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The biological performance of purified supercoiled p53 plasmid DNA in different cancer cell lines

J.F.A. Valente, A. Sousa, V.M. Gaspar, J.A. Queiroz, F. Sousa*

CICS-UBI – Health Sciences Research Centre, Universidade da Beira Interior, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal

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ABSTRACT

Tumor suppressor p53 remains one of the most interesting therapeutic targets in cancer gene therapy due to its consistent mutation in numerous cancers. Thus, the reinstatement of the p53 expression and function can be seen as an effective alternative for cancer treatment, motivating research in this field. In this study, L-methionine matrix was used to purify the supercoiled topoisoform of a plasmid DNA encoding the p53 protein. This pure biopharmaceutical was conjugated with liposomes to comprehensively analyze its *in vitro* performance and therapeutic potential in different cancer cell lines, including the lung and cervix models. A different profile of cellular responses was attained after the transfection of these cancer cell lines with the p53-pDNA. Actually, the *in vitro* transfection with pure sc p53-pDNA resulted in a higher expression of the tumor suppressor protein in cancer cells when compared with the native pDNA samples (oc + sc topoisoforms). Also, wild-type p53 expression following transfection was significantly higher in HeLa cervix cancer cells compared to that obtained in A549 lung cancer cells. Overall, our findings emphasize the potential of sc pDNA gene-based therapy, also raising awareness of the need to adjust the therapeutics, considering the feature of high heterogeneity of cancer cells.

1. Introduction

Tumorigenesis is a process that combines the sequential accumulation of genetic and epigenetic modifications in key oncogenes and tumor suppressor pathways. Usually, these transformations are responsible for the growth of cancer cells with distinct characteristics that include: (i) self-sufficiency to growth signals, (ii) insensitivity to anti-growth signals, (iii) evasion from programmed cell death, (iv) unlimited replicative potential, (v) sustained angiogenesis, and (vi) the ability to invade and metastasize – the so-termed Cancer Hallmarks [1]. Regarding the plethora of genes involved in tumorigenesis, the p53 transcription factor (encoded by the TP53 human gene) is one of the most important [1,2]. Most of the cancers known until now present mutations in TP53 with rates that vary between 10% (*e.g.*, in hematopoietic malignancies) and close to 100% (*e.g.*, in high-grade serous carcinoma of the ovary) [3].

The tumor suppressor p53, also termed the guardian of genome, is involved in key physiological processes including DNA damage response, upon which it triggers cells senescence and apoptosis [4]. In fact, the pathways whereby p53 leads to execution of the apoptosis program are complex; however, Bax was proven to be transcriptionally activated by p53 in human cell lines [5]. Also, an increase of Bax protein expression was found in several cell lines following p53 overexpression, and it has been confirmed that Bax can inhibit Bcl-2 activity, thus accelerating apoptosis [6]. The ablation of TP53 could be due to the single base substitution and loss of alleles, mediated by viral and cellular proteins, which are reported to play a major role in specific cancers. Some cancers including breast carcinomas, sarcomas, brain tumors, and adrenal cortical carcinomas, Li-Fraumeni (LFS), and Li-Fraumeni-like (LFL) syndromes have already demonstrated an early genetic predisposition to TP53 mutations. Moreover, since TP53 is extremely polymorphic in coding and non-coding regions, some of these polymorphisms have been related to an increase in cancer susceptibility [7]. Another important element is that other tumor suppressors are usually inactivated by frameshift or nonsense mutations, with the TP53 missense mutations being caused by single amino acid changes at many different positions [2]. Due to its biological relevance, p53 is therefore a very valuable therapeutic target, and so far, different strategies have been explored to mimic or reinstate its activity.

The use of pDNA (pDNA) transgene expression vectors in non-viral cancer gene therapy has emerged as a valuable methodology to reinstate the expression of wild-type p53 into malignant cells and restore its tumor suppressive function. Regarding the use of p53 as a therapeutic target, our research group has recently demonstrated that a

* Corresponding author at: Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail address: fani.sousa@fcsaude.ubi.pt (F. Sousa).

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purified supercoiled (sc) isoform of p53-encoding pDNA is the most efficient isoform for promoting non-viral pDNA-based transgene expression [8]. To obtain highly pure biopharmaceutical formulations, different strategies have been reported so far (like size exclusion, anion exchange, hydrophobic interaction, reversed phase, thiophilic adsorption, and affinity chromatography) [9,10]. In this focus, our group has been exploring amino acids as ligands for DNA/RNA affinity chromatography, as a successful method for the isolation of the sc isoform of different plasmids namely the pcDNA3-FLAG-p53 vector. For this specific transgene expression cassette, two different commercial matrices with L-arginine and L-methionine ligands have been investigated [8,11].

Herein, an agarose matrix functionalized with L-methionine ligands was employed in order to promote the isolation of a sample composed only by sc pDNA. The final sample quality should be analyzed and the results should be in agreement with the parameters established by the regulatory agencies such as Food and Drug Administration (FDA), USA, and European Medicines Agency (EMA) [11]. By using this purification technology, pure sc p53-pDNA was successfully recovered and its biological performance was investigated following liposome-mediated delivery in different cancer cells. The use of different cell lines can help for a better understanding of the behavior of this suggested therapy *in vivo,* mainly because tumors are very different and even the same cancer is not homogeneous, being composed of different cells that are highly permutable, which makes the use of strategies able to efficiently target all the modified spots mandatory. Regarding this approach, and to the best of our knowledge, this is the first time that a comprehensive analysis of pure sc pDNA transfection by using commercial cationic delivery systems is performed in different cancer cell lines. To investigate such potential, several experiments were designed in order to initially verify the biocompatibility of the pDNA after the chromatographic procedure. Then, the amount of p53 expressed in the different cell lines was analyzed and correlated with the induced apoptosis (Fig. 1), considering the specific characteristics and the p53 metabolic pathways of the cells under study. Overall, sc pDNA transgene expression efficacy has higher than native pDNA samples and the therapeutic effect of the p53 encoded transgene was dependent on cancer type. Such pre-clinical findings may influence future applications of this approach.

2. Materials and methods

2.1. Materials

The NZYtech Maxi Prep Kit was purchased from NZYTech (Lisbon, Portugal). Ammonium sulphate ($(NH_4)_2SO_4$) was purchased from VWR and tris(hydroxymethyl) aminomethane (Tris) was obtained from Merck (Darmstadt, Germany). The 6.07 kbp pcDNA3-FLAG-p53 Addgene plasmid 10838 [12] was purchased from Addgene (Cambridge, MA, USA), Resazurin sodium salt, L-methionine agarose matrix and all the reagents used in bacterial amplification were obtained from Sigma-Aldrich (St. Louis, M.O., USA). The DNA ladder was obtained from Bioline (London, UK). The Annexin V-FITC/PI apoptosis kit was purchased from Calbiochem (La Jolla, CA, USA). The Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich and the Lipofectamine 2000 was purchased from Thermo Fisher Scientific (Inc., Lisbon, Portugal). All reagents were of research grade and used without further purification.

2.2. Methods

2.2.1. Plasmid production and pre-purification

pcDNA3-FLAG-p53, a plasmid with 6.07 kbp from Addgene, Cambridge, MA, USA (plasmid 10838) was amplified in a cell culture of *E. coli* DH5α. This *E. coli* was grown at a temperature of 37 °C in an Erlenmeyer flask containing 250 mL of Terrific Broth medium (20 g/L[1] of tryptone, 24 g/L of yeast extract, 4 m L of glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) and 30 μg/mL of ampicillin. In advanced log phase (OD_{600} ≈ 9), the bacteria growth was suspended and cells were recovered by centrifugation. The native plasmid DNA (sc + oc) was recovered and pre-purified with the NZYtech Maxi Prep Kit, according to the manufacturer's instructions.

