# Intracellular delivery of NF-κB small interfering RNA for modulating therapeutic activities of classical anti-cancer drugs in human cervical cancer cells

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## Abstract

Cervical cancer is the second most common cancer and fourth leading cause of cancer-related deaths among women. Advanced stage of the disease is treated with radiation therapy and chemotherapy with poor therapeutic outcome and adverse side effects. NFκB, a well-known transcription factor in the control of immunity and inflammation, has recently emerged as a key regulator of cell survival through induction of antiapoptotic genes. Many human cancers, including cervical carcinoma, constitutively express NF-κB and a blockade in expression of its subunit proteins through targeted knockdown of the gene transcripts with small interfering RNAs (siRNA) could be an attractive approach in order to sensitize the cancer cells towards the widely used anti-cancer drugs. However, the inefficiency of the naked siRNA to cross the plasma membrane and its sensitiveness to nuclease-mediated degradation are the major challenges limiting the siRNA technology in therapeutic intervention. pH-sensitive carbonate apatite has been established as an efficient nano-carrier for intracellular delivery of siRNA, due to its strong electrostatic interaction with the siRNA, the desirable size distribution of the resulting siRNA complex for effective endocytosis and the ability of the endocytosed siRNA to be released from the degradable particles and escape the endosomes, thus leading to the effective knockdown of the target gene of cyclin B1 or ABCB1. Here, we report that carbonate apatite-facilitated delivery of the siRNA targeting NF-κB1 and NF-κB2 gene transcripts in HeLa, a human cervical adenocarcinoma cell line expressing NF-κB, led to a synergistic effect in enhancement of chemosensitivity to doxorubicin, but apparently not to cisplatin or paclitaxel.

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## Introduction

Cervical cancer represents the second most common cancer among women worldwide with approximately 250,000 annual deaths according to the report of the World Health Organization, making it the fourth leading cause of cancer death among women. More than 96% of cervical cancers are positive for high-risk human papillomaviruses (HPVs) and the persistent viral infection is involved in the development of invasive carcinoma.[1-5] The E6 and E7 oncoproteins of HPVs inactivate major tumor suppressors, p53 and retinoblastoma protein (pRb) and also induce genomic instability resulting in transformation of normal cervical cells into malignancy.[6-9] Early-stage tumor is successfully treated with surgery or radiation whereas the standard protocol for the advanced stages relies on combination of radiation therapy and chemotherapy with the platinum-based chemotherapy considered to be preferable.[10,11]

HPV genome is usually found to be integrated into the chromosomes of cervical cancer cells leading to high-level expression of E6 and E7.[12] E6 induces expression of human telomerase reverse transcriptase (hTERT), the rate-limiting component of telomerase activity involved in suppressing senescence in specific germ-line cells, proliferating stem cells and cancer cells.[13] By interacting with E6 associated protein (E6AP), E6 promotes degradation of p53, thereby preventing growth arrest and apoptosis of the cancer cells following moderate and severe DNA damage, respectively.[13] In addition, E6 inhibits apoptosis by interfering with other pro-apoptotic proteins, such as Bak, FADD and procaspase [8,14,15] On the other hand, E7 binds and inactivates pRb and its family members, p107 and p130, leading to constitutive activation of E2F and consequential expression of a number of genes involved in DNA replication in S phase.[16] E7 can induce Akt activation through sequestering of PP2A phosphatase, thus maintaining its signalling for cell proliferation by blocking its dephosphorylation.[17] Moreover, E7 has been shown to target the promyelocytic leukemia (PML) protein and circumvents cellular senescence involving pRb and p53 tumor suppressor pathways.[18] Both E6 and E7 can independently induce genome instability via centrosome over-duplication,[19] polyploidy formation or chromosomal rearrangements (translocations, additions and deletions),[20,21] resulting in altered expression of various genes.

