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The role of doxorubicin in non-viral gene transfer in the lung

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ABSTRACT

Proteasome inhibitors have been shown to increase adeno-associated virus (AAV)-mediated transduction *in vitro* and *in vivo*. To assess if proteasome inhibitors also increase lipid-mediated gene transfer with relevance to cystic fibrosis (CF), we first assessed the effects of doxorubicin and *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal in non-CF (A549) and CF (CFTE29o-) airway epithelial cell lines. CFTE29o- cells did not show a response to Dox or LLnL; however, gene transfer in A549 cells increased in a dose-related fashion ($p < 0.05$), up to approximately 20-fold respectively at the optimal dose (no treatment: $9.3 \times 10^4 \pm 1.5 \times 10^3$, Dox: $1.6 \times 10^6 \pm 2.6 \times 10^5$, LLnL: $1.9 \times 10^6 \pm 3.2 \times 10^5$ RLU/mg protein). As Dox is used clinically in cancer chemotherapy we next assessed the effect of this drug on non-viral lung gene transfer *in vivo*. CF knockout mice were injected intraperitoneally (IP) with Dox (25–100 mg/kg) immediately before nebulisation with plasmid DNA carrying a luciferase reporter gene under the control of a CMV promoter/enhancer (pCIKLux) complexed to the cationic lipid GL67A. Dox also significantly ($p < 0.05$) increased expression of a plasmid regulated by an elongation factor 1α promoter (hCEFI) approximately 8-fold. Although administration of Dox before lung gene transfer may not be a clinically viable option, understanding how Dox increases lung gene expression may help to shed light on intracellular bottle-necks to gene transfer, and may help to identify other adjuncts that may be more appropriate for use in man.

1. Introduction

Proteasome inhibitors have been shown to increase adeno-associated virus (AAV)-mediated transduction *in vitro* and *in vivo* [1–3]. The exact mechanism remains unclear and does not appear to be related solely to the inhibition of vector degradation through the ubiquitin–proteasome degradation pathway. In contrast to viral transductions, the pathways involved in cell and nuclear entry of non-viral gene transfer agents, and movement of these complexes within the cell, are poorly understood and the contribution (if any) of proteasomes to this process remains unclear. Two studies have reported that proteasome inhibitors increased non-viral gene transfer *in vitro* [4,5].

Cystic fibrosis (CF) is a chronic disease which will require treatment with gene transfer agents over the life-time of the patient. Problems associated with repeat administration of most viral vectors have been well described [6,7]. Because of this, repeat administration of non-viral gene transfer agents is currently the most promising approach for the development of CF gene therapy. The cationic lipid GL67A [8] is, in our hands, the most efficient non-viral vector for lung transfections. GL67A consists of three components: (1) the cationic lipid GL67 [Cholest-5-en-3-ol (3β)-,3-[(3-aminopropyl)[4-[(3-aminopropyl)amino]butyl]carbamate], consisting of an amine (spermine) and a lipid (cholesterol) component linked together via a carbamate linker, (2) DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) a naturally occurring neutral lipid, (3) DMPE-PEG5000 (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylenegly col)5000]) which is 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine

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linked to polyethylene glycol monomethylether (average molecular weight ca. 5000) via a carbamate linkage. To generate the gene transfer agent the three lipids are formulated at 1:2:0.05 (GL67:DOPE:DMPE-PEG5000) molar ratios and complexed to DNA [8]. We have achieved partial correction of the chloride transport defect in the lungs of CF patients after GL67A-mediated gene transfer of plasmid DNA carrying the cystic fibrosis transmembrane conductance regulator (*CFTR*) cDNA [9]. However, the efficiency of non-viral gene transfer is comparatively low compared to viral vectors, such as for example Sendai virus [10], and it is currently unclear, if non-viral gene therapy can ameliorate the chronic lung infection and inflammation that causes most morbidity and mortality in CF subjects. The clinical trial programme currently carried out by the UK CF Gene Therapy Consortium (www.cfgenetherapy.org.uk) aims to assess if GL67A-mediated *CFTR* gene transfer is able to ameliorate CF lung disease. In parallel, however, we are investigating a variety of adjuncts that may further increase GL67A-mediated lung gene transfer as part of our pre-clinical development programme.

Here, we assessed if proteasome inhibitors could improve GL67A-mediated gene transfer in the mouse lung. Doxorubicin, an anthracyclin derivative, and *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL), a tripeptide aldehyde inhibitor, have previously been shown to increase AAV-mediated gene transfer *in vitro* [11]. Importantly, doxorubicin is in clinical use for cancer chemotherapy, and in support of our translational research programme, we chose this drug to determine, if proteasome inhibition increases non-viral gene transfer in the mouse lung *in vivo*.