2.2.2. Preparative chromatography

For the chromatographic experiments, an ÄKTA purifier system with UNICORN 5.11 software (GE Healthcare, Uppsala, Sweden) was used. L-methionine-agarose gel (Sigma Aldrich) was then packed in a 16 × 40 mm (approximately 8 mL) column. The supplied datasheet characterizes this resin as containing a one-atom spacer and an extent of labelling between 2–10 μmol/mL. A circulating water-bath was used to fix the temperature (5 °C) along the chromatographic runs. A 200 μL loop was used to load the pDNA onto the column at a rate of 1 mL/min. The elution was monitored using an ultraviolet (UV) detection at

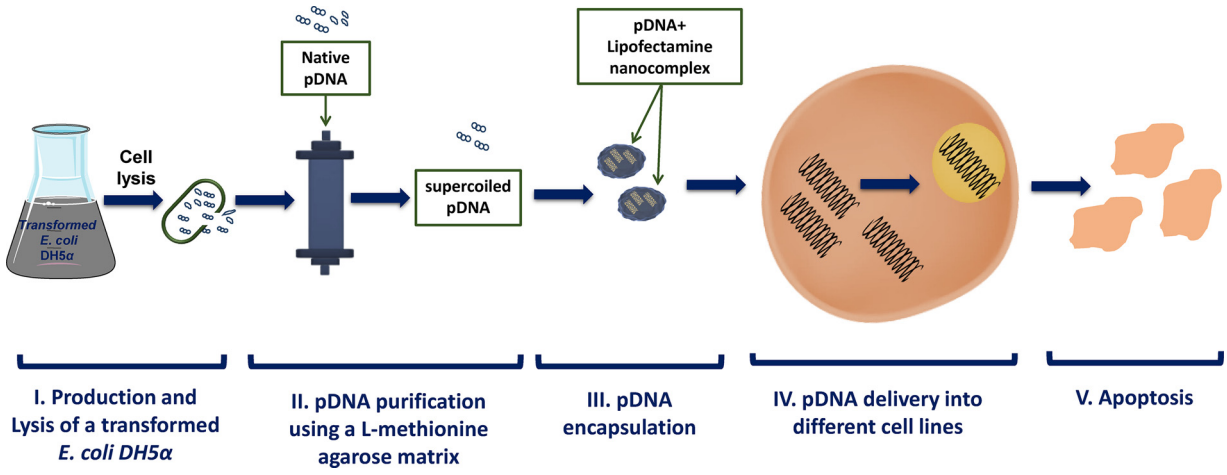


Fig. 1. Schematic representation of the biotechnological approach used in the present work. (I) Production and lysis of an *E. coli* DH5α strain transformed with the pcDNA3-FLAG-p53 vector; (II) pDNA purification and recovery of sc topoisoform by using an L-methionine affinity chromatography agarose-based matrix; (III) DNA complexation with Liposomes using commercially available cationic liposomal formulation Lipofectamine 2000; (IV) pDNA delivery to A549, HeLa cancer cells and normal human dermal fibroblasts (hFIB); (V) Evaluation of sc p53-pDNA mediated apoptosis of cancer cells.

260 nm. To perform the complete isolation of the sc plasmid isoform, a decreasing stepwise gradient comprising initially 2.35 M of $(NH_4)_2SO_4$ in 10 mM Tris − HCl pH 8.0 and then 10 mM of Tris − HCl, pH 8.0, at 5 °C was used. Finally, to check the eluted species, an agarose gel electrophoretic analysis was performed as previously described, using a Uvitec Cambridge Fire-reader UV transilluminator equipped with a CCD camera (Uvitec Cambridge) [11].

2.2.3. Cell culture and transfection

Cell culture experiments were performed with two cancer cell lines of different origins, the A549 non-small lung carcinoma cell line and HeLa cervix cancer cell line, and a non-malignant cell line, the human dermal Fibroblasts (hFib). DMEM-F12 medium supplemented with 10% v/v heat activated FBS and with streptomycin (100 μg/mL)) was used for cell culture at 37 °C, under a 5% CO_2 humidified atmosphere. Initially, cells were seeded in 25 cm^3 T-flasks until confluence was attained. Afterwards, the cells were sub-cultivated by incubation on 0.18% trypsin (1:250) with 5 mM EDTA.

The, *in vitro* transfection experiments were carried out by seeding 2×10^4 cells in a 96-well plate with 200 μL of DMEM-F12 complete medium followed by incubation for 24 h. Then, a medium without FBS and antibiotic was used to promote transfection. The transfection of pDNA was then performed with a commercially available transfection reagent (Lipofectamine 2000 (LP2000)). Briefly, in each well of 96-plate, 0.28 μL of LP2000 and 0.14 μg of DNA were diluted in 5.15 μL of Opti-MEM® I medium, according to the manufacturer's protocol. Before the complexation reaction, LP2000 was incubated for 5 min at room temperature (RT). The LP2000-pDNA complexes were added to cells and then incubated for a period of 6 h after which the medium was changed to DMEM-F12 complete medium.

2.2.4. Plasmid DNA fluorescent labelling

The labeling of pDNA biopharmaceuticals with the FITC dye was performed to allow the follow-up of its cellular uptake and intracellular localization. Briefly, 5 μg of pDNA was added to 71 μL of labeling buffer (0.020 g of sodium (di)tetraborate in 1 mL of H_2O) and 2 μL of FITC (100 mg of FITC in 200 μL of sterile DMSO). After this, the solution was stirred for 4 h at RT, and protected from light. Finally, 85 μL of 3 M NaCl and 212.5 μL of absolute ethanol was added to precipitate FITC labeled pDNA by overnight incubation at − 20 °C.

2.2.5. Cellular uptake analysis by confocal laser scanning microscopy (CLSM)

To evaluate pDNA-lipoplexes cellular uptake kinetics, 1×10^4 cells were seeded in complete DMEM-F12 in Ibidi μ-Slide 8-well cell culture treated chambers (Ibidi GmbH, Germany) and cultured overnight. Transfection was performed upon achieving 70% of cells confluence. Then, cells were incubated for 20 min with Hoechst 33342® (1:1000) (Invitrogen™ Molecular Probes™) and subsequently rinsed 3 times with PBS (pH = 7.4). Transfection was performed during 0, 2, 4 and 6 h, with LP2000 nanosized lipoplexes loaded with native p53-pDNA (sc + oc) or sc p53-pDNA biopharmaceuticals. Following the incubation period, the DMEM-F12 medium was exchanged and 4% paraformaldehyde in PBS was used for transfected cells fixation (for 20 min, at RT). To enable a better visualization, transfected cells were washed three times with PBS. Visualization was finally performed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., USA) equipped with a plane-apochromat 63 ×/DIC objective.

2.2.6. Cytotoxicity

The cytotoxicity of liposomal-pDNA formulations was evaluated by using the resazurin assay. For this purpose, A549, HeLa, and hFIB cells were seeded in 96-well plates as described above. Resazurin (10 μL, 2.5 mM) was added to each well two days after transfection. The plate was then incubated in the dark for 4 h, at 37 °C, in a humidified atmosphere of 5% CO_2. After incubation, resofurin was measured using a plate reader spectrofluorometer (Spectramax Gemini XS, Molecular Devices LLC, US), at an excitation/emission wavelength of λex = 560 nm and λem = 590 nm. Data represents the mean of three independent experiments.

