A80

Microcalorimetric and spectroscopic studies on the mechanism of interaction between novel peptoids and lipid bilayers - effect of length, charge and N-terminal end group

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Biomacromolecules as proteins and nucleic acids are promising drug candidates. However, one problem with biomacromolecules is that they usually have to pass the cell membrane to exert their effect. Utilization of cell penetrating peptides (CPPs) might be a way to transport biomacromolecules across the cell membrane. It is becoming increasingly evident that CPP uptake pathways may vary depending on the physico-chemical properties of the CPP and the cargo they deliver, the specific cell types and the specific experimental conditions. Nevertheless, the interaction between CPPs and membrane is the very first step of the internalization. Analysis of the CPPs interaction with liposomes is expected to provide information about the CPPs interaction with the cell membrane. We have performed a thermodynamic characterization and spectroscopic of the binding between a series of novel CPPs and anionic liposomes. Recently, we described a new class of CPPs, which seem to show superior biological effect compared to the well described CPPs. The molecular design of these alpha-peptide-beta-peptoid chimeras is based on alternating repeats of (-amino acids and (-peptoid residues. The rationale was to benefit from the structure-promoting effects and lipophilicity from the unnatural chiral (-peptoid residues, and the (-amino acid residues providing cationic properties and hydrogen bonding possibilities. The chimeras are very stable toward proteolysis, non-hemolytic, possess antibacterial activity and promising cell-penetrating potential. Interpretation of the data obtained in ITC-experiments showed that an increased number of basic residues in the novel CPPs sequence resulted in a more favorable interaction with the anionic liposomes. Additional experiments revealed that a hydrophobic interaction was a part of the binding. From CD spectra it was concluded, that no major structural changes occurred in the novel CPPs when they were in the presence of anionic liposomes. The initial electrostatic attraction in CPPs internalization mechanism was confirmed by comparing Gibbs free energy ((*G*) with the number of basic residues. Furthermore, it is proposed that the hydrophobic interaction registered could be between hydrophobic groups on the novel CPP and the hydrophobic region of the liposome. Another possibility could be simultaneously increased lipid-lipid interaction in the hydrophobic region of the liposome. In conclusion, when comparing the novel CPPs with results obtained for the well described CPP penetratin it seems, that the binding to anionic liposomes is more favorable for all novel CPPs investigated.

doi:10.1016/j.drudis.2010.09.426

A81

Studies towards improved cell-penetrating peptide-promoted macromolecular drug delivery

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The general concept of drug delivery facilitated by cell-penetrating peptides (CPPs) is well-known; however its practical utility for delivery of biopharmaceuticals necessitates further development concerning *in vivo* stability and efficiency of these peptidic carriers. In the present project, the aim is to increase the stability towards enzymatic degradation as well as to improve membrane translocation properties by incorporating novel unnatural amino acids into the naturally occurring CPP penetratin. The CPP efficiency of these penetratin analogues will be tested upon conjugation to a therapeutic biomacromolecule. Nine novel and unique amino acid building blocks have been synthesized from enantiopure aziridines to form amino acids with additional cationic charges as compared to natural amino acids. An increased number of cationic charges in CPPs have been shown to improve the interaction between CPPs and the cell membrane. The novel amino acids will be incorporated into penetratin to increase its cationic charge and to generate more efficient and stable CPPs. The enzymatic stability of penetratin is estimated by testing its resistance towards degradation by intestinal juice from rats. The metabolites are analyzed by an Orbitrap MS to identify the initial sites of cleavage and the largest non-degradable fragment as well. Thereby the optimal sites for incorporation of the novel amino acids may be revealed. The modified penetratin molecules will be tested for stability and CPP efficiency.

doi:10.1016/j.drudis.2010.09.427

A82

New configuration of an *in vitro* blood–brain barrier model

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It is an undeniable fact that neuroscience has an urgent need for a reliable and translatable *in vitro* model to investigate the human blood–brain barrier (B3). The use of human primary cerebral capillary endothelial cells is considered to provide such a model. The aim of the present study was to compare a B3-model based on two novel immortalized human primary brain endothelial cell (hBEC) lines. The human cerebral cortex microvascular endothelial cell (hCMEC-D3) and the human brain capillary endothelial cell line (NKIM-6) were used. These cell lines were used to investigate the potential transport of large molecules across the cell monolayer. The B3 is unique in that it consists of highly selective endothelial cell interface that create tight junctions around the capillaries separating the bloodstream from the brain parenchyma. Brain endothelial cells in association with astrocytes display complex tight junctions, polarized expression of enzymes, transporters and receptors. In order to take advantage of the influence associated with astrocytes we established an *in vitro* co-culture model of hBECs with primary human astrocytes. The co-culture was performed either by growing the cells on either side of a permeable membrane or growth in direct contact. Using a cell-based kinetic profiling approach

we studied the optimal conditions for attachment and proliferation of the astrocytes and hBECs. Furthermore, we monitored the effect of hBEC growing directly on the surface of an adherent astrocytic monolayer. The tight junctions between the brain endothelial cells forms a diffusion barrier that is responsible for the high paracellular resistance which is a crucial characteristic for any B3-model. In order to test the integrity of this barrier in the B3-model and simultaneously measure the transcellular transport we combined fluorescent compounds and dye labelled large molecules to test the permeability across the barrier. This strategy allows for the discrimination between transcellular and paracellular transport.

