A Huntington's disease embryonic stem cell phenotypic HTS to identify small molecule modulators of mutant Huntingtin

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1. Introduction

- Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG repeat expansion in the huntingtin gene (*HTT*) resulting in the production of toxic mutant huntingtin protein (mHTT).
- HD therapeutic discovery is currently biased towards HTT lowering agents. Encouragingly, a clinical pipeline for DNA/RNA targeted HTT-lowering agents now exists; however, most of these approaches use biological agents that require invasive administration and have limited biodistribution.
- Identification of brain penetrant small molecules with suitable oral dosing and systemic distribution that selectively lower mHTT protein would be potentially advantageous over novel biological therapies, such as ASOs (RG6042; Tabrizi *et al.*, 2019).
- A phenotypic assay in HD-patient derived, polyQ48 embryonic pluripotent stem cells (Genea020; Bradley *et al.*, 2011) was developed to identify small molecules that reduce mHTT protein levels. Using this assay, we screened AstraZeneca's 250k EPEC diverse compound library through the Open Innovation Partnership Scheme and identified a hit compound that was shown to work via a unique HTT RNA-lowering mechanism.
- Herein we present a review of this screening campaign and its output along with the characterisation studies aimed at understanding the mechanism of action of the hit series.

2. HTS assay and downstream screening cascade

- mHTT-lowering activities were measured using a HTRF HTS assay configured in Genea020 cells. Cells were seeded onto collagen coated 384-well plates before compound treatment for 48 hours. Cells were lysed and mHTT levels were measured.
- Compounds showing a reduction of mHTT by >30% at a single concentration were progressed through the screening cascade to characterise protein lowering selectivity and specificity.
- Validation of the screening cascade/assay panel was endorsed by the behaviour of the tool compound Branaplam (LMI070) which is known to lower HTT levels by targeting the RNA through a splicing mechanism that induces nonsense-mediated decay (Keller *et al.*, 2022).

Description	Assay Target / Format	Purpose
Primary HTS assay	**mHTT** / HTRF	Identify compounds that lower mHTT
Protein-lowering counter assays	**tHTT** / HTRF	Determine mHTT – tHTT specificity
	AKT / HTRF	Determine selectivity to another protein
	α-Tubulin / HTRF	Determine selectivity to a HKG
	mHTT, tHTT / MSD	Orthogonal HTT-lowering assay
HTRF Interference assay	**mHTT Interference** / HTRF	Rule out assay interference
RNA-lowering assays	**Htt, HPRT** / qPCR, ViewRNA, branched DNA	Assess RNA mechanism
Cell toxicity assays	**ATP levels** / CellTitre-Glo (CTG)	Determine mHTT lowering activity window over toxicity
	Total Nuclear Stain (TNS) / HCA	
Mechanism deconvolution assay	**Global Protein Synthesis (GPS)** / HCA	Establish global translation MoA

Table 1. Genea020 HTS cell assay portfolio. *Protein-lowering HTRF/MSD formats employed to identify HTT-lowering hits and characterise selectivity towards counter-targets. qPCR & ViewRNA formats employed to assess RNA mechanism. Orthogonal cell toxicity measurements captured using CellTitre-Glo (CTG) and Total Nuclear Stain (TNS) assays to ensure that HTT-lowering is not a direct consequence of toxicity. GPS assay (puromycin labelling) employed to determine whether activity is via a global translation inhibition mechanism.*

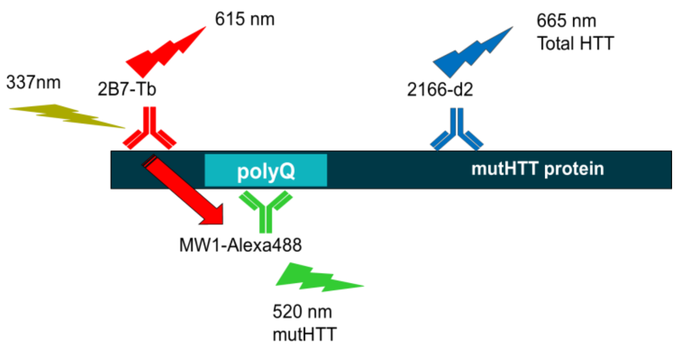

Figure 1. HTRF HTT detection assay principle (Weiss et al., 2009). *mHTT specific signal is generated through proximity of the N-terminal 2B7 and polyQ-specific MW1 antibody pair. Total HTT signal is generated from the 2B7 and 2166 antibody pair. mHTT / tHTT levels can be generated individually or as a multiplex.*