2.2.7. Western blot analysis

The expression of p53 protein mediated by cells transfection with p53-pDNA vectors was evaluated by Western blot. Briefly, following transfection with LP2000-pDNA lipoplexes, cells were rinsed with ice-cold PBS and homogenized in cell lysis buffer: 25 mM Tris − HCl buffer, pH 7.4; 2.5 mM EDTA; 1% Triton X-100; 2.5 mM EGTA; 25 mM phenylmethylsulfonyl fluoride and complete, EDTA-free protease inhibitor cocktail (Roche). Cell extracts were then centrifuged at 11,500 rpm for 7 min at 4 °C and the supernatant was analyzed using Bradford Protein Assay (BioRad) according to the manufacturer's instructions and then fractionated by electrophoresis on 10% SDS-PAGE. Proteins were denatured (95 °C for 10 min) and transferred to polyvinylidene difluoride filter (PVDF) membranes (100 V for 40 min). Then, TBS-T supplemented with 5% BSA was used for the blocking. The anti-p53 primary antibody (1:100 in TBS-T) (Santa Cruz Biotechnology) and the Bax primary antibody (1:1000 in TBS-T) (Cell Signaling) was used to incubate the membranes at 4 °C, overnight. Membranes were then washed three times with TBS-T, and then incubated with the p53 anti-rabbit secondary antibody diluted 1:25,000 in TBS-T. The membrane was then washed and incubated in β-actin primary antibody (1:20,000 in TBS-T) (Santa Cruz Biotechnology) for 2 h and finally incubated in the β-actin secondary antibody (Santa Cruz Biotechnology). ECL substrate (BioRad) was used to signal detection according to manufacturer's instructions and images were acquired by using a ChemiDoc™ XRS system (BioRad) and analyzed with the Image Lab software (BioRad).

2.2.8. P53 expression by ELISA

The p53 ELISA kit (Enzo Life Sciences) was used to assess the p53 protein expression after cells' transfection with p53-pDNA vector. Briefly, following transfection with the different p53-pDNA formulations, cells were rinsed with ice-cold PBS and homogenized in cell lysis buffer: 25 mM Tris − HCl buffer, pH 7.4; 2.5 mM EDTA; 1% Triton X-100; 2.5 mM EGTA; 25 mM phenylmethylsulfonyl fluoride and complete, EDTA-free protease inhibitor cocktail (Roche). Cell extracts were then centrifuged at 11,500 rpm for 7 min at 4 °C and the supernatant was analyzed using Bradford Protein Assay (BioRad) according to the manufacturer's instructions. Then, the ELISA protocol provided by Enzo Life Sciences was applied according to the manufacturer's instructions and the p53 protein expression was finally measured in a plate reader spectrofluorometer (Spectramax Gemini XS, Molecular Devices LLC, US), at 450 nm. Data represent the mean of two independent experiments.

2.2.9. Flow cytometry analysis

Apoptosis in malignant and non-malignant cells transfected with p53 expressing pDNA was evaluated by flow cytometry through Annexin V-FITC/PI staining (Calbiochem, USA). For this purpose, 5×10^5 cells were initially seeded in sterile 6-well culture plates containing DMEM-F12 culture medium supplemented with 10% FBS. Cell growth was then promoted at 37 °C, 5% CO_2, in a humidified atmosphere, for 24 h. In the following day, the culture medium was removed and the cells were transfected with LP2000 cationic liposomes loaded with different pDNA formulations (sc p53-pDNA and native p53-pDNA (oc + sc)), according to the manufacturer's instructions. After 48 h of transfection, cells were detached by using trypsin/EDTA and pelleted by centrifugation (1500 rpm, 5 min, RT). Binding buffer was used to resuspend cells that were then labeled with Annexin V-FITC and PI according to the manufacturer's instructions. Flow cytometry experiments were performed on a BD FACS Calibur flow cytometer (Becton Dickinson Inc., USA) equipped with a 15 mW, 488 nm laser, and a 635 nm red-laser. Different region of interest (ROI) were selected to

acquire the data for the different cell lines used. A total of 1×10^4 events were collected in the FL-1 (530/30 nm) and FL-2 channel (585/42 nm). As controls for ROI delimitation and detectors of gain/voltage adjustment, non-treated cells were used. Hydrogen peroxide (H_2O_2) was used as cell-death-inducing agent to obtain positive controls for apoptosis and necrosis. Briefly, positive control cells were seeded in 24-well culture plates and treated with 160 mM of H_2O_2 for 12 h, at 37 °C, 5% CO_2, in culture medium supplemented with 10% FBS and antibiotics/antimycotics (complete medium). The cells were then recovered as earlier mentioned and analyzed by Annexin V-FITC/PI staining. Data processing was performed in FCS Express version 5 Research Edition (De Novo Software™, LA, USA).

2.2.10. Statistical analysis

Each experience was performed at least three times using independent cell cultures. Data were expressed as a mean ± standard error (S.D.) or standard error means (S.E.M). The statistical analysis performed was one-way analysis of variance (ANOVA), followed by multiple comparison test Turkey. A p-value below 0.05 was considered statistically significant. Data analysis and statistical tests were performed in GraphPad Prism 6 software.

3. Results and discussion

3.1. L-Methionine-based purification of p53 supercoiled pDNA

The p53 tumor suppressor gene is a valuable biological target for gene-based cancer therapies since common small therapeutic molecules are unable to reinstate its activity, rendering p53 as one of the so-termed “undruggable targets”. Recently, several reports have demonstrated that sc topology outdoes the biological performance of native pDNA samples that simultaneously contain oc and sc topoisoforms [8,13]. To ensure the purity of the pDNA, our research group has developed affinity chromatography-based pDNA purification platforms, which allow to obtain purified plasmid preparations that conform to the guidelines of regulatory agencies [14]. In particular, the L-methionine agarose matrix was previously explored and characterized by Valente and co-workers (2014), in order to recover the supercoiled topoisoform of different plasmids, including the p53-encoding plasmid vector (pcDNA3-FLAG-p53), from *E. coli* lysates, accomplishing the quality parameters required by the regulatory agencies [11]. We have reported that the sc p53-encoding plasmid, purified by this matrix, does not present in its composition proteins, RNA or gDNA, the endotoxin level was below the recommended by the regulatory agencies and also the content of the sc isoform was above 97% [11].

Regarding the above mentioned, L-methionine agarose matrix was applied in the present work, and a decreasing stepwise gradient comprising 2.35 M of $(NH_4)_2SO_4$ in 10 mM Tris − HCl pH 8.0 and 10 mM of Tris − HCl, pH 8.0, at 5 °C was applied to recover the sc pDNA specie, as demonstrated in Fig. 2.

3.2. Evaluation of the transfection behavior

After the purification process, pure sc pDNA was successfully conjugated with commercial cationic liposomes (LP2000). This product is commonly used in pDNA delivery into different cells [15–19], and since the main objective of this study is to compare the biologic activity of pure sc pDNA in different cell types, this standard transfection reagent is adequate. The use of different cell lines can help to better understand the behavior of this suggested therapy *in vivo*, mainly because tumors are very different and even the same cancer is not homogeneous, being composed by different cells that are highly permutable, which makes the use of strategies able to efficiently target all the modified spots mandatory. Also, when using the liposystem applied in this research work, it is not possible to redirect it only for the cancer cells being the therapeutic formulation dispersed for all the cells (cancer and non-cancer).

