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Evaluation of a multi-functional nanocarrier for targeted breast cancer iNOS gene therapy

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ABSTRACT

The present study determines whether the novel designer biomimetic vector (DBV) can condense and deliver the cytotoxic iNOS gene to breast cancer cells to achieve a therapeutic effect. We have previously shown the benefits of iNOS for cancer gene therapy but the stumbling block to future development has been the delivery system.

The DBV was expressed, purified and complexed with the iNOS gene. The particle size and charge were determined via dynamic light scattering techniques. The toxicity of the DBV/iNOS nanoparticles was quantified using the cell toxicity and clonogenic assays. Over expression of iNOS was confirmed via Western blotting and Griess test.

The DBV delivery system fully condensed the iNOS gene with nanoparticles less than 100 nm. Transfection with the DBV/iNOS nanoparticles resulted in a maximum of 62% cell killing and less than 20% clonogenicity. INOS overexpression was confirmed and total nitrite levels were in the range of 18 μM.

We report for the first time that the DBV can successfully deliver iNOS and achieve a therapeutic effect. There is significant cytotoxicity coupled with evidence of a bystander effect. We conclude that the success of the DBV fusion protein in the delivery of iNOS *in vitro* is worthy of future *in vivo* experiments.

1. Introduction

The development of hormone refractory or chemotherapy resistant breast cancer cells means that the treatment options for this disseminated disease are mainly palliative. For those patients in this position the outcome is bleak. Thus, there is a pressing need for new and alternative therapies (Bagi, 2005). Gene therapy has been identified by many authors as a promising treatment strategy for metastatic breast cancer (Levy et al., 2006). The use of gene therapy to deliver nitric oxide offers an effective treatment for tumours. The anti-tumour effects of overexpression of the iNOS gene have been well documented. Xie et al. (1995) demonstrated that overexpression of an iNOS cell line reduced the survival and metastatic potential of a murine melanoma cell line. Further work indicated elevated apoptosis in iNOS transfectants coupled with a bystander effect (Xie and Fidler, 1998). Adenoviral-iNOS delivery together with radiation increased apoptosis in HCT116 colorectal cancer cells (Wang et al., 2004). In addition, we have shown that non-targeted liposomal (lipofectamine) delivery of the therapeutic iNOS gene in tumours generates potent cytotoxicity via $NO^{\cdot}$/peroxynitrite-induced apoptosis and radiosensitisation in both rodent and human tumour models *in vitro* and *in vivo* (Worthington et al., 2002, 2004, 2005; McCarthy et al., 2007a,b; Coulter et al., 2008). The iNOS gene has also been delivered under the control of the human osteocalcin promoter where androgen independent prostate cancer was effectively targeted *in vitro* and the levels and cytotoxicity of nitric oxide generated were comparable to those of the constitutively driven CMV/iNOS plasmid (McCarthy et al., 2007a,b). Despite this strong anti-tumour effect iNOS gene therapy is unlikely to reach the clinic because there is no effective systemic delivery system. Primary concerns include controlling expression of the iNOS gene to the target site otherwise possible systemic hypotension could occur (Wang et al., 1999; Halliwill et al., 2000).

Undoubtedly, the progress of gene therapy has been impeded by the lack of a suitable delivery vehicle. Despite the large number of gene therapy clinical trials (1579) since 1989, only 2 have made it to stage IV and only one treatment Gendicine (a p53 replication deficient adenovirus) has been commercially approved in China. Broadly, there is a trade-off between safety and effi-

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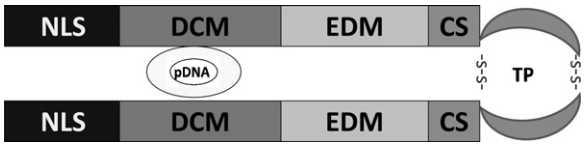

Fig. 1. Schematic diagram illustrating the domains of the designer biomimetic vector (DBV) condensing plasmid DNA (pDNA). These include a nuclear localisation signal (NLS), a DNA condensing motif (DCM), an endosomal disruption motif (EDM), a cathepsin substrate (CS) and a cyclic targeting peptide (TP). The dimer is created through the linkage of cysteine residues in targeting peptide via disulphide bonds. (This figure is a modified reprint with permission from Mangipudi et al., 2009.)

ciency, with safety concerns remaining for viral delivery systems and non-viral systems showing poor efficiency of transfection. For systemic administration of therapeutic genes, the vector should be designed to overcome many biological barriers. Impediments *in vivo* include interaction with blood components, restricted extravasation through blood vessel walls at the target site, lack of specificity and failure to enter target cells (Viola et al., 2010; Escoffre et al., 2010). Furthermore, the ideal delivery system should have the biocompatibility of polyplexes, efficiency of lipoplexes and the engineering capability of viruses. To overcome these issues an alternative strategy for gene delivery may be possible using recombinant multifunctional vectors. Techniques in genetic engineering facilitate the biosynthesis of functional motifs and design of the vector architecture at a molecular level.

