Article

Pro-Apoptotic and Immunotherapeutic Effects of Carbon Nanotubes Functionalized with Recombinant Human Surfactant Protein D on Leukemic Cells

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Abstract: Nanoparticles are efficient drug delivery vehicles for targeting specific organs as well as systemic therapy for a range of diseases, including cancer. However, their interaction with the immune system offers an intriguing challenge. Due to the unique physico-chemical properties, carbon nanotubes (CNTs) are considered as nanocarriers of considerable interest in cancer diagnosis and therapy. CNTs, as a promising nanomaterial, are capable of both detecting as well as delivering drugs or small therapeutic molecules to tumour cells. In this study, we coupled a recombinant fragment of human surfactant protein D (rfhSP-D) with carboxymethyl-cellulose (CMC) CNTs (CMC-CNT, 10–20 nm diameter) for augmenting their apoptotic and immunotherapeutic properties using two leukemic cell lines. The cell viability of AML14.3D10 or K562 cancer cell lines was reduced when cultured with CMC-mwCNT-coupled-rfhSP-D (CNT + rfhSP-D) at 24 h. Increased levels of caspase 3, 7 and cleaved caspase 9 in CNT + rfhSP-D treated AML14.3D10 and K562 cells suggested an involvement of an intrinsic pathway of apoptosis. CNT + rfhSP-D treated leukemic cells also showed higher mRNA expression of p53 and cell cycle inhibitors (p21 and p27). This suggested a likely reduction in cdc2-cyclin B1, causing G2/M cell cycle arrest and p53-dependent apoptosis in AML14.3D10 cells, while p53-independent mechanisms appeared to be in operation in K562 cells. We suggest that CNT + rfhSP-D has therapeutic potential in targeting leukemic cells, irrespective of their p53 status, and thus, it is worth setting up pre-clinical trials in animal models.

Keywords: carbon nanotubes; human SP-D; cancer cells; apoptosis; immunotherapy

1. Introduction

The innate immune system plays a key role in the clearance of pathogens and synthetic compounds including nanoparticles [1,2]. Nanoparticles have numerous biomedical applications [3–6], which can serve as drug delivery carriers or vaccine adjuvants [7]. Among nanoparticles, carbon nanotubes (CNTs) have unique physico-chemical properties, and hence, they are amenable as therapeutic nanocarriers [8–10]. CNTs can be single-walled (SWCNTs) and multiple-walled (MWCNTs), depending on length, diameter, and structure, and the layers of single CNT the wall is composed of [11].

Human surfactant protein D (SP-D) is a humoral, pattern recognition molecule, which is found to be associated with pulmonary surfactant, as well as mucosal surfaces outside the lungs [12,13]. SP-D belongs to the collectin family, a collagen containing C-type (calcium-dependent) lectin [14]. The primary structure of SP-D comprises a cross-linking amino-terminal region, a triple-helical collagen region, a coiled-coil neck region, and a C-type lectin domain or carbohydrate recognition domain (CRD) as a trimeric unit [15,16]. SP-D can bind to various carbohydrate and/or charge patterns on the surface of pathogens and become involved in clearing them by recruiting phagocytic
cells such as neutrophils and macrophages [15,16]. SP-D can also interact with a range of cancer cell lines (leukemic, lung, pancreatic, prostate, ovarian and breast). For example, a truncated form of recombinant human SP-D (rfhSP-D), composed of trimeric neck and C-type lectin domain, has been shown to interfere with tumour progression via apoptosis induction, invasion, and epithelial-to-mesenchymal transition [17–22]. These studies have thus suggested that SP-D has an immune surveillance role against tumors.

SP-D can associate with nanoparticles and modulate their uptake by macrophages [23,24]. SP-D can bind efficiently with oxidized (Ox) DWCNTs via their C-type lectin domain [2,25]. SP-D mediated enhancement of nanoparticle uptake by alveolar macrophages and dendritic cells in mice has been examined using polystyrene, carbon black and silica nanocarriers [23].

