Nuclear accumulation of plasmid DNA can be enhanced by non-selective gating of the nuclear pore

Roosmarijn E. Vandenbroucke, Bart Lucas, Joseph Demeester, Stefaan C. De Smedt* and Niek N. Sanders

Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

Received January 25, 2007; Revised April 19, 2007; Accepted May 17, 2007

ABSTRACT

One of the major obstacles in non-viral gene transfer is the nuclear membrane. Attempts to improve the transport of DNA to the nucleus through the use of nuclear localization signals or importin-β have achieved limited success. It has been proposed that the nuclear pore complexes (NPCs) through which nucleocytoplasmic transport occurs are filled with a hydrophobic phase through which hydrophobic importins can dissolve. Therefore, considering the hydrophobic nature of the NPC channel, we evaluated whether a non-selective gating of nuclear pores by trans-cyclohexane-1,2-diol (TCHD), an amphipathic alcohol that reversibly collapses the permeability barrier of the NPCs, could be obtained and used as an alternative method to facilitate nuclear entry of plasmid DNA. Our data demonstrate for the first time that TCHD makes the nucleus permeable for both high molecular weight dextrans and plasmid DNA (pDNA) at non-toxic concentrations. Furthermore, in line with these observations, TCHD enhanced the transfection efficacy of both naked DNA and lipoplexes. In conclusion, based on the proposed structure of NPCs we succeeded to temporarily open the NPCs for macromolecules as large as pDNAs and demonstrated that this can significantly enhance non-viral gene delivery.

INTRODUCTION

Viral vectors are efficient DNA delivery systems as they possess natural mechanisms to enter cells, to escape from endosomes and to transport their DNA into the nucleus. However, they also display important disadvantages, such as immunogenic response and safety risks when administered to patients. Non-viral carriers lack these disadvantages, but poor transfection efficiencies currently limit the usefulness of these vectors for gene therapy applications. The low gene transfer capacity of non-viral vectors is mainly due to their inability to translocate the therapeutic DNA into the cell nucleus. Indeed, it has been shown that microinjection of plasmid DNA (pDNA) in the cytoplasm of non-dividing cells resulted in <1% gene expression, while a massive gene expression occurred when the pDNA was injected in the nucleus (1–3). In dividing cells the nuclear envelope disassembles on a regular base, which offers an opportunity for DNA to enter the nucleus (4–6). However, the DNA that is waiting in the cytoplasm for the next cell division is sensitive to degradation by nucleases. Therefore, methods that can enhance the nuclear uptake of DNA into nuclei of both non-dividing and dividing cells are urgently needed in non-viral-based gene therapy.

Nucleocytoplasmic transport proceeds through the nuclear pore complexes (NPCs) which form channels in the nuclear envelope with a diameter of ~40 nm (7,8). Vertebrate NPCs have a mass of ~125 MDa and contain 30–50 different proteins, which are called nucleoporins. Small molecules with a molecular weight up to 30 kDa can passively diffuse through the NPC. In contrast, the translocation of larger macromolecules into the nucleus occurs via an active mechanism involving nuclear transport receptors. The majority of the nuclear transport pathways are mediated by receptors of the importin family. Proteins or other cargo molecules that carry a classical nuclear localization sequence (NLS) are recognized by importin-α, which subsequently forms a complex through its importin-β-binding domain with importin-β (9). NLSs can be highly diverse in nature and range from the short bipartite classical NLS to extended protein domains, as is the case for histones or ribosomal proteins (10). To promote the nuclear import of DNA, NLS peptides, NLS-containing proteins and even importin-β (11) have been attached to DNA via several strategies: electrostatic (12–17) or covalent (18–22) binding, via protein–DNA interaction (23,24), via PNA clamps (15,25–27) and coupled to polymers (28–30), lipids (31–36) or recombinant lambda phage (37). Nevertheless, all these

*To whom correspondence should be addressed. Tel: +32-9-2648047; Fax: +32-9-2648189; Email: Stefaan.DeSmedt@UGent.be

© 2007 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
attempts to improve the transport of DNA to the nucleus through the use of NLSs or importin-β have achieved only limited success.

