Citation: Cell Death and Disease (2011) 2, e115; doi:10.1038/cddis.2010.94

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Correspondence

Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease

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Cell Death and Disease (2011) **2,** e115; doi:10.1038/cddis.2010.94; published online 13 January 2011
Subject Category: Neuroscience

Dear Editor,

Huntington's disease (HD) is characterized clinically by movement abnormalities (i.e. chorea), psychiatric symptoms, and cognitive deficits. Mutant Huntingtin (Htt) with expanded (>36) polyQ (glutamine) repeats causes the dysfunction and death of neurons, particularly medium spiny neurons (MSNs) that account for $\sim$90% of striatal neurons, despite an ubiquitous expression.[1,2] The striatal neuronal death correlates with the increment of HD severity, being about $\sim$30% in grade 0 and $\sim$95% of MSNs in grade 4 patients.[3] The mechanism underlying the striatal cell death remains elusive and no effective treatment is available for this fatal disease. Different from well-characterized apoptosis, necroptosis is an emerging alternative cell death mediated by RIP1 kinase.[4] Necrostatin-1 (Nec-1) was recently confirmed to be an allosteric RIP1 kinase inhibitor ($EC_{50} = 0.18\ \mu M$),[5] protective in NMDA-mediated excitotoxicity and acute pathologies including cerebral ischemia.[4] Here we explored the role of necroptosis in HD by studying Nec-1 in immortalized striatal cells and R6/2 transgenic mouse.

ST14A is an immortalized striatal cell line with MSNs characteristics[6] and ST14A 8plx line stably expressing mutant Htt fragment (N548-128Q) was established as a cell model of HD.[7,8] To our surprise, pan-caspases inhibitor zVAD-fmk efficiently induced ST14A 8plx cell to death, which can be almost completely rescued by Nec-1 (Figures 1a and b). Unlike apoptosis, dying striatal cells showed atrophy and shrinkage of the cell body and had no caspase-3-specific cleavage of neuronal cytoskeleton α-fodrin protein compared with the cleavage after staurosporine-induced apoptosis (Supplementary Figure S1a). Further experiments with selective caspase inhibitors revealed that caspase-8 inhibitor (IETD-fmk), but not caspase-3 (DEVD-fmk) or caspase-9 (LEHD-fmk) inhibitor, had the similar necroptosis-inducing effect as zVAD-fmk (Figures 1a and b and Supplementary Figure S1b). The zVAD-fmk or IETD-fmk inhibited RIP1 cleavage dose-dependently in striatal cells (Supplementary Figure S1c), thus facilitating RIP1 kinase activation and subsequent necroptosis, whereas Nec-1 rescued striatal cell death and restored the normal status of RIP1 cleavage in zVAD-fmk/IETD-fmk-treated cells to the similar level as untreated control cells (Figure 1c and Supplementary Figure S1d). Another necroptosis inhibitor, necrostatin-5 did not inhibit zVAD-fmk-induced striatal cell death (data not shown).

In response to the same dose of zVAD-fmk, ST14A 8plx cells were more vulnerable and exhibited more cell death than parental ST14A cells (Figure 1d). We also noticed that there were more full-length RIP1 proteins in 8plx cells compared with parental cells (Supplementary Figure S1e). In addition, culturing 8plx cells in serum-free medium promoted RIP1-mediated cell death following zVAD-fmk treatment (Figure 1e), but had no impact on zVAD-fmk-induced inhibition of RIP1 cleavage (Supplementary Figure S1f). We next assessed the ERK1/2 signaling and found that zVAD-fmk treatment greatly reduced the phosphorylated ERK1/2 and the effect was inhibited by Nec-1 treatment, correlating with its inhibition of necroptosis (Figure 1f). We did not observe the phosphorylation of JNK or p38 MAPK in striatal cells necroptosis (data not shown).

