genes. (For primer list, see **Table 2**). The qPCR was performed using the LightCycler 480 (Roche). Initial denaturation was 10 min. at 95°C, followed by 45 cycles of 10 sec. denaturation at 95°C, 30 sec. annealing at 59°C and 20 sec. elongation at 72°C. The final elongation was performed 5 min. at 72°C.

Primer efficiencies were determined using LinRegPCR v2012.0 with the raw data amplification curves as input. The raw data were baseline corrected and absolute transcript level expressions (N0) were calculated as described previously (Ruijter *et al.*, 2009). All samples were run in triplicate on a plate. On all plates both reference genes were included to correct for inter-plate variance.

Sanger sequencing

Full-length and skipped products were amplified using exon 9 forward or htt exon 16 reverse primer (see **Table 2**). PCR products were loaded on agarose gel and bands were extracted using the QIAquick Gel Extraction Kit (QIAgen). The purified products were re-amplified, purified, and analyzed by Sanger sequencing, using the Applied Biosystems 96-capillary 3730XL system (Life Technologies Corporation, Carlsbad, USA) with the Applied Biosystems BigDyeTerminator v3.1 kit.

Table 2. Primer sequences used for Sanger sequencing and (quantitative) RT-PCR.

Target gene	Species	Primer Name	Application	Sequence (5' - 3')
HTT	Human	hHttEx9Fw1	RT-PCR	GAGCTTCTGCAAACCCTGAC
HTT	Human	hHttEx16Rev1	RT-PCR	CTTCACGCTGACCCTCACAT
Htt	Mouse	mHttEx11Fw1	RT-PCR	TCCAGGTCAGATGTCAGCAG
Htt	Mouse	mHttEx14Rev1	RT-PCR	CTATGGCCCATTCTTTCCAA
Htt	Mouse	mHttEx12Fw1	qRT-PCR	CCACTCCTGGTTCTGTTGGT
Htt	Mouse	mHttEx12Rev1	qRT-PCR	TGGGATCTAGGCTGCTCAGT
Htt	Mouse	mHttEx13Fw1	qRT-PCR	GTTAGATGGTGCCGATAGCC
Htt	Mouse	mHttEx13Rev1	qRT-PCR	GTCCTCCTGTGGCTGTCCTA
Htt	Mouse	mHttEx27Fw1	qRT-PCR	ACGGAAAGGGAAGGAGAAAG
Htt	Mouse	mHttEx27Rev1	qRT-PCR	CACCAACTTTCTTGGGACTCA
Rpl22	Mouse	mRpl22Ex3Fw1	qRT-PCR	AGGAGTCGTGACCATCGAAC
Rpl22	Mouse	mRpl22Ex3Rev1	qRT-PCR	TTTGGAGAAAGGCACCTCTG
Ywhaz	Mouse	mYwhazEx4Fw1	qRT-PCR	TCTGGCCCTCAACTTCTCTG
Ywhaz	Mouse	mYwhazEx4Rev1	qRT-PCR	AGGCTTTCTCTGGGGAGTTC

Abbreviations: HTT, huntingtin; Rpl22, ribosomal protein L22; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

Protein isolation, caspase-6 assay and Western blotting

Cells were detached from the culture surface with a 0.5% Trypsin/EDTA solution three days after transfection. After washing twice with HBSS, cells were resuspended in 200 µl ice cold caspase lysis buffer, containing 50 mM Hepes, 50 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.1% CHAPS. Next, samples were sonicated 3 times for 5 seconds using ultrasound with an amplitude of 60 at 4°C. After 1 hour incubation in a head-over-head rotor at 4°C, the extract was centrifuged for 15 min at 10,000 g and 4°C and supernatant was isolated. Protein concentrations were determined by the bicinchoninic acid kit (Thermo Fisher Scientific, Waltham, USA) using Bovine Serum Albumin as a standard. Samples were snap frozen and stored at -80°C.

