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Therapeutic efficacy of intravenously administered transferrin-conjugated dendriplexes on prostate carcinomas

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STRUCTURED ABSTRACT

Aims: Improved treatments for prostate cancer are critically needed in order to overcome metastasis and lethal recurrence. Intravenously administered gene therapy would be an attractive anti-cancer treatment strategy, however the lack of suitable carrier systems able to selectively deliver therapeutic genes to tumors has so far limited this investigation. Given that transferrin receptors are overexpressed on prostate cancer cells, the purpose of this study is to determine whether transferrin-bearing dendriplex encoding TNF±, TRAIL and IL-12 would suppress the growth of prostate cancer cell lines in vitro and in vivo.

Materials & Methods: Transferrin-bearing dendriplexes encoding TNF±, TRAIL and IL-12 were intravenously administered to mice bearing subcutaneous PC-3 and DU145 tumors.

Results: The administration of the DAB-Tf dendriplex encoding TNF± resulted in tumor suppression for 60% of PC-3 and 50% of DU145 prostate tumors.

Conclusions: These dendriplexes hold great potential as a novel approach for prostate cancer therapy.

KEYWORDS
Prostate cancer; gene therapy; tumor targeting; dendrimer; transferrin
INTRODUCTION
Prostate cancer is one of the most commonly diagnosed malignancies in men and remains the second leading cause of cancer-related deaths in the United States and the European Union. Although radiation therapy, chemotherapy, radical prostatectomy and cryoablation can be efficacious therapies for localized tumors, there is still no effective treatment modality for patients with recurrent or metastatic disease [1]. New treatment approaches are therefore critically needed for these patients.
Among novel experimental strategies, gene therapy holds great promise for the treatment of prostate cancer. The identification of the genetic mutations involved in this disease has led to the development of a wide panel of highly diverse, rapidly evolving gene therapy approaches, such as corrective gene therapy, suicide gene therapy, apoptosis-inducing gene therapy and immunomodulation therapy [1]. These approaches have already shown promise for in situ use. For example, in a phase I/II clinical trial, the in situ delivery of an adenoviral vector delivery of the Herpes Simplex Virus thymidine kinase gene followed by the pro-drug ganciclovir resulted in apoptosis of prostate cancer cells and immune cell activation [2, 3].
However, the use of gene therapy is currently limited by the lack of delivery systems able to selectively deliver the therapeutic genes to the tumors by intravenous administration, without secondary effects to normal tissues. In order to overcome this problem, we recently demonstrated that the conjugation of transferrin (whose receptors are abundantly expressed on cancer cells) to generation 3- diaminobutyric polypropylenimine (DAB) dendrimer led to gene expression principally in the tumors following intravenous administration [4]. As a result, the intravenous administration of DAB-Tf dendrimer complexed to the Tumor Necrosis Factor (TNF±) expression
plasmid led to tumor suppression for 90% of the A431 epidermoid carcinoma tumors, with long-term survival of the mice.

Building on these promising results, we have focused on investigating if this delivery system carrying plasmids encoding for TNF±, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or interleukin-12 (IL-12), could lead to therapeutic effects on prostate cancer following intravenous administration.

TRAIL, a member of the TNF family of cytokines, is particularly promising in the development of anti-cancer therapeutics, as it preferentially induces apoptosis of cancer cells whilst sparing normal cells [5]. Its binding to death receptors DR4 and DR5 leads to recruitment of Fas-associated death domain, resulting in tumor cell death via caspase-dependent apoptotic pathways [5]. As it has a short biological half-life and is rapidly cleared from the circulation after intravenous administration [6], new delivery strategies are particularly needed to improve its therapeutic potency.

Another cytokine, IL-12, has been shown to be efficacious in the induction of potent antitumor immunity [7]. IL-12 has multiple effects on B cells, T cells and natural killer cells and is also a major regulator of Th1 cell differentiation. In animal melanoma models, the administration of IL-12 plasmid resulted in tumor growth inhibition in addition to regression of established tumors [8]. However, the intravenous use of IL-12 is hampered by its potential side effects [9], thus emphasizing the need of tumor-specific delivery.