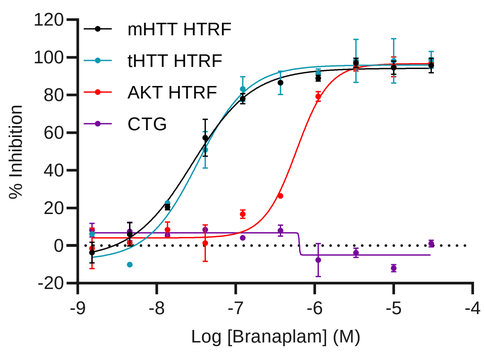

Figure 2. Representative profile of tool compound Branaplam*. Reduction of mHTT (black) is equipotent to tHTT (teal) with a robust window (>30-fold) over AKT-lowering (red) and no evidence of toxicity as determined by CellTitreGlo (purple).*

3. Hit identification

- EPEC Diverse collection contains 250K compounds which provides a broad representation of AZ's industry-leading compound collection.
- Screening cascade identified 36 hit compounds with ≥3-fold window over cell toxicity (Total Nuclear Stain).
- **Only one hit 'Compound 1' showed selectivity for HTT over all counter-assays and so was characterised further.**

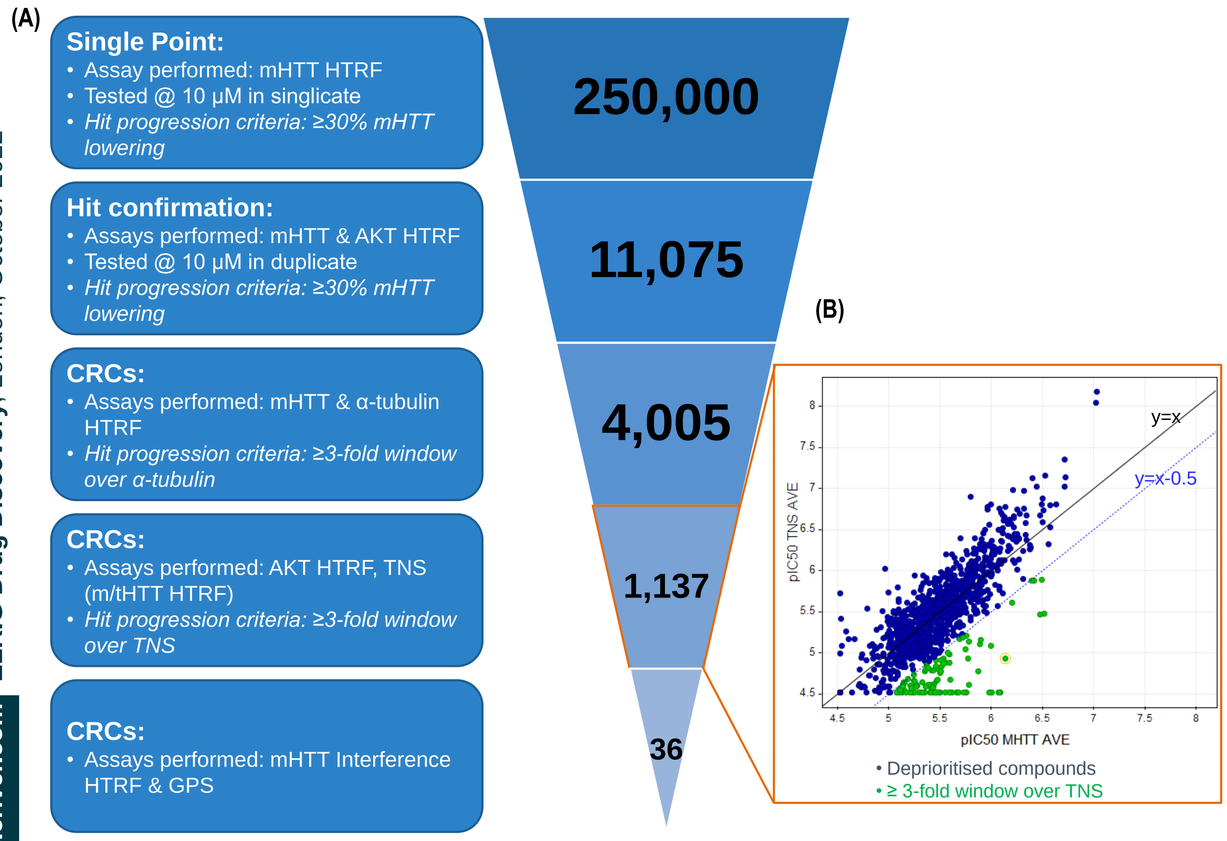

Figure 3. (A) Screening cascade. (B) mHTT-lowering / TNS toxicity correlation plot highlighting 36 progressed hits. *CRCs – Concentration-response curves.*

4. Hit characterisation – HTT protein-lowering

- Compound 1 was HTT specific / selective – no toxicity observed.
  - IC50 between 10 – 30 µM; profile appears to be non-allele selective.
  - Lowering effects are partial (maximal ~50%); mechanism unknown.
- HTT protein-lowering confirmed using orthogonal mesoscale discovery assay.