For *in vitro* caspase cleavage, 30 µg protein lysate was incubated with 0.5 to 1.5 U human recombinant caspase-6 (Calbiochem, Darmstadt, Germany) for 5 hours at 37°C.

Protein extracts were separated by Criterion XT Tris-Acetate Gel, 3–8%, 12-wells (BioRad). For each sample both the PageRuler prestained and Spectra Multicolor High Range protein ladders (Thermo Fisher Scientific) were used as markers. Gels were blotted onto a nitrocellulose membrane using the Transblot Turbo (BioRad) for 30 min at 2.5 A. Membranes were blocked with Tris Buffered Saline (TBS) containing 5% non-fat milk powder (Profitar Plus, Nutricia, Zoetermeer, the Netherlands). The antibodies used for detection of htt were 4C8 (MAB2166) for htt (Millipore, Billerica, USA) dilution 1:1,000. A β-actin antibody (mouse AC-15 (Abcam,Cambridge, UK) , dilution 1:2000) was used as loading control. Secondary antibody was goat α-mouse-IRDye800 (LI-COR Biosciences, Lincoln, USA), diluted 1:5,000 in block buffer. Odyssey scanner (LI-COR) was used to visualize infrared bands. Intensities of protein

bands were quantified using Odyssey software. The skipping efficiencies were calculated as described in calculations and statistical analysis paragraph.

In vivo injection into mice

Mouse htt specific AONs (mAON12.1, mAON12.2, and mAON13) and scrambled control AONs were injected in anesthetized C57bl/6j male mice between the ages of 12-14 weeks (Janvier SAS, France). For AON list, see **Table 1**. Animals were singly housed in individually ventilated cages (IVC) at a 12 hour light cycle with lights on at 7 am. Food and water were available ad libitum. All animal experiments were carried out in accordance with European Communities Council Directive 86/609/EEC and the Dutch law on animal experiments and were approved by the Leiden University animal ethical committee (protocol number: 11203). Animals were anesthetized with a cocktail of Hypnorm-Dormicum-demineralized water in a volume ratio of 1.33:1:3. The depth of anesthesia was confirmed by examining the paw and tail reflexes. When mice were deeply anesthetized they were mounted on a Kopf stereotact (David Kopf instruments, Tujunga, USA). A total of 30 µg AON mix diluted in 2.5 µl sterile saline was bilaterally injected at the exact locations 0.50mm frontal from bregma, ±2.0mm medio-lateral, and -3.5mm dorso-ventral. For injections, customized borosilicate glass micro-capillary tips of approximately 100 µm in diameter, connected to a Hamilton needle (5 µl, 30 gauge) were used. The Hamilton syringe was connected to an injection pump (Harvard apparatus, Holliston, MA, USA) which controlled the injection rate set at 0.5 µl/min. After surgery the animals were returned to the home cage and remained undisturbed until sacrifice, with the exception of daily weighing in order to monitor their recovery from surgery. After 7 days the mice were sacrificed by intraperitoneal injection of overdose Euthasol (ASTfarma, Oudewater, the Netherlands) and brain tissue isolated and snap frozen till further analysis.

Calculations and statistical analysis

RNA and protein skipping percentages were calculated using the following formula: Skipping % = (Molarity skipped product / (Total molarity full length product + skipped product)) * 100%. The 586 N-terminal htt fragment levels were calculated using β-actin as reference. The skipping percentages were analyzed using a paired two-sided Student t test. Differences were considered significant when $P < 0.05$.

4.4. Results

Exon 12 skipping resulted in a shorter htt protein resistant to caspase-6 cleavage

The first amino acid of the 586 caspase-6 site previously implicated in disease pathology (Graham *et al.*, 2006), is encoded in exon 12 and the last three amino acids are encoded in exon 13. Exon 12 also encodes two caspase-3 sites at amino acids 513 and 552 (Wellington *et al.*, 1998; Wellington *et al.*, 2000). Our initial aim was to generate a shorter htt protein lacking these 3 caspase sites by skipping both exon 12 and 13. This double exon skip would be necessary to maintain the open reading frame and subsequently protein translation. Therefore, we transfected various AONs (**Table 1**) in patient derived fibroblasts, total RNA was isolated after 24 hours and cDNA was amplified using htt primers flanking the skipped exon to examine skipping efficiencies.

