Cell-penetrating-peptide-mediated siRNA lung delivery

S.A. Moschos, A.E. Williams and M.A. Lindsay[1]
Biopharmaceutics Research Group, Airways Disease, National Heart and Lung Institute, Imperial College London, London SW3 6LY, U.K.

Abstract

The therapeutic application of siRNA (short interfering RNA) shows promise as an alternative approach to small-molecule inhibitors for the treatment of human disease. However, the major obstacle to its use has been the difficulty in delivering these large anionic molecules *in vivo*. A potential approach to solving this problem is the chemical conjugation of siRNA to the cationic CPPs (cell-penetrating peptides), Tat-(48–60) (transactivator of transcription) and penetratin, which have been shown previously to mediate protein and peptide delivery in a host of animal models. In this transaction, we review recent studies on the utility of siRNA for the investigation of protein function in the airways/lung. We show that, despite previous studies showing the utility of cationic CPPs *in vitro*, conjugation of siRNA to Tat-(48–60) and penetratin failed to increase residual siRNA-mediated knockdown of p38 MAPK (mitogen-activated protein kinase) (MAPK14) mRNA in mouse lung *in vivo*. Significantly, we will also discuss potential non-specific actions and the induction of immunological responses by CPPs and their conjugates and how this might limit their application for siRNA-mediated delivery *in vivo*.

Introduction

The knockdown of protein expression using siRNA (short interfering RNA)-mediated RNAi (RNA interference) is now commonly used to investigate the function of genes during biological responses [1,2]. Furthermore, this strategy is currently being examined for the treatment of diseases such as macular degeneration, hepatitis C infection and cancer [3–6]. However, owing to its relatively large size and anionic charge, one of the key problems in utilizing siRNA as a therapeutic agent, and during studies *in vivo*, is the availability of effective delivery systems. Initial investigations suggest that siRNA delivery may only be possible in cells/tissues that are amenable to topical applications such as the eye and the vagina or, largely due to the first-pass effect, it is possible to target the liver following intravenous administration.

The structure of the lung permits direct access to the branching airways and alveoli and therefore might represent an ideal organ for application of siRNA-based therapeutics. Indeed, this contention is supported by studies showing safe and efficacious siRNA-mediated modulation of a range of diseases in animal models, including acute lung injury, bleomycin-induced fibrosis and allergen-induced airway hyperresponsiveness (Table 1). These actions have been mediated through down-regulation of a variety of targets including haem oxygenase [7,8], discoidin domain receptor [9], IL (interleukin)-13 receptor [10], Fas [11], caspase 8 [11], MIP (macrophage inflammatory protein)-2 [12], KC (keratinocyte-derived cytokine) [12], caveolin-1 [13] and arginase I [14]. Interestingly, siRNAs have also been shown to effectively reduce viral titre and symptoms in mice infected with influenza, para-influenza and respiratory syncytial virus following the targeting of viral proteins [15–18]. Furthermore, Li et al. [19] have demonstrated siRNA-mediated attenuation of SARS (severe acute respiratory syndrome) following coronavirus infection in rhesus macaques using either a prophylactic or therapeutic regime.

In the majority of these studies, siRNA was administered either via the i.n. (intranasal) or the i.t. (intratracheal) route packaged in cationic lipids (e.g. Oligofectamine^{TM}) and polymers (e.g. polyethyleneimine and dendrimers). However, such siRNA formulation approaches suffer drawbacks including toxicity and siRNA-mediated induction of immune responses through TLRs (Toll-like receptors) [20–22]. To address these problems, we and others have investigated the effect of chemical conjugation to the CPPs (cell-penetrating peptides) penetratin and Tat-(48–60) (transactivator of transcription) upon siRNA delivery *in vitro* and *in vivo*. The CPPs Tat-(48–60) and penetratin are short cationic peptide sequences derived from the HIV-1 Tat [23,24] and the insect Antennapedia homeoprotein (penetratin) [25] respectively. Both have been extensively evaluated for the *in vitro* and *in vivo* delivery of biologically active peptides and proteins [26,27]. Significantly, CPPs have been shown to mediate uptake of a range of other biological and non-biological cargoes, which has led to the suggestion that they might represent a universal non-toxic approach for the delivery of oligonucleotides [28–31].