2. Materials and methods

2.1. In vitro transfection

A549 cells, an adenocarcinoma cell line (ATCC, Manassas, USA) and a cystic fibrosis tracheal epithelial cell line (CFTE29o-) [12] were plated onto 6-well plates (100,000 cells/well). Approximately 24 h later cells were transfected with the eukaryotic expression plasmids pCIKLux carrying a luciferase reporter gene under the control of the cytomegalovirus (CMV) immediate/early promoter (1 μg DNA/well) or an irrelevant control plasmid carrying no reporter gene (pCIK-empty). Each was complexed to Lipofectamine 2000 (LF2000, Invitrogen, Paisley, UK) according to manufacturer's recommendations for 6 h. During this time cells were also exposed to various concentrations of doxorubicin (0.5–20 mM, Sigma–Aldrich, Poole, UK) dissolved in sterile water for injection or *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL: 10–100 μM, Sigma–Aldrich) dissolved in ethanol. After 6 h the medium was removed and replaced with fresh MEM medium containing 10% FCS and 1% ampicillin/streptomycin. Cells were harvested 48 h after transfection and resuspended in 500 μl 1× RLB buffer (Promega, Southampton, UK) followed by three freeze/thaw cycles and centrifugation at 13,000 g_{av} for 10 min. The supernatant was frozen for quantification of luciferase expression.

2.2. Mice

Male and female C57Bl/6, *Balb*/C mice (Charles River, UK) or gut-corrected CF-knockout mice on a mixed genetic background [13] (bred in-house) were used at approximately 6–12 weeks of age (for details see Figure legends). All experiments were carried out with approval of the appropriate local Ethics Committee and according to Home Office regulations.

2.3. Doxorubicin administration in vivo

Doxorubicin (Dox, Sigma–Aldrich, Poole, UK) was dissolved in sterile water for injection to 5 mg/ml and administered intraperitoneally (IP) (0–100 mg/kg) immediately before gene transfer. IP injection of proteasome inhibitors has previously been shown to be effective in the context of AAV-mediated gene transfer. In some experiments Dox was administered topically to the lung using our standard nasal sniffing protocol. For this the mice were anaesthetised with isofluorane and a single 100 μl bolus containing 125–500 μg Dox (equivalent to 6–25 mg/kg) was applied to the nose and rapidly sniffed into the lung. 25 mg/kg was the highest dose feasible for topical administration as mice showed signs toxicity above this concentration. Caelyx® (2 mg/ml), a pegylated liposome encapsulated Dox formulation, was injected IP or intravenously (IV) immediately before gene transfer.

2.4. In vivo transfection of murine lung

The cationic lipid GL67A is a mixture of GL67 (Genzyme, Haverhill, UK), DOPE and DMPE-PEG5000 (Avanti Lipids, Alabama, USA) [8]. The lipids were formulated and freeze-dried by OctoPlus N.V. (Leiden, The Netherlands) as previously described [8]. pCIKLux or pG4-hCEFI-soLux, which is an advanced fourth generation plasmid carrying a CpG-depleted, codon optimised luciferase reporter gene regulated by a hybrid promoter consisting of the CMV enhancer and the elongation factor 1α promoter [14].

Immediately after Dox administration mice were placed into an exposure chamber and exposed to an aerosol generated by a PARI LC+ nebuliser (PARI GmbH, Starnberg, Germany) at a pressure of 22 psi for 1 h. GL67A/pDNA complexes for aerosolisation were prepared as previously described [14]. Briefly, 25 mg plasmid DNA (5 mg/ml) were complexed with 5 ml GL67A to a final molar ratio of 6:8 in a total volume of 10 ml. Reporter gene expression was quantified 24 h after transfection to detect maximal levels of gene expression.

2.5. Bioluminescence imaging

D-Luciferin (Xenogen Corporation, Alameda, CA) was either administered IP (150 mg/kg in 200 μl) or topically (75 mg/kg in 100 μl), using our nasal sniffing protocol described above, to mice 10 min before imaging. Bioluminescence (photons s^{-1} cm^{-2} sr^{-1}) from living mice was measured using an IVIS50 system (Xenogen Corporation, Alameda, CA, USA) at a binning of 4, over 10 min, using the software programme Living Image (Xenogen). For anatomical localisation a pseudocolour image representing light intensity (blue least intense, red most intense) was generated using Living Image software and superimposed over the greyscale reference image. To quantify bioluminescence in the nose and right and left lungs photon emission in a defined area was measured by marking a standardized area for quantification.

