ORALLY BIOAVAILABLE, SMALL-MOLECULE SPLICING MODIFIERS WITH SYSTEMIC AND EVEN HTT-LOWERING ACTIVITY *IN VITRO* AND *IN VIVO*

Anuradha Bhattacharyya,[1] Kerstin A. Effenberger,[1] Christopher R. Trotta,[1] Jana Narasimhan,[1] Wencheng Li,[1] Matthew G. Woll,[1] Minakshi Jani,[1] Nicole Risher,[1] Shirley Yeh,[1] Yaofeng Cheng,[1] Nadiya Sydorenko,[1] Marla Weetall,[1] Amber Southwell,[2] Michael Hayden,[3] Joseph M. Colacino,[1] and Stuart W. Peltz[1]

1. PTC Therapeutics, Inc. 100 Corporate Court, South Plainfield, NJ, USA; 2. Burnett School Of Biomedical Sciences, College Of Medicine, University Of Central Florida, Orlando, FL, USA; 3. Centre For Molecular Medicine And Therapeutics, Department Of Medical Genetics, University Of British Columbia, Vancouver, Canada.

Abstract

Background:

HD is a hereditary neurodegenerative disorder caused by expansion of cytosine-adenine-guanine trinucleotide repeats in the *HTT* gene. Consequently, the mutant protein is ubiquitously expressed and drives pathogenesis of HD through a toxic gain-of-function mechanism.[1–3] Animal models of HD demonstrate that reducing HTT protein levels alleviates motor and neuropathological abnormalities, supporting huntingtin lowering as a therapeutic approach.[2] Clinical and preclinical stage modalities, including antisense oligonucleotides, virally delivered micro RNAs, and zinc finger protein transcription factors, reduce HTT levels by repressing *HTT* transcription, stability, and/or translation.[1,2] These modalities require invasive procedures to deliver the drug to the CNS and do not achieve broad CNS distribution. These compounds act via a novel mechanism promoting the inclusion of a pseudoexon containing a premature termination codon (stop-codon psiExon), leading to *HTT* mRNA degradation and reduction of HTT levels.

Aims

We aimed to develop a class of small-molecule splicing modifiers specifically synthesised to promote selective splicing and ultimately reduction in huntingtin mRNA and protein levels.

Methods/Techniques

Here we describe the identification of small-molecule splicing modifiers lowering HTT expression by selective modulation of the critical recognition step of pre-mRNA splicing.

Results/Outcome

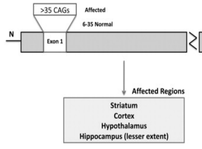
The results presented here demonstrate the potential of orally bioavailable small molecules that effectively lower HTT levels consistently throughout the CNS and periphery to be a non-invasive, effective treatment option for patients.

Conclusions

This work represents the first example of the identification and optimisation of orally bioavailable splicing modifiers that penetrate all tissues and exert their action on huntingtin splicing and lowering evenly throughout the body.

Huntington's Disease

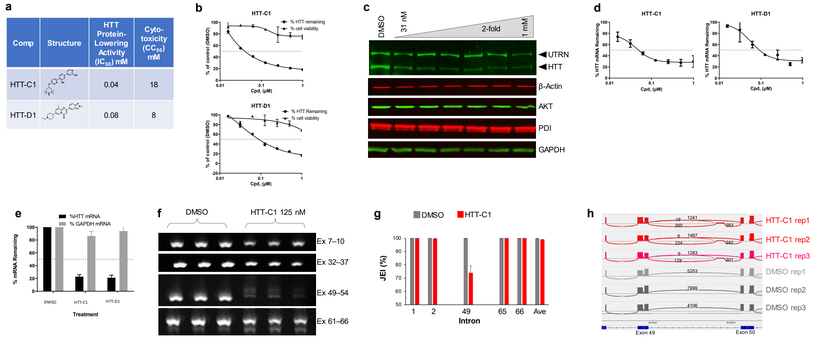
- Caused by a monogenic defect in the *HTT* gene on chromosome 4
- Trinucleotide repeat (CAG – codes for glutamine/Q) expansion at the N-terminus
- Disease status depends on the number of CAG repeats: in general, inverse correlation between mean age of onset and CAG repeat length
- Patient population:
  - ~40,000 patients in the US
  - ~25,000 patients in Europe
- Underreported due to lack of treatment options, >150,000 are at risk in the US
- Currently there are no approved disease-modifying therapies for HD