Hatefi's group has recently reported the structure of a designer biomimetic vector (DBV) that essentially consists of several domains each with a distinct independent function (Mangipudi et al., 2009) (Fig. 1). These domains include: (a) an efficient DNA condensing motif (DCM) obtained from the adenoviral μ peptide, (b) a targeting peptide (TP) which enables cell-specific delivery of the nanoparticles, (c) endosomal disruption via an engineered pH responsive endosome disrupting motif (EDM) that mimics the influenza fusogenic peptide and (d) a nuclear localisation signal (NLS) obtained from a human immune-deficiency virus type 1 (HIV-1) (Keller et al., 2002; Dane et al., 2006; Cochrane et al., 1990; Midoux et al., 1998). We have shown that the DBV is able to condense plasmid DNA encoding green fluorescent protein into nanosize particles, protect DNA from degradation by serum endonucleases, target ZR-75-1 cancer cells, internalize via receptor-mediated endocytosis, disrupt endosomal membranes and exploit microtubules to translocate the genetic material into the nucleus, thus mediating gene transfer (Mangipudi et al., 2009).

We report for the first time that this novel DBV can be used to deliver the iNOS gene to breast cancer cells *in vitro* and achieve a therapeutic effect. We show that DBV/iNOS complexes can be formed and we have characterized these nanoparticles in terms of size and charge. In addition nanoparticles formed at various ratios were further analysed in terms of their ability to induce cytotoxicity in the ZR-75-1 breast cancer cell line. This cell line was selected because the targeting peptide in the DBV is developed specifically to target ZR-75-1 with minimal binding affinity towards MCF-10A normal mammary or MDA-MB-231 breast cancer cells. Given that NO has potent radiosensitising and chemosensitising effects, we believe that, coupled with this exciting novel delivery system, DBV/iNOS provides potential for an exciting gene therapy strategy for breast cancer.

2. Materials and methods

2.1. Expression and purification of DBV

The gene encoding DBV was in the pET21b (Merck, UK) expression vector under the control of T7 promoter as used previously by Mangipudi et al. (2009). The pET21b:DBV expression vector was transformed into *Escherichia coli* BL21(DE3) pLysS (Novagen, UK). Starter cultures (5 ml) were grown in LB media at 37 °C overnight with 50 μg/ml ampicillin. Circlegrow medium (MB Biomedicals, Solon, OH), 500 ml, was inoculated with the starter culture and allowed to grow at 30 °C until the absorbance reached 3.0 (565 nm). The culture was induced with 0.4 mM IPTG for 4 h at 20 °C. The culture broth was centrifuged at 3200 × *g* for 7 min at 4 °C and the pellet frozen till further use.

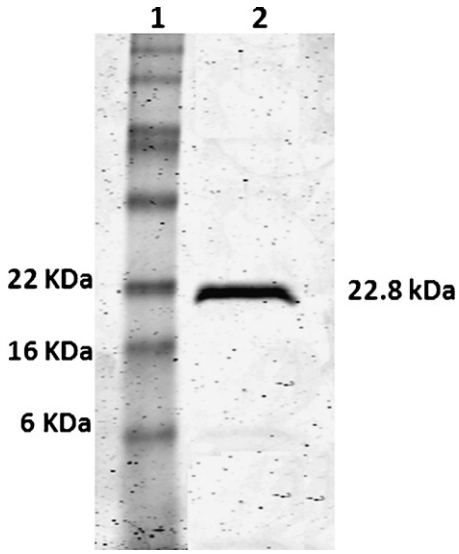

Fig. 2. SDS-PAGE of the purified designer biomimetic vector at 22.8 kDa. Lane 1 is the protein marker and lane 2 is the DBV.

The pellet was suspended in lysis buffer containing 100 mM NaH_2PO_4, 10 mM Tris–HCl (pH 8.0), 6 M guanidine HCl, 3 protease inhibitor tablets (Roche, UK) and 300 μl of cocktail 7 (Calbiochem, UK). After 60 min of gentle agitation the lysate was centrifuged for another 60 min at 3200 × *g*. The supernatant was adjusted to 10 mM immidazole and 1.8 ml of Ni-NTA resin (Qiagen, UK) added followed by 60 min of gentle agitation (25–30 rocks per min) to facilitate binding. Following centrifugation at 3200 × *g* for 5 min, the supernatant was discarded. The resin was loaded in a 0.8 × 4 ml. PolyPrep chromatography column (BioRad, UK), washed with 25 ml of lysis buffer supplemented with 10 mM immidazole, and eluted with 250 mM immidazole. The DBV rich elution was stored in 50% glycerol at −20 °C. The purity and expression of the DBV was determined by SDS-PAGE (10% Bis–Tris gels, Invitrogen, UK) (Fig. 2).