doi:10.1016/j.drudis.2010.09.428

A83
Solid lipid nanoparticles for gene delivery into prostate cancer cells

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Prostate adenocarcinoma is the most common cancer occurring in male. The aim of this study is to develop a gene delivery system based on solid lipid nanoparticles (SLNs) for the transfer of tumor suppressor genes that are able to induce death into prostate cancer cells. Formulations of cationic SLNs, consisting of stearic acid/DOTAP/pluronic, were produced. Additionally, formulations with and without 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in various molar ratios were tested. The SLNs produced were approximately 100 nm in size and showed a positive surface charge (+40 mV) in water. The SLNs showed excellent stability, as evidenced by size, zeta potential, transfection efficiency over 140 days, and possibility of lyophilization and/or sterilization without loss of efficiency. The SLNs were able to protect genetic material against DNase digestion and showed a transfection capacity comparable to that of Lipofectamine 2000®, a commercially available gene carrier. Interestingly, we found that the transfection efficiency of SLNs in prostate cancer PC3 cells was significantly higher when compared to that in normal human prostate PNT2-C2 cells. Further examination revealed that this is due to enhanced endosomal escape rather than enhanced internalization of SLNs in prostate cancer cells. These results indicate that cationic SLNs are a promising tool for gene delivery into prostate cancer cells.

doi:10.1016/j.drudis.2010.09.429

A84
Kinases in cationic lipid/polymer-mediated gene delivery

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Cationic lipids/polymers, complexed with DNA (also called lipo/polyplexes), are promising tools for gene delivery or transfection. Lipo/polyplexes have low toxicity, a relative low immunological response and can be synthesized on large scale. Lipo/polyplexes are internalized by cells via endocytosis. The endocytotic pathway that is used by lipo/polyplexes depends on the cell type and the type of lipo/polyplexes, and likely contributes to transfection efficiency. We have recently shown that adhesion receptors are involved in binding and endocytosis of lipoplexes. Cell receptors also have been described for the endocytosis of polyplexes. Receptor occupation can initiate signaling cascades, commonly mediated by kinases, which in turn tightly regulate endocytosis and endocytotic processing. The elucidation of cellular signaling signatures, initiated by lipo/polyplexes and/or those that allow or preclude gene delivery, will be instrumental in understanding the interaction between lipo/polyplexes and cells at the molecular level and contribute to the design of protocols with improved gene delivery efficiency. In this study we have performed a screen with a wide range of validated pharmacological kinase inhibitors, and evaluated their effects on lipo/polyplex transfection efficiency. In this screen a kinase is identified that specifically influences the transfection efficiency of a polyplex. It is further demonstrated that, as a part of the underlying mechanism, this kinase regulates the endocytotic processing of the polyplex and, as a consequence, controls its endosomal escape.

doi:10.1016/j.drudis.2010.09.430

A85
Peptide-based nano-particle for *in vivo* delivery of siRNA

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The development of short interfering RNA (siRNA), has provided great hope for therapeutic targeting of specific genes responsible of pathological disorders. However their clinical application remains limited by their poor cellular uptake, low bioavailability, and insufficient capability to reach targets *in vivo*. We have designed a novel approach, based on short amphipathic peptides 'CADY' that promotes efficient delivery of siRNA into wide variety of mammalian cell lines and *in vivo* upon systemic and topical administrations. This carrier consisting of a balance between hydrophobic and hydrophilic domains and forms stable discrete 'nanoparticles' with siRNA, through non-covalent interactions. Cellular uptake mechanism of CADY/siRNA nanoparticles is dependent on the size of the particle and involves membrane potential and dynamic, which enables a rapid release of the siRNA into the cytoplasm and promotes a robust down-regulation of target mRNA. CADY-carriers were applied to the delivery of siRNA targeting the cell cycle regulatory protein Cyclin B1 into cancer cells. We demonstrated that when associated with CADY, sub-nanomolar concentrations of siRNA Cyclin B1 significantly knocked down Cyclin B1 protein levels resulting in cell cycle arrest in G2 arrest and blocked cancer cell proliferation. The surface of CADY particles can be functionalized and addition of cholesterol-moiety significantly improves siRNA stability *in vivo*, thereby enhancing the efficiency of this technology for systemic administration following intravenous injection. We have validated the therapeutic potential of this strategy for cancer treatment by targeting cyclin B1 in various mouse tumour models and demonstrate that CADY-mediated delivery of cyclin B1 siRNA prevents tumour growth *in vivo* following systemic intravenous injection. Moreover, we showed that functionalization of CADY particles with other chemical groups or biological moieties can be applied to generate formulations to target specific cell types or tissues which can