2.6. Tissue homogenate-based luciferase assay

Mice were culled by cervical dislocation. The mouse lungs were harvested and frozen in liquid nitrogen. To extract luciferase protein lungs were placed in 100 μl and 300 μl 1× RLB buffer (Promega), respectively. The lung was homogenized using a Fast-Prep homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) set to 40 m/s for 45 s followed by 15 min incubation at room temperature. The supernatant was removed and transferred to a QiaShredder column (Qiagen, Crawley, Sussex) and centrifuged (1 min at 16,000 g_{av}) followed by an additional centrifugation (5 min at 16,000 g_{av}). Luciferase activity was measured in the supernatant using a standard luciferase assay kit (Promega) and the TD-20e luminometer (Turner, Sunnyvale, CA, USA). Total protein per sample was determined using the BioRad protein assay kit (BioRad laboratories, Hercules, CA) and luciferase activity was expressed as arbitrary relative light units (RLU)/mg total protein.

2.7. Statistical analysis

Statistical analyses were performed by ANOVA and Kruskal–Wallis followed by post hoc analysis appropriate for parametric and non-parametric data. The null hypothesis was rejected at $p < 0.05$.

3. Results

3.1. Proteasome inhibitors in vitro

To establish proof-of-principle, we first assessed the effects of proteasome inhibitors on Lipofectamine 2000-mediated (LF2000) gene transfer in two airway epithelial cell lines (A549 and CFTE29o- cells). Cells were exposed to various concentrations of Dox (0.5–20 mM) and LLnL (10–100 μM) for 6 h during transfection with pCIKLux complexed to LF2000. CFTE29o- cells did not show a response to Dox or LLnL (data not shown), but gene transfer in A549 cells increased in a dose-related fashion approximately 20-fold respectively, at the optimal dose ($p < 0.05$, $n = 8$/group) (Fig. 1). We reproducibly observed a *U*-shaped dose-response with Dox.

3.2. Doxorubicin effects in the murine lung

To assess the effect of proteasome inhibitors on non-viral lung gene transfer *in vivo* CF knockout mice were injected intraperitoneally (IP) with Dox (0, 25, 50 and 100 mg/kg, $n = 8$ mice/group) immediately before nebulisation of pCIKLux complexed to the cationic lipid GL67A, and compared to animals transfected without

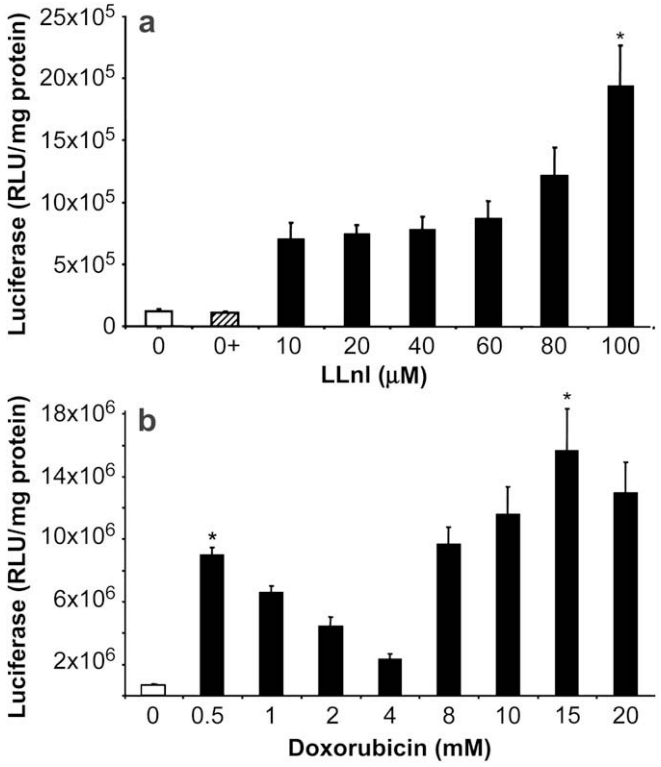

Fig. 1. Proteasome inhibitors increase lipid-mediated gene transfer in A549 cells *in vitro*. A549 cells were exposed to the proteasome inhibitors LLnL (1–100 μM) and doxorubicin (0.5–20 mM) during transfection with pCIKLux complexed to Lipofectamine 2000. (a) LLnL exposure, (b) Doxorubicin exposure. Bars represent mean ± S.E.M ($n = 8$ wells/group). 0 represents cells exposed to no drug or solvent, 0+ represents cells exposed to 28 μl ethanol per well to control for the LLnL solvent. $^{*}p < 0.05$ compared to no proteasome inhibitor control. A representative of 2 independent experiments is shown.