However, after transfection of one of the exon 12 AONs, AON12.1, that targets an ESE in the 3' part of exon 12, a 135 base pair partial skip of exon 12 was observed (**Figure 1a**). This in-frame skip was confirmed by Sanger sequencing (**Figure 1b**). The highest skipping percentage of AON12.1 in patient derived fibroblasts was 59.9% (±0.7%) at a concentration of 50 nM (**Figure 1c**). The partial exclusion of the 3' part of htt exon 12 can be explained by activation of a cryptic 5' splice site present in exon 12 (AG|GTCAG (Zhang, 1998)) (**Figure 1d**). A thus modified htt protein also lacks the active caspase-3 site at amino acid 552 (DLND), and the isoleucine (I) of the active caspase-6 site at amino acid 586 (IVLD) is replaced by a glutamine (Q).

Western blot analysis using the 4C8 antibody indeed revealed a 5 kDa shorter htt protein (**Figure 2a**), which is in concordance with the predicted 45 amino acid skip. Three days after a single AON12.1 transfection, 27.7% (±5.4%) of total htt protein levels consisted of this shorter htt protein (**Figure 2b**). There was no decreased cell viability after AON transfection *in vitro* (**Figure S1**).

To show a reduction in the amount of the 586 N-terminal htt protein fragments that are normally formed after caspase-6 cleavage, we performed an *in vitro* caspase-6 assay. Protein was isolated from human fibroblasts three days after treatment with 50 nM of AON12.1. After samples were incubated with recombinant active caspase-6, the 586 N-terminal htt protein fragment was detected at 98 kDa by Western blot (**Figure 2a**). Samples treated with AON12.1 resulted in a 48.9% (±11.2%) reduction of these 586 N-terminal htt protein fragments (**Figure 2c**). Furthermore, changing the first amino acid of the amino acid 583 to 586 caspase-6 motif is sufficient to prevent the formation of the toxic 586 N-terminal htt protein fragments.

