Nitric Oxide Synthase Silencing by Bimodal Liposomes may Reduce Perfusion in Tumours as Assessed by DCE-MRI

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Introduction: The up regulation and production of nitric oxide (NO) by nitric oxide synthases (NOS), of which the inducible (iNOS) produces the largest amounts of NO, has been associated with tumour grade and poor outcome (1,2). Studies by Robinson *et al.* 2006, utilizing subcutaneous tumours grown from DLD-1 human colon adenocarcinoma cells, previously transfected to either constitutively overexpress murine iNOS (iNOS-19) or the empty vector (pBAN2R) (3) showed that overexpressing tumours grew more rapidly in vivo, had an increased degree of functionally perfused vasculature and were resistance to vascular shut down from vascular disrupting agents (3,4), making iNOS an interesting target for anti-angiogenic therapy. Liposome nanoparticle formulations are now very attractive in drug delivery especially in gene therapy, as they can be formulated to express a range of imaging tracers and accumulate within tumour tissue due to the endothelial perfusion and retention (EPR) effect. However, showing therapeutic out come has been a problem due to the degree of siRNA delivery. In this study, liposomes that have both gadolinium and rhodamine imaging tracers and containing murine iNOS siRNA will be investigated *in vitro* by incubation with the pBAN2 and iNOS-19 cell lines over a period of 72 hours, or *in vivo*, by inoculating iNOS-19 tumour bearing mice where the T_1 over 24 hours will be used to assess liposome delivery, and tumour volume and Dynamic Contrast Enhanced MRI (DCE-MRI) will be used to therapeutic out come.

Methods: Liposome formulations: Cationic liposomes were formulated Gd.DOTA.DSA/DOPC/CDAN/DOPE-Rhodamine for either *in vitro* (0% PEG): 30/38.5/31/0.5 mol%, or *in vivo* applications (7.5% PEG): 30/31/31/7.5/0.5 mol%. The liposomes contain a previously synthesized paramagnetic lipid Gd.DOTA.DSA which has been previously described by Kamaly *et al.* 2008 (5). Briefly; appropriate amounts of each stock lipid was added to a round-bottom flask and the solution evaporated to produce a thin film, which was then re-hydrated with 4mM HEPES buffer and the solution sonicated for 2h at 30 ^{o}C to produce empty liposome (containing no siRNA). Mm_Nos2_2_HP siRNA (target sequence CCG ATT TAG AGT CTT GGT GAA), or AllStars negative siRNA was then added to liposome solutions under vigorous vortexing at a 1:12 siRNA: lipid ratio. The liposomes were further sonicated for 5 minutes and then sized by photon correlations spectroscopy and were in the size range 70-120 nm. MR was carried out on phantoms to insure similar relaxivities for each liposome preparation.

***In vitro* experiments:** pBAN2 and iNOS-19 expressing cells (5 x 10^5) were grown in 6 well plates, or slides for histology, and incubated with 40 µl of liposomes (iNOS siRNA liposomes, AllStars negative siRNA liposomes or empty liposomes) for either MRI cell labeling, FACs analysis, nitrite production (Greiss Reaction as described by Robinson *et al.* 2006) or fluorescent microscopy experiments (slides were stained with DAPI prior to imaging) at 24, 48, and 72 hours.

MRI: Cells where harvested, washed, and the pellets resuspended in 1% agarose. Cell pellets were then placed into a quadrature ^{1}H volume coil and imaged using a 4.7T Varian Direct Drive MRI scanner. A spin-echo sequence with the following parameters was used to assess T_1 relaxation, TR = 50, 100, 200, 300, 500, 700, 1200, 3000, 2800 ms, TE = 10 ms, FOV = 45 x 45 cm, averages = 1: matrix size = 256 x 128: and a 2.0 mm thickness.

***In vivo* experiments:** 1 x 10^6/0.1ml iNOS-19 cells were inoculated into the flanks of 6-8 weeks old Balb/c nude mice. When the tumours reached approximately 10 mm^3 mice were anaesthetized with an isoflurane/O_2 mix and then placed into a quadrature ^{1}H volume coil as described above. A spin-echo sequence was used to obtain T_1-weighted images using the parameters: TR = 400, 700, 2800, and 5000 ms: TE = 15 ms: FOV = 45 x 45 mm: averages = 1: matrix size = 256 x 128: 2.0 mm thickness, and 20 consecutive transverse slices covering the whole abdomen. The mice were then removed from the magnet and a tail vein cannulated for the administration of 200µl iNOS siRNA liposomes, AllStars negative siRNA liposomes, empty liposomes or water as a control (n=3 in each group). Mice were then scanned with the same parameters 2 hours, 12 hours and 24 hours post injection.When tumours reached approximately 300mm^2 mice were cannulated for administration of 100 µl 0.05 mM Gd.DTPA and placed into a quadrature ^{1}H volume coil as described above. First a multi-slice gradient echo with varying flip angle (10 to 90) was used to gain T_1 maps: TR = 65 ms, TE = 2.86 ms, FOV = 40 x 40 mm: averages = 4: matrix size = 128x128: 2.0 mm thickness, and 10 consecutive transverse slices covering the whole abdomen. For dynamic contrast acquisitions the same multi-gradient echo was used with a fixed flip angle of 40, a total of 70 volumes, where Gd.DTPA was administered directly after the first 10 baseline volumes. T-One weighted Perfusion imaging Parameter Calculation Toolkit (TOPPCAT) using the operating system ImageJ, was used to create quantitative maps of K^{trans} and fV from dynamic T_1-weighted perfusion images. After the final scan mice were injected with 15 mg/kg of the perfusion marker Hoechst 33342 was inoculated i.v, the mice sacrificed and the tumours and selective organs were excised and frozen and sections where taken for Heamotoxylin and Eosin staining.