2.2. Maintenance of cells and transfections

ZR-75-1 (ATCC, Manassas, VA) breast cancer cells were grown as monolayers in RPMI 1640 (Invitrogen, UK) supplemented with 10% foetal calf serum (PAA, UK). ZR-75-1 cells were seeded in 96 well tissue culture plates at a density of 5×10^4 cells per well for transfection. Cells were approximately 80% confluent at the time of transfection. One hour before transfection, ZR-75-1 cells were conditioned in media enabling maintenance during transfection, RPMI-1640 supplemented with insulin–transferin–selinium (ITS) (Sigma–Aldrich, UK), dexamethasone (Sigma–Aldrich, UK) and fibronectin human plasma (Millipore, UK). A 100 μl aliquot of either DBV/pEGFP (pEGFP-N1, Clontech, UK) complexes at N:P ratios (4–10) containing 1 μg pEGFP or DBV/iNOS complexes at N:P ratios (8–12) containing 1 μg of iNOS gene was added to each well (N:P the molar ratio of nitrous atoms of the DBV vector to phosphates in pDNA). The plates were then incubated for 4 h in a CO_2

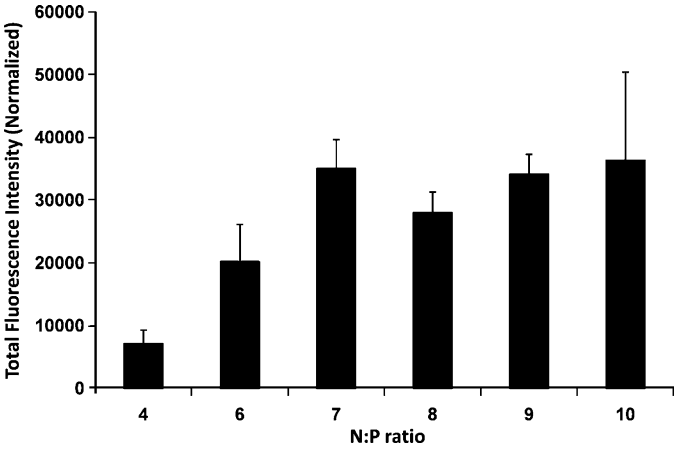

Fig. 3. Total green fluorescent protein expression for ZR-75-1 cells transfected with DBV/pEGFP complexes at range of N:P ratios. Data are the means of three independent experiments ±SEM.

incubator at 37 °C. After 4 h, the medium was removed and replaced with RPMI 1640 supplemented with 10% foetal calf serum.

2.3. Quantification of fluorescent intensity

ZR-75-1 cells that were transfected with DBV/pEGFP complexes were trypsinised and washed twice with 2% formaldehyde in phosphate buffered saline. The expression of green fluorescent protein was measured by flow cytometry using FACS calibur system (BD Biosciences, UK). The data was analysed using the Flo-jo software program and fluorescent intensity reported at 4% gating. The data are reported as means ± SEM, $n = 3$ (Fig. 3).

2.4. Neutralization of the iNOS gene by DBV

An electrophoretic gel mobility assay was performed to examine the ability of DBV in neutralizing the plasmid DNA negative charges. The DBV was complexed with the iNOS gene at various N:P ratios. Various amounts of vector solution in bis–tris propane buffer (pH 7.4) were added to an equal volume of H_2O at 22 MΩ (Millipore, UK) containing 1 μg of DNA. Bis–tris propane buffer consists of 10 mM bis–tris propane, 5 mM NaCl, 0.25 mg/ml BSA and PH to 7.0. Previous studies have indicated that a N:P ratio of 8–10 is optimal for DBV/pEGFP. However, for DBV/iNOS complexes with a N:P ratio of 4–14 were made and run on a 1% agarose gel to determine neutralization of particle charge (Fig. 4a).

2.5. Particle size, charge and distribution analysis of DBV/iNOS

The mean hydrodynamic particle size and charge measurements for DBV/iNOS complexes were performed using Dynamic Light Scattering (DLS) by a Malvern Nano ZS90 instrument and DTS software (Malvern Instruments, UK). The data are reported as mean of each N:P ratio ±SEM, $n = 3$ (Fig. 4b). The size distribution of the nanoparticles from N:P ratios 6–12 is also reported (Fig. 4c).

2.6. Transmission electron microscopy

The DBV/iNOS nanoparticles were prepared at a N:P ratio of 9 and stained with 5% uranyl acetate for 5 min at room temperature. The nanoparticles were imaged using JEOL 1200 EX II (120 kV) at the University of Idaho Centre for Electron Microscopy and Microanalysis (Fig. 4d).

2.7. Cell toxicity assay

This assay was performed in conditioned medium on ZR-75-1 cells for the DBV (uncomplexed), DBV/pEGFP and DBV/iNOS complexes. ZR-75-1 cells were seeded at a density of 5×10^4 cells per well in 96 well plates. Cells were treated with a serial dilution of DBV only, DBV/pEGFP and DBV/iNOS complexes for 4 h. The conditioned medium was removed and replaced with fresh RPMI supplemented with 10% serum followed by overnight incubation at 37 °C humidified CO_2 atmosphere. The control well with 0 μg/ml concentration received PBS. After 48 h, WST-1 reagent (Roche Applied Science, Indianapolis, IN) was added, incubated for 4 h, and absorbance was measured at 440 nm. The measured absorbance for test groups is expressed as percent of the control where the control is defined as 100% viable. The data are reported as the mean of each condition ± SEM, $n = 3$. The statistical significance was determined using an ANOVA ($p < 0.05$) (Fig. 5a).