Repeat Count	Classification	Disease Status
<28	Normal	Unaffected
28–35	Intermediate	Unaffected
36–39	Reduced penetrance	+/- Affected
40–above	Full penetrance	Affected

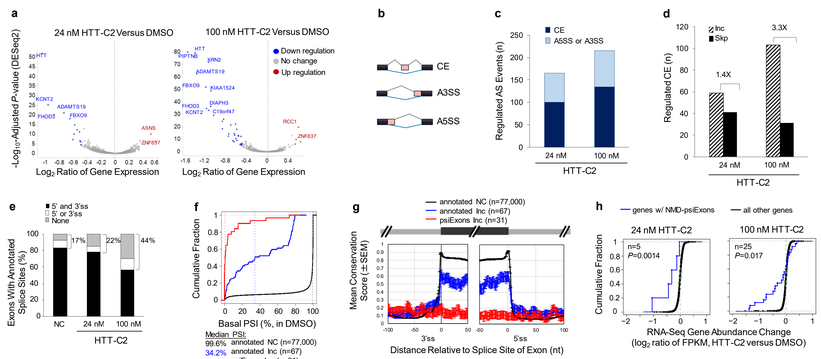
Results

Fig. 1. Novel Splicing of Human *HTT* Pre-mRNA and HTT-Lowering Activity *in vitro*



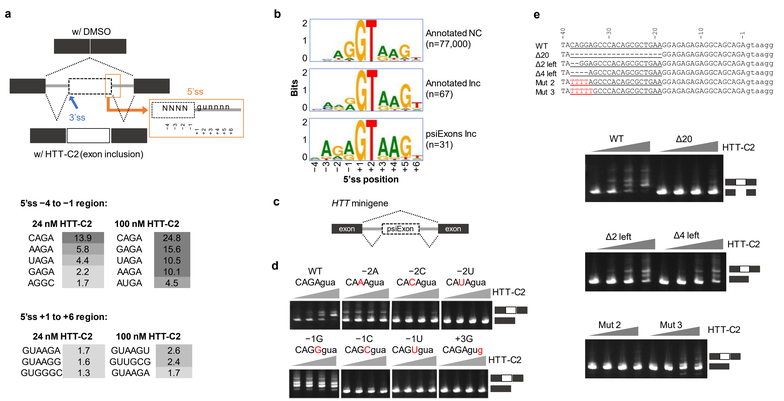
(a) Chemical structures of HTT-C1 and HTT-D1. **(b)** ECL analysis of mutant HTT protein from fibroblasts isolated from a homozygous patient with HD (GM04857) after 96 hours of treatment with HTT-C1 and HTT-D1 (0.01–1.0 μM). Representative graphs show percent HTT remaining relative to the DMSO control. Cell viability assays were performed in parallel. **(c)** Western blot of HTT protein and housekeeping proteins, beta-actin, AKT, PDI, and GAPDH in HD fibroblasts after 96 hours of continuous treatment with HTT-C1 (0.015–1.0 μM). UTRN was also used as a loading control. **(d)** RT-qPCR analysis of *HTT* mRNA in patient fibroblasts after 24 hours of treatment with HTT-C1 and HTT-D1 (0.01–1.0 μM). Representative graphs show percent *HTT* mRNA remaining relative to DMSO control; normalised to housekeeping gene, TATA-box binding protein. **(e)** RT-qPCR analysis of *HTT* mRNA in B-lymphocytes from the same patient (GM04856 cells) after 24 hours of treatment with HTT-C1 and HTT-D1 (0.25 μM). Representative graphs show percent *HTT* mRNA remaining relative to DMSO control; normalised to housekeeping gene GAPDH. **(f)** RT-PCR analysis of *HTT* mRNA after 24 hours of treatment with 125 nM HTT-C1 or DMSO in patient-derived B-lymphocytes (GM04856). **(g)** JEI of intron 49 and a selection of other introns. The JEI of intron 49 was significantly reduced, indicating a splicing event (>25% reduction; $P<0.05$). Error bars represents standard deviation. Data were based on three biological replicates of NGS data. **(h)** Sashimi plot of AS within intron 49 of the *HTT* pre-mRNA using NGS data. A minimum threshold of five reads was used to visualise these data in IGV.

