

Summary

Biopharmaceuticals typically display high selectivity and high potency towards specific targets, but also often display poor stability and limited permeation across biologic barriers. A strategy to enhance their therapeutic potential is to employ cell-penetrating peptides (CPPs) as delivery vectors. These carriers are short, often cationic peptide sequences, which can permeate cell membranes and deliver cargo. Research with CPPs *in vitro* often comprises modifications of the parent CPP, with the aim of enhancing the stability, detection and delivery potential of the investigated compounds. However, modifying the parent CPP may change the interaction between the CPP, the cargo, and the targeted cell when examined *in vitro*. As a consequence, the therapeutic effect of the CPP may be difficult to replicate *in vivo* and in clinical trials. Thus, the aim of the current study was to examine the role that subtle, structural differences in a CPP conferred on the interaction of CPPs with cargo, individual cells and epithelial membranes *in vitro*. Penetratin (PEN) was used as a model CPP, and fluorophore-conjugation and stereochemistry as examples of relevant alterations. The natural L-form of PEN was labeled with a panel of fluorophores and these, along with the corresponding PEN D-enantiomer, were exposed to a panel of *in vitro* cell models.

The CPPs and fluorophore conjugates were evaluated regarding their cytotoxicity (cell viability, membrane integrity, ultrastructure), their translocation (intracellular distribution, translocation propensity, mode of action) and cargo delivery potential (intracellular, transepithelial and cargo dependency). Conjugation of PEN to an array of fluorophores resulted in significant detrimental effects observed in multiple cell models, evident by a rapid, pronounced reduction in cell metabolism and reduction in IC_{50} values, altered cell morphology and increased cell membrane permeability. In general, cationic, bulky fluorophores were notably more cytotoxic as compared to neutral or anionic fluorophores.

Furthermore, the conjugates altered the mode of translocation of the non-labeled PEN inconsistently, as the examined fluorophore-CPP conjugates displayed distinctly different distribution patterns as well as different degrees of cell internalization. Upon exposure to mammalian cells, it was demonstrated that D-PEN induces a rapid reduction in cell metabolism in addition to the pronounced reduction in plasma membrane integrity and changes in the cellular ultrastructure. By comparison, L-PEN induced no cytotoxicity at equimolar concentrations. It was observed that the examined enantiomers were internalized by different pathways: L-PEN translocated by endocytosis, and D-PEN by direct translocation. This observation was consistent with an increased paracellular delivery mediated by D-PEN, which was ascribed to non-specific effects on tight junctions as a result of membrane perturbation. In conclusion, it was demonstrated that fluorophore-conjugation and stereochemistry comprise critical determinants of the therapeutic value of PEN as both fluorophore-conjugation and stereochemistry played a significant role in the interaction of the parent CPP with the cargo and the cell and epithelium.