Key words: lung, p38 mitogen-activated protein kinase (p38 MAPK), penetratin, short interfering RNA (siRNA), transactivator of transcription (Tat).

Abbreviations used: CPP, cell-penetrating peptide; IL, interleukin; i.n., intranasal; i.t., intratracheal; KC, keratinocyte-derived cytokine; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; SARS, severe acute respiratory syndrome; siRNA, short interfering RNA; Tat, transactivator of transcription; TLR, Toll-like receptor; TNFα, tumour necrosis factor α.

[1]To whom correspondence should be addressed (email m.lindsay@imperial.ac.uk).

Table 1 | siRNA-mediated changes of biological activity in the lung

GFP, green fluorescent protein; HVJ, haemagglutinating virus of Japan (Sendai virus); i.v., intravenous; RSV, respiratory syncytial virus; shRNA, short hairpin RNA.

Target	Delivery approach	Biological action in animal model	Reference
Haem oxygenase-1	Unmodified siRNA (i.n.)	Protected against oxidant-induced lung injury (mouse)	[8]
Discoidin domain receptor	Unmodified siRNA (i.n.)	Inhibited bleomycin-induced lung inflammation (mouse)	[9]
IL-13 receptor	Unmodified siRNA encapsulated in HVJ-envelope vector (i.t.)	Inhibited bleomycin-induced pulmonary fibrosis (mouse)	[10]
GFP/Fas/caspase 8	Unmodified siRNA (i.t.)	Inhibited acute lung injury (mouse)	[11]
GFP/MIP2/KC	Unmodified siRNA (i.t.)	Inhibited acute lung injury (mouse)	[12]
Caveolin-1	Unmodified siRNA in liposomes (i.v.)	Increased vascular permeability; targets endothelial cells (mouse)	[13]
SARS virus	Unmodified siRNA (i.n.)	Inhibited SARS infection (mouse and rhesus monkeys)	[19]
Arginase I	Plasmid expressing shRNA in ExGen500 (i.t.)	Inhibited IL-13-induced airway hyperresponsiveness (mouse)	[14]
Haem oxygenase-1	Unmodified siRNA (i.n.)	Enhanced reperfusion-induced lung apoptosis (mouse)	[7]
Influenza	Unmodified siRNA in polyethyleneimine (i.v.)	Inhibited influenza virus infection (mouse)	[15]
Influenza (nucleoprotein or acidic polymerase)	Unmodified in siRNA Oligofectamine™ (i.v.)	Inhibited influenza virus infection (mouse)	[16]
Respiratory syncytial virus (*NS1* gene)	Plasmid expressing shRNA in NG042 (i.n.)	Inhibited RSV infection (mouse)	[17]
Respiratory syncytial virus and para-influenza virus	Unmodified siRNA in OptiMEM® with/without TransIT-TKO® (i.n.)	Inhibited RSV and para-influenza virus infection (mouse)	[18]

In vitro siRNA delivery and CPPs

A number of investigators have demonstrated penetratin-mediated delivery of siRNA and antisense oligonucleotides into isolated neurons [32–34] and knockdown of eGFP (enhanced green fluorescent protein) and CDK9 (cyclin-dependent kinase 9) expression in HeLa cells using siRNA conjugated to Tat-(48–60) [35], with biological activity reported at the sub-micromolar range. In all these studies, the CPP was linked to the siRNA via a disulfide bond; in the case of penetratin, this was to the 5′-end of the sense strand [32–34], whereas Chiu et al. [35] attached the Tat-(48–60) residue to the 3′-end of the antisense strand.