Dox. CF knockout mice were used because we routinely use these for gene transfer studies and have a large in house breeding colony. Gene expression was analysed 24 h after gene transfer. Dox increased gene transfer in a dose-related fashion (Fig. 2). Mice receiving the lowest dose (25 mg/kg) did not show an increase in luciferase expression, but in mice receiving 50 or 100 mg/kg luciferase was significantly (approximately 20-fold, $p < 0.05$) increased compared to no Dox administration. Administration of 100 mg/kg did not increase gene transfer compared to 50 mg/kg, but toxicity was observed at this dose, with 1 out of 8 mice dying within the 24 h period and 4 out of 7 mice showing hunching and pilo-erection. In contrast all mice treated with 50 mg/kg dose were outwardly indistinguishable from mice not treated with Dox. Subsequent experiments were, therefore, carried out with a dose of 50 mg/kg.

To assess reproducibility of the results the experiment was repeated using the optimal dose of 50 mg/kg Dox IP with higher *n* numbers showing reproducible results (Fig. 3). Although we would not expect there to be difference between CF and non-CF mice, we also assessed the effects of Dox in wild-type *Balb*/C and C57Bl/6 mice and consistently demonstrated a significant ($p < 0.05$) increase in GL67A-mediated lung gene expression after IP Dox administration, respectively (Fig. 3).

Since the optimal dose (50 mg/kg) is approximately 3 times higher than that used clinically, we attempted to increase the local concentration of Dox in the lung by administering the drug (6–25 mg/kg) topically via nasal sniffing. However, this did not increase luciferase expression compared to mice receiving no Dox (data not shown).

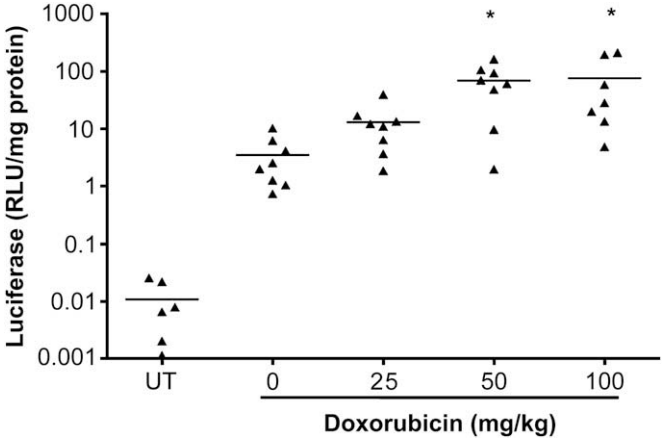

Fig. 2. Doxorubicin increased lipid-mediated gene transfer in the murine lung *in vivo* in a dose-related fashion. CF knockout mice were treated with doxorubicin (25–100 mg/kg) intraperitoneally immediately before nebulisation of pCIKLux complexed to the cationic lipid GL67A. 24 h after gene transfer luciferase expression was quantified in the lung. Symbols represent individual animals and the horizontal bar represents the group mean ($n = 6$–8 mice/group). UT = untransfected mice, $^{*}p < 0.05$ compared to no doxorubicin control (0 mg/kg).

3.3. In vivo bioluminescence imaging

We have previously shown that cationic lipid GL67A-mediated luciferase gene transfer can be detected with *in vivo* bioluminescence imaging (BLI) in lungs of mice following nasal sniffing of lipid/DNA complexes into the lung [15]. However, nebulisation is generally less efficient than nasal sniffing and, as shown here, insufficient (only 2 out of 9 mice showed a weak signal) to allow BLI using standard IP administration of the luciferin substrate (150 mg/kg) (Fig. 4a). Interestingly, we showed that (a) a significantly ($p < 0.001$) stronger BLI signal can be visualised in 7 out of 9 mice after GL67A/pCIkLux nebulisation by administering the luciferin substrate topically (75 mg/kg) to the lungs of *Balb*/C mice by nasal sniffing instead of using the standard IP route (Fig. 4b) despite the fact that only half of the IP dose was administered and that (b) IP administration of Dox (50 mg/kg) further significantly ($p < 0.05$) increased the BLI signal (Fig. 4c,d).