The objectives of this study were therefore to investigate if transferrin-bearing DAB dendrimer complexed with TNF±, TRAIL or IL-12 expression plasmids had a therapeutic effect on prostate cancer cells in vitro and in vivo after intravenous administration.
MATERIALS & METHODS

Cell lines and reagents

Generation 3- diaminobutyric polypropylenimine dendrimer (DAB), iron-saturated human holo-transferrin, dimethylsuberimidate and the other reagents were from Sigma Aldrich (Poole, UK). The expression plasmids encoding β-galactosidase (pCMVsport β-galactosidase) and TNF± (pORF9-mTNF±) were purchased respectively from Invitrogen (Paisley, UK) and InvivoGen (San Diego, CA), while the expression plasmids encoding TRAIL (pORF9-mTRAIL) and IL-12 (pORF-mIL12) came from Source BioScience (Nottingham, UK). These plasmids were purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Label IT® Cy3 Nucleic Acid Labeling kit was purchased from Cambridge Biosciences (Cambridge, UK). Passive lysis buffer came from Promega (Southampton, UK). Androgen-irresponsive DU145 and androgen-responsive LNCaP human prostate carcinomas were obtained from the European Collection of Cell Cultures (Salisbury, UK). Firefly D-luciferin and Bioware® androgen-irresponsible PC-3M-luc-C6 human prostate adenocarcinoma that expresses the firefly luciferase were from Caliper Life Sciences (Hopkinton, MA). Tissue culture media were obtained from Invitrogen (Paisley, UK).

Preparation of transferrin-bearing polypropylenimine dendrimer

Generation 3- diaminobutyric polypropylenimine dendrimer (DAB, 24 mg) was conjugated to transferrin (Tf, 6 mg) by cross-linking with dimethylsuberimidate (DMSI, 12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL), as previously described [4, 10]. The conjugation reaction took place at 25°C for 2 h whilst stirring. DAB-Tf dendrimer was then purified by size exclusion chromatography using a Sephadex
G75 column, freeze-dried, before having its identity confirmed by $^1$H NMR spectroscopy, using a Jeol Oxford NMR AS 400 spectrometer [4].

**In vitro studies**

**Cell culture**

PC-3M-luc-C6, DU145 and LNCaP prostate cell lines overexpressing Tf receptors were grown in either MEM (for PC-3 and DU145 cells) or RPMI medium (for LNCaP cells), at 37°C in a humidified atmosphere under 5% CO$_2$. Fetal bovine serum (10% (v/v), L-glutamine (1% (v/v) and penicillin-streptomycin (0.5% (v/v) were added to both media. In addition, RPMI medium was also supplemented with 10 mM HEPES (5 mL) and 1 mM sodium pyruvate (5 mL).

**Cellular uptake of DNA**

Plasmid DNA encoding $^2$-galactosidase was labeled with the fluorescent probe Cy3 using a Label IT® Cy3 Nucleic Acid Labeling kit, as described by the manufacturer. PC-3, DU145 and LNCaP cells were seeded on coverslips in 6-well plates ($10^4$ cells/well) and grown at 37°C for 24 h. They were then incubated with Cy3-labeled DNA (2.5 µg DNA / well) complexed by mixing to DAB-Tf and DAB at their optimal dendrimer: DNA weight ratios (leading to complete DNA condensation and highest transfection) respectively of 10:1 for DAB-Tf and 5:1 for DAB [10, 11]. Control slides were treated with naked DNA. Following 24 h treatment, the cells were then washed three times with PBS and fixed with methanol for 10 min. DAPI was then used to stain the nuclei and the cells were examined using a Leica TCS SP5 confocal microscope. DAPI was excited with the 405 nm laser line (bandwidth: 415-491nm), whereas Cy3 was excited with the 543 nm laser line (bandwidth: 550-620 nm). Cy3-
related fluorescence intensity was then quantified in each of the confocal microscopy pictures by using Adobe Photoshop Elements software. Results were expressed as fluorescence intensity (in arbitrary units) per cell.

In vitro transfection

Transfection efficacy of the plasmid DNA encoding β-galactosidase and carried by DAB-Tf dendrimer was evaluated by a β-galactosidase transfection assay. Following seeding at a density of 2000 cells/well in 96-well plates, PC-3, DU145 and LNCaP cells were left to grow at 37°C for 72 h. They were then incubated for a further 72 h with DNA (10 µg/mL), alone or complexed to DAB-Tf and DAB (dendrimer: DNA weight ratios of 10:1 for DAB-Tf and 5:1 for DAB). The cells were then lysed with 50 µL/well of 1X passive lysis buffer (PLB) for 20 min, before being incubated with 50 µL/well of assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL ζ-nitrophenol-β-galactopyranoside, 200 mM sodium phosphate buffer, pH 7.3) for 2 h at 37°C. The absorbance of the samples was then read at 405 nm with a plate reader (Thermo Lab Systems, Multiscan Ascent, UK) [4].