NFκB, a well-known transcription factor in the control of immunity and inflammation has recently emerged as an important player in the regulation of cell survival through induction of the antiapoptotic genes.[22-24] NF-κB1 and NF-κB2 are first synthesized as large precursors and subsequently processed as the mature NF-κB subunits, p50 and p52, respectively. In cytoplasm, the Rel/NFκB protein complex remains inactive in association with its inhibitory subunit, IκB and upon stimulation of specific membrane receptors or cytotoxic drugs, is activated following proteasomal degradation of the phosphorylated form of IκB and translocate to the nucleus to induce transcription of many target genes.[25-27] Many human cancers including cervical carcinoma constitutively express NF-κB and blockade of its activation results in enhanced sensitivity towards the proapoptotic effect of TNF, drugs and radiation.[28-30] Thus, targeted knockdown of the gene transcripts for NF-κB subunits through intracellular delivery of specific siRNAs (small interfering RNAs) could be a highly promising treatment option in order to sensitize the cancer cells towards the classical chemotherapy drugs.[31,32] Since anti-cancer drugs induce apoptosis of cancer cells usually by interfering with cellular metabolism or cell cycle, selective knockdown of the genes for the enzymes directly or indirectly

involved in cell proliferation or survival, could not only slow down the growth of cancer cells, but also make them more vulnerable to the cytotoxic drugs. siRNA, being unstable and anionic in nature, is subjected to condensation with a cationic vector prior to intracellular delivery. The existing barriers in proper condensation, cellular uptake and endosomal escape of the siRNA significantly influence the overall silencing potency of the delivered siRNA. We have recently established pH-sensitive carbonate apatite as a powerful tool to efficiently deliver siRNA across the cell membrane and silence the gene expression of cyclin B1 and ABCG2/ABCB1 in order for inducing apoptosis of cervical and breast cancer cells, respectively, in combination with conventional anticancer drugs.[33,34] Here, we report that carbonate apatite-mediated delivery of the siRNA targeting NF-κB1 and NF-κB2 gene transcripts in HeLa, a human cervical adenocarcinoma cell line known to express NF-κB led to a significant increase in chemosensitivity to doxorubicin while no such synergistic effect was so evident for cisplatin or paclitaxel.[30,35]

## Materials and Methods

### Reagents

Dulbecco's modified Eagle medium (DMEM) was purchased from BioWhittaker (Walkersville, USA). DMEM powder, foetal bovine serum (FBS) and trypsin-ethylenediamine tetraacetate (trypsin-EDTA) were obtained from Gibco BRL (California, USA). Calcium chloride dehydrate ($CaCl_2.2H_2O$), sodium bicarbonate, dimethyl sulphoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich (St Louis, MO, USA). The chemotherapy drugs, doxorubicin, paclitaxel and cis-diammineplatinum (II) dichloride are from Sigma Aldrich (St. Louis, USA). Both doxorubicin and cis-diammineplatinum (II) dichloride were dissolved in distilled water and 2 mM stock solutions were prepared whereas paclitaxel was dissolved in DMSO and 10 mM stock solution was prepared.

### siRNA design and sequence

The validated anti-NF-κB1 and anti-NF-κB2 siRNAs were purchased from QIAGEN (California, USA) with the target sequence of 5'-GACGCCATCTATGACAGTAAA-3' and 5'-AACCCAGGTCTGGATGGTATT-3', respectively. siRNA was received in lyophilised form and subsequently reconstituted with RNase-free water to obtain a stock solution of 20 μM. The siRNA solution was allocated into multiple reaction tubes (as repeated thawing might affect the silencing efficiency of siRNAs),