CNTs, when opsonized with rfhSP-D, can provoke a differential pro-inflammatory immune response [26]. Surface modifications of hydrophobic CNTs are used for their good dispersion via covalent or non-covalent surface coatings [27]. For instance, the dispersion of MWCNTs via oxidation (Ox-CNT), or with carboxymethyl-cellulose (CMC-CNT), has been reported [27]. Soluble complement components, such as factor H and C1q, opsonize functionalized CNTs, suggesting that key innate immune molecules can bind CNTs and alter inflammatory response [27].

This study was aimed at examining the ability of CNT + rfhSP-D to induce apoptosis using an eosinophilic cell line, AML14.3D10 [28], and a chronic myelogenous leukemia cell line, K562, to assess if CNT + rfhSP-D nanomaterials are worth testing in animal models.

2. Results

2.1. CNT + rfhSP-D Treatment Reduces Cell Viability of AML14.3D10 and K562 Leukemic Cell Lines

First, we analysed and confirmed the stable binding of purified rfhSP-D with CMC-MWCNTs, as evident from the SDS-PAGE (Figure 1). Supernatant after centrifugation was also loaded, which did not show presence of rfhSP-D. rfhSP-D (10 µg/mL), without the addition of CNT, was used as a positive control. The quantitative analysis of viability in treated (cells + CNT + rfhSP-D; 5, 10, and 20 µg/mL in serum-free RPMI medium; cells + CNT as control) leukemic cells was carried out using trypan blue (Figure 2) and MTT (Figure 3) assays at 24 h time point. Trypan blue exclusion assay revealed a significant reduction in the cell viability in CNT + rfhSP-D treated cell lines (AML14.3D10: ~48%; K562: ~56%) at 24 h in a dose-dependent manner (Figure 2). This was confirmed by the MTT assay: AML14.3D10 (~37%) and K562 (~55%) (Figure 3). As evident by the MTT assay, rfhSP-D (20 µg/mL) alone was also able to reduce cell viability in both AML14.3D10 (~51%) and K562 (~69%) cell lines.

2.2. Proliferation of AML14.3D10 and K562 Cell Lines Is Reduced following CNT + rfhSP-D Treatment

Experiments were carried out to determine whether CNT + rfhSP-D (20 µg/mL) affected AML14.3D10 and K562 cell proliferation (Figure 4). Mouse anti-Ki-67 antibody staining was used to measure the percentage proliferation. CNT + rfhSP-D treated AML14.3D10 cells showed ~29% cell proliferation compared to rfhSP-D alone (20 µg/mL) (~57%) (Figure 4). However, a higher percentage of cell proliferation was noted in CNT-treated AML14.3D10 cells (~88%). In the case of K562 cell line (Figure 4B), approximately ~34% cell proliferation was noticed following CNT + rfhSP-D treatment (compared to CNT alone; ~107% proliferative cells stained with Ki-67 antibody), suggesting that cells treated only with CNT continued to proliferate and grow unhindered. rfhSP-D alone (20 µg/mL) treatment was also capable of reducing proliferation of AML14.3D10 (~57%) and K562 (~63%) cells when compared to CNT alone. These data suggested that CNT + rfhSP-D was more effective in reducing cell proliferation of both AML14.3D10 and K562 cell lines, indicating its therapeutic potential against acute and chronic leukemic cell lines.
Figure 1. Purified rfhSP-D (10 µg/mL) or carboxymethyl cellulose-coated carbon nanotubes (rfh-SP-D-CNTs) coupled-rfhSP-D (10 µg/mL) samples were subjected to an SDS-PAGE (15% v/v). Lane 1: Supernatant (10 µL/well) taken after centrifugation of rfhSP-D-CNT. Lane 2: purified rfhSP-D. Lane 3: rfhSP-D-CNT. The original image is available as a Supplementary Material.