In vitro anti-proliferative activity

Following seeding at a density of 2000 cells/well in 96-well plates, PC-3, DU145 and LNCaP cells were left to grow at 37°C for 72 h. They were then incubated with DAB-Tf dendriplexes encoding TNF±, TRAIL and IL-12, at final DNA concentrations of 1.28x10⁻³ to 100 µg/mL. After 72 hours incubation with the treatment, anti-proliferative activity was assessed by using a standard MTT assay [4] (three independent experiments with n=5 for each concentration level). The incubation of the treatments with the cells was maintained for 72 h, in order to allow comparison
with our previous cytotoxicity results, obtained with DAB-Tf dendrimer but with different therapeutic DNA and different cancer cells.

**In vivo tumoricidal activity**

The *in vivo* experiments described below were approved by the local ethics committee and performed in accordance with the UK Home Office regulations. PC-3M-luc-C6 and DU145 cancer cells in exponential growth were subcutaneously implanted to both flanks of male immunodeficient BALB/c mice (1 x 10^6 cells per flank). When tumors became palpable, vascularized and reached a diameter of 5 mm, the mice were split into groups of five before being intravenously injected with the treatments, namely Tf-bearing DAB dendrimer complexed with TNF±, TRAIL or IL-12 expression plasmids, the targeted dendrimer and naked DNA (50 µg of DNA) once daily for 10 days. The weight of the mice was measured every day to evaluate the toxicity of the treatments and tumor volume was determined by caliper measurements (volume = d^3 x À/6).

Bioluminescence imaging using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA) was also used to monitor the therapeutic efficacy of the treatments. To this end, mice bearing subcutaneous PC-3M-luc-C6 tumors were intravenously injected with treatments as described above. Ten minutes before imaging, they were intraperitoneally injected with the luciferase substrate D-luciferin (150 mg/kg body weight), before being anaesthetized by isoflurane inhalation on Days 1, 3, 5, 7, 9 of the experiment. The light emitted from the bioluminescent tumors was detected for 2 min using Living Image® software and displayed as a pseudo-color overlay onto a gray scale image of the animal. Identical illumination settings were used for acquiring all images [12].
**Statistical Analysis**

Results were expressed as means ± standard error of the mean (S.E.M). Statistical significance was assessed by one-way analysis of variance (ANOVA) and Bonferroni multiple comparison post-test (GraphPad Prism software). *P* values lower than 0.05 were considered statistically different.
RESULTS

Cellular uptake of DNA

Confocal microscopy experiments confirmed that Cy3-labelled DNA was taken up by PC-3, DU145 and LNCaP cells (Figure 1). The fluorescently-labeled DNA was found to be disseminated in the cytoplasm following treatment with DAB-Tf and DAB dendriplexes in the three tested cell lines. However, the DNA uptake appeared to be much more pronounced after treatment with Tf-bearing dendriplex than with the non-targeted dendriplex, corresponding to an increase in fluorescence intensity of respectively 1.5-fold in PC-3 cells, 6-fold in DU145 cells and 15-fold in LNCaP cells (Figure 1). DNA appeared to be more efficiently taken up by PC-3 cells when delivered by means of the targeted dendriplex, compared to LNCaP and DU145 cells. However, these results need to be taken with caution, as they correspond to the quantification of one single representative confocal microscopy picture. No statistical analysis can therefore be applied to these results.

Co-localization of DNA in the nuclei was visible in the 3 cell lines following treatment with Tf-bearing dendriplex, as well as in PC-3 cells following treatment with DAB dendriplex, but to a lesser extent. By contrast, no Cy3-derived fluorescence was visible in cells treated with naked DNA. The conjugation of Tf to DAB therefore improved the uptake of DNA by PC-3, DU145 and LNCaP prostate cancer cells.

In vitro transfection

Treatment with DAB-Tf dendriplex led to an increase in gene expression on both PC-3 and LNCaP cells (Figure 2). On PC-3 cells, gene expression following treatment with DAB-Tf dendriplex \((2.69 \times 10^{-3} \pm 0.09 \times 10^{-3} \text{U/mL})\) was 1.3-fold higher than with unconjugated DAB dendriplex \((2.08 \times 10^{-3} \pm 0.09 \times 10^{-3} \text{U/mL})\). Similarly, it was slightly
higher following treatment with DAB-Tf dendriplex ($1.77 \times 10^{-3} \pm 0.11 \times 10^{-3}$ U/mL) than that observed with DAB dendriplex ($1.53 \times 10^{-3} \pm 0.05 \times 10^{-3}$ U/mL) on LNCaP cells. However, there was no significant difference between gene expression following treatment with DAB-Tf dendriplex and DAB dendriplex on DU145 cells ($1.47 \times 10^{-3} \pm 0.10 \times 10^{-3}$ U/mL and $1.29 \times 10^{-3} \pm 0.11 \times 10^{-3}$ U/mL respectively). Treatment with control dendrimer and naked DNA only resulted in weak levels of gene expression, as expected.