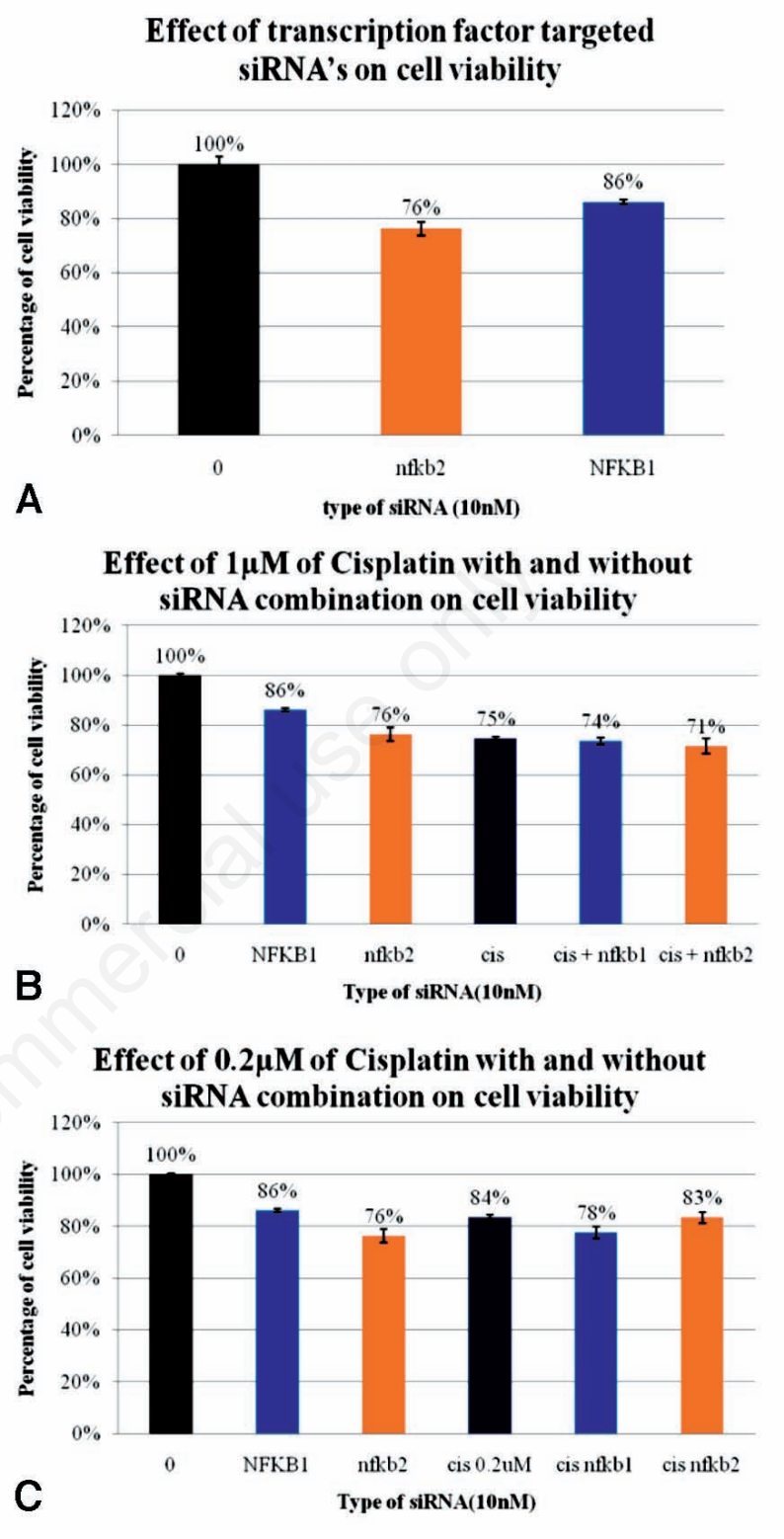


**Figure 1. Effects of silencing of NF-kB 1 and NF-kB 2 expression on cancer cell viability (A) and on viability of cancer cells under higher (B) and lower (C) dose of cisplatin. 50,000 of HeLa cells from the exponentially growth phase were seeded in each of the wells of a 24-well plates the day before the siRNA/apatite complexes were prepared by mixing 3 L of 1 M $CaCl_2$ with 10 nM of siRNA in 1 mL of fresh serum-free $HCO_3$- (44 mM)-buffered DMEM medium (pH 7.5) and incubating at 37°C for 30 min. The medium containing the siRNA/apatite complexes and supplemented with 10% FBS was added onto the rinsed cells either with or without 1 μM of cisplatin and the cells were cultured for a consecutive period of 48 h prior to the assessment of cell viability based on MTT assay. Each experiment was done in triplicate and the data represent mean value ± SE (n=3).**

before being stored at -20°C as recommended by Qiagen.