2.3. Apoptosis Induction by CNT + rfhSP-D in AML14.3D10 and K562 Cell Lines

The quantitative analysis of apoptosis induction by CNT + rfhSP-D was performed using flow cytometry. A significant proportion of AML14.3D10 or K562 (Figure 5) cells treated with CNT + rfhSP-D (20 µg/mL), or rfhSP-D (20 µg/mL) alone, resulted in increased apoptosis induction at 24 h, compared to CNT alone (untreated control). CNT + rfhSP-D was effective in inducing the maximum apoptosis at 24 h; AML14.3D10 (~71%) and K562 (~66%), when compared to CNT alone [AML14.3D10 (~12%) and K562 (~7%)]. rfhSP-D (20 µg/mL) alone was also able to reduce cell viability in both AML14.3D10 (~43%) (Figure 5) and K562 (~37%) cell lines (Figure 5; Supplementary Materials). This assay is based on the ability of annexin V/FITC to bind to phosphatidylserine (PS) on apoptosing cells. A higher percentage of PI positive AML14.3D10 compared to K562 cells appeared to suggest that these cells were late apoptotic/necrotic. Staurosporine (1 µM/mL), used as a positive control for triggering apoptosis, brought about ~72% apoptosis at 24 h.

2.4. Up-Regulation of Cell-Cycle Inhibitors by CNT + rfhSP-D Treatment

To further understand the mechanism of apoptosis induced by CNT + rfhSP-D in AML14.3D10 or K562 cells, we analysed the expression of cell cycle inhibitors by qRT-PCR. p21 was upregulated in CNT + rfhSP-D treated AML14.3D10 (log_{10} 5.7-fold) and K562 (log_{10} 2.7-fold) (Figure 6) [compared to CNT alone: AML14.3D10 (log_{10} 1.2-fold) and K562 (log_{10} 1-fold)]. p27 transcripts were also upregulated in CNT + rfhSP-D challenged AML14.3D10 (log_{10} 2.5-fold) and K562 (log_{10} 2-fold) cells. The level of upregulation was considerably higher compared to CNT or rfhSP-D alone that were negative and positive controls, respectively, suggesting that coating rfhSP-D on CNTs enhanced rfhSP-D potency for targeting tumors.
Figure 2. Cell viability following treatment with CNT + rfhSP-D-CNT in AML14.3D10 (A) and K562 (B) cell lines via trypan-blue-dye exclusion assay. Cells (0.1 \( \times 10^5 \)) were treated with CNT + rfhSP-D (5, 10, 20 \( \mu \)g/mL), rfhSP-D (20 \( \mu \)g/mL) or CNT alone (20 \( \mu \)g/mL) for 24 h at 37 °C. The data has been normalized with cells only as 100% of the cell viability. * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \) compared to CNT only group.

2.5. rfhSP-D Upregulates p53 Expression in AML14.3D10 Cell Line

p53, a transcription factor, regulates oncogenic responses including DNA damage, cell cycle arrest, and apoptosis. CNT + rfhSP-D or rfhSP-D alone treated AML14.3D10 cells showed increased transcript levels of p53 when compared to untreated cells. CNT + rfhSP-D treated cells showed log\(_{10}\) 8.2-fold increased mRNA levels, compared to rfhSP-D treated cells (approximately log\(_{10}\) 5.2-fold) (Figure 7). p53 transcripts were not measured in K562 cells as these cells do not express wild type p53. These data suggest that CNT + rfhSP-D treatment can induce apoptosis in these cell lines regardless of their p53 status.

2.6. Apoptosis Induction in AML14.3D10 and K562 Cells by rfhSP-D-CNT via Intrinsic Pathway

Since apoptosis can be initiated via intrinsic or extrinsic pathways, expression of caspases was examined in AML14.3D10 or K562 cell lines treated with CNT + rfhSP-D (20 \( \mu \)g/mL) or rfhSP-D alone (20 \( \mu \)g/mL), using a fluorogenic substrate to detect the activation of caspase 3 and 7 (Figure 8). Higher levels of caspase 3 and 7 were observed...
in CNT + rfhSP-D treated AML14.3D10 (Figure 8A) and K562 (Figure 8B) cells, when compared to rfhSP-D or CNT alone-treated cells. There was a time-dependent increase in caspase 3 and 7 activation, which peaked at 24 h. Cleaved caspase 9 level was observed in CNT + rfhSP-D (or rfhSP-D-treated) AML14.3D10 or K562 cells at 12h, reflecting an intrinsic pathway (Figure 9).