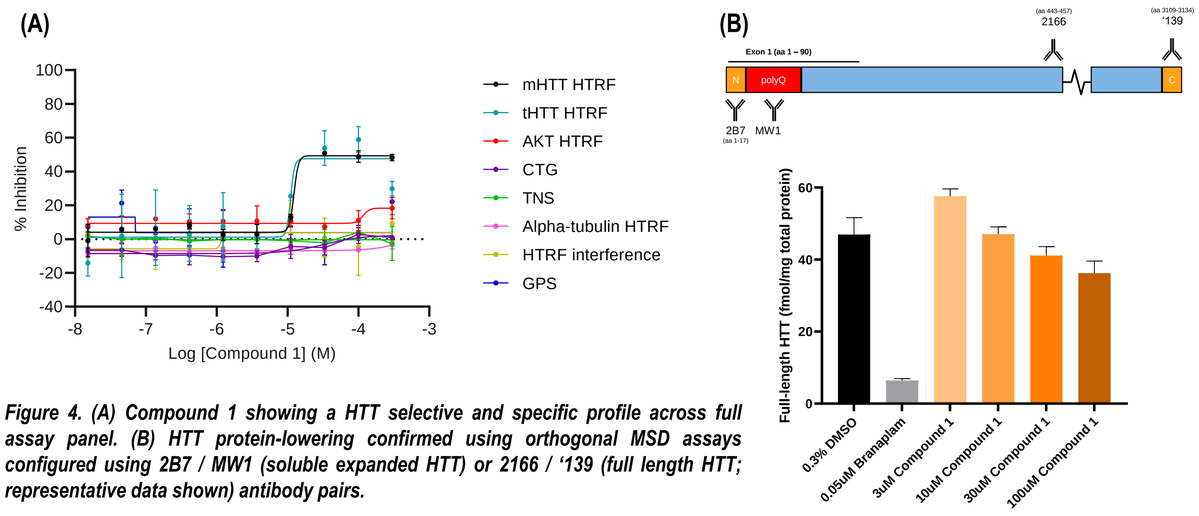

Figure 4. (A) Compound 1 showing a HTT selective and specific profile across full assay panel. (B) HTT protein-lowering confirmed using orthogonal MSD assays configured using 2B7 / MW1 (soluble expanded HTT) or 2166 / '139 (full length HTT; representative data shown) antibody pairs.

5. Hit expansion

- Compound 1 and a small array of close analogues (~30) were synthesized.
- A small but significant improvement in activity (~3-fold) was achieved for several of the explored structural modifications with emerging SAR.

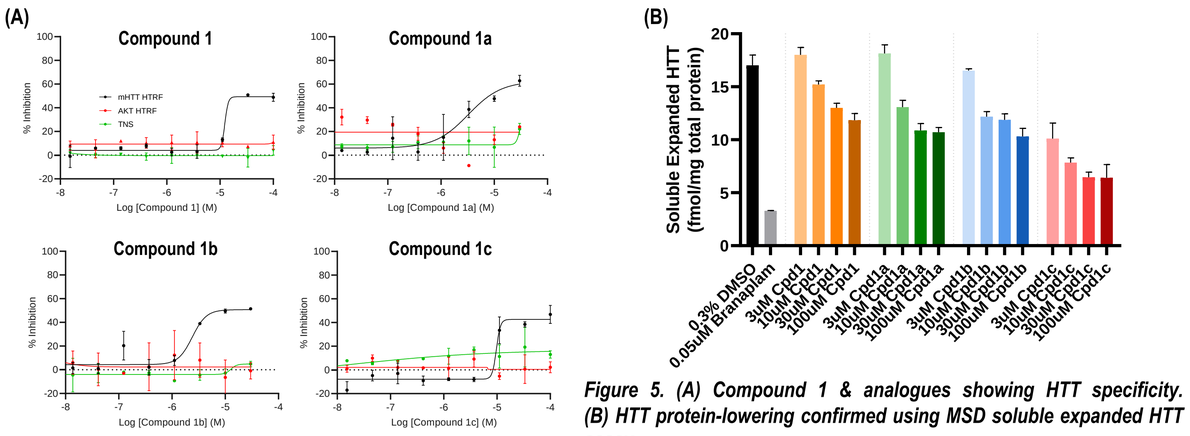

Figure 5. (A) Compound 1 & analogues showing HTT specificity. (B) HTT protein-lowering confirmed using MSD soluble expanded HTT assay.