It has recently been shown that the nuclear uptake of macromolecules can be enhanced significantly by addition of the amphipathic molecule trans-cyclohexane-1,2-diol (TCHD) (38). The mechanism by which TCHD causes nuclear localization of macromolecules can be explained based on the inner channel properties of the nuclear pores. It is believed that these nuclear pores are filled with a hydrophobic phase through which importins, but not inert hydrophilic substrates, can dissolve. The addition of TCHD causes a temporary, non-selective gating of the pore and allows passage of molecules which would otherwise be rejected from passage. In other words, a non-selective gating of the nuclear pore channel by TCHD renders the actual translocation through the pore channel independent of nuclear transport receptors. This can be explained by the fact that TCHD causes disruption of the hydrophobic interactions between the hydrophobic phenylalanine–glycine repeats of the nucleoporines, which consequently causes collapsing of the permeability barrier of the NPCs. Importantly, the effect of TCHD is reversible and does not cause damage of the nuclear pores (38).

In this paper we studied whether a non-selective gating of nuclear pores by amphipathic molecules like TCHD could also be used as an alternative method to facilitate nuclear entry of plasmid DNA. Therefore, we examined the effect of TCHD (a) on the nuclear import of macromolecules and pDNA and (b) on the transfection efficiency of naked pDNA and non-viral nanoparticles, such as poly- and lipoplexes. In summary, we found that TCHD was able to make the nuclear membrane permeable for both high molecular weight dextrans and pDNA at non-toxic concentrations. Furthermore, TCHD enhanced the transfection efficiency of both naked pDNA and DOTAP:DOPE-based lipoplexes, but had no effect on the linear PEI-based polyplexes.

MATERIALS AND METHODS

Materials

Dulbecco’s modified Eagle’s medium (DMEM), l-glutamine (l-Gln), heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline (PBS) and penicillin/streptomycin (P/S) were supplied by GibcoBRL (Merelbeke, Belgium). The secreted alkaline phosphatase (SEAP) expression plasmid was a kind gift from Prof. Dr Wagner (University of Munich, Germany). The pGL3-control plasmid and luciferase assay kit were obtained from Promega (Leiden, The Netherlands). One hundred fifty-eight kilodaltons of tetramethylrhodamine isothiocyanate-labeled dextran (TRITC-dextran) and TCHD were purchased from Sigma Aldrich. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) chloride salt, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Amino(Polyethylene Glycol)2000] (DSPE-PEG2000) were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Cell culture

A549 (lung carcinoma cells; ATCC number CCL-185) and Vero (African green monkey cells; ATCC number CCL-81) cells were cultured in DMEM containing 2 mM l-Gln, 10% heat-inactivated FBS, 100 U/ml P/S and grown at 37°C in a humidified atmosphere containing 5% CO2.

Fluorescent labeling of pDNA

Plasmid DNA was covalently labeled with Cy5 using the LabelIT kit of Mirus Corporation (Madison, WI, USA) according to the manufacturer’s recommendations. Briefly, LabelIT reagent, containing Cy5, and 100 μg DNA were mixed in 1 ml Hepes buffer (20 mM Hepes, pH 7.4) at Cy5:DNA ratio (w:w) of 0.5:1 and incubated at 37°C for 1 h. Subsequently, the labeled pDNA was separated from unattached label by precipitation in the presence of ethanol and 0.5 M NaCl and reconstituted in 20 mM Hepes buffer (pH 7.4).

Microinjection studies

Microinjection studies were conducted using a Femtojet® microinjector and an Injectman® NI 2 micromanipulator (Eppendorf, Hamburg, Germany). Vero cells were chosen for these microinjection experiments as they have a well-defined nucleus and large cytoplasm. Vero cells (2.5 × 10⁴ cells/cm²) were plated onto sterile glass bottom culture dishes (MatTek Corporation, MA, USA) and allowed to adhere for 1 day. The cells were then washed with PBS and transferred into 2 ml serum-free medium supplemented with 20 mM Hepes (pH 7.4) to improve the buffering capacity of the medium during microinjection. Five microliters 158 kDa TRITC-dextran (1 mg/ml) or Cy5-labeled pDNA (1 mg/ml) was back-loaded into Femtotop II microinjection needles and cells were injected using an injection pressure of 100 psi, a back-pressure of 30–50 psi and injection duration of 0.5 s. Where mentioned, the medium was replaced after microinjection by TCHD-containing serum-free medium, supplemented with 20 mM Hepes (pH 7.4).