**In vitro anti-proliferative activity**

DAB-Tf complexed to TNF±, TRAIL and IL-12 expression plasmids significantly enhanced the *in vitro* anti-proliferative efficacy on the three prostate cancer cell lines when compared to the unconjugated dendriplex (Table 1). When complexed to TNF± expression plasmid, it significantly increased the *in vitro* therapeutic effect by 5.1-fold in PC-3 cells ($IC_{50}: 0.27 \pm 0.04 \mu g/mL$), 1.7-fold in DU145 cells ($IC_{50}: 1.26 \pm 0.11 \mu g/mL$) and by more than 100-fold in LNCaP cells ($IC_{50}: 1.01 \pm 0.07 \mu g/mL$), compared to the unconjugated dendriplex. It was most efficacious on PC-3 cells, followed by LNCaP cells and then DU145 cells.

When complexed to TRAIL expression plasmid, DAB-Tf also increased the anti-proliferative activity, this time by 21-fold in PC-3 cells ($IC_{50}: 0.85 \pm 0.03 \mu g/mL$), 3.5-fold in DU145 cells ($IC_{50}: 0.81 \pm 0.04 \mu g/mL$) and 5.2-fold for LNCaP cells ($IC_{50}: 1.16 \pm 0.24 \mu g/mL$), compared to the unconjugated dendriplex. DAB-Tf dendriplex expressing TRAIL was most efficacious on DU145 cells, closely followed by PC-3 cells and then LNCaP cells.

Finally, DAB-Tf complexed to IL-12 expression plasmid led as well to an increased anti-proliferative activity in comparison with the unmodified dendriplex, by 1.7-fold for
PC-3 cells (IC$_{50}$: 1.11 ± 0.16 µg/mL), 1.5-fold for DU145 cells (IC$_{50}$: 0.80 ± 0.09 µg/mL) and more than 30-fold for LNCaP cells (IC$_{50}$: 3.31 ± 0.25 µg/mL). It was most efficacious on DU145 cells, followed by PC-3 cells and then LNCaP cells, as observed with TRAIL-expressing DAB-Tf dendriplex.

Overall, the most efficacious treatment observed in this in vitro study was DAB-Tf dendriplex expressing TNF± on PC-3 cells. By contrast, uncomplexed DAB-Tf, DAB and naked DNA did not exert any cytotoxicity to the cells at the tested concentrations, thus demonstrating that 1) DAB-Tf and DAB dendrimers were safe for the cells in the range of concentrations used in our experiments and 2) that a tumor-targeted delivery system is needed for the effective delivery, transfection and subsequently efficacy of the therapeutic DNA on prostate cancer cells.

**In vivo study**

The intravenous administration of DAB-Tf dendriplex encoding TNF± resulted in PC-3 tumor regression within 24h (Figure 3). This effect was maintained for 4 days, allowing the tumors to shrink to nearly half their initial size. From Day 5, some tumors kept regressing while others started growing, resulting in an overall slowdown of PC-3 tumor growth compared to the other treatments (Figure 3). From Day 15, the mice bearing growing tumors had to be euthanized due to their tumors reaching the maximum allowed size. The remaining mice, whose tumors were regressing or had completely disappeared, were kept until the end of the study.

The replacement of TNF± by TRAIL expression plasmid led to a different tumor growth pattern following administration of DAB-Tf dendriplex. A smaller number of PC-3 tumors were regressing compared to that observed with TNF± gene therapy, resulting in an overall slowdown of PC-3 tumor growth compared to controls.
Following administration of DAB-Tf dendriplex encoding IL-12, PC-3 tumors slowly started regressing until Day 5. From that day, some of them started growing, resulting in an overall slowdown of PC-3 tumor growth compared to controls. Unlike that observed with TNF±, none of the tumors completely disappeared.

Treatment of the DU145 tumors with DAB-Tf dendriplex encoding TNF± resulted in an overall slowdown of tumor growth compared to controls, although some tumors were regressing (Figure 4). From Day 20, only the mice bearing regressing tumors or no longer any tumors were still in the study.

DAB-Tf dendriplex encoding TRAIL initially led to an overall stable response for 9 days, followed by tumor growth.

The administration of DAB-Tf dendriplex encoding IL-12 resulted in an initial tumor regression for 5 days, followed by a very slow tumor growth. By contrast, all the PC-3 and DU145 tumors treated with DAB-Tf, naked DNA or left untreated, were growing throughout the experiment.