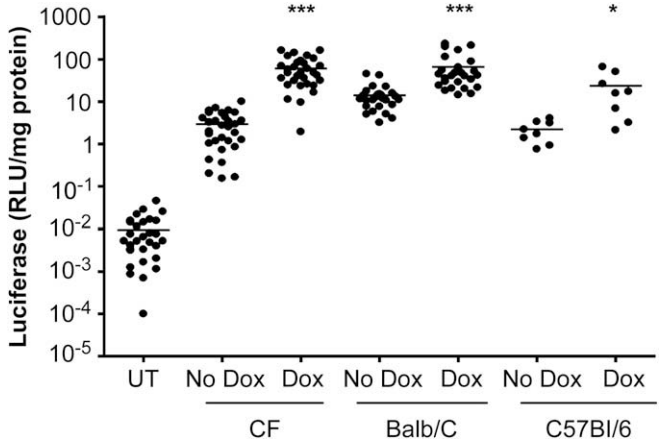

Fig. 3. Doxorubicin-mediated increase in gene transfer is reproducible and also occurs in non-CF mice. CF knockout mice or wild-type *Balb*/C and C57Bl/6 mice were nebulised with GL67A/pCIKLux complexes and received either doxorubicin (50 mg/kg IP) immediately before administration, or were left untreated. 24 h after gene transfer luciferase expression was quantified in the lung. Symbols represent individual animals and the horizontal bar represents the group means ($n = 8$–31 mice/group). UT = untransfected mice, $^{***}p < 0.005$ and $^{*}p < 0.05$ compared to no doxorubicin controls.

3.4. Promoter-independent increase in gene expression

In addition to the pCIKLux plasmid which is regulated by the immediate/early cytomegalovirus (CMV) promoter/enhancer, we also assessed the effects of Dox on a eukaryotic luciferase expression plasmid regulated by a hybrid promoter consisting of the CMV enhancer and the elongation factor 1α promoter (pG4-hCEFI-soLux). Similar to results obtained with pCIKLux, Dox significantly ($p < 0.005$) increased pG4-hCEFI-soLux-mediated expression in *Balb*/C mice (Fig. 5).

3.5. Effects of Caelyx® on lung gene expression

The most advanced Dox formulation currently in clinical use is a pegylated liposome encapsulated formulation [Caelyx® (UK) or Doxil® (USA)]. We, therefore, assessed the effects of Caelyx® on GL67A/pCIKLux-mediated lung gene transfer. We administered either 4, 16.5 or 40 mg/kg IP to CF knockout mice ($n = 8$/group) mice. These doses were chosen because 16.5 mg/kg is equivalent to 50 mg/m^2 body surface, the latter being dose used in man, and 40 mg/kg being equivalent to 400 μl of a 2 mg/ml solution, the maximum volume allowed for IP injection under Home Office license regulations. All mice tolerated the treatment well and were culled 24 h after gene transfer. IP administration of Caelyx® did not increase reporter gene expression in the lung (Fig. 6a).

For clinical use Caelyx® is administered intravenously (IV) and we, therefore, also assessed the effects of IV administration on lung gene expression. Caelyx® (4 or 20 mg/kg) was injected into the tail vein of mice immediately before nebulisation of GL67A/pCIKLux. The 20 mg/kg, equivalent to 200 μl of a 2 mg/ml solution, is the maximum volume allowed for IV injection under Home Office license regulations. All mice tolerated the treatment well and were culled 24 h after gene transfer. IV injection of Caelyx® modestly increased gene expression, but this did not reach significance ($p = 0.05$) (Fig. 6b).

4. Discussion

Here, we show that proteasome inhibitors increase cationic lipid-mediated gene transfer *in vitro* and in murine lung *in vivo*. However, the clinically relevant agent doxorubicin shows a narrow efficacy-toxicity window, which was not improved by variations in route of administration. We conclude, it is unlikely that Dox will be suitable for clinically relevant gene therapy in CF patients.

Proteasome inhibitors have been shown to increase AAV-mediated gene transfer in various *ex vivo* and *in vivo* models of lung gene transfer, including air–liquid interphase cultures, xenograft models and the murine lung [3,11]. However, the mechanism is unclear; possibilities including (a) prevention of virus degradation, (b) improved nuclear uptake, (c) improved genome uncoating, (d) enhanced capsid processing, (e) improved second strand synthesis and (e) reduced degradation of the recombinant protein. The effect of proteasome inhibitors in the context of non-viral gene transfer has, to the best of our knowledge, only been described in two *in vitro* studies. Kim J et al. reported that proteasome inhibitors increased non-viral peptide-mediated gene transfer and Kim KI et al. showed that doxorubicin increases lipid-mediated gene transfer [4,5].

In our hands the benefits of LLnL and doxorubicin on non-viral gene transfer *in vitro* were cell type specific. Although A549 cells, an adenocarcinoma cell line with type II cell characteristics responded to both proteasome inhibitors with a 20 to 30-fold increase in gene expression, the drugs did not increase gene expression in CFTE29o- cells, derived from tracheal epithelium of a CF subject. In the context of AAV transduction A549 cells also responded to LLnL and doxorubicin [11] whereas as shown by Kim et al. [4] proteasome inhibitors did not increase LF2000-mediated gene transfer in CF/T1 cells, another CF airway epithelial cell line. Similarly Yan et al. showed that the response to LLnL varies in different airway cell lines when transduced with AAV [11]. It is unlikely that the unresponsiveness of CFTE29o- cells to proteasome inhibitors is due to the CF genotype of these cells, because we also demonstrated that non-viral gene transfer in both CF-knockout mice and non-CF mice responded similarly to Dox pre-treatment.