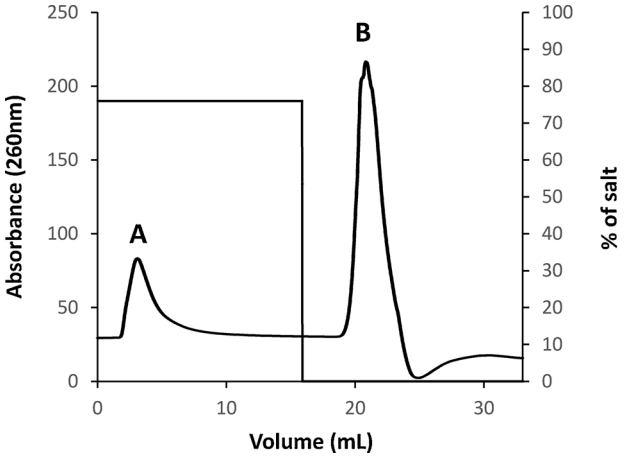
Concerning the above mentioned, the cellular uptake kinetics (at 0, 2, 4 and 6 h of transfection) of different pDNA lipoplexes was evaluated in different cell lines to study possible differences in cellular entry and delivery among formulations. The obtained profiles after 6 h of transfection with sc pDNA lipoplexes are presented in Fig. 3. In this image, it is possible to observe the presence of FITC-labeled pDNA in the nucleus of different cell lines after 6 h of transfection with LP2000. Therefore, it was also inferred that these transfection conditions are suitable for further experiments with the cell lines under study.

In addition, a time course transfection study was performed at different time-frames, 0, 2, 4 and 6 h, respectively, in order to evaluate the internalization profile of pDNA /lipoplexes in each cell line (Fig. 4).

As see in Fig. 4, after 2 h of transfection, all cell lines presented some cellular uptake of FITC/sc pDNA-lipoplexes, being more evident in hFIB. However, it is also visible that among the cancer cells, pDNA-lipoplexes seems to be faster and more internalized in HeLa cancer cells than in A549 cells. Regarding A549, it was demonstrated through the relative mean fluorescence intensity (M.F.I.) graphs (Fig. 4) that the amount of fluorescence in these cells is lower when compared with HeLa or hFIB cells. Previous research works had also demonstrated low transfection results for A549 for both viral and non-viral vectors, showing that these cells are harder to transfect even when commercial liposomal formulations [8,20,21] are used. Concerning the therapeutic application, perhaps it should be necessary to adjust and optimize the transfection to reach a more effective result.

3.3. Plasmid DNA-loaded lipoplexes cytotoxicity

To evaluate the cytotoxic profile of pDNA liposomal formulations,



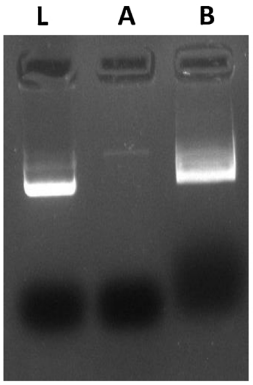


Fig. 2. Chromatographic profile and respective agarose gel electrophoresis of the plasmid isoforms separation from a pre-purified pDNA sample (sc + oc) (lane L) in an L-methionine agarose column at 5 °C. The elution was performed by a stepwise gradient of 2.35 M of $(NH_4)_2SO_4$ in 10 mM Tris − HCl pH 8.0 (A) to 10 mM Tris − HCl pH 8.0 (B), as represented by the dashed line. In the electrophoresis gel, lane L represents the pre-purified plasmid sample (sc + oc) and lanes A and B represent the respective peaks of each chromatogram.

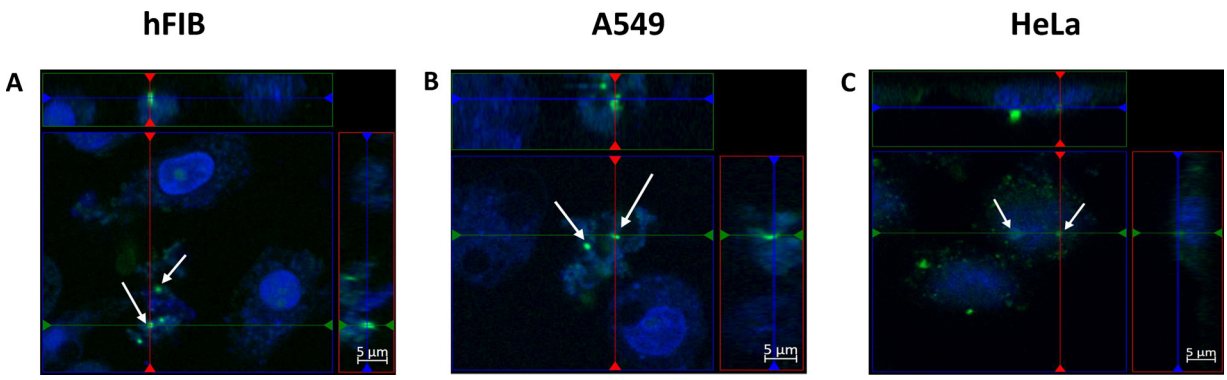


Fig. 3. Orthogonal view of live cell imaging six hours after pDNA complexes addition to the different cell lines (A- hFIB; B- A549; C- HeLa). The blue staining represents the cell's nucleus and the green staining represents FITC-labeled pDNA.

Resazurin assays were performed at 48 h and 72 h after transfection. As demonstrated by Fig. 5A, the treatment with sc p53-pDNA and native p53-pDNA (sc + oc) in normal hFib did not elicit any cytotoxic effect. In Fig. 5B, there is a negligible difference between the cytotoxic effect of native and sc-pDNA transfected A549 cells. On the contrary, the administration of sc p53-pDNA/LP2000 lipoplexes in HeLa cancer cells elicits a slight cytotoxicity (Fig. 5C). Moreover, the results from 72 h are in accordance with the tendency presented at 48 h after transfection. Actually, these results could indicate a possible contribution of p53 expression to the decrease in cell viability that was obtained, since as demonstrated in the previous section, this kind of cells presented the highest internalization of lipoplexes. The verified cell death can be a signal of apoptosis induced by the p53 expression, instead of the cytotoxic effect of the pDNA formulation. The validation of this hypothesis can be a good result as it may indicate the success of the delivery and expression of p53-pDNA, as well as it can confirm the importance of obtaining and using the sc topoisoform of pDNA to achieve a more significant biological effect. A similar effect was also previously described by Gaspar and collaborators, where at 24 h after transfection no cytotoxicity was observed, while at 72 h a decrease in HeLa cell viability was obtained. Also, in that research work no changes have been verified for fibroblasts viability [8].

To further evaluate if the observed cytotoxicity in cancer cell lines is correlated with the p53 expression, a Western blot analysis of the expressed wild-type protein was performed.

3.4. Analysis of p53 transgene expression

As demonstrated in Fig. 6, the expression of the p53 tumor suppressor protein is significantly higher when the purified sc pDNA vector is used. It should also be noted that, for all the malignant cell lines tested, the purified sc pDNA isoform yielded a higher amount of p53 when compared with that obtained with native pDNA (oc + sc) formulations. These results are in agreement with the results of previous studies regarding the improved biological performance of pDNA vectors administered in the supercoiled topoisoform [8,22].