After demonstrating that Nec-1 inhibited striatal cell necroptosis efficiently *in vitro*, we next evaluated the Nec-1 in well-studied R6/2 transgenic mouse model of HD, which expresses exon 1 of mutant human *htt* gene.[9] Nec-1 can cross the blood-brain barrier easily but has a short half-life, about $\sim$1 h.[10] So we delivered Nec-1 intracerebroventricularly with Alzet osmotic pump by neurosurgery to ensure continuous supply of the drug. The treatment was started from 5 weeks of age as we observed increased full-length RIP1 protein in R6/2 mice at this time point compared with age-matched wild-type littermates (Supplementary Figure S1g). The mouse motor function was monitored by Rotarod test at the speed of 5 and 15 r.p.m. (Figures 2a and b). The disease onset was determined when animal failed to run over 7 min at 15 r.p.m.[8] Nec-1 treated mice retained much better motor performance at the age of 11 weeks (Figure 2a). The disease onset was about 78.0 ± 4.4 days in Nec-1-treated mice ($n = 7$)

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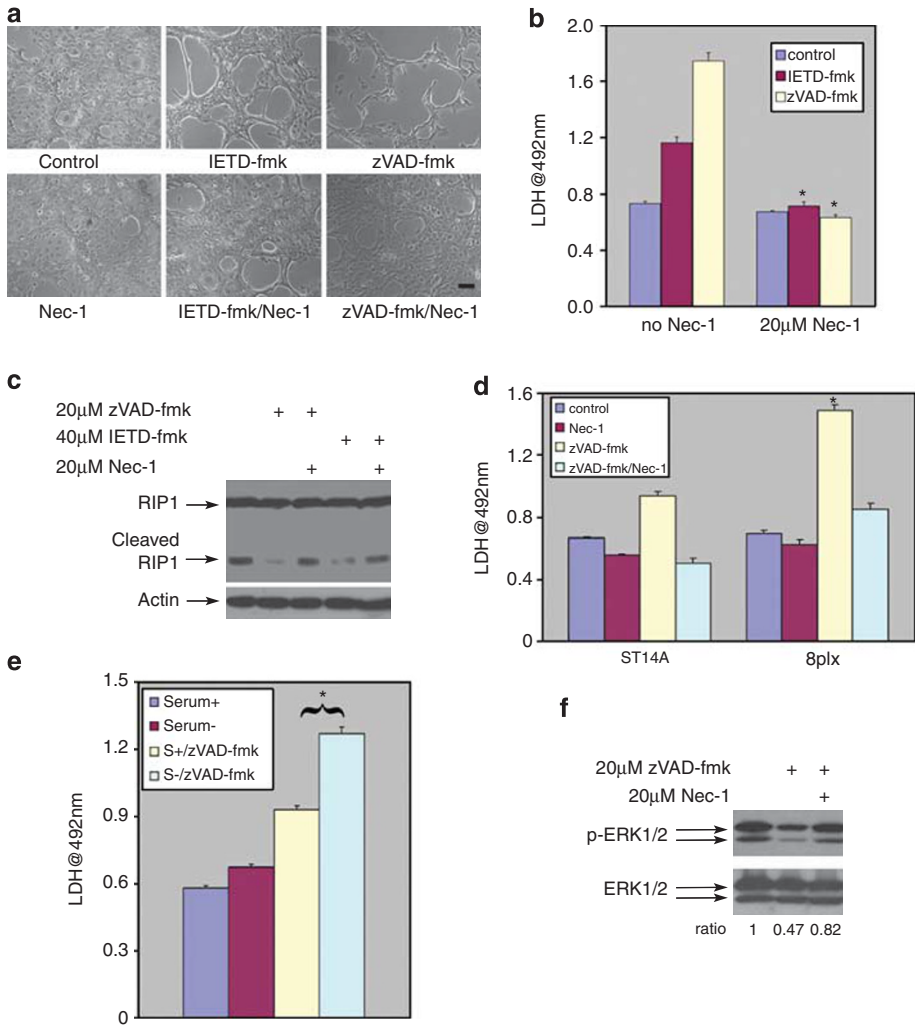
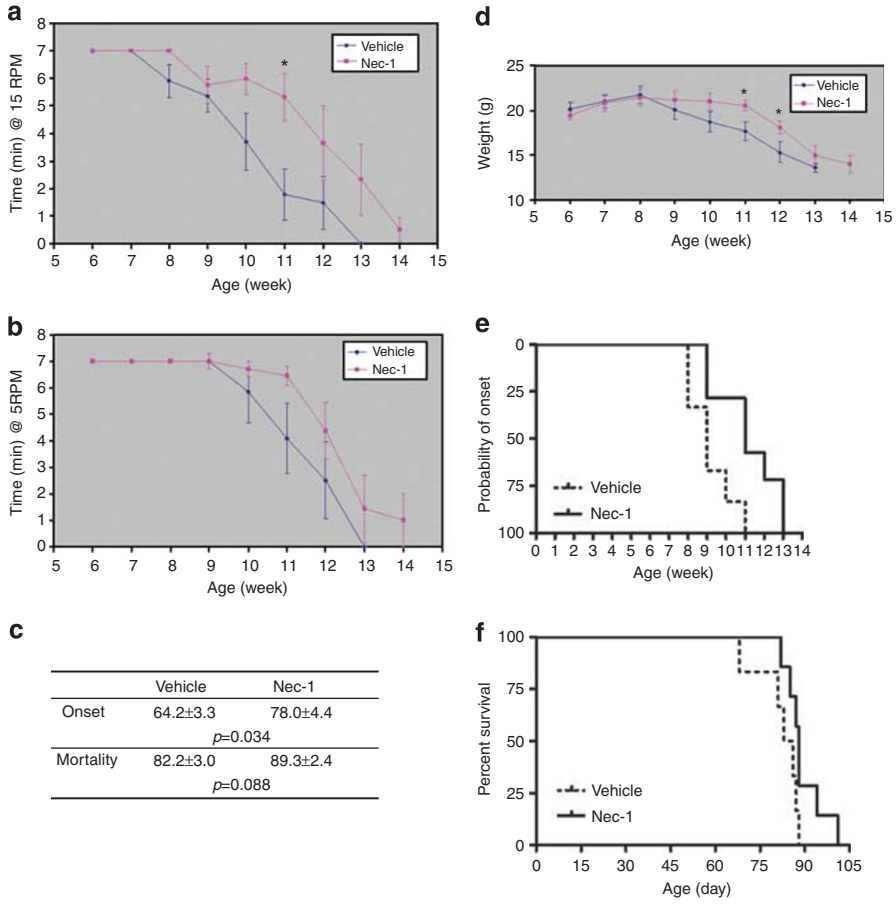