### Cell culture and seeding

HeLa cells were grown in 25 $cm^2$ culture flask in DMEM supplemented with 10% heat-inactivated FBS in a humidified atmosphere containing 5% $CO_2$ at 37°C. The exponentially growing cells were trypsinised and centrifuged at 10,000 rpm for 5 min. Fresh medium was added to resuspend the pellet, followed by counting of the cells with a hemocytometer. Appropriate dilutions were made using culture medium to produce a cell suspension with the concentration being $5.0\times10^4$ cells/mL. One mL of the prepared cell suspension was subsequently added into each of the wells of a 24-well plate and allowed to attach and grow for overnight at 37°C and 5% $CO_2$ prior to siRNA transfection.

### Generation of target siRNA/carbonate apatite complexes and transfection of HeLa cells

On the day of siRNA transfection, 100 mL of DMEM was prepared using 1.35 g of DMEM powder and 0.37 g of sodium bicarbonate, followed by pH adjustment to 7.4 using 0.1 M hydrochloric acid. The prepared DMEM solution was filtered across the 0.2 μm syringe filter and 1 mL of the medium was transferred into 1.5 mL microcentrifuge tubes. Three μL of 1 M $CaCl_2$ was mixed with 10 nM of siRNA in 1 mL of fresh serum-free $HCO_3^-$ (44 mM)-buffered DMEM medium (pH 7.5), followed by incubation at 37°C for 30 min for complete generation of siRNA/carbonate apatite particles. Ten percent FBS and (depending on the experimental conditions) 0.2 to 1 μM drugs (cisplatin, doxorubicin, paclitaxel) were mixed with the medium containing the siRNA/apatite complexes and the medium was added onto the rinsed cells. The cells were cultured for a consecutive period of 48 h prior to the assessment for cell viability.

### Cell viability assessment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Following incubation of HeLa cells with the siRNA/apatite complexes either in presence or absence of the cancer drugs, the fraction of viable HeLa cells was determined using MTT assay. Briefly, 30 μL from a MTT stock solution (5 mg/mL in PBS) was added into each of the wells, followed by incubation at 37°C and 5% $CO_2$ for 4 h. Medium containing MTT was then aspirated and the purple formazan crystals at the bottom of each well were dissolved by pipetting with 300 μL of DMSO solution. Absorbance of the resulting formazan solution was determined at wavelength 595 nm using microplate reader (Dynex Opsys MR, US) with reference to 630 nm. Each experiment was performed in triplicates and the data were plotted as mean ± standard deviation (S.D.) of three independent experiments.

## Results and Discussion

### Roles of NF-kB 1 and NF-kB 2 in the proliferation/survival of cervical cancer cells

In order to investigate the potential roles of NF-kB 1 and NF-kB 2 in the proliferation or survival of HeLa cells that express both of the protein subunits,[36] specific validated siRNA (10 mM) against NF-kB 1 or NF-kB 2 mRNA was added together with $Ca^{2+}$ (3 mM) to the bicarbonate-buffered DMEM prior to the incubation at 37°C for 30 min to form carbonate apatite/siRNA complexes. Figure 1A shows the cell viability as assessed by MTT assay following consecutive 2 day incubation of HeLa cells with the apatite complexes carrying either anti-NF-kB 1 or anti-NF-kB 2 siRNA. The MTT result shows that NF-kB 2 siRNA is more effective than NF-kB 1 siRNA, as it killed 24% of the cells in comparison to 14% by NF-kB 1 siRNA.