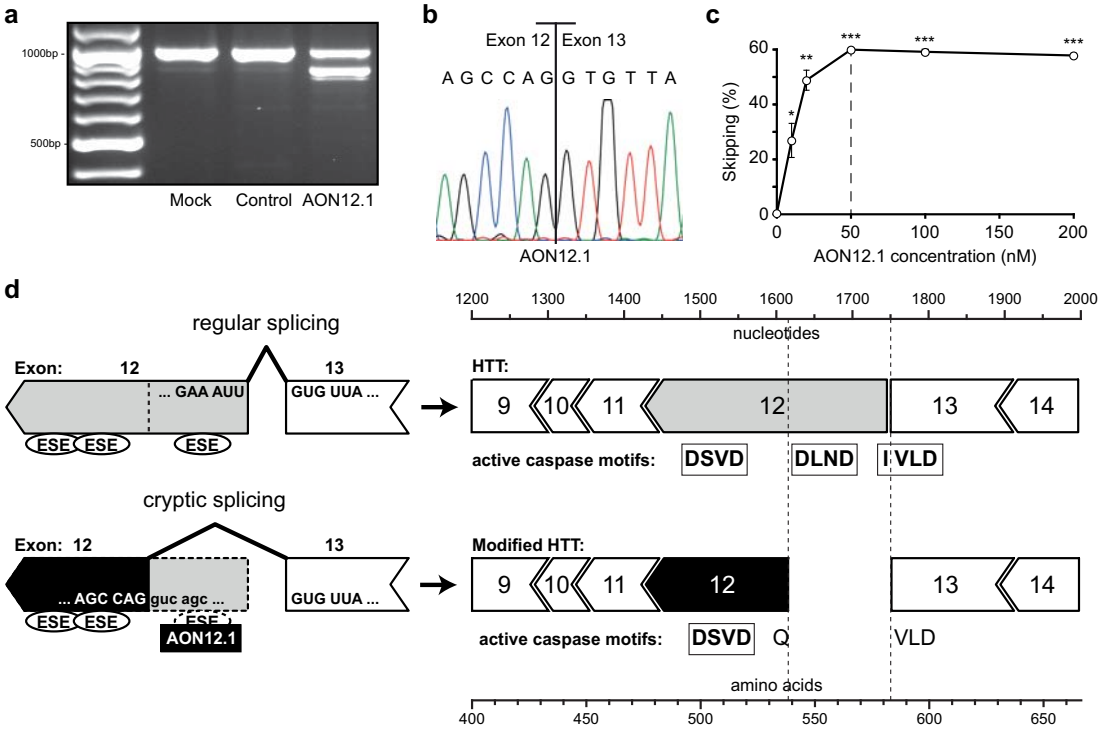

Figure 1. In-frame partial skip of human htt exon 12. Patient-derived fibroblasts were transfected with a htt AON targeting exon 12 (AON12.1), control AON, and non-transfected cells (Mock) and RNA was isolated after 24 hours. **(a)** Agarose gel analysis of the htt transcript with primers flanking exon 12 and 13. Transfection with 100 nM AON12.1 resulted in a product lacking the 3' part of exon 12. **(b)** Partial skip of exon 12 by AON12.1 was confirmed by Sanger sequencing. **(c)** Lab-on-a-Chip analysis was performed to calculate skip levels with AON12.1 concentrations ranging from 10 to 200 nM. Mean ± SD, data were analyzed using paired student t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, relative to mock transfection, n = 4. **(d)** Schematic representation of partial htt exon 12 skipping and subsequent changes in the 552 and 586 caspase cleavage motifs on the htt protein. AON12.1 targets an exonic splicing enhancer site (ESE) cluster in the 3' part of htt exon 12, activating a 5' cryptic splice site and an in-frame exclusion of the 3' part of exon 12. Skipping of the distal part of exon 12 results in the translation of a modified htt protein, lacking the caspase-3 site at amino acid 552 (DLND), and the isoleucine **(I)** of the active caspase-6 site at amino acid 586 (IVLD) is replaced by a glutamine **(Q)**.

In vivo htt exon 12 skipping

To investigate the potential of htt exon skipping *in vivo* and to test if removal of the amino acid sequence surrounding the 586 caspase-6 cleavage site could be harmful *in vivo*, we designed AONs homologous to the mouse sequence. Since mice do not exhibit the cryptic splice site responsible for the partial skip in human cells, we could only investigate the in-frame full skip of both exon 12 and 13. This required the combined application of three AONs: mAON12.1, mAON12.2, and mAON13.

As proof of principle, murine C2C12 cells were transfected with 200nM of mAON12.1, mAON12.2, and mAON13. This resulted in a skip of both exons with an efficiency of 86.8% (±5.6) (**Figure S2**).