Fig. 2. Selectivity of Compound-Induced Splicing



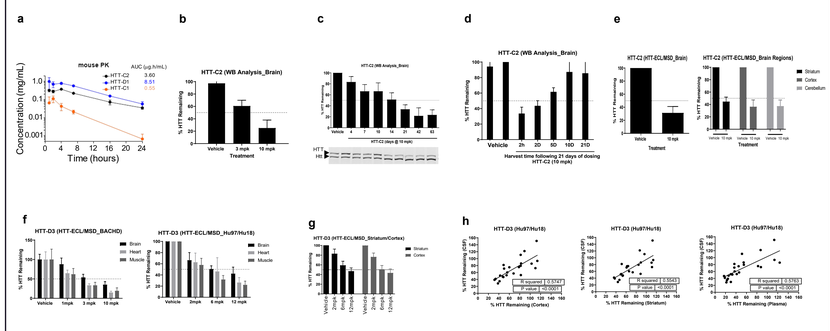
(a) Volcano plot of RNA-Seq analysis comparing gene expression in SH-SY5Y cells treated with either 24 nM or 100 nM of HTT-C2 with DMSO treatment. mRNAs with significant changes in expression (>1.5-fold, false discovery rate <5%) are shown as blue and red dots for down- and upregulation, respectively. **(b)** A schematic of AS events. **(c)** Number of regulated AS events in SH-SY5Y RNA-Seq data following treatment with 24 nM and 100 nM HTT-C2. **(d)** Number of CEs Inc or Skp after HTT-C2 treatment; ratio of Inc/Skp are shown in text. **(e)** Percentage of exons with 3'ss and 5'ss annotated by public databases (Refseq, Ensembl, or UCSC-Known Genes) for NC or Inc exons. **(f)** Cumulative distribution function curves of basal PSI index (average PSI in DMSO samples). Graph shows data for exons separated into three groups; Inc is based on ΔPSI >20% and Fisher's Exact Test $P<0.001$ in any one of the two conditions (24 nM or 100 nM HTT-C2 versus DMSO). Median values are shown as dashed vertical lines for each group. **(g)** Sequence conservation of 3'ss and 5'ss region. Conservation is based on phastCons score for 46-way placental mammals. Mean (SEM) conservation scores are shown. **(h)** Cumulative distribution function curves of RNA-Seq mRNA abundance change for genes with predicted NMD-psiExons. NMD-psiExons are psiExons whose inclusion in mRNA introduces a premature termination codon or causes frameshift or both and are included following HTT-C2 treatment. Number of genes (n) and *P*-value are indicated. *P*-value is based on comparison with "all other genes" group using Wilcoxon rank-sum test.

Fig. 3. Regulation of Compound-Induced Splicing



(a) 5'ss sequence at regions (−4 to −1 and +1 to +6) were studied for enrichment of Inc versus NC exons. Significance scores are shown. **(b)** Schematic of 5'ss sequence logo in the three exon groups: annotated NC, annotated Inc, and psiExons Inc. **(c)** Diagram illustrating the design of *HTT* minigene constructs for studying compound-induced splicing. **(d)** PCR analysis of RNA extracts from HEK293 cells transfected with WT human *HTT* minigene or constructs with point mutations in the −2 to +3 region of the 5'ss; cells were treated with DMSO or HTT-C2 (0.010–1 μM). **(e)** Sequence of the 20-nucleotide region upstream of the 5'ss of *HTT* Stop-Codon psiExon49a showing partial deletions and mutations performed on this region, and their effects on HTT-C2-induced splicing (lower panel). Through partial deletion or mutation of the nucleotides CAGGA at positions −38 to −34, this region was shown to be important in regulating splicing events.