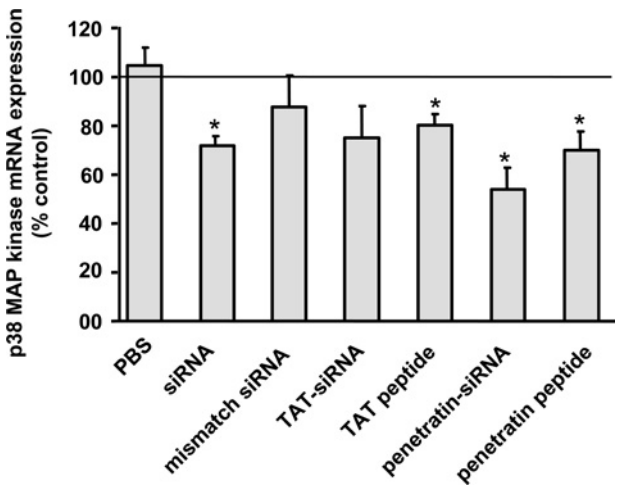
In vivo lung siRNA delivery and CPPs

In the absence of reports on Tat-(48–60)- or penetratin-mediated siRNA delivery *in vivo*, we investigated the utility of this approach in the mouse lung. Specifically, we targeted p38 MAPK (mitogen-activated protein kinase) (also known as MAPK14), whose activation is known to be important in the release of multiple pro-inflammatory mediators including TNFα (tumour necrosis factor α) and IL-1 [36,37]. In these studies, we used a commercially available siRNA sequence (Dharmacon) which we found to exert potent p38 MAPK knockdown when transfected into mouse fibrobasts (L929) in culture (IC_{50} ~140 pM). We conjugated Tat and penetratin to the 5′-end of the sense strand via a disulfide linkage between a cysteine residue on the peptide and a C_6-thiol linker on the oligonucleotides, followed by denaturing HPLC purification, and showed a limited shift in IC_{50} to ~800 pM. In the absence of transfection, unconjugated siRNA failed to show p38 MAPK knockdown *in vitro* across the concentrations evaluated (0.3–10 μM). In contrast, both the Tat-(48–60)– and penetratin–siRNA conjugates, but not CPP alone, achieved an ~40% down-regulation at the highest concentration, without loss of cell viability. However, these micromolar concentrations were significantly higher than those reported previously to give biological activity using CPP–siRNA conjugates *in vitro* [32–35]. Although we are uncertain of the reason for these differences, these investigators do not appear to have carried out post-conjugation HPLC purification in the presence of denaturing agents in order to remove excess cationic CPP from the siRNA–peptide conjugate. Thus, as with non-conjugated cationic lipids and polymers, increased delivery could have resulted from the formation of complexes [38].

To determine their biological activity *in vivo*, we administered siRNA duplexes into mice by i.t. instillation under mild anaesthesia. As reported previously with unmodified non-formulated siRNA [7,9,11,12], we observed a significant down-regulation of p38 MAPK expression using non-conjugated C_6-thiol linker-modified siRNA alone

Figure 1 | Effects of various siRNAs and CPPs on MAPK14 mRNA expression in mouse lung

Effects of MAPK14 siRNA, 4-base mismatch siRNA, Tat-(48–60) peptide, penetratin peptide and siRNA conjugated to either Tat-(48–60) (TAT-siRNA) or penetratin (penetratin-siRNA) upon MAPK14 mRNA expression in mouse lung, 6 h after i.t. administration. Results are means ± S.E.M., $n = 6$–18 animals per group. Samples were analysed using Student's t test. $^{*}P < 0.05$ compared with PBS-treated controls.