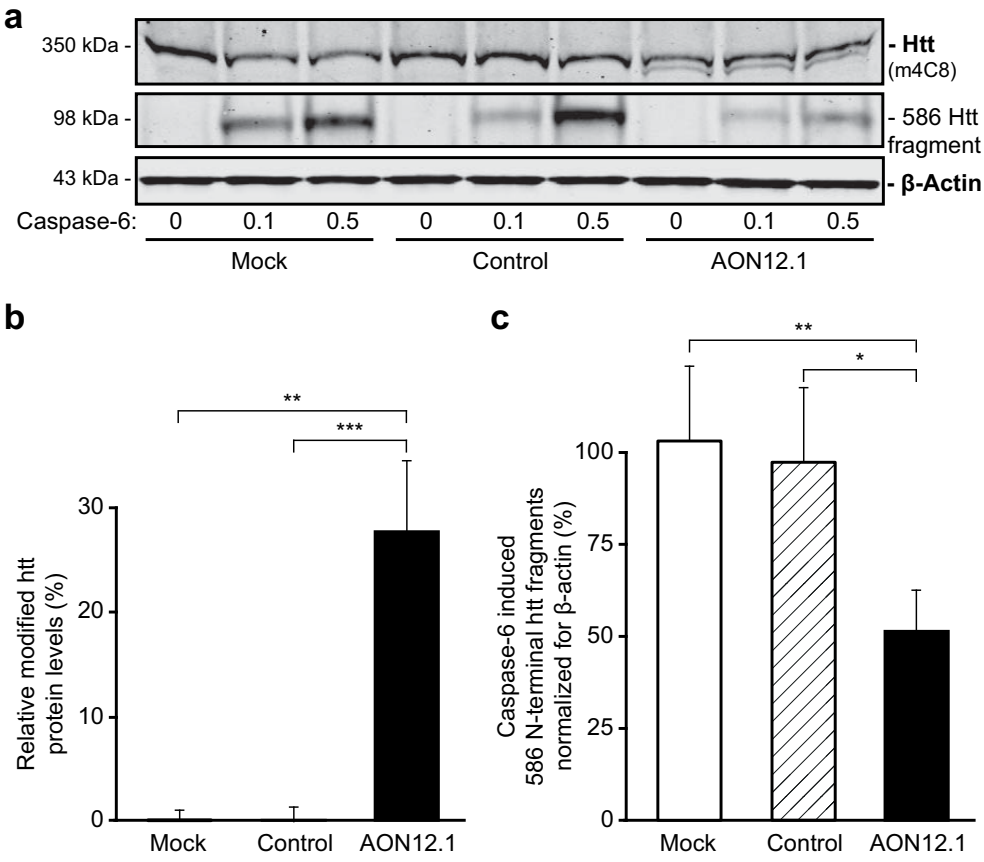

Figure 2. Modified htt protein after partial exon 12 skipping is resistant to caspase-6 cleavage. Patient-derived fibroblasts were transfected with 50nM AON12.1 and control AONs. **(a)** Transfection with AON12.1 resulted in the appearance of a modified htt protein of approximately 343 kDa. *In vitro* caspase-6 cleavage assay shows that the 586 N-terminal htt fragment (98 kDa) increases with increasing concentration of caspase-6. In samples from cells treated with AON12.1 this 586 N-terminal htt fragment is reduced, while the loading control β-actin remains unchanged. **(b)** Levels of modified htt protein after transfection with AON12.1 determined by Odyssey software quantification, normalized for β-actin. **(c)** Quantification of the 586 N-terminal htt fragment, determined by Odyssey software, normalized for β-actin. Mean ± SD, data were evaluated using paired student t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 6.

Next, a single dose of 30 µg scrambled AON or 30 µg mAON12.1, mAON12.2, and mAON13 (10 µg per AON) was injected bilaterally into the mouse striatum. After 7 days the mice were sacrificed and expression levels of exon 12 and 13 in the mouse htt transcript were assessed by qRT-PCR (**Figure 3**). Exon 12 was significantly reduced by 21.5% (±8.5%) and exon 13 was significantly reduced by 23.1% (±8.3%). Exon 27, downstream of the area targeted for skipping, was not reduced.

This reduction in htt exon 12 and 13 after single injection in the mouse striatum further supports the concept that exon skipping could be a potential therapeutic approach to prevent formation of toxic 586 N-terminal htt fragments.