While both of the siRNAs were validated by the manufacturer (QIAGEN) using quantitative RT-PCR to confirm their knockdown efficiency of 79% and 84% for NF-κB1 and NF-κB2, respectively, the relatively low efficacy of either treatment in killing cancer cells as compared to the free particles (positive control) was possibly due to the constitutive expression of the genes in spite of the cleavage of substantial amount of the respective mRNAs and the active roles being played by MAP kinase and PI-3 kinase pathways in cell survival or proliferation through other downstream signaling molecules. The higher knockdown efficacy of anti-NF-κB2 siRNA than that of anti-NF-κB1 siRNA could account for its more pronounced cytotoxic effect than the latter.

### Influences of NF-kB 1 and NF-kB 2 gene knockdown on cisplatin-induced cell toxicity

Cisplatin is one of the most effective anticancer drugs for solid tumors, including ovarian, testicular, cervical, and small cell lung cancers.[37,38] Treatment of HeLa cells with apatite/siRNA complexes in presence of 1 μM of cisplatin for 2 consecutive days led to 26% and 29% of the total cells killed for NF-κB1 and NF-κB2 siRNAs, respectively while 25% of the cells were killed with cisplatin alone compared with the particles only (Figure 1B), suggesting an additive effect on cell death probably owing to the lack of any cross-talk existing between the pathways of NF-κB signaling and cisplatin-induced toxicity.

On the contrary, the combined treatment with apatite/siRNA complex and a lower dose of cisplatin (200 nM) resulted in a slight decrease in cell viability only for NF-κB1 siRNA, but not for NF-κB2 siRNA compared with the respective apatite/siRNA complex or

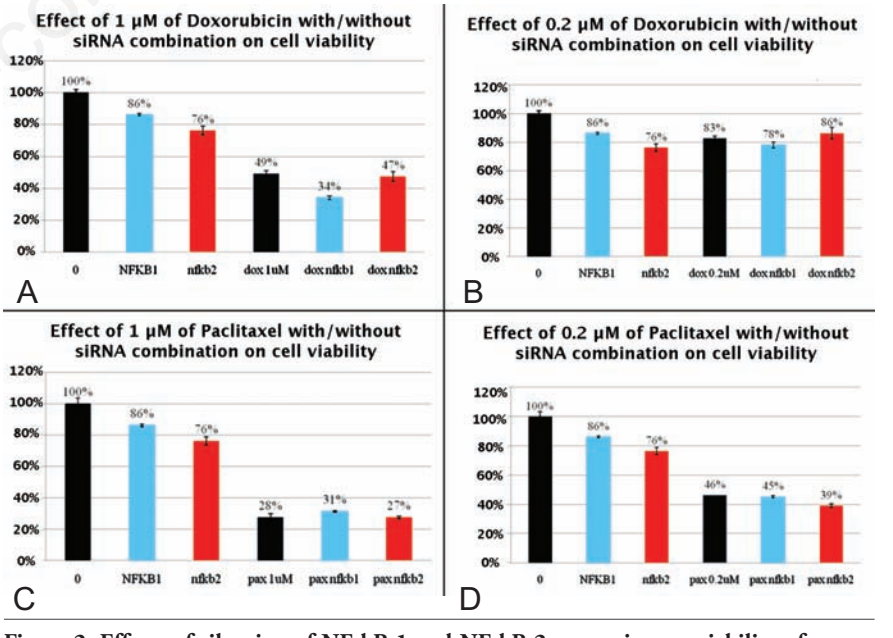


**Figure 2. Effects of silencing of NF-kB 1 and NF-kB 2 expression on viability of cancer cells under A) higher dose of doxorubicin; B) lower dose of doxorubicin; C) higher dose of paclitaxel, D) lower dose of paclitaxel. Parameters are the same as the experiments in Figure 1. Y Axis: percentage of cell viability. X Axis: type of SiRNA (10 nm).**

cisplatin (Figure 1C), indicating that apatite/NF-κB1 siRNA complex and cisplatin at that particular dose individually exert their cytotoxic effects without involvement of any cross-communicating signaling loop between them.