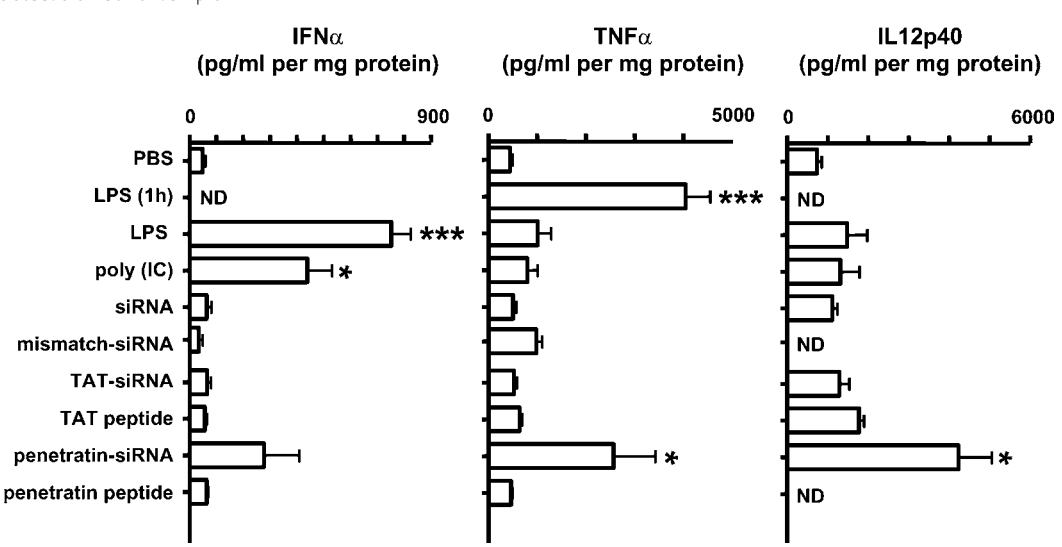


(Figure 1). This is despite the fact that siRNAs have been shown to be degraded rapidly in serum by enzymes such as RNase A [39] and implies that lung provides a more tolerant environment. Administration of the CPP–siRNA failed to show an effect of conjugation to Tat-(48–60) or penetratin upon either the distribution, magnitude or duration of lung p38 MAPK knockdown (Figure 1). In surprising contrast with our *in vitro* observations, penetratin peptide alone induced a significant knockdown in p38 MAPK mRNA expression 6 h after administration and, in the case of Tat-(48–60), this was also observed at 12 and 24 h (results not shown), suggesting a non-selective action of these peptides upon gene expression. The mechanism and extent of this phenomenon is still unclear; however, in the absence of adverse behaviour or gross histological changes at 24 h following administration, lung toxicity in the form of necrosis or apoptosis can be eliminated. Indeed, exposure of the lung epithelial A549 cell line to Tat-(48–60) or penetratin at concentrations of up to 30 μM does not induce toxicity [40]. Alternatively, the observed reduction in expression of p38 MAPK mRNA could occur following the non-specific interaction of CPPs with extracellular or intracellular targets. In this respect, both CPPs in our study have been reported to induce clathrin-dependent uptake of the TNFα and EGF (epidermal growth factor) receptors, without their activation [41]. Additionally, as these highly positively charged CPPs are major constituents of transcription factor DNA-binding domains, interference with gene transcription could account for our observations. Interestingly, this tight binding to both plasma membranes and genomic DNA has been shown previously to result in erroneous conclusions about the mechanism of CPP uptake [42,43].

Notwithstanding these non-selective actions of Tat-(48–60) and penetratin, we have additionally observed the elicitation of an innate immune response by the penetratin–siRNA, but not the Tat-(48–60)–siRNA conjugate or the siRNA alone (Figure 2). On the basis of the cytokine profile documented, this response could be a result of the

Figure 2 | Effects of various siRNAs and CPPs on pro-inflammatory cytokine levels in mouse lung

