Summary

We show that in *S. cerevisiae*, α-syn over-expression is less toxic in cells expressing stress inducible Ssa3 or Ssa4 as the only source of Hsp70 than the constitutive ones. The reduction in toxicity reported here does not attributes to increase in level of Hsp104 or Hsp90 in A3 strain, as deletion of Hsp104 and Hsp90 in A3 strain also reduces α-syn associated cytotoxicity. Hsp70 has two functional domains; NBD (Nucleotide-Binding Domain) and SBD (Substrate-Binding Domain). We showed that NBD of Ssa Hsp70 governs their functional distinction with regard to α-syn toxicity, as the hybrid A32 reduced toxicity better than the hybrid A23. In vitro fibrillisation assay showed that Ssa3 has little effect on α-syn fibrillisation implying that reduction in Ssa3 strain is not due to refolding. Surprisingly Ssa23 behaves similar to Ssa3 in vitro. This can be explained based upon the previous results that SBD is involved in reduction of fibrillisation in vitro. The knockout of gene encoding Atg5, key regulator of Autophagy, restored toxicity in Ssa3 strain suggesting Ssa3 reduces α-syn mediated toxicity through modulation of Autophagy. Western results show that α-syn level increased in Ssa3 upon deletion of ATG5. Higher autophagic flux in A3 strain also attributes to its ability to reduce amyloid associated toxicity better than its constitutive counterpart. GSH is a known player in relation with α-syn associated toxicity (Flower et al., 2005). Increase in intracellular Glutathione level makes cell better armoured for combating oxidative stress. A3 and A32 strains showed higher total Glutathione level compared to A2 and A23 strains. Our data show that Hsp70 mediated reduction in α-syn toxicity is a combined effect of Autophagy and increased Glutahione level.

Using ThT assay 5 BMs were screened against α-syn fibrillation in vitro. BM.4 was found to be the most potent one. BM.4 was further validated using other methods like TEM imaging, CD spectroscopy and SDS-PAGE analysis. To confirm that decrease in fluorescence in ThT

assay is due to decrease in fibril formation or not, direct imaging using TEM was done to visualise fibrils. TEM images clearly showed absence of any fibrils. As it is well known fact that amyloids form β-sheet structures, CD spectroscopy was performed to detect presence of any β-sheet structures in presence of BM.4 in α-syn after 5 hours of incubation at $37^{O}C$. α-syn alone showed β-sheet profile while in presence of BM.4 α-syn retained random coil profile. SDS-PAGE analysis also went along with the other results confirming that BM.4 has the potential to reduce α-syn fibril formation. Further validation by in vivo analysis using PC12 cells further proved ability of BM.4 to reduce amyloid associated toxicity. Visualisation of α-syn-GFP punctae in yeast model of α-syn toxicity showed solubilisation of aggregates in presence of BM.4 derivatives. Probable mechanism of inhibition of α-syn fibril formation by BM.4 could be its interaction with α-syn monomers in 1:1 stoichiometry and retaining the protein in its monomer form. Interaction of BM.4 with α-syn was checked by BLI experiment. The results showed that BM.4 has affinity in μM range with α-syn which infers inhibition of α-syn fibrillation by BM.4 might be through its interaction with the same. Thus, the present molecule could be used as a potent therapeutic against α-synucleinopathies including PD.

Phage display has always been used as a powerful technique to screen peptide interactors for various biomolecules in question. Here we employed phage display technique to identify strong interacting cyclic peptides of α-syn which could also inhibit its fibril formation. After 6 rounds of screening and with gradually increasing washing conditions we narrowed down to around 1000 phages from a vast library of 10^{12} phages. The interacting phages were further screened for their anti-fibrillation activity against α-syn. The most potent one was sequenced and synthesised for validation as a potent inhibitor. In vitro fibrillation assay using ThT, CD analysis, SDS-PAGE analysis and TEM imaging technique were used to successfully validate Pep.3 and CycPep.3 as novel peptides for inhibition of α-syn fibrillation. To check the effect

of Pep.3 and CycPep.3 in vivo we used PC12 cells and performed MTT assay in presence of α-syn amyloids. Increase in absorbance in presence of peptides proved that the peptides have capability to reduce toxicity in neuronal cell line model as well. Also the peptides at 4 times higher concentration were found be non-toxic to PC12 cells. Deducing the active motif of Pep.3 by trimming the peptide from both N-terminal and C-terminal yielded Pep.3c as the motif required for inhibition of α-syn fibrillation. Thus, all the above results showed that Pep.3 and CycPep.3 are a potent inhibitor of α-syn fibrillation. As it is already known that all amyloid diseases like AD, HD, Prion diseases, DM2, ALS etc are because of deposition of amyloids and these amyloids share similar biophysical properties. Thus, the inhibitor found in this study can further be explored for other amyloid associated disorders.