Although in previous results we obtained a higher cellular uptake of sc pDNA lipoplexes in hFIB in comparison to the cancer cells, the level of p53 protein expression obtained in Western blot analysis was relatively lower. This could be correlated with the mechanisms of p53 expression in hFib and other normal cells [23] where the primary control of the p53 levels is performed through its ubiquitin-mediated proteasomal degradation. Regarding this, there are until now three different studies published, were the Mdm2 was identified as the master endogenous E3-ligase with high specificity for p53 [23]. Thus, the results suggest that in normal conditions, if the cell does not require the p53 function, it can modulate its expression or induce p53 degradation, even after the transgene delivery.

Interestingly, there is a significant difference in the reinstatement of p53 expression in HeLa and A549 cell lines with the latter yielding a lower amount of tumor suppressor protein, which is in accordance with the previous results of cellular uptake. The lower expression of p53 in A549 cells can be a result of the lower transfection, indicating that these cells are more resistant to the formulation entrance into the cells, making difficult the therapeutic intervention. Also, it should be emphasized that in A549 cell line, a negligible p53 gene expression was observed at the basal state when the cells were non-treated. These results are in accordance with the literature since A549 could present some basal levels of wt-p53 protein [24]. This basal level comes from a deletion on the CDKN2A locus that harbors p16 and p14ARF genes in A549 cells. Without p14ARF, Mdm2 levels are high, which keep p53 low. Even though p53 levels are low, there is still a basal level of the

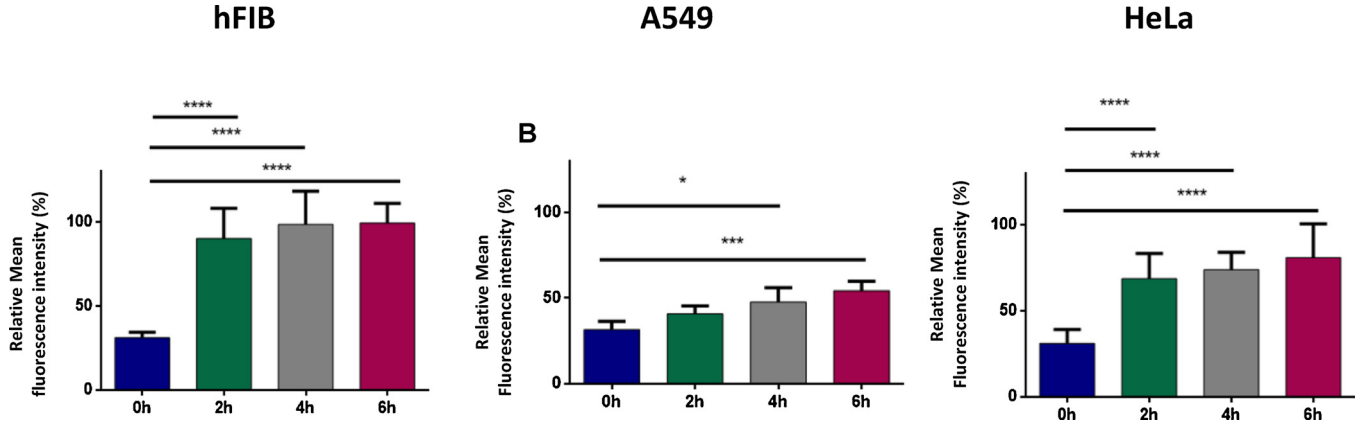


Fig. 4. M.F.I. values of p53 labelled FITC in the different cell lines (A- hFIB; B- A549; C- HeLa). Data is represented as mean ± S.D., n = 10.

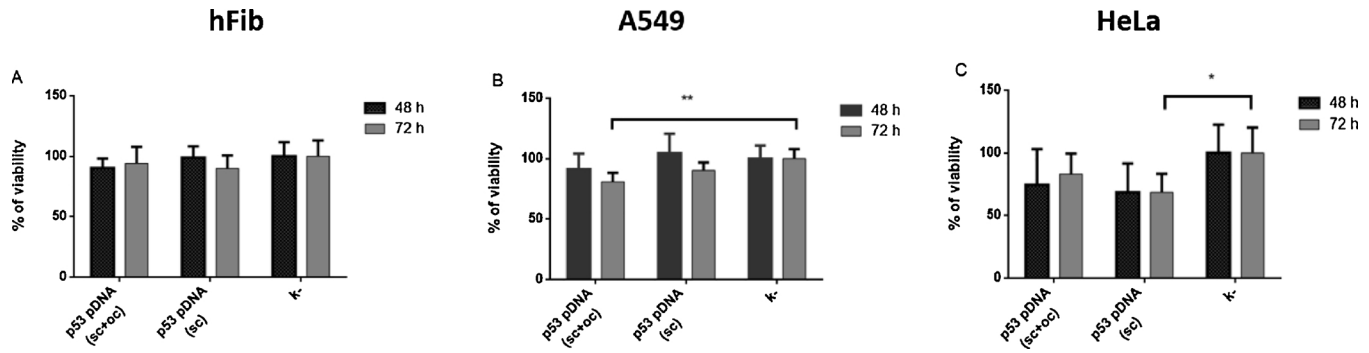


Fig. 5. Evaluation of cell viability following transfection with LP2000 lipoplexes loaded with p53-pDNA in different cell lines, after 48 h and 72 h. (A) Human dermal fibroblasts; (B) A549 non-small lung cancer; (C) HeLa cervix carcinoma. Non-transfected cells were used as negative controls for cytotoxicity (K−). Ethanol treated cells were used as positive controls for cytotoxicity (K+). Data is represented as mean ± S.D., n = 3.

protein [25].

In HeLa cancer cells, the oncoproteins E6 and E7 from HPV virus are responsible for p53 degradation being this, the reason for the non-existence of a basal level of p53 in these cells [26]. Regarding the p53 expression, the highest level amongst the cell lines under study was verified, which can be correlated with the pDNA uptake since this cell line is fairly easy to transfect and very receptive to vectors, making it a popular research tool.

ELISA analysis was also performed in order to confirm the results provided by the Western blot analysis. The results provided for the p53 protein expression for the different cell lines following transfection with the p53-pDNA lipoplexes are presented in Fig. 7.

Considering these new results, it is possible to observe that they are in accordance with the Western blot analysis, presenting exactly the same tendency. Once again, the HeLa cells revealed higher levels of p53 expression, correlating well with the other results of apoptosis. Moreover, it is relevant to underline that for all the cell lines under study, the higher p53 expression level was achieved when the supercoiled isoform was used for transfection, which corroborates other studies describing this specie as the most biologically active [8,27]. In fact, Gaspar and collaborators (2011) observed that the sc pDNA transfected cells exhibited the highest p53 expression levels when compared with other formulations [8], emphasizing the relevance to establish a suitable biotechnological process to prepare the sc pDNA biopharmaceuticals.

3.5. p53-mediated cell apoptosis

Apoptosis is measured in terms of binding of externalized phosphatidylserine to phospholipid binding protein Annexin V conjugated with fluorochromes [28]. Concerning that, to further evaluate the influence of p53 tumor suppressor protein expression in cells, the apoptosis was investigated by flow cytometry of different cancer cell lines following transfection with p53-encoding pDNA lipoplexes. Regarding the hFIB, the flow cytometry results are in agreement with those obtained by Resazurin and Western blot assays, as only a relatively low number of apoptotic cells were obtained following transfection, with more than 91% of healthy cells being obtained (Fig. 8). These low apoptotic levels could be due to the self-regulation mechanisms presented in non-tumoral cells as previously mentioned [23].