2.8. Clonogenic assay

ZR-75-1 cells were plated and transfected as in the cell toxicity assay. Following overnight incubation the transfected cells were plated and seeded at 500 and 1000 cells per well. Non-transfected control cells were also plated out under identical conditions to transfected cells. Plates were incubated for 14 days at 37 °C in 5% CO^2/95% air. Colonies were fixed and stained with 0.4% crystal violet in 70% methanol and counted. Three independent experiments were carried out for each cell line and the mean value reported ±SEM (Fig. 5b).

2.9. Determination of iNOS expression via Western blotting

The lysates of ZR-75-1 cells transfected with DBV/iNOS at N:P ratios of 8–10 were collected to determine iNOS expression. Protein concentration was measured using UV spectrometry 595 nm. Each sample (20 μg) was electrophoresed through a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel, transferred onto a nitrocellulose membrane (Hybond-C, Amersham, UK) and probed with a rabbit monoclonal anti-iNOS antibody at 1:1000 (Santa Cruz, CA). Levels of gene expression were assessed using the Pierce Supersignal kit (MSC, Dublin, Ireland) with a Canon Powershot 7.1 mega pixel camera and ID Scan Ex v3.1 software (Fig. 6a).

2.10. Total nitrite (NO_2^-) detection

The three step Griess test (Active Motif Inc., Rixensart, Belgium) converts nitrate (NO_3^-) into nitrite (NO_2^-) giving a total NO_2^- concentration that can be quantified by construction of a standard curve. The addition of the Griess reagent then converts the NO_2^- into a purple coloured azo compound which can be assessed by photometric measurement. Media samples were taken 24 h after transfection. The RPMI1640 cell culture medium is unsuitable for Griess test analysis because of its intrinsically high levels of NO_2^-/NO_3^-. This problem was overcome by plating out in RPMI 1640, transfecting and replacing the medium with MEM for the duration of the time course. Three independent experiments were carried out for the ZR-75-1 cell line and the mean value is reported ±SEM. The statistical significance was determined using an ANOVA ($p < 0.05$) (Fig. 6b).

3. Results

The objective of this research was to determine whether the novel designer biomimetic vector could deliver the cytotoxic iNOS gene to breast cancer cells with a subsequent therapeutic effect. Many experiments were conducted to demonstrate this objective