4.5. Discussion

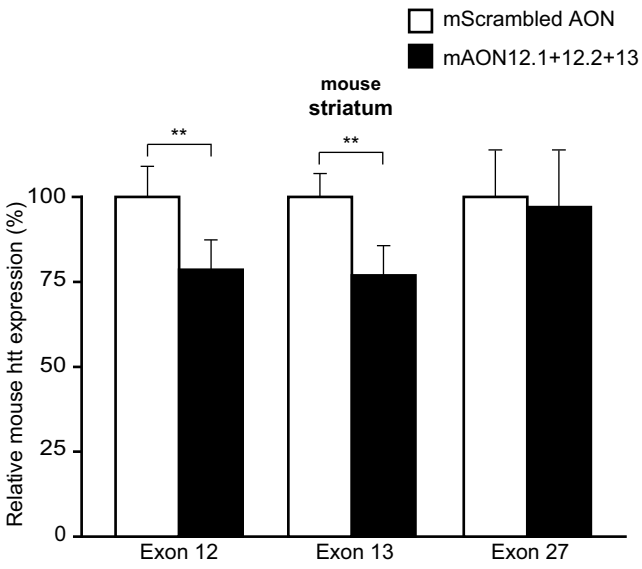

Figure 3. Reduction of mouse htt exon 12 and 13 after a single local injection into the mouse striatum. A single injection consisting of mAON12.1, mAON12.2 and mAON13 (10 µg each) or 30 µg scrambled AON was injected bilaterally into the mouse striatum. After 7 days the mice were sacrificed and the presence of exon 12, 13 and 27 in the htt transcript was examined by qRT-PCR. Mean + SD, data were evaluated using paired student t-test, * $P < 0.05$, n = 5.

Our results provide proof of concept that AON-mediated exon skipping can remove the caspase-3 and caspase-6 motifs from the htt protein, both *in vitro* and *in vivo*. *In vitro* skipping, at least in the human transcript, counteracted the formation of the 586 N-terminal htt protein fragment implicated in toxicity of expanded htt. This htt protein modification is therefore a novel potential therapeutic approach. Our results in patient-derived fibroblasts show that a single AON can remove the 3' part of exon 12 from the human htt mRNA through activation of a cryptic splice site in exon 12. This results in the formation of a shorter htt protein resistant to caspase-6 cleavage. A single injection of AONs targeting htt exon 12 and 13 into the striatum of control mice already resulted in a 22% reduction of htt exon 12 and 13. *In vivo* skipping efficiencies are known to be lower than *in vitro* and 20 to 25% AON-induced splicing already has been shown to result in phenotypic improvements in DMD (Tanganyika-de Winter *et al.*, 2012) and Usher syndrome mice (Lentz *et al.*, 2013).

Possibly, not only this 586 N-terminal htt fragment, but smaller fragments may be toxic entities (Sathasivam *et al.*, 2013; Lunkes *et al.*, 2002; Waldron-Roby *et al.*, 2012). However, the formation of the 586 N-terminal mutant htt protein fragments by caspase-6 cleavage was found to be crucial in the pathogenesis of HD (Graham *et al.*, 2006; Wellington *et al.*, 2000). Notably, caspase-6 is activated in the striatum and frontal cortex of (pre-symptomatic) HD patients and this activation inversely correlates with the age of disease onset, as well as with the CAG repeat size (Graham *et al.*, 2010). Mice expressing the 586 N-terminal htt expanded polyQ fragment develop symptoms similar to mouse models with shorter N-terminal polyQ fragments (Warby *et al.*, 2008; Tebbenkamp *et al.*, 2011; Waldron-Roby *et al.*, 2012) and removal of the 586 caspase-6 site from the full-length mutant htt protein, prevents this phenotype (Graham *et al.*, 2006; Pouladi *et al.*, 2009), underscoring the significance of this particular htt protein fragment, and suggesting that modifying the htt protein using AONs to prevent the formation of the N-terminal 586 htt fragment would be beneficial. That caspase-6 is not exclusively responsible

for the formation of the 586 N-terminal htt fragment was concluded from experiments where a transgenic HD mouse model was crossed with a caspase-6 knock-out mouse. These mice did show the same 586 amino acid N-terminal htt fragment, suggesting that other proteases can also cleave the caspase-6 motif (Gafni *et al.*, 2012). Our exon skip approach does not target the proteases, but removes the proteolytic motif proper, implicated in enhanced toxicity from the htt protein (Warby *et al.*, 2008; Tebbenkamp *et al.*, 2011; Waldron-Roby *et al.*, 2012).