Figure 3. CNT + rfhSP-D treatment reduced viability of AML14.3D10 (A) and K562 (B) cells, as measured by MTT assay. The data have been normalized with cells only as 100% of the cell viability. Values are means ± SEM (n = 3) * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to CNT only group.
Figure 4. Anti-proliferative effects of CNT + rfhSP-D on AML14.3D10 (A) and K562 (B) cell lines. Values are means ± SD. ** p < 0.01, and *** p < 0.001 compared to CNT group only. The raw data are available as Supplementary Materials.
Figure 5. Flow cytometry analysis of apoptosis induction in AML14.3D10 (A) or K562 (B) cell lines treated with CNT + rfhSP-D. For Annexin V/FITC and DNA/PI staining, 12,000 cells were acquired and plotted. Values are means ± SEM (n = 3). ** p < 0.01 and *** p < 0.001 compared to CNT only group. The raw data are available in the Supplementary Materials.
Figure 6. CNT + rfhSP-D treatment causes the upregulation of p21 and p27 cell cycle inhibitors in AML14.3D10 (A) and K562 (B) cell lines. AML14.3D10 or K562 (0.4 × 10⁶) cells, treated with CNT + rfhSP-D (20 µg/mL) or rfhSP-D (20 µg/mL), plus untreated control (cells+ CNT) (20 µg/mL), were used for RNA extraction, cDNA synthesis and RT-qPCR, using 18S as an endogenous control. The RQ value was calculated using the formula: RQ = 2^{−ΔΔCt}. Values represent means ± SEM (n = 3). * p < 0.05 and ** p < 0.01 compared to CNT only group.
Figure 7. CNT + rfhSP-D treated AML14.3D10 cells show upregulation of the mRNA transcript levels of p53. AML14.3D10 (0.4 × 10⁶) cells were treated with CNT + rfhSP-D or rfhSP-D alone, along with an untreated control (cells + CNT) (20 μg/mL each). The RQ value was calculated using the formula: RQ = 2 − ΔΔCt. * p < 0.05 and ** p < 0.01 compared to CNT only group.

A

Figure 8. Activation of caspase 3/7 in AML14.3D10 (A) or K562 (B) cell lines following CNT + rfhSP-D treatment. AML14.3D10 or K562 cells (0.1 × 10⁵) were seeded and challenged with CNT + rfhSP-D (20 μg/mL) or rfhSP-D (20 μg/mL). Cells + CNT was used as an untreated control. **** p < 0.0001 versus control group (n = 3).
3. Discussion

The involvement of innate immune mechanisms in cancer progression and resistance has opened up opportunities for using innate immune molecules as a part of anti-tumour therapeutic strategies. Immune system, innate as well as adaptive, is a double-edged sword that can either foster tumour progression via immunosuppression, angiogenesis, and metastasis, or resist oncogenesis [29,30]. SP-D, especially the trimeric CRDs in its recombinant form (rfhSP-D), has recently been shown to be protective against a range of cancer, based on in vitro studies. Coupling rfhSP-D with nanoparticles triggers a differential immune response [26]. rfhSP-D-bound CNTs upregulate the pro-inflammatory response (IL-1β, TNF-α, IL-6 and IL-12) in U937 and THP-1 cells [26]. Here, we examined the ability of CNT + rfhSP-D to act as a potent inducer of apoptosis in leukemic AML14.3D10 or K562 cell lines. CNT + rfhSP-D treatment reduced the cell viability of AML14.3D10 and K562 cells and induced apoptosis at 24 h in a dose- and time-dependent manner, peaking at 24 h and 20 µg/mL. A significant reduction in viability was observed in

Figure 9. CNT + rfhSP-D treatment upregulates the levels of cleaved caspase 9 on AML14.3D10 (A) or K562 (B) cell lines at 24 h. AML14.3D10 or K562 cells (0.4 × 10⁶) were treated with rfhSP-CNT or rfhSP-D, along with an untreated control (cells + CNT). Values are expressed as mean ± SD (n = 3). ** p < 0.01, and *** p < 0.001 versus control group.
CNT + rfhSP-D treated AML14.3D10 (~37%) and K562 (~55%) cells compared to untreated control (cells + CNT), based on trypan blue and MTT assays.