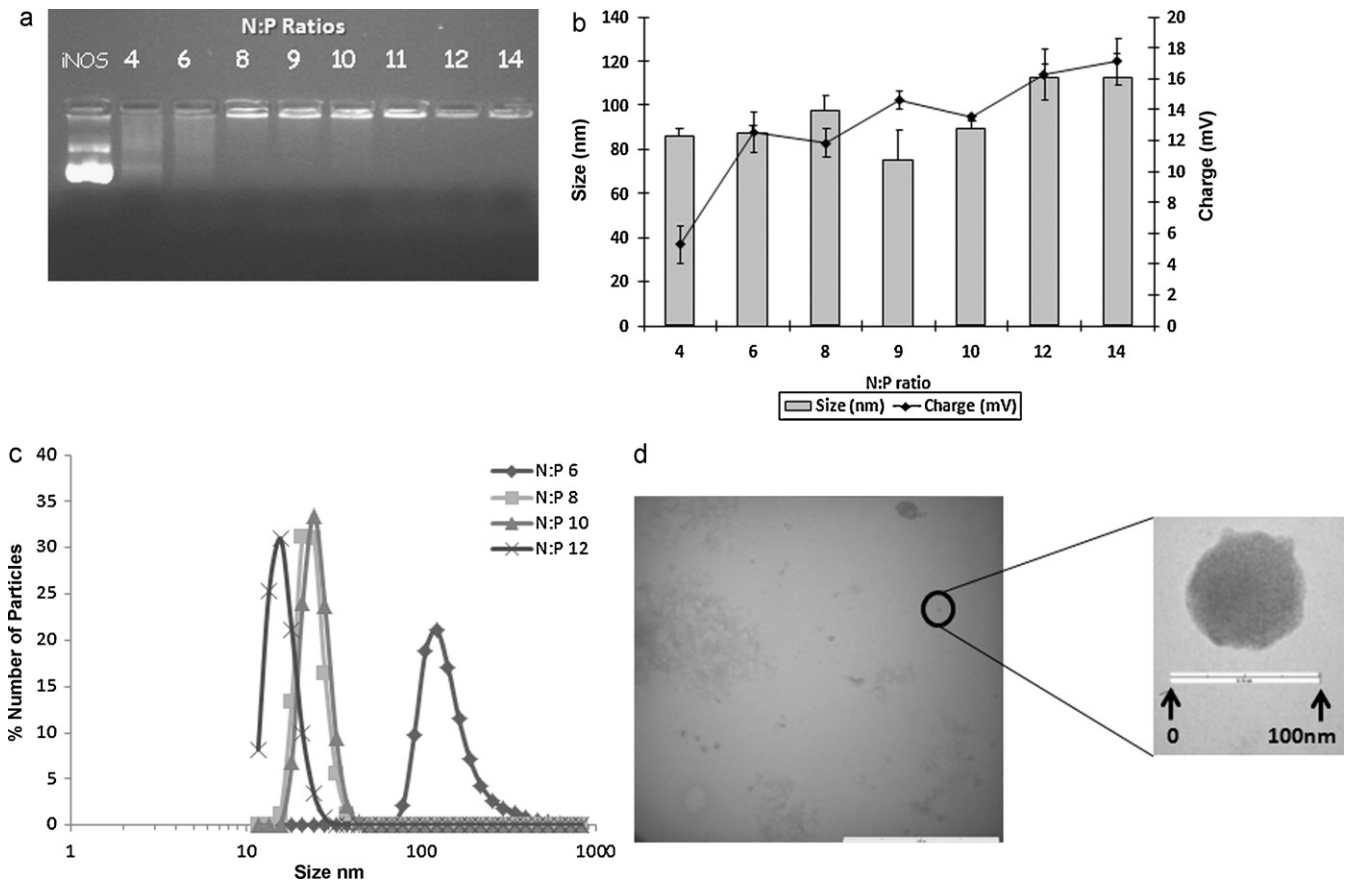

Fig. 4. Physiochemical characterisation of the DBV/iNOS nanoparticles. (a) Representative agarose gel retardation of the DBV/iNOS nanoparticles over a range of N:P ratios. (b) The particle size and charge analysis of the DBV/iNOS nanoparticles over a range of N:P ratios. (c) The DBV/iNOS particle size distribution over a range of N:P ratios. (d) The TEM picture of the spherical nanoparticles formed at N:P 9. Means are the average of 15 independent measurements ±SEM.

in terms of efficient iNOS condensation, determination of the optimal N:P ratio, particle size, charge and distribution of DBV/iNOS, evidence of cell toxicity via the WST-1 and clonogenic assay and finally proof of iNOS overexpression via Western blotting and the Griess test.

3.1. Production of DBV and pEGFP transfection

The DBV protein was expressed in *E. coli* BL21 (DE3) plysS with a 3 mg/l yield. The purity of DBV was confirmed by SDS-PAGE and the dimer (molecular weight: 22.8 kDa) was used in all subsequent experiments (Fig. 2). As a proof of principle the DBV was also complexed with the reporter pEGFP construct in order to visualise successful transfection. Previous work has shown that the DBV is specific for ZR-75-1 cells, internalizes via the receptors specific for the targeting peptide and does not transfect normal mammary cells MCF-10A that are devoid of this receptor (Mangipudi et al., 2009). Transfection efficiencies of 16% were achieved within the ZR-75-1 breast cancer cell line following delivery of a pEGFP construct using the DBV at N:P ratios of 4–10 (data not shown). Following flow cytometry, the total pEGFP fluorescent intensity displayed was normalised to the lipofectamine pEGFP positive control. For all of the N:P ratios with DBV/pEGFP there was a significant increase in fluorescent intensity from N:P ratios 7–10 (Fig. 3).

3.2. DNA neutralization, particle size, charge and distribution analysis

The gel retardation assay also indicated that the iNOS gene was fully neutralized from N:P ratio of 8 upwards (Fig. 4a). The extent of iNOS condensation with DBV was characterized in terms of shape by transmission electron microscopy with size, charge and distribution analysis by dynamic light scattering technique. The results indicate that the DBV is able to condense iNOS into nanoparticles with average sizes less than 100 nm at N:P ratios between 4 and 10 (Fig. 4b and d). It was observed at N:P ratio of 9, the iNOS gene was fully neutralized with an average size of 75.1 nm and +14.6 mV surface charge (Fig. 4b). Furthermore the distribution of the nanoparticles was over a very narrow range with N:P ratios of 8–12 with no evidence of aggregation (Fig. 4c). Therefore N:P ratios of 8 upwards could confidently be selected for the iNOS *in vitro* experiments.

3.3. Cell toxicity assay

The results of the WST-1 cell toxicity assay in ZR-75-1 cells indicate that neither the control (DBV only) nor the DBV complexed with pEGFP caused any cellular toxicity (Fig. 5a). Previous studies have also shown that DBV alone is not cytotoxic (Mangipudi et al., 2009). Indeed no signs of stress on cellular morphology were observed following transfection with DBV/pEGFP complexes. As expected from previous studies the liposomal delivery of iNOS resulted in 66% cytotoxicity. Furthermore, when the DBV was used to deliver iNOS to the ZR-75-1 cells there was considerable toxicity comparable to that of liposomal delivery. Transfection with DBV/iNOS complexes at N:P ratios from 8 to 12 resulted in survival percentages of 47, 48, 44, 44, 38 respectively. The toxicity of all the N:P ratios was significant when compared to the DBV/pEGFP controls ($p < 0.001$).

