

## **Exosite Binding confers high specificity and detection capacity to fluorogenic substrates for BoNT/A**

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BoNT/A is widely used as a therapeutic agent as well as in wrinkle reduction at very low doses and its quantification in pharmaceutical preparations therefore requires precise and sensitive methods. To date, the gold assay remains the pharmacotoxicological mouse LD50 assay, which provides the *in vivo* toxicity of any given toxin sample, a true reflection of the three steps essential to toxin action which are binding, translocation and substrate hydrolysis. This assay is however costly, time consuming, requires the use of a large number of animals and is therefore subject to numerous criticism. With mounting pressure emanating from governmental authorities, there is a growing interest in alternative methods, like the endopeptidase assay which measures and quantifies the active, metalloprotease moiety of the toxin, corresponding to the Light Chain (LC). Based on this protease activity and using the Internal Collision-Induced Fluorescence Quenching technique, Pharmaleads has developed PL50, a high-affinity mimetic peptide substrate for BoNT/A (Poras et al., Appl. Environ. Microbiol., 2009, 75: 4382-4390). The sequence of this 48-mer, Ac-156-203 SNAP-25-NH<sub>2</sub> (Nop<sup>197</sup>, Pya<sup>200</sup>, Nle<sup>202</sup>), is based on that of SNAP-25, the natural substrate of BoNT/A and spans through to the previously identified BoNT/A □-exosite (Breidenbach and Brunger, Nature, 2004, 432:925-929). Shorter peptides containing the natural cleavage site of BoNT/A, like PL63 (Ac-187-203 SNAP-25-NH<sub>2</sub> (Nop<sup>197</sup>, Pya<sup>200</sup>, Nle<sup>202</sup>) or the SNAPtide substrates (List Biological), are also commonly used substrates. In order to compare these different substrates, their binding constants were measured as well as their limits of detection of BoNT/A LC. Results of these experiments show that PL50 has a the highest binding affinity towards BoNT/A with a Km 0.95 μM versus a Km of 15 and 20 μM for PL63 and SNAPtide respectively. Consistent with the recognition of allosteric binding exosites on BoNT/A (Ouimet et al., Toxicon, 2008, 51: Suppl.1:17). These specificity constants also confer higher detection limits to the longer PL50 peptide substrate, which is able to detect as low as 3 pg of BoNT/A LC, compared to 300 pg for SNAPtide. Interestingly, whereas the osmolyte trimethylamine *N*-oxide (TMAO) significantly increased the affinity of the shorter peptides, it decreased the one of PL50.