FACS analysis revealed a significant increase in the percentage of Annexin V-/PI-positive leukemic cells following CNT + rfhSP-D treatment, characterized by the disruption of the asymmetric arrangement of the membrane, and appearance of PS on the outer side of the cell membrane in the cells undergoing apoptosis [31]. Annexin V, a 36 kDa protein, can bind PS, and also enter the entire plasma membrane in necrotic cells. CNT + rfhSP-D triggered the maximum apoptosis at 24 h [AML14.3D10 (~71%) and K562 (~66%)], when compared to CNT alone [AML14.3D10 (~12%) and K562 (~7%)]. However, no significant difference in terms of cell viability reduction/apoptosis induction was noticed following rfhSP-D treatment at 48 h in AML14.3D10 and K562 cells, suggesting recovery of the cells after 24 h. Apoptosis induction in AML14.3D10 and K562 cell lines by CNT + rfhSP-D may occur through the intrinsic pathway, supported by increased levels of caspase 3, 7 and cleaved caspase 9. This validates earlier studies on AML14.3D10, prostate and breast cancer cells [20,21,32], and the involvement of a mitochondrial pathway [20,21,32].

We also tried to understand the underlying mechanism of apoptosis induction by CNT + rfhSP-D and the associated signaling pathways. CNT + rfhSP-D caused increased transcript level of p53 in AML14.3D10 cell line, probably due to oxidative stress [17,33]. The upregulation of p53 in CNT + rfhSP-D treated AML14.3D10 cells may downregulate pAkt pathway, increasing Bad and Bax, which in turn, causes the release of the cytochrome c, and caspase 9 cleavage. In addition, the increased expression of p53 and cell cycle inhibitors (p21/p27) can cause inactivation of the cyclin B–cdc2 complex, crucial for G2/M cell cycle transition [17]. The existence of a lack of p53 wild type gene in K562 cell line, and its increased susceptibility to CNT + rfhSP-D, the protective effects of rfhSP-D bound to CNTs seem p53 independent. An involvement of cellular receptors expressed by these cancer cell lines is of paramount importance. SP-D interaction with HMGA1, CD14, CD91-calreticulin complex, SIRPα, EGFR, and GRP78 has been reported [20–22,33,34]. The presence of rfhSP-D on CNT as an array of therapeutic molecule is likely to have a clustering effect on these putative receptors, enhancing the potency of rfhSP-D.

In conclusion, CNT + rfhSP-D nanomaterial seems to be an attractive and novel therapeutic approach for targeting intracellular signaling cascades. There is a clear therapeutic potential of rfhSP-D against tumour cells. The advantage here is that the enhanced glycosylation of oncogenic targets can evade natural or therapeutic antibodies. Having established the specific nature of interactions between CNT + rfhSP-D and receptors found on leukemic cancer cells, we can hope to investigate host response in the murine models of cancer using wild type and SP-D knock-out mice.

4. Materials and Methods

4.1. Cell Culture

AML14.3D10 and K562 cells (ATCC) were cultured in RMPI media containing 10% v/v fetal calf serum (FCS), 2 mM L-glutamine, and penicillin (100 U/mL)/streptomycin (100 µg/mL) (ThermoFisher Scientific, Oxford, UK). Cells were grown at 37 °C under 5% v/v CO₂ until 80–90% confluency was reached.