6. Hit characterisation – HTT RNA-lowering

- HTT RNA-lowering mechanism shown for Compound 1 series by ViewRNA.
- Excellent correlation with HTRF protein lowering activity; also displaying partial (maximal ~50%) effects.

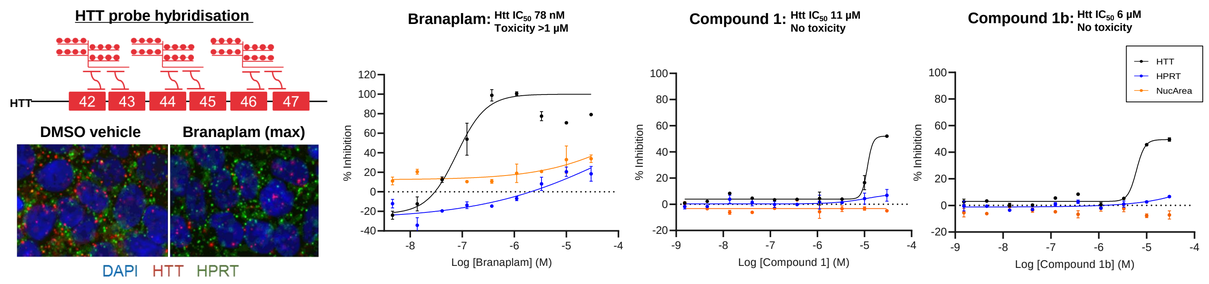

Figure 6. Compound 1 series (and Branaplam) show HTT RNA-lowering effects (24 hours) via ViewRNA format which combines fluorescence and sequential branched-DNA amplification to visualise mRNA transcripts with single-molecule sensitivity in individual cells.

- HTT RNA-lowering mechanism confirmed by qRT-PCR at multiple canonical exon boundaries.
- Compound 1 series mechanism is distinct from the Branaplam intron49 alternative splicing event.

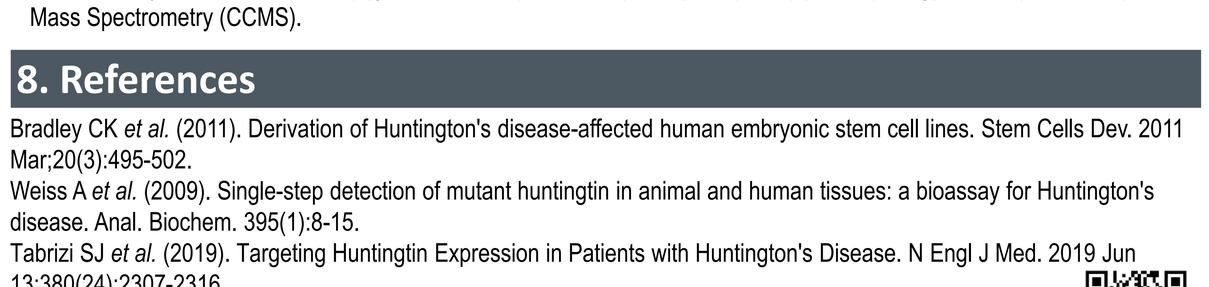

Figure 7. (A) Compound 1 series (and Branaplam) show HTT RNA-lowering effects (24 hours) via qRT-PCR across multiple canonical exon boundaries (ex49-50 shown). (B) Compound 1 series HTT-lowering mechanism is distinct from Branaplam which induces an alternative splicing event through incorporation of a pseudo-exon (i49b).

7. Conclusions and next steps

- Compound 1 profile in HD-patient derived, polyQ48 embryonic pluripotent stem cells is very promising given the observed specificity and selectivity with qPCR / ViewRNA data indicating a novel RNA-based mechanism.
- Several analogues demonstrated improved HTT-lowering potency & clear SAR with selectivity over AKT-lowering & cell toxicity (TNS).
- HTT RNA-lowering appears to be reasonably specific, as assessed by Taqman Genecard and branched-DNA arrays (data not shown).
- Activity in HD ESC-derived neuronal systems has proved challenging with only one analogue showing very weak activity (data not shown).
- Next steps involve a small medicinal-chemistry effort to identify more potent analogues to support mechanism of action studies, likely to include RNASeq [global RNASeq – transcriptome], AmpliSeq [HTT splicing] and Capture Compound® Mass Spectrometry (CCMS).

8. References

Bradley CK *et al.* (2011). Derivation of Huntington's disease-affected human embryonic stem cell lines. Stem Cells Dev. 2011 Mar;20(3):495-502.

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