Effects of MAPK14 siRNA, 4-base mismatch siRNA, Tat-(48–60) peptide, penetratin peptide and siRNA conjugated to either Tat-(48–60) (TAT-siRNA) or penetratin (penetratin-siRNA) upon pro-inflammatory cytokine levels in the mouse lung 6 h after i.t. administration. Results are means ± S.E.M., $n = 5$–6 animals per group. Samples were analysed using the one-way ANOVA and Tukey post-analysis. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with PBS-treated controls. IFN, interferon; ND, not determined because of lack of sample.



activation of TLR-3, -7 and/or TLR-8 in the endosomal pathway [44]. As siRNA, Tat-(48–60) peptide or penetratin peptide alone did not have an impact upon inflammatory biomarker levels, we believe that the mechanics of penetratin-mediated siRNA delivery might differ from that of the Tat-(48–60) conjugate.

Overall, these studies and those of other investigators suggest that the airways and lung are an ideal target for siRNA-based therapeutics. Thus even unmodified and non-formulated siRNA produce protein knockdown following i.t. and i.n. delivery. Under these circumstances, it may not be surprising that conjugation to CPPs such as Tat-(48–60) and penetratin were unable to increase knockdown of p38 MAPK mRNA expression, although this approach may be beneficial for delivery in other systems. However, our results imply that, when using Tat-(48–60) and penetratin as a delivery vehicle, it will be important to consider non-specific actions of these CPPs, as well as the possible induction of immunological responses by the penetratin–siRNA conjugates.