4.2. Dispersion and Functionalization of CNTs

The CNTs used in this study were characterized and functionalized as previously described [26,27]. Briefly, CNTs (diameters 10–20 nm, length 5–20 µm; Array Nano) were dispersed using CNT sulfuric acid/nitric acid (3:1 ratio) via sonication and functionalized using CMC (Sigma-Aldrich/Merck, Dorset, UK) in a 1:2 mass ratio [26,27].

4.3. Expression and Purification of rfhSP-D

A recombinant fragment of human SP-D (rfhSP-D) was expressed and purified as described previously [17,32]. Affinity purified rfhSP-D was then subjected to endotoxin
level measurement using QCL-1000 Limulus amebocyte lysate system (Lonza, Slough, UK); the endotoxin levels were found to be ~5 pg/µg of rhfSP-D.

### 4.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The binding of rhfSP-D to CMC-CNTs was assessed via SDS-PAGE (12% v/v). CNT + rhfSP-D samples were boiled in a treatment buffer containing SDS and β-mercaptoethanol at 95 °C for 10 min before loading on to the gel. The SDS-PAGE gel was stained for 2 h using brilliant blue stain containing methanol (50% v/v) and acetic acid (10% v/v). This followed submersion of the stained gel with gentle shaking with de-staining solution (staining solution without brilliant blue).

### 4.5. Trypan-Blue-Dye Exclusion Assay

AML14.3D10 or K562 cells (0.1 × 10^6) were seeded in a 12-well plate in complete RPMI complete medium overnight under 5% CO2 at 37 °C. Next, the cells were washed with PBS and treated with CNT + rhfSP-D (5, 10 or 20 µg/mL), or rhfSP-D alone (20 µg/mL), in serum-free RPMI for 24 h. Cells + CNT and Staurosporine (1 µM/mL) were used as an untreated/negative and positive control, respectively. Cells were then washed, detached using 5 mM EDTA, and centrifuged (1200 × g). The cell pellet, re-suspended in RPMI, was treated with Trypan blue (10 µL) (60%), and viable cells were counted using hemocytometer in 5 different optical fields with a threshold value of 200 cells per field.

### 4.6. MTT Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, Dorset, UK) assay was performed to assess the cell metabolic activity (cells + CNT + rhfSP-D; cells + CNT). AML14.3D10 or K562 cells (0.1 × 10^6) were seeded in 96-well plates in RPMI complete medium until 85% confluency, and treated with CNT + rhfSP-D (5, 10 or 20 µg/mL), or rhfSP-D (20 µg/mL), in serum free RPMI medium for 24 h. MTT (50 µg/µL) per well was added for 4 h at 37 °C. 25 µL medium per well was then mixed with 50 µL DMSO (10', 37 °C), and the absorbance was read at 570 nm using an ELISA plate reader.

### 4.7. Flow Cytometry

For apoptosis assays, AML14.3D10 or K562 cells (0.4 × 10^6) were seeded in culture petri dishes (Nunc) in complete RPMI medium for 24 h and treated with CNT + rhfSP-D (20 µg/mL), or rhfSP-D (20 µg/mL), in serum-free RPMI medium for 24 h. Other controls were used as described above. Detached, centrifuged and PBS washed cells were incubated with Alexa Fluor 488 (1:200) (Sigma-Aldrich/Merck, Dorset, UK) (15', RT) in dark, and the extent of apoptosis was measured using Novocyte Flow Cytometer. Compensation parameters were acquired using unstained, untreated FITC stained, and untreated PI-stained samples for all the cell lines.

For proliferative studies, AML14.3D10 or K562 cells (0.4 × 10^6) were washed with PBS, probed with anti-mouse Ki-67 (BioLegend, San Diego, CA, USA) diluted in permeabilization reagent of the FIX&PERM kit (Fisher Scientific), and incubated for 30 min at room temperature (RT). Goat anti-mouse-FITC conjugate (1:200) (Fisher Scientific) was used as a probe at RT in the dark for 30 min. Cells (12,000) were acquired for each experiment and compensated before plotting the acquired data.

