Cell Death and Differentiation (2013) 20, 366
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Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase

Cell Death and Differentiation (2013) **20,** 366; doi:10.1038/cdd.2012.133; published online 30 November 2012

Dear Editor,

Necroptosis has emerged as an important form of regulated necrotic cell death, which can occur during mouse development and is also an important contributor to necrotic tissue injury in a variety of mouse models of human pathologies, including brain trauma, ischemia-reperfusion injuries, viral infections and acute inflammatory responses.[1] Necrostatin-1 (Nec-1; methyl-thiohydantoin-tryptophan (MTH-Trp)), identified by us in a cell-based small-molecule necroptosis screen,[2] was found to selectively target the kinase activity of RIP1, a key mediator of necroptosis.[3] Nec-1 is commercially available and has been used extensively both *in vitro* and *in vivo* by multiple groups to elucidate the role of necroptosis. However, because this molecule is also known to inhibit indoleamine-2,3-dioxygenase (IDO),[4] we feel that it is important to revisit and summarize some of the key published and new data regarding activity and specificity of Nec-1.

We assessed inhibition of RIP1 and IDO by Necs, including Nec-1, original molecule identified in our screen,[2] 7-Cl-O-Nec-1, optimized analog of Nec-1,[3,5] inactive analogs of both Necs (denoted by 'I') and an IDO inhibitor, 1-methyl-D, L-tryptophan (1-MT) (Supplemantary Figure S1A). With respect to RIP1 inhibition, our data confirmed excellent correlation of Nec's activity against RIP1 kinase *in vitro* and necroptosis in Jurkat cells (Supplemantary Figure S1B). Notably, 1-MT failed to inhibit necroptosis, confirming the specific role of RIP1 kinase, rather than IDO, in necroptosis. Conversely, only the original Nec-1, but none of the other Necs, inhibited IDO activity (Supplemantary Figure S1C). These results clearly distinguish the SAR of Necs against the two different enzyme targets.

As we pointed out in our papers, widely used and commercially available Necrostain-1 (MTH-Trp), a molecule originally identified in a necroptosis screen, is not optimal.[2,3,5] In particular, metabolic stability of Nec-1 is very limited ($T_{1/2} < 5$ min in mouse microsomal assay) because of the presence of sulfur of thiohydantoin.[5] Chemical optimization of Nec-1 (>200 derivatives analyzed to date) led to the development of a much improved analog, termed 7-Cl-O-Nec-1 (or 7-Cl-O-MH-Trp) (Supplemantary Figure S1A),[5] which showed a robust improvement in metabolic stability to $T_{1/2}$ of ~ 1 h in liver microsomal assay and in *in vivo* pharmacokinetics (PK) study.[5]

Target specificity is a critical consideration for any small-molecule tool. Notably, optimized 7-Cl-O-Nec-1 displayed exclusive selectivity towards RIP1 in a screen of a >400 human kinases, including several other RIP family members.[6] Furthermore, we observed perfect correlation between inhibition of RIP1 kinase and cellular necroptosis by Nec-1 analogs.[3] We also found, using wild-type and RIP1-deficient Jurkat and mouse fibroblast cells, that the ability of Nec-1 to inhibit cell death is entirely dependent on the expression of RIP1 kinase.[3,6] Thus, Nec-1 and, especially, 7-Cl-O-Nec-1 represent useful chemical probes for defining the role of RIP1 kinase in cellular regulation. It is also worth noting that very closely related inactive analogs of both Nec-1 and 7-Cl-O-Nec-1 have also been described to facilitate confirmation of on-target activity of the inhibitors *in vitro* and *in vivo* (Supplemantary Figures S1A and B).[3]

Overall, these data lead us to suggest that in interpreting the results obtained using original Nec-1, a limited bioavailability and inhibitory activity against IDO should always be considered. It is also useful to bear in mind that Nec-1i, commercially available inactive derivative of Nec-1, cannot be used alone to differentiate between targeting IDO and RIP1. Optimized 7-Cl-O-Nec-1 represents a superior tool, especially, *in vivo*. Additional tools, such as described in the literature RIP3 and IDO-deficient animals and/or short hairpin RNA knockdowns, can help further distinguish between these two pathways.

Conflict of Interest

The authors declare no conflict of interest.

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