Although doxorubicin treatment increased non-viral gene transfer by up to 20-fold these levels in our experience are still too low to allow detection of recombinant protein with immunohistochemistry or X-gal-based methods. Thus, we are currently unable to determine which cells have been transduced and if the number of cells expressing the recombinant protein, or the amount of protein per cell, increased in response to doxorubicin treatment. This question is also difficult to address in the existing AAV studies. Although the number of reporter gene expressing cells increased in response to proteasome inhibition, it is unclear if negative cells were untransduced, or if cells were transduced but levels of reporter gene expression were below the detection limit of the assay [2]. Kim et al. suggested that proteasome inhibition did not affect the number of cells transfected with peptide-based gene transfer agents, but increased the amount of protein made per cell [4].

Proteasome inhibitors have been shown to increase AAV-mediated transduction in C57Bl/6 mice and Rag-deficient mice [2,11]. Here, we show that proteasome inhibitors increase lipid-mediated gene transfer in a variety of strains including wild-type *Balb*/C mice, C57Bl/6 mice and gut-corrected CF knockout mice on a mixed genetic background. Thus, the drug action appears to be strain and CF genotype independent. By spiking homogenized lung tissue with Dox immediately before performing a luciferase assay we excluded a direct effect of the drug on photon emission (data not shown).

We also show that Dox pre-treatment improved *in vivo* bioluminescence imaging (BLI) of reporter gene expression after nebulisation of GL67A/DNA complexes to the mouse lung. The whole body nebulisation set-up used in the study is comparatively inefficient in delivering gene transfer agents to the murine lung and it was, therefore, not surprising that photon emission was too low to generate a visible BLI signal in most animals after IP injection of luciferin substrate. Importantly, however, topical administration of luciferin directly to the lung increased photon emission significantly and generated a BLI signal in 7 out of 9 mice despite the fact that only half of the dose recommended for IP injections was delivered. Not surprisingly a combination of luciferin administration directly to the lung and doxorubicin pre-treatment further increased photon emission. The enhanced photon emission after topical administration of luciferin is likely due to a higher local concentration of the substrate in transfected lung epithelium. Buckley et al. have shown that topical administration of luciferin also increased lung photon emission after virus-mediated gene transfer [16]. More extensive dose-optimisation may significantly reduce the amount of luciferin required and, thereby, reduce costs of BLI studies.

Although it is generally assumed and probably most plausible that the beneficial effects of doxorubicin on viral and non-viral gene transfer are due to proteasome inhibition, it is worth mentioning that doxorubicin has additional effect. The drug intercalates into DNA and inhibits topoisomerase II activity [17] and the implications of these actions on gene expression after gene transfer are not understood. Importantly, we show that the drug action is promoter/enhancer-independent as doxorubicin increased CMV and elongation factor 1α(hCEFI)-dependent gene expression. Doxorubicin is also known to activate reactive oxygen species (ROS)