As demonstrated by the results of Fig. 9, pDNA transfection promotes A549 cells apoptosis after 48 h, with malignant cells transfected, with sc pDNA exhibiting a slightly higher amount of early and late apoptotic cells in comparison to native pDNA formulations. However, it is important to emphasize that no significant difference between sc and native transfected cells were obtained at this time point. In addition, the transfection of HeLa cells with p53 transgene elicits significant cell death with more than 35% of necrotic/early apoptotic cells (Fig. 10). Interestingly, as demonstrated by the flow cytometry data, the p53 tumor suppressor appears to have a more pronounced therapeutic effect in HeLa cells in comparison with A549 non-small lung cancer cells, which is in agreement with the amount of p53 protein expression obtained in Fig. 6. As mention above, these findings could be correlated with the fact that A549 cells are recognized to be more resistant to the

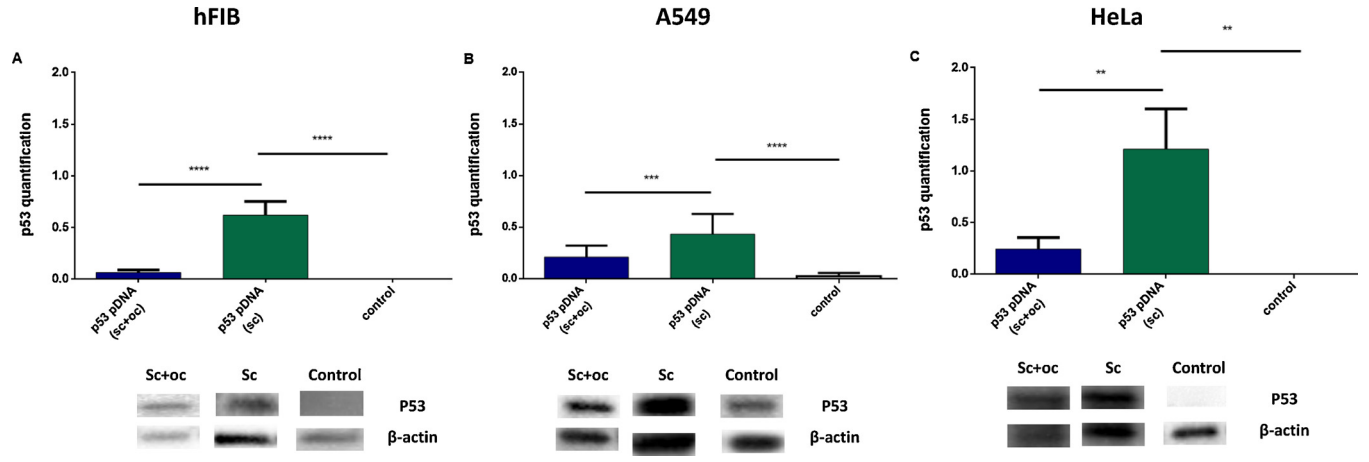


Fig. 6. Evaluation of p53 protein expression through Western Blot analysis after 48 h of different cell lines transfection with the p53-pDNA lipoplexes. (A) Human dermal fibroblasts; (B) A549 non-small lung cancer cells; (C) HeLa cervix carcinoma. Non-transfected cells were used as controls. Data are represented as mean ± S.D., n = 3.

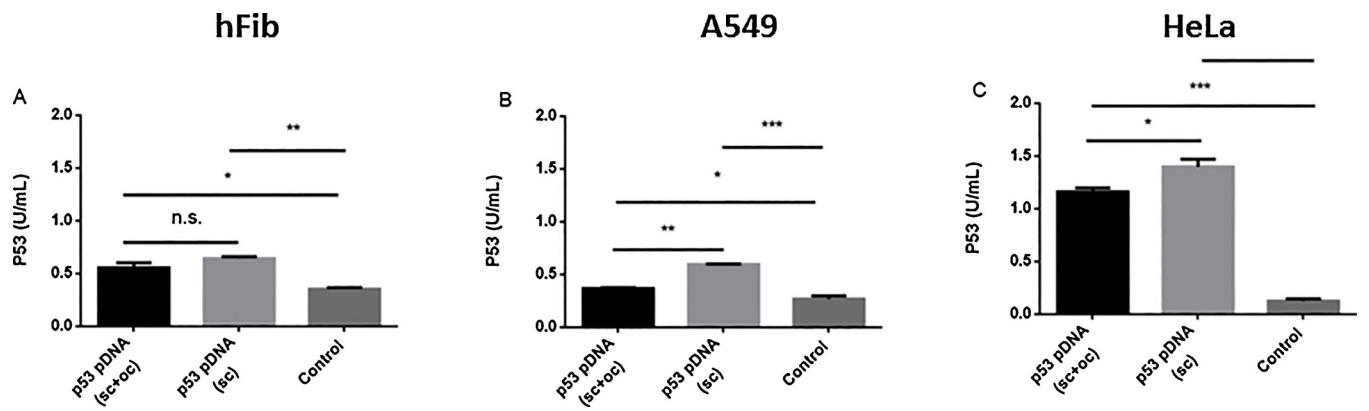


Fig. 7. Evaluation of the p53 protein expression through ELISA test after 48 h of different cell lines transfection with the p53-pDNA lipoplexes. (A) Human dermal fibroblasts; (B) A549 non-small lung cancer cells; (C) HeLa cervix carcinoma. Non-transfected cells were used as controls. Data are represented as mean ± S.D., n = 2.

action of p53 tumor suppressor since these cells express wild-type p53 [29,30]. Recent gene therapy experiments using replication-defective adenovirus vectors to express p53 revealed that tumor cells with wt p53 are highly resistant to the apoptotic effect of Ad-p53 both in culture and in tumor xenograft models [31,32]. Resistance to p53-mediated apoptosis in A549 may be attributable to tolerance to high-level p53 expression or efficient degradation of exogenous p53. In fact, Lu et al. found that inhibition of Mdm2 expression in A549 using antisense oligonucleotide induces growth arrest and accumulation of p53, suggesting that although Mdm2 is not overexpressed in this cell line it is still important for regulating endogenous p53. Therefore, they also successfully tested the possibility of Mdm2 may play a role in causing resistance to exogenous p53 expression and consequent function [30].

As revealed by Western blot analysis, the production of p53 following gene transfer of sc pDNA is lower than the attained for HeLa cells. This may impose a lower cytotoxic activity from the p53 tumor suppressor, and consequently a lower therapeutic outcome. Likewise, this behavior could also be correlated with the lower cellular uptake of pDNA-liposome formulations as displayed in Fig. 4.

Regarding the obtained results, it was verified that the p53-pDNA delivered was not able to promote the desirable apoptosis in this kind of cells. The low cellular uptake is a critical point in this result, which can be improved by using more effective delivery technologies [8]. Concerning that, it is reasonable to associate the lower expression of p53 protein, when compared with HeLa and hFib, to the scarce pDNA cell entrance.