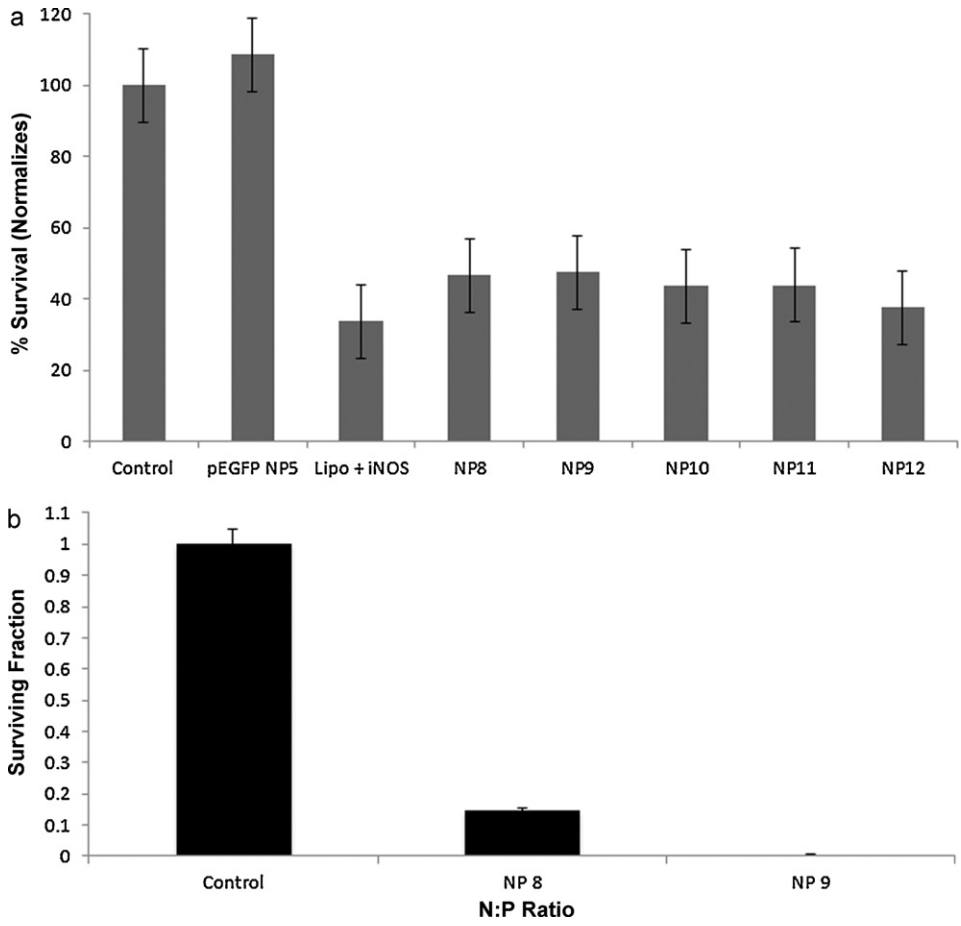

Fig. 5. Evaluation of cytotoxicity of DBV/iNOS complexes via (a) WST-1 cell toxicity assay showing ZR-75-1 cell survival following a 4 h transfection with DBV/iNOS nanoparticles. Absorbance was read at 440 nm. (b) Clonogenic assay showing surviving fraction of ZR-75-1 cells after 2 weeks following a 4 h transfection with DBV/iNOS nanoparticles. Data are the means of three independent experiments ±SEM. (*$p < 0.001$ compared to DBV/pEGFP control.)

3.4. Clonogenic assay

Transfection of ZR-75-1 cells with DBV/iNOS gave a significant ($p < 0.01$) reduction in clonogenicity at N:P ratios 8 and 9 (Fig. 5b). Surviving fractions were 14.5% and 0.4% respectively. Again this indicates evidence of a bystander effect and the inability of the breast cancer cells to form colonies following treatment at the optimal N:P ratio.

3.5. Determination of iNOS overexpression

Fig. 6a shows a representative Western blot 24 h following transfection with DBV/iNOS together with GAPDH for sample loading control. There was no detectable iNOS expression in the control, DBV only or DBV/pEGFP transfected samples. iNOS overexpression was detected in the ZR-75-1 cells transfected with either liposome/iNOS or DBV/iNOS at an N:P ratio of 9. This is an indication that the DBV/iNOS nanoparticles were stable and overcame the intracellular barriers resulting in significant iNOS expression in transfected ZR-75-1 cells. Further confirmation of the cytotoxic effect is seen by the reduction in GAPDH in those cells transfected with the iNOS gene.

Having established the overexpression of iNOS by Western blot it was important to demonstrate NO generation, which is responsible for the observed cytotoxic effect in ZR-75-1 cells *in vitro*. NO accumulation was measured using the Griess test. Twenty-four hours after transfection, 200 μl of medium was used to quantify the NO_2^- levels. The typical endogenous range of NO can be in the pM range and so the production of NO in the μM range is a clear indication that NO is being generated (Fig. 6b). Both liposomal delivery of iNOS and DBV/iNOS at a N:P ratio of 9 had significantly higher levels of NO_2^- compared to control, pEGFP and DBV only ($p < 0.001$). This is clear evident that the DBV vector can deliver iNOS to the cells with upregulation of nitric oxide production.