For caspase 9 and 8 studies, AML14.3D10 or K562 cells (0.4 × 10^6) were treated with CNT + rhfSP-D or rhfSP-D (cells + CNT as a control) for 24 h at 37 °C, and probed with rabbit anti-human cleaved caspase 9 or 8 (Cell Signaling Technology, Danvers, MA, USA) (1:200) for 1 h at RT. Cells were washed in PBS, incubated with Alexa Fluor 488 (1:200) (Sigma-Aldrich) at RT in dark for 30 min, acquired and compensated (12,000) prior to plotting the data.
4.8. Caspase-3/7 Analysis

AML14.3D10 or K562 cells (0.1 × 10^5) were seeded in 96 well plates in RPMI complete medium until 80% confluency. The cells were then treated with CNTs, as described above, in serum-free RPMI medium containing CellEvent™ Caspase-3/7 Green Detection Reagent (5 µM; Thermo-Fisher) (0, 10, 20, 30 or 40 h). Cells + CNT was used as an untreated/negative control. CellEvent™ Caspase-3/7 Green Detection Reagent is a fluorogenic substrate for activated caspases 3 and 7 in cells undergoing apoptosis. The plates with treated and untreated samples were incubated at 37 °C with 5% CO2 to detect the levels of Caspase 3/7 using a Clariostar plus microplate reader (BMG Labtech, Cary, NC, USA).

4.9. Quantitative RT-PCR

AML14.3D10 or K562 cells (0.5 × 10^6) were incubated with CNT + rfhSP-D (20 µg/mL) or rfhSP-D (20 µg/m in serum-free RPMI medium for 18 h and RNA was isolated using GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich) and treated with DNase I. 2 µg of total RNA was used for cDNA synthesis using High Capacity kit (Applied Biosystems/ThermoFisher, Abingdon, UK). Primer sequences were designed using Primer-BLAST software (Table 1). Each PCR reaction, carried out in triplicates, contained SYBR Green (5 µL) MasterMix (Applied Biosystems), primers (75 nM), and cDNA (500 ng) (7900HT; Applied Biosystems). The cycle involved 2′/50 °C and 10′/95 °C, and 40 cycles (15 s/95 °C; 1′/60 °C). Human 18S rRNA was used as a housekeeping gene control. Relative quantification (RQ) value and formula: RQ = 2 − ∆∆Ct were used to calculate the relative expression of each target. Cells + CNT was used as an untreated/negative control.

Table 1. Target genes and terminal primers used in the RT-qPCR analysis.

| Target Gene | Forward Primer | Reverse Primer |
|-------------|----------------|----------------|
| 18S | 5′-ATGGCCGTTCTTAGTTGGTG-3′ | 5′-CGCTGAGCCAGTCAGTGAG-3′ |
| P53 | 5′-AGCACTGTCCAACAAACCA-3′ | 5′-CTTCAGGTGGCTGGAGTGAG-3′ |
| p21 | 5′-TGGAGACTCTCAGGGTCGAAA-3′ | 5′-CGGCCATTGGAGTGTGGAG-3′ |
| p27 | 5′-CCGTTGACCACAAGAGT-3′ | 5′-GCTCGCTCTTCCATGTCTC-3′ |

4.10. Statistical Analysis

The graphs were generated using the GraphPad Prism 6.0 software. A one-way ANOVA test was carried out for statistical significance analysis. values less than 0.05 were considered as statistically significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms221910445/s1.

Author Contributions: Conceptualization, H.A.K., U.K.; Data curation, U.K. and H.M.A.; Formal analysis, U.K. and S.H.A.; Funding acquisition, H.A.K.; Investigation, H.A.K., U.K. and S.H.A.; Methodology, U.K., H.M.A. and S.H.A.; Project administration, H.A.K.; Resources, H.A.K., U.K.; Software, U.K., H.M.A.; Writing—review & editing, H.A.K. and U.K. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award Number (15-NAN-3664-02).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.
Acknowledgments: Technical assistance of Valarmathy Murugaiah, Khalid Ibrahim, Ashraf Hatamihal, and Adnan Khan is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

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