To determine the cellular distribution, the fluorescence in the cells was visualized on different time points after microinjection using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium). With a 60× water immersion objective and the 561 and 638 nm laser lines for the excitation of the TRITC and Cy5 label, respectively. A non-confocal diascopic DIC (differential interference contrast) image was collected simultaneously with the confocal images.

For the z-scan analysis of the fluorescence after cytoplasmic microinjection of the Cy5-labeled pDNA (Cy5-pDNA), the confocal volume (~1 fl) of a BioRad MRC 1024 CLSM (Hemel Hempstead, UK) equipped with the Confocor 2 fluorescence correlation spectroscopy (FCS) setup (LSM510 Confocor 2, Zeiss, Göttingen, Germany) was positioned in a randomly selected site in the nucleus. The fluorescence, along the
z-axis at this selected XY site and perpendicular to the cell surface, was recorded with the avalanche photodiodes of the ConfoCor 2 system before and every 10 min after addition of 1% (w/v) TCHD dissolved in serum-free medium supplemented with 20 mM Hepes (pH 7.4).

**Preparation of polyplexes and lipoplexes**

Polyplexes consisting of 22 kDa linear PEI were prepared as described by Fayazpour et al. (39). Briefly, polyplexes were prepared in 20 mM Hepes pH 7.4 by adding the PEI all at once to the pDNA at a N/P ratio of 10. Subsequently, the mixture was vortexed for 10 s and the polyplexes were allowed to equilibrate for 30 min at room temperature prior to use. The final pDNA concentration in the polyplexes was 0.126 μg/μl.

Liposomes composed of DOTAP:DOPE:DSPE-PEG<sub>2000</sub> (molar ratio 5:5:0.2) were prepared as described previously (40). Briefly, appropriate amounts of lipids were dissolved in chloroform and mixed. The chloroform was subsequently removed by rotary evaporation at 37°C followed by flushing the obtained lipid film with nitrogen during 30 min at room temperature. The dried lipids were then hydrated by adding Hepes buffer till a final lipid concentration of 10.2 mM. After mixing in the presence of glass beads, liposome formation was allowed overnight at 4°C. Thereafter, the formed liposomes were extruded 11 times through two stacked 100 nm polycarbonate membrane filters (Whatman, Brentfort, UK) at room temperature using an Avanti Mini-Extruder (Avanti Polar Lipids). The extruded liposomes were subsequently mixed with pDNA in a ±charge ratio of 4 and incubated at room temperature for 30 min prior to use. The final pDNA concentration in the lipoplex dispersion was 0.126 μg/μl.

**Size and zeta potential measurements**

The average particle size and zeta potential of the liposomes, lipoplexes and polyplexes were measured by photon correlation spectroscopy (PCS) (Autosizer 4700, Malvern, Worcestershire, UK) and particle electrophoresis (Zetasizer 2000, Malvern), respectively. The liposome, lipoplex and polyplex dispersions were diluted 40-fold in Hepes buffer before the particle size and zeta potential were measured. The average (±standard error) of the liposomes and lipoplexes was 118 ± 1 and 242 ± 6 nm, respectively and their average zeta potential equaled 26 ± 4 and 14 ± 1 mV, respectively. The diameter and zeta potential of the linear PEI polyplexes were 165 ± 4 nm and 33 ± 2 mV.

**Cell viability assay**

The influence of TCHD on the cell viability was determined using the CellTiter-Glo<sup>®</sup> Assay (Promega) according to the manufacturer’s instructions. Briefly, 2.5 × 10<sup>4</sup> cells/cm<sup>2</sup> were seeded in a 96-well plate and allowed to adhere. After 24 h, cells were washed with PBS and incubated with serum-free medium containing increasing amounts of TCHD. After 1 h, the TCHD was removed and replaced by culture medium. After 48 h, the plate was incubated at room temperature for 30 min and 100 μl CellTiter-Glo<sup>®</sup> reagent was added to each well. After shaking the plate for 10 and 2 min incubation at room temperature, the luminescence was measured on a GloMax<sup>™</sup> 96 luminometer with 1 s integration time.