The evaluation of the expression of Bax protein in the different cells used in this research work could provide a more downstream analysis not dependent on mRNA, as these could be affected by different cell-specific self-regulation mechanisms, namely RNAi. Moreover, the

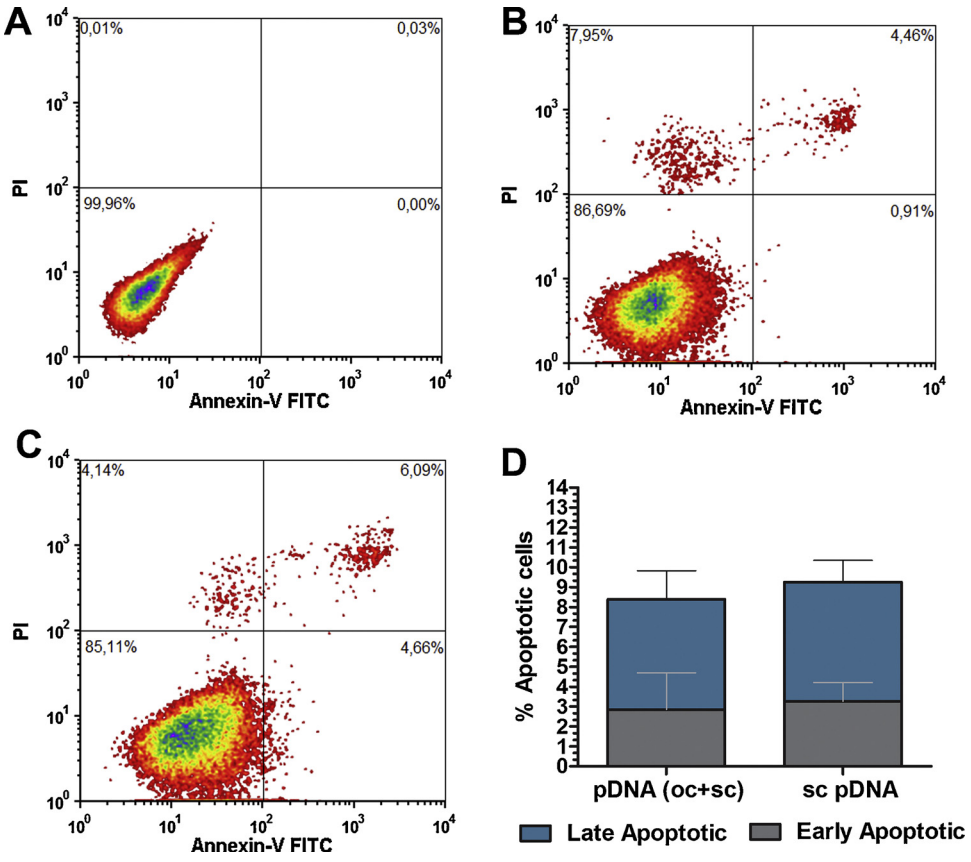


Fig. 8. Flow cytometry analysis of non-tumoral human dermal fibroblast (hFIB) apoptosis following transfection with different formulations of p53-pDNA gene expression vectors. (A) Density dot plot of non-transfected cells, autofluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by L-methionine affinity chromatography. (D) Analysis of apoptotic cells in hFIB cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., n = 3.

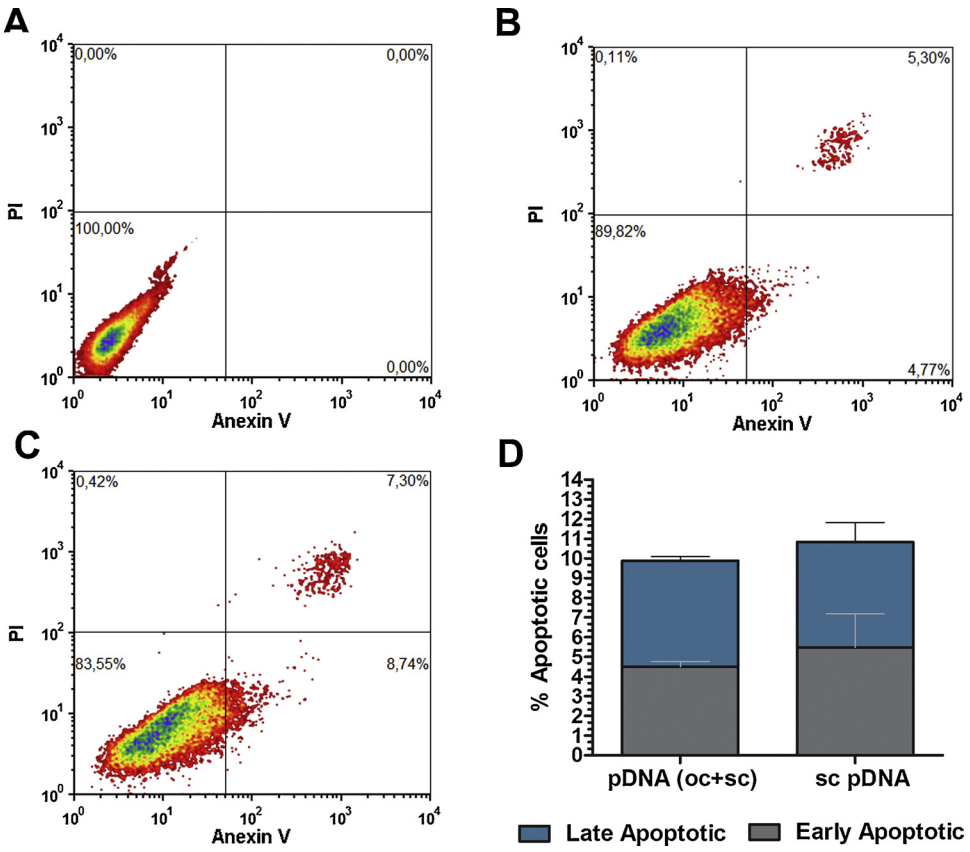


Fig. 9. Flow cytometry analysis of A549 non-small lung cancer cells apoptosis following transfection with different formulations of pDNA gene expression vectors encoding the tumor suppressor p53. (A) Density dot plot of non-transfected cells, auto-fluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by affinity chromatography. (D) Analysis of apoptotic cells in A549 cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., n = 3.