Results: For *in vitro* results, MRI showed that there was a reduction in T_1 (~ 10%) in cells incubated with liposomes (siRNA or empty) compared to that of the control cells, indicating cellular uptake which was confirmed by both the microscope data and FACS, which was shown to be the same for both cell lines. Over expression of nitrite for the iNOS-19 cells was significantly higher than that of the pBAN2 cell line ($p< 0.001$) at each time point and was similar to that shown by Robinson *et.al* 2006 (4). However, iNOS-19 cells after incubation with iNOS siRNA liposomes showed a reduction in nitrite production at the 42 h time point compared to control iNOS-19 cells, AllStar negative siRNA liposomes or empty liposomes which was significant at the 72h time point ($p<0.01$), suggesting that the iNOS siRNA caused silencing of the murine iNOS siRNA and had an reducing effect on NO production (FIG 1). For *in vivo* results, MRI showed that both the empty liposomes and the siRNA containing liposomes had similar changes in T_1 (~ 35% ± 29) associated with an increased accumulation of paramagnetic material over the 24 hour time course, consistent with slow accumulation of liposomal nanoparticles into the tumour tissue by EPR effect similar to that shown by both Kamaly *et.al.* 2008 and Kenny *et.al.* 2009 (5,6). The tumour growth curve shows that liposomes containing iNOS siRNA did not seem to show growth delays directly after administration (FIG 2, arrow a), after approximately 5 days two tumours in the iNOS siRNA liposome treated tumours presented growth delay and in one of these tumours, at approximately 7 days, the growth regressed. Whereas tumours treated with AllStar negative or empty liposome did not show any growth retardation. However, DCE-MRI did not show any significant changes in the mean perfusion for groups treated with iNOS siRNA liposomes when compared to AllStar negative or empty liposome tumours at the tumour size taken. However, as indicated by the tumour grow curve, the tumour seems to reach 300 mm^3 prior to regressing and therefore the time point may be too early (FIG 2, arrow b). However, further data analysis may indicate if there are subtle changes in vascular perfusion at this time point or if the degree of tumour perfusion is indicative of iNOS siRNA response.

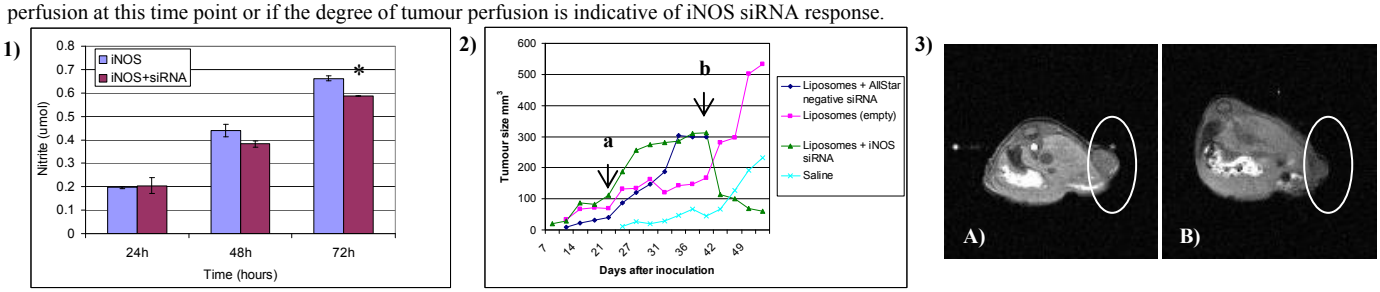

Figure 1: The comparison of nitrite accumulation in the iNOS-19 cell line with and with out incubation of iNOS siRNA liposomes. Data points ± standard deviation. * = $p<0.01$. **Figure 2:** iNOS-19 tumour grow curves for mice administered with either iNOS siRNA liposomes, AllStar negative liposomes or empty liposomes where arrow a indicates administration and arrow b indicates DCE-MRI. **Figure 3:** T_1-weighted images taken at similar anatomical points the same responding tumour (denoted by the circle) at A) 20 days and B) 36 days, after iNOS siRNA liposome administration.

Conclusions These results show that siRNA liposomes can be passively taken up into tumour cells and release siRNA causing translation and effect *in vitro* and *in vivo*. However, although the liposomal uptake can be monitored over 24 hours by assessing changes in T_1 assessing suitable changes in mean tumour perfusion by DCE-MRI may not be sensitive enough to evaluate changes leading to tumour regression and further investigation is needed longitudinally to address the timing of therapeutic outcome associated with siRNA gene therapy by liposomal delivery mechanisms. Nonetheless, these data provide evidence that liposome mediated delivery is capable of gene therapy and gives a better understanding of how imaging technologies can be used to track it.

References 1) Reveneau *et al.* Lab. Invest. 1999;79;1215. 2) Loibl *et al.* Eur. J. Cancer. 2005;41;265. 3) Jenkins *et al.* Proc. Natl. Acad. Sci. U.S.A. 1995;92;4392. 4) Robinson *et al.* Microvascular Res. 2006;71;76. 5) Kamaly N *et al.* Bioconjugate Chemistry. 2008;19;118. 6) Kenny *et al*. Proc. ISMRM p3125 (2009).