**Transfection experiments**

Cells were seeded into 24-well plates at 2.5 × 10<sup>4</sup> cells/cm<sup>2</sup> and allowed to attach overnight. Subsequently, the culture medium was removed, and after two washing steps with serum-free medium, 0.4 μg pDNA, polyplexes or lipoplexes (both containing 0.4 μg pDNA) were added to each well. After 2 h the pDNA or non-viral nanoparticles were removed from the cells and the cells were post-incubated for 1 h with serum-free medium containing increasing amounts of TCHD. Subsequently, this medium was replaced by culture medium and the cells were further incubated at 37°C. After 48 h both the SEAP (or luciferase) activity, as well as the total cellular protein concentration were measured.

To determine the SEAP activity, 100 μl of the culture medium above the cells was taken and incubated at 65°C for 30 min. Subsequently, 100 μl dilution buffer (0.1 M glycine, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, pH 10.4) and 15 μl 4-methylumbelliferyl phosphate (4-MUP, 5.1 μg/μl in distilled water) was added. The obtained mixtures were then incubated at 37°C and the fluorescence was measured on a Wallac Victor<sup>®</sup>2 fluorescence plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA) using an excitation and emission wavelength of 360 and 449 nm, respectively.

To determine the luciferase activity of the cells, cells were lysed with 80 μl 1 × CCLR buffer (Promega) and luciferase activity was determined with the Promega luciferase assay kit according to the manufacturer’s instructions. Briefly, 100 μl substrate was added to 20 μl sample and after a 2 s delay, the luminescence was measured during 10 s with the GloMax<sup>™</sup> 96 luminometer.

To correct for the amount of cells per well, the protein concentration was determined with the BCA kit (Pierce, Rockford, IL, USA). Two hundred microliters mastermix, containing 50 parts reagent A to 1 part B, was mixed with 20 μl cell lysate and incubated at 37°C for 30 min and the absorbance was measured on a Wallac Victor<sup>®</sup>2 absorbance plate reader (Perkin Elmer-Cetus Life Sciences) at 590 nm.

**Statistics**

The experimental data in this report are expressed as mean ± standard deviation (SD). One way ANOVA was used to determine whether data groups differed significantly from each other. A P-value <0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

TCHD facilitates the nuclear accumulation of dextran and plasmid DNA

It has been demonstrated that TCHD enhances the rate of nuclear entry of the maltose binding protein (38). However, this protein has a rather low molecular weight
(40 kDa) and is consequently not totally excluded from the nucleus. Therefore, we studied whether TCHD could induce nuclear entry of higher molecular weight compounds like 158 kDa dextrans and especially pDNA, since nuclear transport of therapeutic genes forms an important bottle neck in non-viral gene delivery.

In a first approach we microinjected 158 kDa TRITC-dextrans in the cytoplasm of Vero cells and followed their nuclear influx in the absence and presence of TCHD by confocal laser scanning microscopy (CLSM). In the absence of TCHD, no TRITC-dextran could be detected in the nucleus, not even after 1 h of incubation (Figure 1A and B). This is as expected, since it is well-known that molecules larger than ~70 kDa cannot move passively through the NPC network (41). When TRITC-dextran microinjected cells were incubated with 1% (w/v) TCHD-containing medium, a rapid nuclear localization of the TRITC-dextrans was detected (Figure 1C till 1G). Indeed, as soon as 10 s after addition of TCHD to the cells, TRITC-dextran was already detected in the nucleus. After 10 min the TRITC-dextran fluorescence was homogeneously distributed throughout the cell. These data clearly demonstrate that TCHD opens the NPCs what results in nuclear passage of macromolecules that otherwise are excluded from the nucleus. We also co-injected TCHD (2% w/v) and TRITC-dextran (158 kDa) in the cytosol, but under these conditions we could not observe nuclear localization (data not shown). One likely explanation is that TCHD, which is an amphiphilic compound and contains a polar ethylene glycol moiety and an apolar butylene moiety, can rapidly cross cell membranes and thus becomes rapidly diluted in the surrounding medium (38).