On the last day of the experiment, 60% of PC-3 and 50% of DU145 tumors treated with DAB-Tf dendriplex encoding TNF± had completely disappeared, while another 10% of DU145 tumors showed a partial response (Figures 3 and 4). As a result of treatment with DAB-Tf dendriplex encoding TRAIL, 10% of PC-3 tumors showed a complete disappearance, 20% of these tumors were regressing, while no DU145 tumors were responsive to this treatment. Finally, treatment with DAB-Tf dendriplex encoding IL-12 did not lead to any tumor disappearance on both tumor types. Only 20% PC-3 tumors showed a partial response, while another 20% remained stable. For DU145 cell line, 20% of the tumors also showed a partial response, while 10% were stable. All the tumors treated with DAB-Tf, naked DNA or left untreated were progressive for both tumor types.
The therapeutic effect resulting from treatment with DAB-Tf dendriplexes was also qualitatively confirmed by bioluminescence imaging on mice bearing subcutaneous PC-3M-luc-C6 tumors (Figure 5). Following treatment with DAB-Tf dendriplex encoding TNF±, luciferase expression in the tumors gradually decreased from Day 1 to Day 11, to completely disappear at Day 11 as a result of the suppression of the entire tumor. Following treatment with DAB-Tf dendriplex encoding TRAIL, the tumors were regressing without completely disappearing at Day 11. As a result of the treatment with DAB-Tf dendriplex encoding IL-12, the tumors appeared to remain stable. All the other treatments led to an increase of luciferase expression in the growing tumors.

The improved therapeutic efficacy observed with DAB-Tf dendriplexes treatment resulted in an extended survival of 22 days compared to untreated tumors, for both cell lines (Figures 3 and 4). Treatment with naked DNA only extended the survival of the animals by 1 or 2 days compared to untreated mice.

No apparent sign of toxicity or significant weight loss were observed, suggesting that all treatments were well tolerated by the mice (Figures 3 and 4).
DISCUSSION

The use of gene therapy for the treatment of recurrent prostate cancer and its metastasis is currently hampered by the lack of safe and efficacious gene delivery systems able to deliver therapeutic genes to their site of action following intravenous administration, without secondary effects to normal tissues. In order to remediate to this problem, we hypothesized that a generation 3- diaminobutyric polypropylenimine (DAB) dendriplex encoding TNF±, TRAIL and IL-12 and conjugated to transferrin, would improve the delivery of therapeutic DNA and suppress the growth of prostate cancer cell lines in vitro and in vivo. Transferrin receptors are abundantly expressed on prostate cancer cells [13] and have been successfully used for the delivery of liposomes to prostate tumors [14], thus making a transferrin-based tumor targeting strategy particularly interesting to explore on prostate cancer cells with the DAB dendrimer delivery system. In addition, the combination of transferrin-based active targeting and passive targeting due to the accumulation of dendriplexes resulting from the enhanced permeability and retention effect should provide a tumor-selective targeting to these dendriplexes.

Furthermore, generation 3-DAB dendrimer presents the advantage of having 100% protonable nitrogens [15], making it particularly suitable for binding the negatively charged DNA by electrostatic interactions [16].

In this study, we successfully demonstrated that the conjugation of Tf on the dendriplexes improved DNA uptake by the three prostate cancer cell lines studied, compared to control dendriplexes and naked DNA treatment. Tf receptor-mediated uptake of DNA and drugs has been extensively studied on numerous cancer cell lines [17-22], but rarely on prostate cancers. Although the difference between treatments was less pronounced in our study, our results were in accordance with
previous data obtained by Sahoo and colleagues [23], who demonstrated that the uptake of Tf-conjugated, paclitaxel-loaded nanoparticles was about 3-fold higher than that of unconjugated nanoparticles in PC-3 cells. The enhanced DNA uptake resulting from treatment of the cells with a transferrin-bearing delivery system could be explained by the level of transferrin receptor protein expression on these prostate cancer cell lines. Liu previously demonstrated that transferrin receptors were overexpressed on PC-3, DU145 and LNCaP cells, with a higher level of expression on the surface of PC-3 cells [13].

As a consequence of this improved uptake, the treatment of the cells with DAB-Tf dendriplex resulted in an increase in gene expression on PC-3 and LNCaP cells, the improvement on DU145 being not significantly different from the gene expression level obtained with unconjugated DAB dendriplex. Our results were in agreement with the similarly improved gene expression observed by Yu et al. [24] when using immunolipoplexes targeting Tf receptors on prostate cancer cells.