A key question for translating genetic therapies into clinical applications for neurodegenerative disorders is how to administer AONs into the human brain. Since AONs do not cross the blood-brain-barrier, a more invasive delivery method was applied by intracranial injection (Miller *et al.*, 2013). AONs thus infused have been shown to diffuse throughout the non-human primate brain and could be detected in the nuclei and cell bodies of neurons and glial fibrillary acidic protein (GFAP)-positive astrocytes in the striatum, hippocampus, cerebellum, cortex, and spinal cord (Kordasiewicz *et al.*, 2012). The stability, potency, and broad distribution of AONs in the brain marks them as good candidate for potential htt lowering therapeutic for HD.

In our approach, AON-mediated reduction of the toxic N-terminal htt fragment is achieved without lowering of overall htt expression. This would be an advantage over non-allele-specific htt reduction approaches. In HD there is a gain of toxic function of the mutant htt protein, but regular htt is important for normal cellular function (Dragatsis *et al.*, 2000) and is essential during development (Zeitlin *et al.*, 1995). Kordasiewicz *et al.* showed that 4 months repression of total htt of around 50% to 75% did not produce side effects in rats and non-human primates (Kordasiewicz *et al.*, 2012). However, the effects of non-allele-specific lowering of htt over longer time periods has not yet been studied. This is important since HD carriers or patients probably have to be treated lifelong.

For this AON-mediated htt protein modification a single AON would be applicable to the entire HD patient population. Furthermore, by specifically removing critical caspase motifs in htt, there is less chance of unwanted side effects that could result from pharmacological inhibition of overall caspase-6 activity. On the other hand, while it is plausible to expect that the removal of a small stretch of 45 internal amino acids from htt will not, or only modestly, affect htt function - and only after the start of treatment - the extent of this remains to be established. We aim to study this further, as well as the effect on the HD-phenotype, by sustained intraventricular infusion of exon skipping AONs in HD animal models.

In conclusion, in the current manuscript we provide proof of principle for a novel approach to reduce mutant htt toxicity by modifying the protein proper, without changing its protein level. This would provide a valuable addition to the emerging field of AON treatment strategies for neurodegenerative disorders.

4.6. Acknowledgements

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4.7. Supplementary Material

Supplementary Materials and Methods

Cell culture mouse cells

Mouse myoblasts C2C12 (ATCC, Teddington, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% FBS, 1% glucose, 2% Glutamax and 100 U/ml P/S. For AON sequences, see **Table 1**.

PCR mouse huntingtin

PCR was performed with forward and reverse primer in respectively exon 9 and 14 of the mouse htt sequence (see **Table 2**).

Cell viability assay

Two days after transfection in a 96-wells plate, cells were prepared for Cellomics multiparameter cytotoxicity version 3 (Thermo Fisher Scientific) measurements, according to manufacturer's instructions. Cell viability was analyzed on the Array Scan VTI reader (Thermo Fisher Scientific) using the following absorption/emission filter sets: (1) total nuclear intensity: 350/461 nm, (2) lysosomal mass: 646/674 nm, and (3) cell permeability: 491/509 nm. All transfections were performed in triplicates on one plate.