Next, we tested whether TCHD can also facilitate the nuclear uptake of pDNA, since pDNAs are much larger (2–10 MDa) than 158 kDa TRITC-dextran and have dimensions in the range of the inner diameter of the channels formed by the NPCs. When Cy5-pDNA was microinjected in the cytoplasm of Vero cells, a fluorescent spot was visible at the injection site (Figure 2A, position 1). After 1 h incubation with TCHD, we could not observe accumulation of the pDNA inside the nucleus by CLSM (data not shown). Importantly, during that time the fluorescent microinjection spot became more diffuse but remained visible, indicating a restricted mobility of pDNAs in the cytoplasm. This is in agreement with the observations by Lukacs et al. (42) who showed that the diffusion of pDNA in the cytoplasm may be an important rate-limiting barrier in gene delivery utilizing non-viral vectors. Hence, after 1 h only a small fraction of the microinjected pDNA will have reached the nuclear membrane. Additionally, as stated earlier the inner diameter of the NPC is in the size range of pDNA. Therefore, even in the presence of TCHD the number of pDNA molecules that enter the nucleus is probably low and beyond the detection limit of the CLSM.

To monitor nuclear pDNA influx with higher sensitivity, we used a CLSM equipped with a fluorescence correlation spectroscopy (FCS) set up, which can detect as few as ~1 fluorescent molecule in a femtoliter range confocal volume (43). We performed time-dependent z-scans, perpendicular to the slide surface and through position 2 (Figure 2A) before and after microinjection of Cy5-pDNA in the cytoplasm at position 1 (Figure 2A), hereby crossing first a part of the cytoplasm beneath the nucleus, then the nucleus and finally the cytoplasm above the nucleus (Figure 2B). The black squares in Figure 2C show that after cytoplasmic microinjection of the Cy5-pDNA, a fluorescence signal could be detected in the cytoplasm, but not in the nucleus. The difference in fluorescence intensity detected below and above the nucleus is most likely due to a difference in distance from the cytosolic injection site. Subsequently, 1% (w/v) TCHD was added to the cells and z-scans through

Figure 1. Non-confocal DIC (A, C and inserts in E till G) and confocal images (B and D till G) of Vero cells after cytosolic microinjection of 158 kDa TRITC-dextran. Images (A) and (B) represent a microinjected cell 60 min after microinjection. Images (C) till (G) represent another microinjected cell after 10 s (D), 40 s (E), 5 min (F) and 10 min (G) incubation with 1% (w/v) TCHD.
position 2 were performed every 10 min. The z-scan after 60 min, represented by gray circles in Figure 2C, shows a clear elevated Cy5-pDNA fluorescence signal in the nucleus compared to the fluorescence profile before addition of TCHD. Subsequently, we studied the pDNA influx in the nucleus in the presence and absence of TCHD. Therefore, the confocal volume of the FCS set up was parked in the middle of the nucleus (at \( z \)-value -0.048 mm). A gradual increase of Cy5-pDNA was observed when TCHD was added to the cells (Figure 2D; black circles). In contrast, when no TCHD was present, no increase in fluorescence could be detected in the nucleus (Figure 2D; gray squares). This demonstrates that the time-dependent increase in fluorescence after addition of TCHD is not a result of passive diffusion of small degradation products of the Cy5-pDNA into the nucleus.

**Cytotoxicity of TCHD**

It has been demonstrated that the effect of 7% (w/v) TCHD on the NPC permeability is reversible and that it does not cause denaturation or leakage of nucleoporins out of the NPCs (38). Nevertheless, the reversible non-selective opening of NPCs may result into an unwanted leakage of cellular biomacromolecules from the cytoplasm into the nucleus or vice versa and hence interfere with essential cellular processes. Therefore, to ascertain that TCHD does not cause cytotoxic effects, we determined the cell viability 48 h after exposure to TCHD by the CellTiter-Glo\textsuperscript{®} assay (Promega). This assay assesses the cytotoxicity by quantifying the intracellular ATP levels, which is a sensitive marker of cell viability as within minutes after a loss of membrane integrity, cells lose the ability to synthesize ATP and endogenous ATPases destroy any remaining ATP. Figure 3 shows that incubation of Vero and A549 cells during 1 h with 1% (w/v) TCHD slightly reduced the viability of these cells. These results demonstrate that the TCHD does not cause drastic cytotoxic effects under the conditions of the nuclear uptake experiments above, i.e. incubation of the cells with 1% (w/v) TCHD for 1 h. Furthermore, we noticed a cell-dependent TCHD sensitivity. Indeed, TCHD at concentrations above 1% (w/v) significantly decreases the viability of Vero cells, whereas almost no cytotoxic effects are observed in A549 cells incubated with up to 3% (w/v) TCHD.