4. Discussion

The ideal strategy for cancer gene therapy should incorporate a therapeutic transgene that exerts a considerable cytotoxic effect and an effective delivery system that can be used for systemic delivery without evoking an immune response. In addition, there must be an element of specificity built into either the transgene or the delivery vehicle in order to prevent non-specific toxicities.

Nitric oxide has been well documented for its anti-cancer properties and several studies have elucidated that the overexpression of NO exerts substantial cytotoxicity coupled with radio and chemo sensitisation (Worthington et al., 2002, 2004; De Ridder et al., 2008). Therefore nitric oxide is ideally placed to become an adjuvant therapy to many existing regimens. Many of our previous studies have shown impressive tumour growth delay following intra-tumoural injection of CMV/iNOS complexed with lipofectamine (Worthington et al., 2002, 2004, 2005; McCarthy et al., 2007a,b; Coulter et al., 2008). Despite these encouraging data the application of this technology is somewhat limited for systemic delivery and has not fulfilled its clinical potential. This is due to the significant limitations that surround conventional delivery sys-

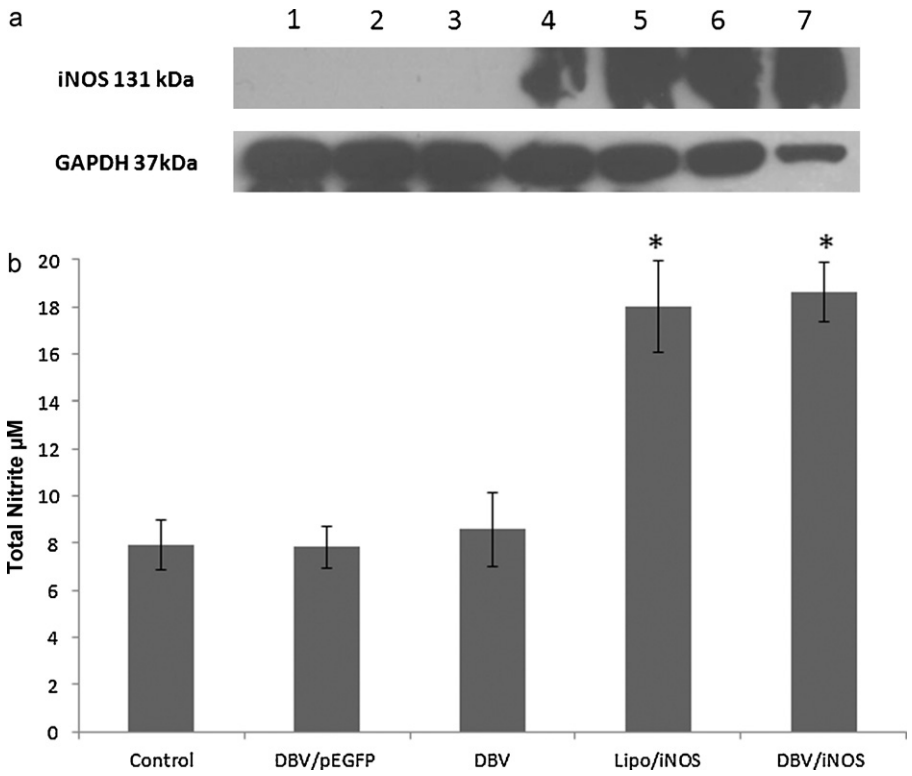

Fig. 6. Determination of iNOS overexpression via (a) Western blotting and (b) total nitrite concentrations following DBV/iNOS transfection in ZR-75-1 cells. 5a: lane 1—control, lane 2—DBV only, lane 3—DBV/pEGFP N:P 5, lane 4—lipo/iNOS, lane 5–DBV/iNOS N:P 8, lane 6—DBV/iNOS N:P 9, and lane 7—DBV/iNOS N:P 10. Data are the means of three independent experiments ±SEM. (*$p < 0.001$ compared to DBV/pEGFP control.)

tems. For example, although the high positive surface charge of liposomes may improve cellular internalization it also results in large aggregates from binding to negatively charged serum proteins (Lv et al., 2006). While PEGylation can stabilize liposomes by masking the charge *in vivo*, recent reports have indicated that multiple injections of PEGylated liposomes into rodents induced PEG specific IgM/IgG antibodies (Ambegia et al., 2005; Ishida et al., 2006).

The advent of designer biomimetic vectors heralds a new era of vectors that are composed of several distinct functional motifs that can be changed at a molecular level to carry out a distinct function. The DBV used in this study was designed to overcome some of the major intracellular barriers associated with gene delivery. One of the major advantages of the DBV over other non-viral systems is the presence of a nuclear localisation signal which eliminates the chance effect of traversing the nuclear membrane during cellular replication. The Rev sequence has previously been shown to actively facilitate the transport of nucleic acids through the nuclear pore via the importin pathway (Hammarskjold et al., 1989; Malim et al., 1989). It is possible that the Rev sequence in the DBV also facilitates iNOS transport. Previous studies have shown functionality of the NLS in the DBV by demonstrating a reduction in gene expression following treatment with bafilomycin (which is known to block microtubules) (Mangipudi et al., 2009). In addition the DBV has an extremely efficient DNA condensing Mu sequence that is rich in arginines and binds plasmid DNA within milliseconds (Murray et al., 2001). Furthermore the design of this DBV is such that the Mu sequence (DNA condensing motif) and the Rev sequence (Nuclear Localisation Sequence) are taken from core proteins within the virus that have not been shown to illicit an immunogenic response. The presence of an endosome disrupting fusogenic sequence and a targeting peptide confer further delivery advantages. The present study examined the feasibility of using the DBV to deliver the therapeutic iNOS transgene to ZR-75-1 breast cancer cells.