The use of DAB-Tf as a delivery system for TNF±, TRAIL and IL-12 expression plasmids significantly enhanced the in vitro therapeutic efficacy of the plasmids on the three prostate cancer cell lines studied, with improvements of up to 100-fold in LNCaP cells compared to the unconjugated dendriplex. The most efficacious treatment observed in this study was DAB-Tf dendriplex expressing TNF± on PC-3 cells, which resulted in an IC50 of 0.27 ± 0.04 µg/mL. Our results were in accordance with previously published works. Kaliberov and colleagues [25] have demonstrated that an adenoviral vector encoding the TRAIL gene under the control of the vascular endothelial growth factor receptor FLT1 promoter significantly increased DU145 cell death in comparison with the PC-3 cell line, following a similar trend to that observed in our results. In another study [26], adenoviral-mediated TRAIL treatment led to a
significant increase in cytotoxicity on PC-3 cells, with 85.5% of cells dead. However, the lack of IC₅₀ values for these cell lines prevents comparison of the anti-proliferative effects of the viral and non-viral strategies. Furthermore, we could not find any studies describing the anti-proliferative effect of TNF±- and IL-12-mediated gene therapy on PC-3, DU145 and LNCaP prostate cancers to allow a comparison with our results.

In our experiments, the IC₅₀ obtained following administration of DAB-Tf dendriplex expressing TNF± was higher than those of TRAIL and IL-12 in DU145 cells, despite being the lowest in PC-3 and LNCaP cells. This may be explained by the sensitivity of these cell lines to the Fas ligand. TNF± is well known for its ability to induce apoptosis in a wide variety of cells. However, Rokhlin and colleagues [27] previously reported that Fas-sensitive PC-3 cell line was more sensitive to TNF± mediated apoptosis and growth inhibition than Fas-resistant DU145 and LNCaP cell lines. These results, in line with our observations, suggested that TNF± mediated apoptosis might be determined by factors that are common in both Fas and TNF± pathways.

In vivo, we demonstrated that the intravenous administration of DAB-Tf dendriplex encoding TNF± resulted in tumor eradication of 60% of PC-3 and 50% of DU145 tumors. In addition, treatment with DAB-Tf dendriplex encoding TRAIL also led to tumor eradication of 10% of PC-3 tumors. To our knowledge, it is the first time that the intravenous administration of tumor-targeted dendriplexes encoding TNF±, TRAIL and IL-12 on mice bearing prostate tumors, inhibited tumor growth and even led to a complete tumor suppression in some cases. Other researchers have previously demonstrated the ability of TNF±, TRAIL and IL-12-encoding DNA to induce a therapeutic effect on prostate tumors, but following intratumoral injection rather than intravenous administration, while using a viral delivery system rather than a non-viral
one, and often in conjunction with other therapeutic modalities, such as radiotherapy [28], co-administration with oncolytic herpes simplex viruses [29], with adenoviral vector-mediated Herpes Simplex Virus / thymidine kinase and ganciclovir [30], or in addition with the drug mifepristone [31]. For example, the intratumoral administration of an adenoviral vector encoding the TRAIL gene under the control of FLT1 promoter, in combination with radiation treatment, was shown to produce significant slowdown of the growth of DU145 tumor xenografts in athymic nude mice [25]. In another work, the intratumoral administration of a recombinant adenovirus expressing IL-12 was shown to significantly slowdown the growth of RM-9 prostate tumors [32]. Similar results were obtained by Saika and colleagues [33] following the intratumor administration of an adenovirus-mediated IL-12 on a 178-2 BMA prostate cancer model. The effects observed in these studies were generally a slowdown of tumor growth, rather than the tumor regression or suppression observed in some instances in our experiments.

In the DU145 xenograft model, the therapeutic effect of DAB-Tf dendriplex encoding TNF± was more pronounced than that obtained with TRAIL and IL-12, contrarily to what was observed in our anti-proliferative assay in vitro. This could be explained by the fact that TNF± exerts its potent cytotoxic effects on tumors in vivo via the death receptor-dependent apoptotic pathway, like TRAIL, but also via its anti-angiogenic effects, believed to be critical for its anti-cancer activity [34, 35]. It actually highlights the limitation of in vitro experiments for predicting the anti-cancer outcome of novel therapeutic systems in vivo.

In this study, we have chosen to use, among other therapeutic agents, a plasmid DNA encoding IL-12. The biological effects of IL-12 encompass stimulation of unspecific immunity, such as via activation of NK cells, and stimulation of specific
immunity as mediated by cytotoxic T lymphocytes. In our experiments, we had to use immunodeficient BALB/c mice unable to produce T cells in order for them to grow subcutaneous tumors of human origin. We therefore hypothesize that the therapeutic effect observed could be further improved by using a fully immunocompetent mouse bearing a murine prostate tumor model [36].