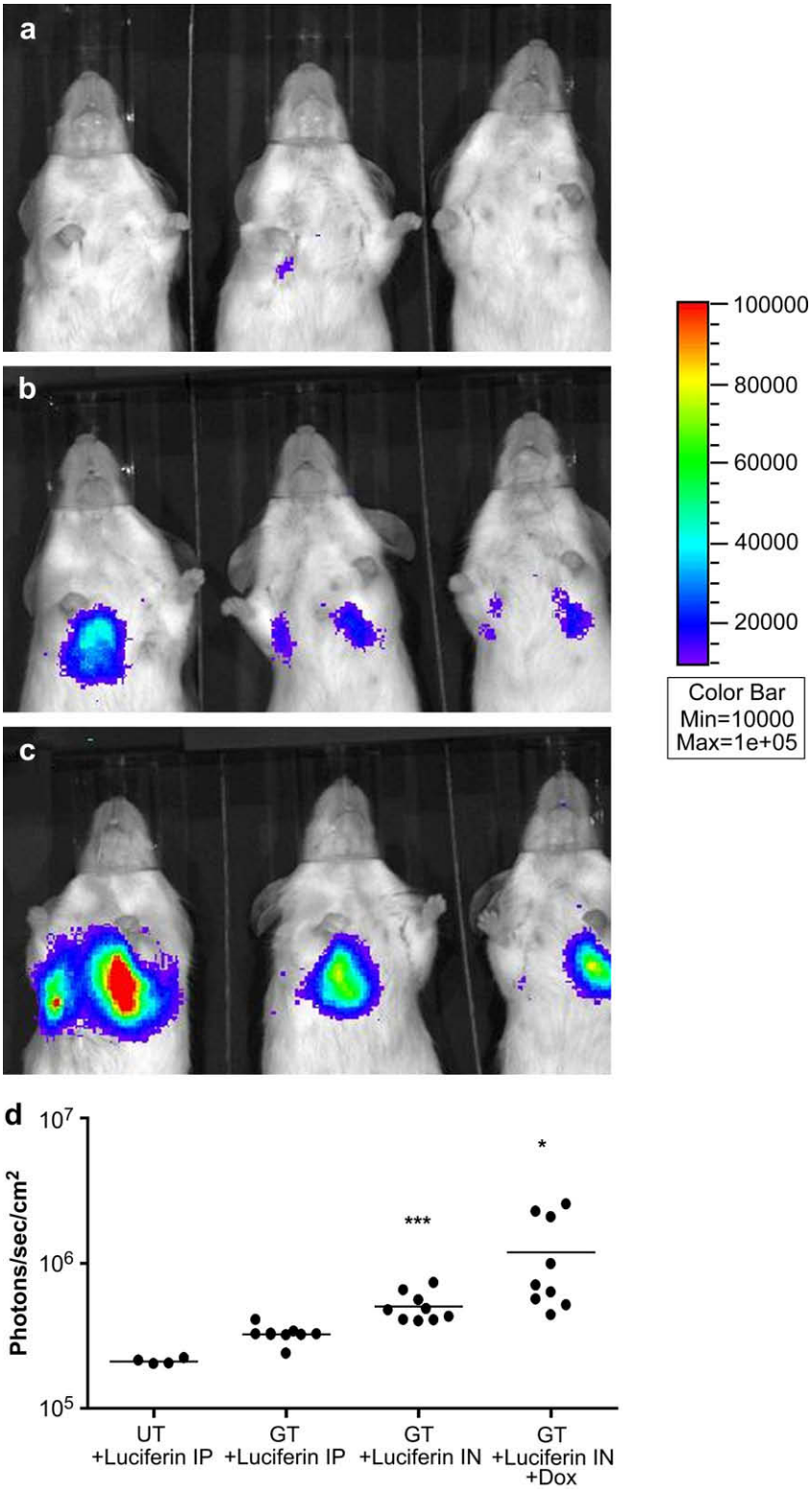

Fig. 4. Doxorubicin improves bioluminescence *in vivo* imaging of lung gene transfer after nebulisation. Wild-type *Balb*/C were nebulised with pCIKLux/GL67A complexes and bioluminescence imaging was carried out 24 h after gene transfer. Luciferin was either administered intraperitoneally (IP) (a) or via nasal sniffing (IN) topically to the lung (b). The bioluminescent signal was further enhanced through doxorubicin (Dox) administration (50 mg/kg) immediately before gene transfer (c). Representative images of $n = 9$ mice/group are shown. *In vivo* photon emission was quantified (d). Symbols represent individual animals and the horizontal bar represents the group mean ($n = 4$–9 mice/group). UT = untransfected mice, GT = mice after gene transfer. $^{***}p < 0.005$ and $^{*}p < 0.05$ compared to mice receiving luciferin IP.

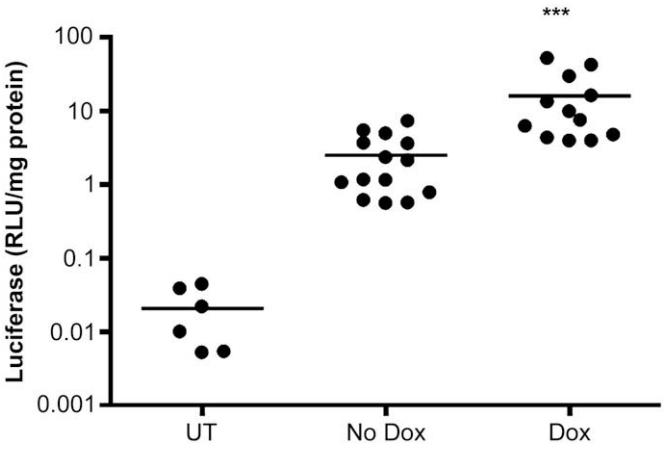

Fig. 5. Doxorubicin-mediated increase in gene expression is not promoter specific. Wild-type *Balb*/C mice were nebulised with a eukaryotic luciferase expression plasmid regulated by a hybrid promoter consisting of the CMV enhancer and the elongation factor 1α promoter (pG4-hCEFI-soLux) complexed to GL67A. 24 h after gene transfer luciferase expression was quantified in the lung. Symbols represent individual animals and the horizontal bar represents the group mean ($n = 12$–14 mice/group). UT = untransfected mice, $^{***}p < 0.005$ compared to no doxorubicin controls.