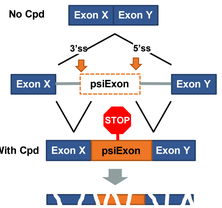
Fig. 4. Compounds Eliciting HTT Lowering *in vivo*



(a) Plasma levels of HTT-C1, HTT-D1, and HTT-C2 over 24 hours in BACHD mice after a single 10 mg/kg dose. **(b)** Western blot analysis of human HTT protein within the brain tissue of BACHD mice treated with HTT-C2 (3 mg/kg or 10 mg/kg) once daily for 14 days; graph shows percent lowering relative to vehicle control and normalised to mouse Htt protein. Example western blot shown below. **(c)** Western blot analysis of 10 mg/kg HTT-C2-induced lowering of human HTT protein within brains of BACHD mice over time. Graph shows percent lowering relative to vehicle control and normalised to mouse Htt protein. Example western blot shown below graph with mouse Htt as a loading control. **(d)** Western blot analysis of human HTT protein expression levels in brain tissue over time following cessation of 10 mg/kg HTT-C2 treatment in BACHD mice. Graph shows percent lowering of human HTT protein relative to vehicle control and normalised to mouse Htt protein. **(e)** ECL analysis of human HTT protein expression levels within different parts of the brain from BACHD mice treated with 10 mg/kg HTT-C2. Graphs show percent lowering relative to vehicle control and normalised to UTRN. **(f)** ECL analysis of human HTT protein expression levels within different tissues from Hu97/18 mice (bottom graph) and BACHD mice (top graph) treated with HTT-D3. Graphs show percent lowering relative to vehicle control and normalised to KRAS. **(g)** ECL analysis of human HTT protein expression levels within the striatum and cortex of the brain from Hu97/18 mice treated with 2/6/12 mg/kg HTT-D3. Graphs show percent lowering relative to vehicle control, normalised to KRAS. **(h)** Regression analysis to show the correlation between HTT lowering of HTT-D3 in the cortex, striatum, and plasma of Hu97/Hu18 mice relative to the CSF.

Summary

HD is a devastating disorder with no approved treatment. Several preclinical studies support the hypothesis that targeting the expression of *HTT* may prevent and/or slow disease progression. To our knowledge, this work represents the first example of the identification and optimisation of orally bioavailable splicing modifiers that penetrate all tissues (including every cell type throughout the brain and periphery) and exert their action on *HTT* splicing and lowering evenly throughout the body. A molecule from our HD drug discovery programme entered a Phase 1 clinical trial in November 2020.



Next Steps: PTC518 Clinical Trials

- In November 2020, PTC initiated a Phase 1 clinical trial to evaluate PTC518 in healthy volunteers
- PTC518 is an orally bioavailable molecule with broad central nervous system and systemic distribution, and has been designed to target Huntingtin protein expression with high selectivity and specificity
- PTC518 has favourable pharmaceutical properties and has demonstrated uniform lowering of the Huntingtin protein throughout the brain in animal models
- The Phase 1 study included both single and multiple ascending dosing regimens that will help establish the safety, pharmacology, and dose selection for the Phase 2 study – selected results from this study are being presented by PTC at EHDN 2021
- PTC is aiming to initiate a Phase 2 study in HD patients towards the end of 2021. Further details regarding this trial will be announced in due course

References

1. Wild EJ, Tabrizi SJ. Therapies targeting DNA and RNA in Huntington's disease. *Lancet Neurol.* 2017;16:837–847.
2. Tabrizi SJ, Ghosh R, Leavitt BR. Huntingtin Lowering Strategies for Disease Modification in Huntington's Disease. *Neuron.* 2019;101:801–819.
3. Nopoulos PC. Huntington disease: a single-gene degenerative disorder of the striatum. *Dialogues Clin Neurosci.* 2016;18:91–98.

A, adenine; aa, amino acid; AKT; alpha serine/threonine-protein kinase; AS, alternative splicing; CC_{50}, half cytotoxic concentration; CE, cassette exon; C, cytosine; CNS, central nervous system; CSF, cerebrospinal fluid; DMSO, dimethyl sulphoxide; ECL, electrochemiluminescence; G, guanine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, Huntington's disease; HTT/*HTT*, human huntingtin protein/gene; Htt, mouse huntingtin; IC_{50}, half maximal inhibitory concentration; IGV, integrated genomics viewer; Inc, exon inclusion event; JEI, junction expression index; KRAS, Kirsten rat sarcoma viral oncogene homologue; mRNA, messenger RNA; NC, exon unchanged; NGS, next-generation sequencing; NMD, nonsense-mediated decay; PCR, polymerase chain reaction; PDI, oxidoreductase-protein disulfide isomerase; PSI, percent spliced in; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SEM, standard error of the mean; Skp, exon skip event; ss, splice site; UCSC, University of California, Santa Cruz; UTRN, utrophin; wt, wild-type.