**Figure 2.** Effect of TCHD on the nuclear entry of microinjected Cy5-pDNA microinjected in the cytoplasm of Vero cells. A schematic overview of the experimental set up is shown in (B). (A) Represents a microinjected cell with arrow (1) representing the place of cytoplasmic microinjection and arrow (2) the place were the z-scan was performed. The black squares in (C) represent the z-scan through the cytosol and nucleus of a microinjected Vero at time point zero, so just after microinjection and before addition of TCHD. The gray circles represent the z-scan 60 min after addition of 1% (w/v) TCHD. In image (C), the center of the cell nucleus is located at \( z \)-value -0.048. The time-dependent increase of Cy5-pDNA fluorescence in the nucleus after cytoplasmic injection of Cy5-pDNA without (gray squares) and with (black circles) TCHD is shown in (D). The black circle in (A) indicates the nuclear membrane.
Influence of TCHD on the transfection efficiency of non-viral vectors

Since nuclear uptake of pDNA is considered as one of the major barriers in non-viral gene delivery and since we showed that TCHD could cause nuclear uptake of Cy5-pDNA, we wondered whether TCHD could enhance the transfection efficiency of naked pDNA, cationic polyplexes and lipoplexes. All transfection experiments were performed in two cell types, namely A549 cells (lung carcinoma cell line) and Vero cells (kidney epithelial cell line), using different TCHD concentrations.

The effect of TCHD on the transfection efficiency of naked pDNA was evaluated by incubating Vero and A549 cells with naked pDNA for 2 h. Subsequently, pDNA that was not incorporated into the cells was removed by washing, and the cells were exposed to increasing percentages (w/v) of TCHD for 1 h (Figure 4). The incubation with TCHD clearly increased the gene expression. This increase reached a maximal value at a TCHD percentage of 0.5 and 1.5% in Vero (Figure 4A) and A549 cells (Figure 4B), respectively. At these optimal concentrations, a 3- and 66-fold increase in gene expression was observed in Vero and A549 cells, respectively. At higher percentages no further increase and even a drop in gene expression was observed. Most likely this indicates that TCHD at these concentrations affects cellular processes that are not detected by the MTT assay. Indeed, it has been shown that the sensitivity of the MTT assay depends on the mechanism causing cytotoxicity (44). Between 0 and 1.5% (w/v) TCHD, a gradual increase in gene expression was observed in A549 cells (Figure 4B), which may indicate that the extent of NPC opening by TCHD is concentration dependent. Whether at 1.5% (w/v) TCHD a maximal opening of the NPCs is reached is not certain, since above this concentration also cytotoxic effects can play a role. This also explains the lower effects of TCHD in Vero cells. Indeed, in these cells the optimal concentration of TCHD to increase gene transfer is 0.5%. Based on the results in A549 cells, we can deduce that at such low TCHD concentration the opening of the NPC has not reached its maximum.

When TCHD was incubated together with the naked pDNA, we also observed an increase in transfection efficiency, but the effect was less pronounced compared to the post-incubation experiments described earlier (data not shown). This could be expected as it takes some time for pDNA to become internalized by the cells and released from endocytotic vesicles into the cytoplasm. Additionally, as cytosolic nucleases degrade pDNA, the optimal moment to add TCHD to the cells is immediately after the release of the pDNA in the cytoplasm.

To exclude that the higher gene expression was due to a perforation of the cell membrane by TCHD, which would allow an increased internalization of pDNA through the plasma membrane, we tested whether TCHD causes cytoplasmic entry of 158 kDa TRITC-dextrans. Figure 5 shows that TRITC-dextrans did not enter the cells in the absence nor in the presence of TCHD (1% w/v). Consequently, we can state that after 1 h incubation TCHD does not cause membrane perforation.