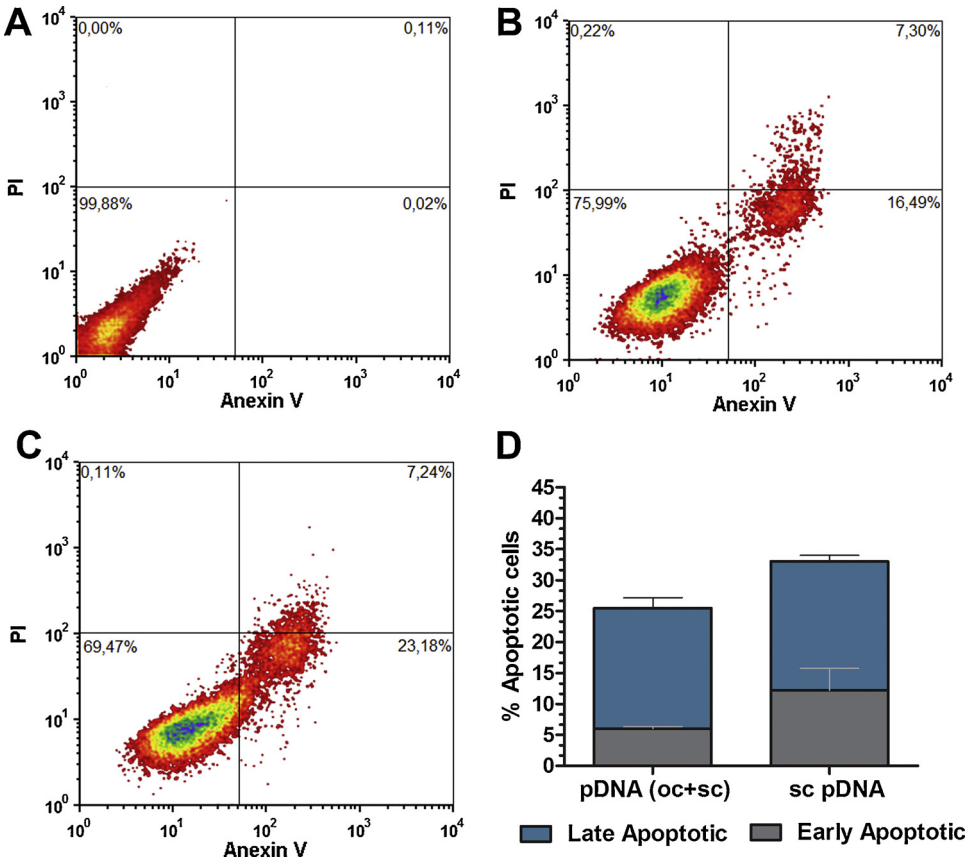


Fig. 10. Flow cytometry analysis of HeLa cells apoptosis following transfection with different formulations of pDNA gene expression vectors encoding the tumor suppressor p53. (A) Density dot plot of non-transfected cells, auto-fluorescence. (B) Density dot plot of cells transfected with pDNA formulation containing native pDNA topoisoforms (sc + oc). (C) Density dot plot of cells transfected with sc pDNA topoisoform previously purified by affinity chromatography. (D) Analysis of apoptotic cells in HeLa cells transfected with different pDNA biopharmaceuticals. Data are presented as mean ± s.e.m., *n* = 3.

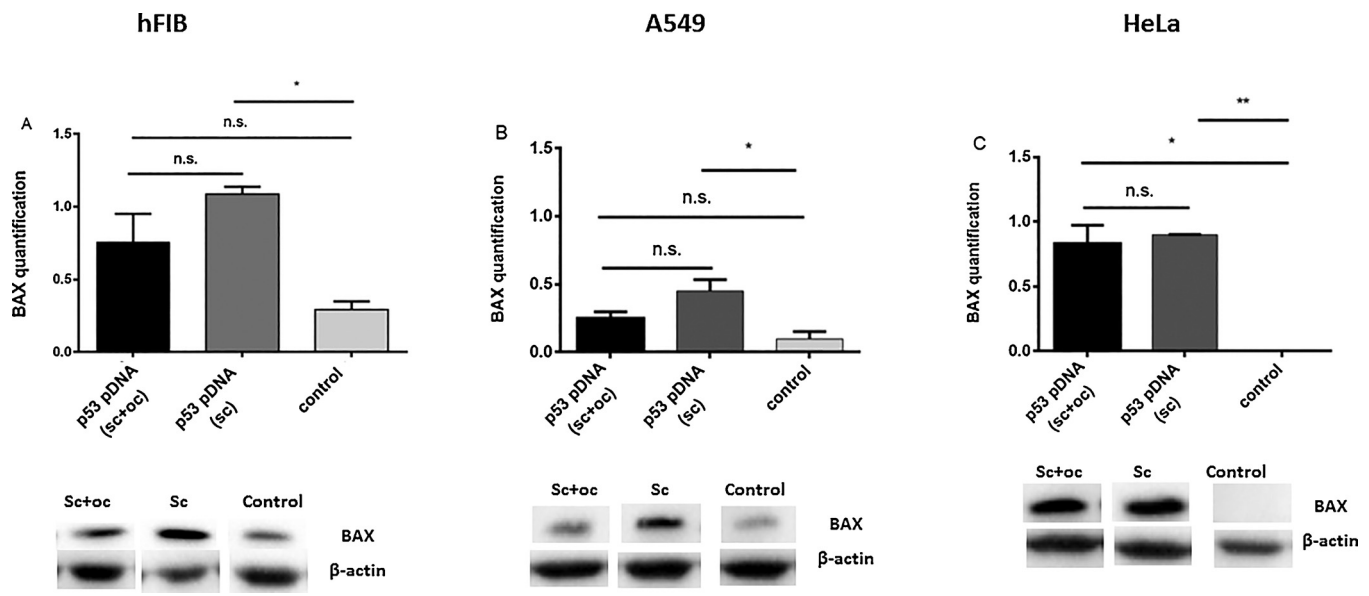


Fig. 11. Evaluation of Bax protein expression after 48 h treatment of different cell lines with p53-pDNA lipoplexes. (A) Human dermal fibroblasts; (B) A549 non-small lung cancer cells; (C) HeLa cervix carcinoma. Non-transfected cells were used as controls. Data are represented as mean ± S.D., n = 2.

pathways whereby p53 leads to execution of the apoptosis program are very complex, however, Bax was proven to be transcriptionally activated by p53 in human cell lines [6]. Also, an increase of Bax protein expression was found in several cell lines following p53 overexpression, and it has been confirmed that Bax can inhibit Bcl-2 activity, thus accelerating apoptosis [5,6]. Moreover, Lai et al. demonstrated that cell cycle arrest and apoptosis were two genetically separable functions of p53 because the up-regulation of Bax expression induced apoptosis but not cell cycle arrest, concluding that p53-mediates apoptosis is, at least in part, through the activation of Bax-dependent pathway [33].

Regarding this information, a study on the Bax levels of the different cells treated with the pDNA formulations was performed (Fig. 11).

Analyzing these results, it was possible to observe that HeLa cancer cells presented a higher Bax expression. In general, a slightly higher Bax expression was achieved for cells transfected with the sc pDNA, but no significant difference was achieved between cells transfected with the sc or a native (sc + oc) p53 encoding pDNA samples. This result could suggest that although the sc p53 encoding pDNA promoted a higher expression of the p53 protein in HeLa cancer cells, it could lose some effect along the apoptotic cascade. However, it is also noteworthy that the difference achieved between the sc or native pDNA is more pronounced in A549 cell line, which is the more apoptosis-resistant cell line. This can suggest that actually the quality of the pDNA biopharmaceuticals can have a great impact on more resistant cancer cells. These results are also in accordance with the results from Annexin V as well as with the results from the p53 protein expression studies. Overall, and comparing this information with other works, namely the findings of Lai and collaborators, it is possible to observe that HeLa are the cancer cells presenting a higher apoptotic behavior but was in A549, where the apoptotic effect promoted by the sc p53 encoding pDNA was more pronounced [33].

4. Conclusions

p53 is one of the most well-established tumor suppressor proteins and an overwhelming amount of data suggests that p53 inactivation is virtually necessary for tumor development and progression. Without p53 inactivation, oncogenic cells would inevitably undergo rapid cell cycle arrest and/or apoptosis. In roughly half of all tumors, p53 is mutated, which abolishes or greatly inhibits its normal cellular functions. In the remaining tumors, the activities of p53 are most likely inhibited by other means such as inactivation of downstream signaling or increased p53 degradation.

In this study, we took advantage of amino acid-nucleic acid biorecognition affinity chromatography to isolate sc pDNA vectors and evaluated their biological performance in different cell lines, using a commercially available liposomal delivery system. As demonstrated, the biological performance of pure sc p53 pDNA was higher than the native samples which could be seen by the reinstatement of p53 protein expression. Moreover, it was observed that transgene expression is highly dependent on cancer type and on the purity of the pDNA biopharmaceutical. These findings are important for raising awareness on the importance of the topoisoform of pDNA preparations in the development of more effective p53-based cancer therapies. Overall, it was found that the best results were achieved when using the sc pDNA for cells transfection and that the p53 expression was significantly higher in HeLa cancer cells, also followed by an higher apoptosis level, suggesting that p53-based transgene therapy may be particularly effective in this cancer type. In the future, it could be interesting to evaluate the performance of sc p53-pDNA in more cell lines and with different non-viral delivery systems so as to ascertain the realistic potential of this therapy.

Declarations of interest

None.

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