Figure 1 Nec-1 inhibited RIP1 mediated necroptosis in striatal cell model of HD *in vitro*. (**a**) Addition of zVAD-fmk (20 μM) or IETD-fmk (40 μM) to ST14A 8plx cells resulted in cell death, which was inhibited by RIP1 inhibitor Nec-1 (20 μM). Photos were taken 36 h after treatment, Bar = 50 μm; and (**b**) supernatant was collected for evaluating cell death by LDH assay. (**c**) Lysates from ST14A 8plx cells treated with zVAD-fmk/IETD-fmk/Nec-1 for 36 h were blotted by RIP1 antibodies. (**d**) Parental ST14A cells and ST14A 8plx cells were treated with 20 μM zVAD-fmk in the presence/absence of 20 μM Nec-1. Cell death was measured 36 h after treatment. (**e**) Mutant Htt expressing ST14A 8plx cells were treated with 20 μM zVAD-fmk in the presence/absence of serum. Cell death was measured 24 h after treatment. (**f**) Lysates from ST14A 8plx cells treated with 20 μM zVAD-fmk for 24 h were analyzed in western blot by antibodies against phospho- or total-ERK1/2. $^*P<0.05$

and 64.2 ± 3.3 days in vehicle-treated ones ($n=6$), respectively (Figure 2c). Vehicle-treated R6/2 mice started to lose weight since 9 weeks of age whereas Nec-1-treated R6/2 mice maintained the body weight even at 11 weeks of age (Figure 2d). The curve of probability of onset of the disease clearly showed the difference with significantly delayed behavior deterioration in Nec-1 treated mice (Figure 2e, $P=0.023$, logrank test). The drug extended the life span of the R6/2 mice modestly (Figure 2c and f).

The zVAD-fmk is widely used as a pan-caspase inhibitor in apoptosis research. However, inhibition of death receptor (Fas/TNFR) signaling by zVAD-fmk leads to RIP1 kinase activation and subsequent necroptosis.[4] The fact that necrostatin-5 can inhibit RIP1 activation induced by extrinsic death receptor signaling[5] but not striatal necroptosis in our model suggests the existence of an alternative intrinsic RIP1 activation pathway in striatal cells. ST14A 8plx striatal cells are more sensitive to necroptosis, possibly due to the effect of mutant Htt on post-translational modifications of RIP1, which include phosphorylation, ubiquitination and caspase cleavage, thus tipping the intracellular balance of RIP1 protein and intrinsic kinase activation pathway(s). Of note, zVAD-fmk itself has shown cytotoxicity in non-neuronal cell lines,[11] and also promotes necrosis in mitochondrial toxin MPP-treated dopaminergic neurons, which are selectively depleted in the patients' brain with Parkinson's disease (PD).[12] Therefore, further studies of predisposed zVAD-fmk/IETD-fmk toxicity in striatal cells may provide useful insights into mechanisms underlying neuronal loss not only in HD, but also in other neurodegenerative diseases like PD.