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References

1 Dorsett, Y. and Tuschl, T. (2004) Nat. Rev. Drug Discovery **3**, 318–329
2 Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature **411**, 494–498
3 Jones, S.W., Souza, P.M. and Lindsay, M.A. (2004) Curr. Opin. Pharmacol. **4**, 522–527
4 Mahanthappa, N. (2005) Pharmacogenomics **6**, 879–883
5 Whelan, J. (2005) Drug Discovery Today **10**, 1014–1015
6 Karagiannis, T.C. and El Osta, A. (2005) Cancer Gene Ther. **12**, 787–795
7 Zhang, X., Shan, P., Jiang, D., Noble, P.W., Abraham, N.G., Kappas, A. and Lee, P.J. (2004) J. Biol. Chem. **279**, 10677–10684
8 Zhang, X., Shan, P., Jiang, G., Zhang, S.S., Otterbein, L.E., Fu, X.Y. and Lee, P.J. (2006) FASEB J. **20**, 2156–2158
9 Matsuyama, W., Watanabe, M., Shirahama, Y., Hirano, R., Mitsuyama, H., Higashimoto, I., Osame, M. and Arimura, K. (2006) J. Immunol. **176**, 1928–1936
10 Fichtner-Feigl, S., Strober, W., Kawakami, K., Puri, R.K. and Kitani, A. (2006) Nat. Med. **12**, 99–106
11 Perl, M., Chung, C.S., Lomas-Neira, J., Rachel, T.M., Biffl, W.L., Cioffi, W.G. and Ayala, A. (2005) Am. J. Pathol. **167**, 1545–1559
12 Lomas-Neira, J.L., Chung, C.S., Wesche, D.E., Perl, M. and Ayala, A. (2005) J. Leukocyte Biol. **77**, 846–853
13 Miyawaki-Shimizu, K., Predescu, D., Shimizu, J., Broman, M., Predescu, S. and Malik, A.B. (2006) Am. J. Physiol. Lung Cell Mol. Physiol. **290**, L405–L413
14 Yang, M., Rangasamy, D., Matthaei, K.I., Frew, A.J., Zimmmermann, N., Mahalingam, S., Webb, D.C., Tremethick, D.J., Thompson, P.J., Hogan, S.P. et al. (2006) J. Immunol. **177**, 5595–5603
15 Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N. and Chen, J. (2004) Proc. Natl. Acad. Sci. U.S.A. **101**, 8676–8681
16 Tompkins, S.M., Lo, C.Y., Tumpey, T.M. and Epstein, S.L. (2004) Proc. Natl. Acad. Sci. U.S.A. **101**, 8682–8686
17 Zhang, W., Yang, H., Kong, X., Mohapatra, S., Juan-Vergara, H.S., Hellermann, G., Behera, S., Singam, R., Lockey, R.F. and Mohapatra, S.S. (2005) Nat. Med. **11**, 56–62
18 Bitko, V., Musiyenko, A., Shulyayeva, O. and Barik, S. (2005) Nat. Med. **11**, 50–55
19 Li, B.J., Tang, Q., Cheng, D., Qin, C., Xie, F.Y., Wei, Q., Xu, J., Liu, Y., Zheng, B.J., Woodle, M.C. et al. (2005) Nat. Med. **11**, 944–951
20 Kariko, K., Bhuyan, P., Capodici, J. and Weissman, D. (2004) J. Immunol. **172**, 6545–6549
21 Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A. et al. (2005) Nat. Med. **11**, 263–270
22 Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K. and MacLachlan, I. (2005) Nat. Biotechnol. **23**, 457–462
23 Frankel, A.D. and Pabo, C.O. (1988) Cell **55**, 1189–1193
24 Green, M. and Loewenstein, P.M. (1988) Cell **55**, 1179–1188
25 Berlose, J.P., Convert, O., Derossi, D., Brunissen, A. and Chassaing, G. (1996) Eur. J. Biochem. **242**, 372–386
26 Lindsay, M.A. (2002) Curr. Opin. Pharmacol. **2**, 587–594
27 Dietz, G.P. and Bahr, M. (2004) Mol. Cell. Neurosci. **27**, 85–131
28 Snyder, E.L. and Dowdy, S.F. (2001) Curr. Opin. Mol. Ther. **3**, 147–152
29 Schwarze, S.R. and Dowdy, S.F. (2000) Trends Pharmacol. Sci. **21**, 45–48
30 Gait, M.J. (2003) Cell. Mol. Life Sci. **60**, 844–853
31 Zatsepin, T.S., Turner, J.J., Oretskaya, T.S. and Gait, M.J. (2005) Curr. Pharm. Des. **11**, 3639–3654
32 Muratovska, A. and Eccles, M.R. (2004) FEBS Lett. **558**, 63–68
33 Davidson, T.J., Harel, S., Arboleda, V.A., Prunell, G.F., Shelanski, M.L., Greene, L.A. and Troy, C.M. (2004) J. Neurosci. **24**, 10040–10046
34 Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M. and Greenberg, M.E. (2006) Nature **439**, 283–289
35 Chiu, Y.L., Ali, A., Chu, C.Y., Cao, H. and Rana, T.M. (2004) Chem. Biol. **11**, 1165–1175
36 O'Neill, L.A. (2006) Nat. Rev. Drug Discovery **5**, 549–563
37 Saklatvala, J. (2004) Curr. Opin. Pharmacol. **4**, 372–377
38 Turner, J.J., Arzumanov, A.A. and Gait, M.J. (2005) Nucleic Acids Res. **33**, 27–42
39 Turner, J.J., Jones, S.W., Moschos, S.A., Lindsay, M.A. and Gait, M.J. (2007) Mol. Biosyst. **3**, 43–50
40 Jones, S.W., Christison, R., Bundell, K., Voyce, C.J., Brockbank, S.M., Newham, P. and Lindsay, M.A. (2005) Br. J. Pharmacol. **145**, 1093–1102
41 Fotin-Mleczek, M., Welte, S., Mader, O., Duchardt, F., Fischer, R., Hufnagel, H., Scheurich, P. and Brock, R. (2005) J. Cell Sci. **118**, 3339–3351
42 Richard, J.P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M.J., Chernomordik, L.V. and Lebleu, B. (2003) J. Biol. Chem. **278**, 585–590
43 Green, I., Christison, R., Voyce, C.J., Bundell, K.R. and Lindsay, M.A. (2003) Trends Pharmacol. Sci. **24**, 213–215
44 Sioud, M. (2006) Trends Mol. Med. **12**, 167–176

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