### Influences of NF-kB1 and NF-kB2 gene knockdown on doxorubicin-induced cell toxicity

Doxorubicin, a chemotherapy drug widely used for the treatment of breast cancer and other malignancies,[39,40] is a topoisomerase II inhibitor and produces DNA damage, thereby signaling for NFkB activation and inducing NF-kB-driven genes which are involved in cell proliferation and survival.[41,42] As shown in Figure 2A, silencing of NF-kB1 gene transcript following intracellular delivery of apatite/siRNA in presence of doxorubicin clearly sensitized the cells to doxorubicin at that particular concentration (1 μM) killing more than 66% of the cells due to the apparent synergistic effect of the drug and the gene knockdown. This could be due to the blockage of expression of the anti-apoptotic genes following knockdown of NFkB1 and as a result, cytotoxic effect of doxorubicin was significantly enhanced.[22-24] Silencing of NFkB2 gene transcript, however, did not show any significant increase in the therapeutic effect of doxorubicin as the number of cells killed was similar to that affected by doxorubicin.

When the dose of doxorubicin was reduced to 200 nM, only 17% of the cells were killed in presence of the drug alone (Figure 2B) compared to 51% of cell death induced by 1 μM of the drug (Figure 2A). As a result, apatite-mediated intracellular delivery of either anti-NF-κB1 or anti-NF-κB2 siRNA in presence of the drug was not accompanied by any noticeable increase in cytotoxicity compared with the drug alone.

Since doxorubicin as a topoisomerase inhibitor could activate NFkB, a potent inducer of anti-apoptotic genes by producing single- or double-stranded DNA breaks and thereby cause the cancer cells to resist the drug therapy, suppression of NFkB activation by siRNA-induced silencing would be an attractive approach to further investigate in other cancer cell lines as well as in cancer models for enhancement of the chemotherapeutic outcome.[39-42]

### Influences of NF-kB1 and NF-kB2 gene knockdown on paclitaxel-induced cell toxicity

Paclitaxel as a microtubule stabilizer is used for the treatment for various cancers including cervical cancer in combination with cisplatin and other cancer drug(s).[43,44] Curcumin has been reported to enhance paclitaxel-induced cell toxicity through downregulation of NF-kB and Akt pathways.[45,46]

As shown in Figure 2C, 1 μM paclitaxel when incubated with HeLa cells continuously for 2 days, killed more cells (over 70%) than cisplatin (Figure 1B) or doxorubicin (Figure 2A) and consequently, nanoparticle assisted-delivery of either NF-kB1 or NF-kB2 siRNA in presence of paclitaxel (1 μM) demonstrated no additional effect on cell death compared with free paclitaxel, indicating the dominant role of paclitaxel in induction of massive cell death under the experimental condition (Figure 2C).

On the other hand, when the concentration of paclitaxel was lowered to 200 nM, silencing of NF-kB1 or NF-kB2 gene expression in presence of the drug was associated with a statistically significant increase in toxicity only for NF-kB2 siRNA with an average cytotoxicity of 61%, compared with the drug alone which killed an average of 54% of the total cells, indicating the potential cross-talk between the pathway of NF-kB pathway and that of paclitaxel-mediated cell toxicity (Figure 2D). The lack of any such effect with anti-NF-kB1 siRNA could be due to its lower gene silencing efficacy than anti-NF-kB2 siRNA as noted earlier in *Materials and Methods* section.

## Conclusions

NF-kB protein complex is a potential target for gene knockdown in the chemotherapy of cervical cancer based on the classical anti-cancer drugs. Therefore, pre-clinical study in animal models of cervical cancer should be carried out through tumor-specific delivery of the siRNAs targeting the subunits of NF-kB in combination with passively diffusible anti-cancer drugs.

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