Our previous work, carried out with a therapeutic DNA encoding TNF± on A431 epidermoid carcinoma tumors, demonstrated the tumor targeting capability of Tf-bearing DAB dendrimer and led to a more pronounced therapeutic effect than that observed when using prostate tumor models [4]. These results highlight the difficulties encountered for the treatment of prostate tumors, independently of the therapeutic strategy chosen, and explain why so many studies use combinatorial modalities to improve the overall therapeutic effect. In this study, we wanted to evaluate the therapeutic efficacy of Tf-bearing DAB dendriplexes encoding TNF±, TRAIL and IL-12 on prostate tumor models following intravenous administration. Tumor-targeted TNF± gene therapy was more efficacious than TRAIL and IL-12, leading to tumor regression and even some tumor disappearance. Still, TRAIL and IL-12-mediated gene therapy actually improved the therapeutic efficacy on prostate tumor models, compared to the slowdown of tumor growth generally observed following intratumoral administration of adenovirus-based gene medicines, and this without any combinatorial treatment. In addition, these tumor-targeted systems are intravenously delivered, thereby allowing them to reach metastases as well as primary tumors, which is particularly important for the treatment of prostate tumors.
CONCLUSIONS

In this study, we have demonstrated for the first time that a tumor-targeting, dendrimer-based gene delivery system complexed to a plasmid DNA encoding TNF± and TRAIL can lead to eradication of some prostate tumors after intravenous administration.

*In vitro*, DAB-Tf complexed to TNF±, TRAIL and IL-12 expression plasmids significantly enhanced the anti-proliferative efficacy on PC-3, DU145 and LNCaP prostate cancer cells when compared to the unconjugated dendriplex, by up to 100-fold in LNCaP cells.

*In vivo*, the intravenous administration of DAB-Tf dendriplex encoding TNF± resulted in tumor suppression for 60% of PC-3 and 50% of DU145 tumors. Treatment with of DAB-Tf dendriplex encoding TRAIL led to tumor suppression of 10% of PC-3 tumors. IL-12 mediated gene therapy resulted in tumor regression of 20% of both types of prostate tumors. By contrast, all the tumors treated with DAB-Tf, naked DNA or left untreated were progressive for both tumor types. The animals did not show any signs of toxicity.

Transferrin-bearing DAB dendriplexes encoding TNF±, TRAIL and IL-12 therefore hold great potential as a novel approach for the gene therapy of prostate cancer and should be further investigated to optimize their therapeutic potential.
FUTURE PERSPECTIVE

While gene therapy has been widely studied in laboratory settings for its therapeutic potential against cancer, little has been done regarding its intravenous, targeted administration to prostate tumors. This is a particularly important issue in the case of this cancer, as there is still no efficacious treatment modality for patients suffering from a recurrent disease or metastasis. In this study, we demonstrated for the first time that the intravenous administration of Tf-bearing DAB dendriplex encoding TNF± resulted in tumor suppression for 60% of PC-3 and 50% of DU145 tumors when using a TNF± encoding plasmid, and for 10% of PC-3 tumors when using a TRAIL encoding plasmid. We therefore hope that this breakthrough will lead to the development of tumor-targeted gene therapy for the treatment of prostate cancer.

In addition, transferrin-bearing DAB dendriplexes encoding TNF±, TRAIL and IL-12 should be further studied to optimize their therapeutic potential against cancer.
EXECUTIVE SUMMARY

Introduction:
- Gene therapy would be an attractive anti-cancer treatment strategy against advanced prostate cancer
- Its use is limited by its failure to reach tumors in a specific way after intravenous administration

In vitro therapeutic efficacy:
- In vitro, transferrin-bearing dendriplex encoding TNF±, TRAIL and IL-12 improved the anti-proliferative efficacy of the therapeutic DNA on PC-3, DU145 and LNCaP prostate cancer cells

In vivo tumoricidal activity:
- In vivo, the intravenous administration of DAB-Tf dendriplex encoding TNF± resulted in tumor disappearance for 60% of PC-3 and 50% of DU145 tumors. Treatment with of DAB-Tf dendriplex encoding TRAIL led to tumor disappearance for 10% of PC-3 tumors. Consequently, the survival of the treated mice was increased by 22 days compared to untreated tumors, for both cell lines.

Conclusion:
- Transferrin-bearing DAB dendriplexes encoding TNF±, TRAIL and IL-12 therefore hold great potential as a novel approach for the gene therapy of prostate cancer and should be further investigated to optimize their therapeutic potential.
REFERENCES


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**REFERENCE ANNOTATIONS**

Publications of special note have been highlighted as:

* of interest

** of considerable interest


*Demonstrates for the first time that the intravenous administration of transferrin-bearing DAB dendriplex encoding TNF± leads to tumor suppression of 90% of A431 human epidermoid carcinoma tumors in mice*


*Demonstrates that the intratumoral administration of an adenoviral vector encoding the TRAIL gene under the control of FLT1 promoter, in combination with radiation treatment, produces significant slowdown of the growth of DU145 tumor xenografts in athymic nude mice*

Demonstrates that the intratumoral administration of a recombinant adenovirus expressing IL-12 leads to a significantly slowdown of the growth of RM-9 prostate tumors


Demonstrates that the intratumoral administration of an adenovirus-mediated IL-12 leads to a significant slowdown of the growth of a 178-2 BMA prostate cancer model.


