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siRNA versus pharmacological inhibition of endocytic pathways for studying cellular uptake of cell penetrating peptides and other drug delivery vectors

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Cell-penetrating peptides (CPPs) have the potential to deliver numerous therapeutic macromolecules into cells including peptides, proteins, and nucleic acids. Under defined conditions endocytosis is thought to be of significant importance for CPP entry but identifying the exact uptake mechanism and pathway(s) involved has been difficult. Multiple pathways have been reported to contribute to uptake, including macropinocytosis and those regulated by clathrin and cavaeolin-1. This project aims enhance the use of cell penetrating peptides as drug delivery vectors by developing new technologies to study their mechanisms of uptake. Traditionally studies investigating the uptake of these molecules, and other drug delivery vectors, have been performed using chemical inhibitors but these are often toxic and lack specificity [1]. We have developed siRNA-based assays to silence endocytic proteins that have previously been shown to regulate distinct endocytic pathways. The effect of depleting these proteins was then assessed to investigate their roles in mediating the uptake of well characterised endocytic probes and CPPs. Two cell lines were predominantly used, HeLa (cervical cancer epithelial) and A431 (human epithelial carcinoma). Endocytic proteins clathrin heavy chain, flotillin-1, dynamin II, caveolin-1 and P21-activated kinase (PAK-1) were depleted using single siRNA sequences; siRNA against GFP was used as a control. In siRNA treated cells the uptake of fluorescent endocytic markers including; Alexa488-transferrin (clathrin mediated endocytosis), 40 kDa FITC Dextran (fluid phase uptake and macropinocytosis), FITC conjugated anti-CD59 antibody (flotillin-1 dependent uptake) and the uptake of Alexa488 CPPs (RRRRRRRRGC-Alexa488-R8, and GRKKRRQRRRPPQ-Alexa488-HIV-TAT) were measured by flow cytometry. Protein depletion was assessed from protein lysates using SDS PAGE and Western blotting. Overall, the data shows that siRNA transfection method could effectively reduce expression of clathrin heavy chain, caveolin-1 and flotillin-1 from HeLa cells and this then allowed for us to study effects on endocytosis of various probes. PAK-1 has been shown to regulate macropinocytosis and we show that the induction of macropinocytosis and PAK-1 expression are highly cell line dependent. Paralleled with this was our findings that cationic CPPs induce an increase in fluid phase uptake of dextran and the extent of this was cell line dependent. Comparative analysis of these experiments with those performed using pharmacological inhibitors, allowed us to determine the usefulness of this approach for drug delivery research.

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SAINTargs, a novel lipid-based targeting device for siRNA delivery

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The endothelium represents an important therapeutic target because its pivotal role in many diseases such as chronic inflammation and cancer and its accessibility for systemic administration. RNA interference by small interfering RNA (siRNA) has become in the last decade a very powerful tool in basic research, and has huge potential to become an important new class of therapeutics for humans. However, due to their size and charge, siRNAs have no bioavailability to enter unperturbed cells. To overcome this problem, our laboratory developed a non-viral lipid-based targeting device which efficiently and specifically delivers siRNA into endothelial cells. Molecular determinants expressed on the surface of inflammation-activated endothelial cells, like certain adhesion molecules and receptors involved in endocytosis, are excellent candidates to increase carrier-mediated siRNA uptake. Therefore we conjugated monoclonal anti-E-selectin antibodies to the cationic amphiphilic lipid, 1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride (SAINT-18) which was complexed in a 1:2000 molar ratio with the transfection agent SAINT-MIX (SAINT:DOPE, 1:1), and siRNA, resulting in a siRNA containing lipoplex called anti-E-selectin-SAINTarg [1]. Our findings demonstrate that anti-E-selectin-SAINTargs maintained the antigen recognition capacity of the parental antibody and showed increased siRNA uptake in otherwise difficult-to-transfect primary human umbilical vein endothelial cells (HUVEC) as compared to non-targeted SAINT-MIX. Moreover, anti-E-selectin-SAINTargs superior binding and uptake efficiency was corroborated by improved silencing of both gene- and protein expression of VE-cadherin in activated HUVEC. The VE-cadherin gene expression could be silenced up to 95% by VE-cadherin specific siRNA, at low siRNA concentrations (30 pmol/ml). Furthermore, no non-specific silencing by scrambled or VE-cadherin specific siRNA was observed. To optimize siRNA delivery into activated endothelial cells we also synthesized anti-VCAM-1-SAINTargs which were as efficient in VE-cadherin silencing as anti-E-selectin-SAINTargs. Because of the heterogeneous expression of adhesion molecules on inflammation-activated endothelial cells *in vivo*, a combination of these two SAINTargs may result in enhanced siRNA effects. Taken together, SAINTargs demonstrate specific and efficient targeting to inflammation-activated difficult-to-transfect primary endothelial cells and results in strong siRNA specific gene silencing at low siRNA concentrations.

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