In a next step, we analyzed the effect of TCHD on the transfection efficiency of linear PEI-based polyplexes, a quite efficient non-viral pDNA carrier that induces endosomal release via the proton sponge mechanism (45). Surprisingly, none of the incubation protocols with TCHD resulted in a significant increase in gene expression mediated by linear PEI-based polyplexes even not when the Vero and A549 cells were post-incubated with 2% (w/v) TCHD (Supplementary Figure 1A and B). These data may indicate that, in agreement with Grosse et al. (46), linear PEI-based polyplexes are mainly released from the endosomes as intact complexes. Because of the latter and taking into account the diameter of the linear PEI-based polyplexes (~165 nm), TCHD is not expected...
to be able to enhance the nuclear transport and hence transfection efficacy of such linear PEI-based polyplexes.

The endosomal escape mechanism of DOTAP:DOPE-based lipoplexes is based on a different mechanism and results in the release of uncomplexed pDNA in the cytosol (47). This implies that, when the free pDNA reaches the nuclear membrane, TCHD should be able to induce its nuclear translocation, similar to the naked pDNA transfections. Therefore, we analyzed the effect of TCHD on the transfection efficiency of DOTAP:DOPE-based lipoplexes. The same incubation protocols and cells were used as in the experiments above. Incubating the cells with TCHD for 1 h immediately after incubation with the lipoplexes, indeed caused an increase in transfection efficiency in both Vero and A549 cells (Figure 6A and B). A maximal increase was observed when the Vero and A549 cells were incubated with 0.5 and 1.5% (w/v) TCHD, respectively. This increase is lower than observed with naked pDNA transfection, which can be attributed to cell division. Compared to naked pDNA transfection, transfection with lipoplexes introduces a higher amount of pDNA in the cytoplasm, which can translocate to the nucleus with higher probability either by entry through the pores or during cell division. Hence, the few extra copies that enter the nucleus after treating the cells with TCHD will not tremendously increase gene expression. In contrast, the amount of pDNA that reaches the cytoplasm and subsequently the nucleus is extremely low in case of naked pDNA transfection. Therefore, if TCHD can cause nuclear translocation of only one pDNA molecule, this effect is much more spectacular.

CONCLUSION

In conclusion, our study demonstrates for the first time that the amphipathic alcohol TCHD can be employed to enhance the nuclear uptake of pDNA by reversibly collapsing the permeability barrier of the NPCs at non-toxic concentrations. Furthermore, our transfection data show that TCHD has huge potential to enhance especially naked gene transfer. Although less efficient than carrier-mediated gene delivery, naked gene transfer is currently

Figure 4. Transfection efficiency of naked pDNA in Vero (A) and A549 (B) cells post-incubated for 1 h with different TCHD concentrations. The asterisk (*) represents data that significantly differ \((P<0.05)\) from the data point 0% (w/v) TCHD.
the most investigated non-viral gene delivery strategy in gene therapy clinical trials because of the higher safety profile, the simplicity of delivery, the lack of immune responses and non-specific interactions in the body (with e.g. extracellular matrices) (48). Naked gene transfer is considered for DNA vaccination, Duchenne muscular dystrophy, peripheral limb ischemia, and cardiac ischemia (49–52). Therefore, strategies that can enhance naked gene transfer by enhancing nuclear uptake of DNA are of huge interest. Additionally, we also want to remark that TCHD is a metabolite of the drug candesartan cilexetil (53) and of the solvent cyclohexanone, a contaminant of intravenous dextrose and parenteral feeding solutions (54), which possibly makes TCHD a clinical acceptable adjuvant for naked gene transfer. However, toxicology and in vivo gene transfer studies are needed to confirm the usefulness of this approach for clinical applications.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
Niek Sanders is a Postdoctoral Fellow with the Fund for Scientific Research – Flanders (Belgium). The European Commission is thanked for funding through the Integrated 6th Framework Programme MediTrans. The LSM510 ConfoCor 2 experiments were performed at the Advanced Light Microscopy Facility (Prof. R. Pepperkok) at the European Molecular Biology Laboratory (EMBL) in Heidelberg (Germany). Prof. K. Ribbeck is thanked for carefully reading the manuscript. Funding to pay the Open Access publication charges for this article was provided by Ghent University.

Conflict of interest statement. None declared.
REFERENCES