Demonstrates that the expression of IL-12 further enhances the efficacy of the oncolytic herpes simplex viruses NV1023 in two murine prostate cancer models.


Describes the therapeutic effect of combination gene therapy with adenoviral vector-mediated Herpes Simplex Virus / thymidine kinase and ganciclovir on prostate cancers.

*Demonstrates that combinatorial approaches using an intratumorally administered recombinant adenovirus expressing IL-12 in conjunction with the drug mifepristone (RY486) improve the therapeutic efficacy in comparison to controls.*
FIGURE LEGENDS

Table 1. Anti-proliferative efficacy of TNF±-, TRAIL- and IL-12-encoding DNA complexed with DAB-Tf and DAB in PC-3, DU-145 and LNCaP prostate cancer cells, expressed as IC₅₀ values (n=15)

Figure 1. A) Confocal microscopy imaging of the cellular uptake of Cy3- labeled DNA (2.5 µg / well) either complexed with DAB-Tf, DAB or in solution, after incubation for 72 hours with PC-3 (top), DU145 (medium) and LNCaP cells (bottom) (Blue: nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415-491nm), green: Cy3-labeled DNA (excitation: 543 nm laser line, bandwidth: 550-620 nm) (Bar: 10 µm). B) Quantification of Cy3-related fluorescence intensity per cell (in arbitrary units a.u.) in the confocal microscopy pictures obtained as described above (n=1).

Figure 2. Transfection efficacy of DAB-Tf (“cplx DT”) and DAB dendriplexes (“cplx D”) in PC-3 (A), DU145 (B) and LNCaP cells (C). DAB-Tf and DAB dendriplexes were dosed at their optimal dendrimer: DNA ratio of 10:1 and 5:1 respectively. (Controls: DAB-Tf (“DT only”), DAB (“D only”) and naked DNA). Results are expressed as the mean ± SEM of three replicates (n=15). *: P <0.05 vs the highest transfection ratio.

Figure 3. A) Tumor growth studies in a PC-3 xenograft model after intravenous administration of transferrin- bearing DAB dendriplex carrying plasmid DNA encoding TNF± (green), TRAIL (red), IL-12 (blue) (50 µg DNA / injection), uncomplexed DAB-Tf (brown), naked DNA encoding TNF± (pale green), naked DNA encoding TRAIL (orange), naked DNA encoding IL-12 (pale blue), untreated tumors (back) (n=10).
B) Variations of the animal body weight throughout the treatment (Color coding as in A).

C) Overall tumor response to treatments at the end of the study, classified in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) [37].

D) Time to disease progression. The Y axis gives the proportion of surviving animals over time. Animals were removed from the study once their tumor reached 12 mm diameter (Color coding as in A).

**Figure 4.** A) Tumor growth studies in a DU145 xenograft model after intravenous administration of transferrin- bearing DAB dendriplex carrying plasmid DNA encoding TNF± (green), TRAIL (red), IL-12 (blue) (50 µg DNA / injection), uncomplexed DAB-Tf (brown), naked DNA encoding TNF± (pale green), naked DNA encoding TRAIL (orange), naked DNA encoding IL-12 (pale blue), untreated tumors (back) (n=10).

B) Variations of the animal body weight throughout the treatment (Color coding as in A).

C) Overall tumor response to treatments at the end of the study.

D) Time to disease progression. The Y axis gives the proportion of surviving animals over time. Animals were removed from the study once their tumor reached 12 mm diameter (Color coding as in A).

**Figure 5.** Bioluminescence imaging of the tumoricidal activity of transferrin- bearing DAB dendriplex carrying plasmid DNA encoding TNF± (“cplx DT-TNF”), TRAIL (“cplx DT-TRAIL”), IL-12 (“cplx DT-IL12”) in a PC-3M-luc-C6 tumor model. Controls: naked DNA and untreated tumors. The scale indicates surface radiance (photons/s/cm²/steradian).
FINANCIAL DISCLOSURE / ACKNOWLEDGEMENTS

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The authors have no affiliation or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.
A

Cplx DAB-Tf  |  Cplx DAB  |  DNA

PC-3

DU145

LNCaP

B

Fluorescence intensity per cell (a. u.)

PC-3 | DU145 | LNCaP

Cell line

Legend:
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- Cplx DAB
- DNA
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