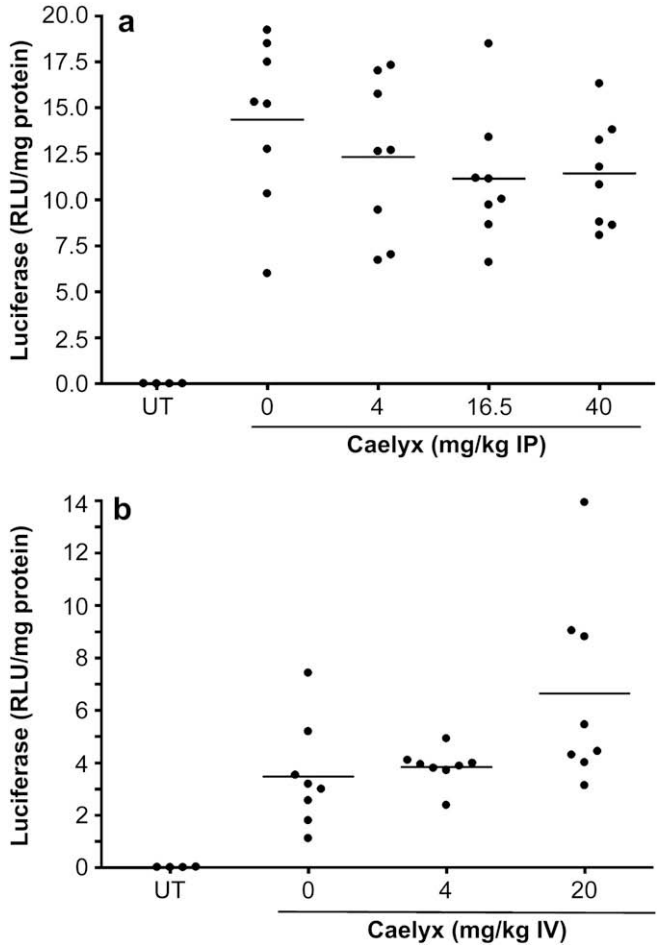

Fig. 6. Caelyx® administration did not significantly increase lung gene expression. Caelyx® is an advanced pegylated liposome encapsulated doxorubicin formulation currently in clinical use. CF knockout mice were treated with Caelyx® (a) intraperitoneally (IP, 4–40 mg/kg) or (b) intravenously (IV, 4 and 20 mg/kg) immediately before nebulisation with pCIKLux/GL67A complexes ($n = 8$/group). Symbols represent individual animals and the horizontal bar represents the group mean ($n = 4$–8 mice/group). UT = untransfected mice.

and one conceivable explanation may be that ROS activate the transcription factor NFκB [18,19].

A more advanced pegylated liposomal formulation of the first-generation doxorubicin is currently in clinical use. The coating prolongs the circulating half-life and slows down plasma clearance compared to free doxorubicin, but most importantly leads to preferential accumulation in tumours, thereby reducing systemic toxicity [20] respectively. We also assessed the effects of Caelyx® IP administration on airway gene transfer, but did not detect any changes in gene expression. Clinically Caelyx® is administered intravenously and, therefore, we assessed its effects via this route of delivery. Although there was a modest increase in lung gene expression, this did not reach significance. These results are perhaps unsurprising given that the formulation was developed for accumulation in tumours. Caelyx® liposomes have an average diameter of approximately 100 nm able to penetrate the discontinuous endothelium of tumour vasculature, but not other tissues [21]. Thus, the drug may not have reached the airway epithelium in sufficiently high concentrations. In contrast Yan et al. showed that IV administration of Doxil®, a formulation identical to Caelyx®, increased AAV-mediated gene expression in a murine tracheal xenograft model [11]. The newly formed vasculature supplying the xenograft may be more discontinuous than the vasculature in the *in vivo* lung, thereby allowing for drug delivery to the target epithelium.

5. Conclusions

As for AAV-mediated gene transfer, doxorubicin increased lipid-mediated gene transfer to the murine lung. The clinically relevant agent doxorubicin shows a narrow efficacy-toxicity window, which was not improved by variations in route of administration. We conclude that although it may be unlikely that Dox will be suitable for clinically relevant gene therapy in CF patients an understanding on how the drug increases lung gene expression may help to shed light on intracellular bottle-necks to gene transfer, and may help to identify other adjuncts which may be more appropriate for use in humans.

Acknowledgements

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular parts of Figure 4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2008.12.037.

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