ERK signaling is involved in the physiological function of striatum in the neural circuitry underlying procedural learning, motor control, and reward as well as in striatal gene transcription induced by BDNF, a critical neurotrophic factor for



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	Vehicle	Nec-1
Onset	64.2±3.3	78.0±4.4
	p=0.034	
Mortality	82.2±3.0	89.3±2.4
	p=0.088	

Figure 2 Nec-1 maintains the body weight and motor function in R6/2 mouse model of HD *in vivo*. Behavior and disease progression data were generated from the same cohort of mice. Motor performance of R6/2 mice was evaluated by recording the time that they remained on a rotarod tuning at 15 r.p.m. (**a**) and 5 r.p.m. (**b**). $^*P<0.05$. Mice were treated with vehicle or Nec-1 by intracerebroventricular delivery using osmotic pump. (**c**) The age (in days) at disease onset and at death was tabulated for both Nec-1- and vehicle-treated animals; data were processed by student's *t*-test. (**d**) The body weight of the R6/2 mice was recorded on a weekly basis. For vehicle treated mice $n=6$ and for Nec-1 treated mice $n=7$. $^*P<0.05$. (**e**) Cumulative probability of onset in Nec-1- and vehicle-treated R6/2 mice. $P=0.023$ by logrank test. (**f**) Cumulative probability of survival in Nec-1- and vehicle-treated R6/2 mice. $P=0.079$ by logrank test

MSNs survival.[13,14] Altered ERK signaling is also implicated in HD and activated ERK signaling is protective to MSNs in HD models in different experimental settings.[7,15,16] This concept is further supported by our observations that Nec-1 prevented the reduction of ERK signaling and increased cell survival in zVAD-fmk-treated striatal cells. In our experiments, striatal cell necroptosis was facilitated by serum-free media, implying that unspecified serum factors inhibiting RIP1 kinase activation under necroptotic stress, and that BDNF, which is deficient in HD,[2] might be one of the factors promoting necroptosis *in vivo*.

Delaying the disease onset by Nec-1 in R6/2 mice further confirmed the involvement of RIP1 signaling in the disease pathogenesis. However, the survival benefit was modest. This discrepancy might be due to different mechanisms involved in early (necroptotic) and late (apoptotic) disease stages as apoptotic characteristics can be detected in late stage (>11 weeks) of R6/2 mouse and in grade 3 and 4 patients' brain.[17] The differentiation between early and late stages of the disease is proposed due to different sensitivity of mutant striatal cells to excitotoxicity as well as the suggestion for the different treatment strategy.[18] Early disease stage with necroptosis signaling might explain the extensive and early involvement of activated astrocytes in HD pathogenesis.[2] In ST14A cells, treatment of Nec-1 increased the cleavage of full-length RIP1 (Figure 1c and Supplementary Figure S1d), indicating the higher basal caspase-8 activity, which might have a side effect in the late apoptotic stage of the disease in mice. As RIP1 protein is also involved in caspase-8 activation in apoptosis, the interplay of apoptosis and necroptosis is even more complicated. It was reported that Nec-1 treatment reverted necroptosis to apoptosis.[19] Hence, concomitant treatment with both apoptosis and necroptosis inhibitors may have better beneficiary effect on the disease, especially regarding the development of caspase inhibitor for HD.[20] Finally, as Nec-1 helped maintaining the body weight and motor functions with significantly delayed disease onset in R6/2 mouse ($\sim$21.5%), it can be considered as a potential treatment of HD patients to ameliorate the symptoms and improve the quality of life.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. SZ acknowledges support by the grant from the Brain Science Foundation, and the support from Dr. Arthur L Day and Dr. Clark C Chen. The authors are also grateful to Dr. Junying Yuan for the critical discussion and suggestion for the experiments. We thank Dr. Lata Menon and Dr. Mingwei Li for the surgical technique help, as well as Dr. Andrew L Kung for the research help. GB and HL are supported by NIH grants NS38077 and R01GM081776. Special thanks to Dr. Robert Friedlander (University of Pittsburg) for the support in the research. Authors are in debt to the insightful comments and suggestions from anonymous reviewers.

Author contributions

SZ designed and performed experiments. YZ helped performing the animal experiment. HL and GB helped designing and performing the experiments with expertise. SZ wrote the manuscript. All authors participated in the discussion and editing of the manuscript.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)