Supplementary Figures

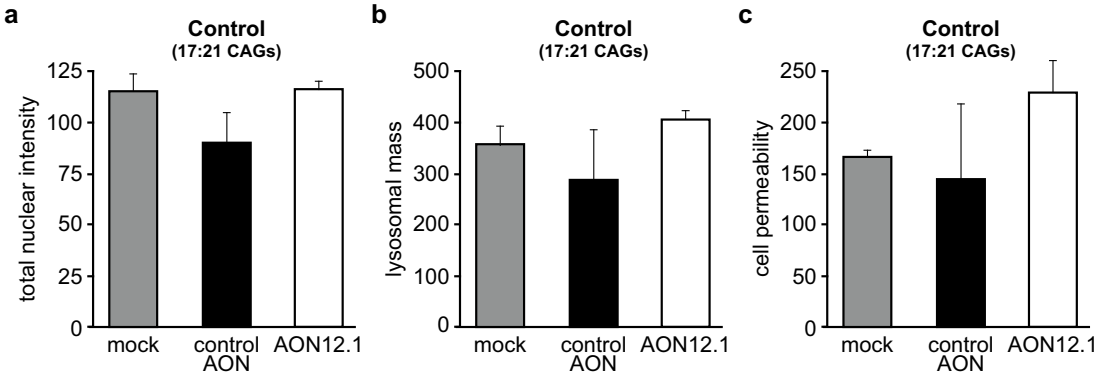

Figure S1. No negative effect htt AON treatment on cell viability *in vitro*. Control fibroblasts were transfected with htt AONs inducing partial skip of exon 12 (AON12.1), control AON, and non-transfected (mock). Mean ± SD, n = 2. The **(a)** total nuclear intensity, **(b)** lysosomal mass, and **(c)** cell permeability were measured. Mean ± SD, n = 2.

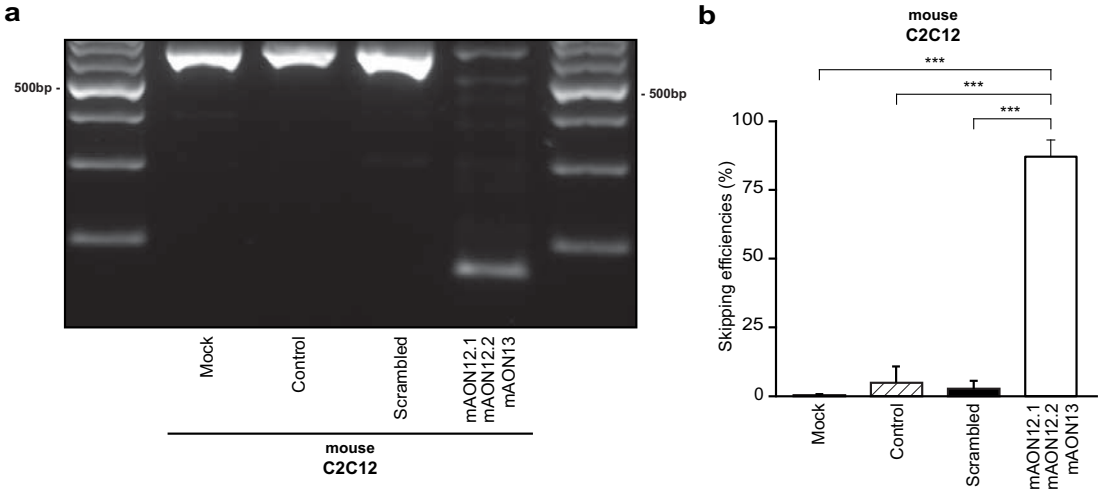

Figure S2. Skipping murine htt exon 12 and 13 *in vitro*. Mouse C2C12 cells were transfected with murine htt AONs, control AON, scrambled AON, and not transfected (Mock). **(a)** Agarose gel analysis of the htt transcript with primers flanking exon 12 and 13. Skipping of htt exon 12 and 13 is seen after transfection with mAON12.1, mAON12.2, and mAON13. **(b)** Lab-on-a-Chip analysis of double-exon skipping after AON treatment. Mean ± SD, data were evaluated using paired student t-test, *** P<0.001, relative to mock transfection, n = 4.