We examined the ability of the DBV to complex with CMV/iNOS and characterised the nanoparticle shape by transmission electron microscopy and size by dynamic light scattering techniques. Both TEM images and size analysis demonstrated the efficient condensation of CMV/iNOS to give nanoparticle sizes less than 100 nm ideal for receptor mediated endocytosis (Fig. 4b and d) (Midoux et al., 1998). Furthermore gel retardation of the DBV/iNOS confirmed neutralisation of the nanoparticles at N:P ratios of 8 and above (Fig. 4a). We have also previously shown that the DBV to be stable following incubation with 10% serum for 90 min at 37 ° (Mangipudi et al., 2009). The potential success of iNOS cancer gene therapy relies on cytotoxicity following cellular delivery. This effect is seen in Fig. 5a where the cell survival 24 h after transfection with DBV/iNOS was analysed. Given that the transfection efficiency was less than 20% there is a significant reduction in cell survival in the region of 60% for all N:P ratios selected. The cell toxicity assay illustrates acute toxicity and with cell survival less than 15% the clonogenic assay may indicate chronic toxicity (Fig. 5b). Previous studies have shown more than 50% reduction in clonogenicity compared with that expected from such relatively low transfection efficiency (McCarthy et al., 2007b).

The unpaired electron, small size and lipophilic nature of NO confers highly reactive and diffusible characteristics ideal for mediating a significant bystander effect. NO reacts with superoxide (O_2^-) and other reactive oxygen species (ROS) within the tumour to form peroxynitrite ($ONOO^-$) and reactive nitrogen oxide species RNOS (Szabó and Ohshima, 2003; Kim et al., 2001; Porasuphatana et al., 2003). As a DNA oxidant, peroxynitrite production results in the accumulation of DNA single strand breaks and cell death. Peroxynitrite is most potent during the process of DNA replication and exerts its effects in rapidly dividing cells. Generation of NO has been demonstrated via the Griess test and iNOS overexpression via Western blotting. High concentrations of peroxynitrite result in

necrosis and subsequent release of cellular contents into the extracellular environment exerting DNA damage on adjacent cells. At lower concentrations peroxynitrite has been shown to cause apoptosis via activation of caspase 3 and subsequent cleavage of caspase 9 (Shacka et al., 2006). Under normal physiological conditions poly-ADP-ribose polymerase (PARP) would be activated to mediate DNA repair. However, the production of RNOS inactivates the zinc fingers of PARP rendering it unable to repair the DNA damage and so cell death ensues (Sidorkina et al., 2003).

Many studies have highlighted the anti-cancer potential from the generation of NO via donor drugs (Griffin et al., 1996; Wink and Laval, 1994). However the concept of systemic delivery of NO donor drugs presents many physiological problems. Yet, overexpression of iNOS holds clinical potential through the inhibition of angiogenesis, reduction in metastatic potential and considerable tumour growth delay (Coulter et al., 2008; Wink et al., 1998). The production of NO via iNOS gene therapy has a targeting advantage over donor drugs thus sparing possible damage to normal tissue. For example the radiation inducible promoters WAF-1 and CArG have successfully driven iNOS expression *in vitro* and intra tumourally *in vivo* (McCarthy et al., 2007a; Coulter et al., 2008). The tumour specific human osteocalcin promoter has been shown to have exquisite specificity for androgen independent prostate cancer cells and has been used to drive iNOS expression (McCarthy et al., 2007b). Nevertheless, these transcriptionally targeted iNOS constructs need to be systemically delivered to be of therapeutic benefit. With a short half-life of 4 s and our transcriptionally targeted iNOS constructs restricting gene expression, we believe that damage to normal tissue will be minimal. Despite excellent cytotoxicity it is not envisaged that iNOS gene therapy will become a stand-alone therapy. The radiosensitising and chemosensitising characteristics make iNOS gene therapy ideally placed as an adjuvant to conventional treatment in the first instance.

The stumbling block thus far to clinical application has been the delivery system and here we have reported for the first time the use of the designer biomimetic vector to successfully condense and deliver the iNOS gene to breast cancer cells. Despite the low transfection efficiency a considerable cytotoxic effect was observed. Undoubtedly, future work will concentrate on the *in vivo* delivery, targeting and immunogenicity of the DBV/iNOS nanoparticles. The success of the DBV fusion protein in the delivery of iNOS represents an exciting step forward in cancer gene therapy.

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