Fabrication and intracellular delivery of siRNA/carbonate apatite nano-composites for effective knockdown of cyclin B1 gene

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Abstract

Gene therapy through intracellular delivery of a functional gene or a gene-silencing element is a promising approach to properly treat critical human diseases like cancer. The ability of synthetically designed small interfering RNA (siRNA) to effectively silence genes at post-transcriptional level has made them attractive options in targeted therapeutics. However, naked siRNA being unable to passively diffuse through cellular membranes, poses difficulty in fully exploiting the potential of the technology. pH-sensitive carbonate apatite has been developed as an efficient tool to deliver siRNA into the mammalian cells by virtue of its high affinity interaction with the siRNA and effective cellular endocytosis. Moreover, internalized siRNA has been found to escape from the endosomes in a time-dependent manner and effectively silenced reporter gene expression. Knockdown of cyclin B1 gene with only 10 nM of siRNA delivered by carbonate apatite has resulted in significant death of cervical cancer cells. Moreover, delivery of siRNA against cyclin B1 gene has led to the sensitization of the cancer cells to both cisplatin and doxorubicin at a particulat drug concentration. Thus, the new method of siRNA delivery is highly promising for pre-clinical and clinical cancer therapy using siRNA therapeutics.

Introduction

Genes encode proteins through messenger RNA (mRNA) to carry out the major functions of a biological system and a disorder either acquired or genetic is usually associated with the suppression or the overexpression of certain genes. Regulation of the gene expression particularly through the delivery of exogenous gene(s) or gene-silencing element(s) could assist in restoring the regular physiological functions for treatment of a genetic or an acquired disease. RNA interference (RNAi) being one of the mechanisms to selectively cleave mRNA transcripts can be harnessed to rapidly develop novel drugs against target genes.[1-6] There are two basic ways of implementing RNAi for selective inhibition of gene expression: i) cytoplasmic delivery of short interfering RNA (siRNA) and ii) nuclear delivery of a short hairpin RNA (shRNA)-expressing plasmid.[7] Silencing by synthetic siRNA, an RNA duplex of 21-23 nucleotides, is more advantageous than shRNA partly due to the difficulty in constructing shRNA expression plasmid prior to the selection and verification of the active sequences[7] and the requirement of the plasmid to cross the nuclear membrane for shRNA expression.[8] The ability of siRNA to potently, but reversibly, silence genes *in vivo* has made them a highly promising drug therapeutic with several different clinical trials ongoing and more poised to enter the clinic.[2,5] However, because of the strong anionic phosphate backbone with consequential electrostatic repulsion from the anionic cell membrane, siRNA is unable to passively diffuse across the membrane.[9] For intracellular delivery purpose, although the viral vectors are highly efficient, they are limited to shRNA delivery and remain highly immunogenic and carcinogenic while the non-viral systems as promising alternatives for siRNA delivery are relatively safe and cost-effective. Being usually polycationic, they are able to form complexes with anionic siRNA, protecting it from nuclease attack and facilitating cellular uptake through electrostatic interactions with negatively charged plasma membrane or through specific interactions between the ligand anchored to the complex and the receptor on the cell membrane.[8] Among the non-viral vectors, polyplexes and lipoplexes have been found efficient for siRNA delivery with significant gene silencing effect both *in vitro* and *in vivo*. However, synthetic non-viral systems are still inefficient with an increase in performance often being associated with an increase in cytotoxicity.[10] The major obstacle for siRNA delivery in the non-viral route is the degradation of a significant portion of the internalized siRNA by nucleases.[11]

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We have recently developed an efficient delivery system based on some fascinating properties of carbonate apatite- ability of preventing crystal growth for generation of nanoscale particles as needed for efficient endocytosis and fast dissolution kinetics in endosomal acidic compartments to facilitate the release of delivered therapeutics from the particles and endosomes.[12-15] Here, we show that pH sensitive carbonate apatite particles having high affinity interactions with siRNAs, mediate efficient endocytosis and subsequent endosomal escape of the siRNAs, leading to the silencing of reporter gene expression more effectively than commercially available lipofectamine. Additionally, nanoparticle-assisted intracellular delivery of validated siRNA against cyclin B1 mRNA results in significant inhibition of cancer cell growth. Moreover, delivery of the anti-cyclin B1 siRNA sensitizes a human cervical cancer cell line (HeLa cell) to cisplatin and doxorubicin as well.

Materials and Methods

Reagents

Plasmid pGL3 (Promega) containing a luciferase gene under SV40 promoter and pEGFP-N2 (CLONTECH Laboratories, Inc.) containing green fluorescence protein gene under CMV were propagated in the bacterial strain XL-1 Blue and purified by QIAGEN plas-

mid kits. LysoTrackerTM Red DND-99, MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) and DMEM were purchased from Molecular Probes, Sigma and Gibco BRL, respectively. The lipofectamine 2000 transfection reagent was obtained from Invitrogen™ corporation, California, USA. Luciferase GL3 siRNA (Target sequence 5'-AACTTACGCTGAGTACTTCGA-3'), GFP-22 siRNA (Target sequence 5'-CGGCAAGCTGACCCTGAAGTTCAT-3'), siRNA against cyclin B1 (Target sequence 5'-AACACTTATACTAAGCACCAA-3') and all Stars Neg. siRNA Fluorescein were purchased from QIAGEN. siRNAs were delivered in the lyophilized form and upon delivery the siRNAs were diluted to obtain a 20 μM solution using RNAse-free water provided by Qiagen. The siRNA solution was then allocated into multiple reaction tubes for storage as repeated thawing might affect siRNA's silencing efficiency. The siRNAs were stored at -20°C as recommended by Qiagen.

Cell culture

HeLa cells were cultured in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 μg penicillin mL^{-1}, 50μg streptomycin mL^{-1} and 100 μg neomycin mL^{-1} at 37°C in a humidified 5% CO_2-containing atmosphere.

Formation of siRNA/carbonate apatite particles and transfection of cells

Cells from the exponentially growth phase were seeded at 50,000 cells per well into 24-well plates the day before transfection. 3-6 μL of 1 M $CaCl_2$ was mixed with 100 pM -100 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. Medium with generated siRNA-containing particles was added with 10% FBS to the rinsed cells. After 4 hr incubation, the medium was generally replaced with serum-supplemented medium and the cells were cultured up to 24-72h depending on the assay. In some experiments, siRNA/apatite particles were continuously incubated with the cells for 48h. siRNA/lipofectamine formulation and transfection were done according to the procedure provided by Invitrogen.

Determination of siRNA loading efficiency

The amount of fluorescein-labeled siRNA adsorbed onto apatite nanoparticles was determined from the fluorescence intensity of the siRNA within the pellet obtained after centrifugation of siRNA/apatite complexes. Following generation of siRNA/apatite particles as described above, using 5 mM Ca^{2+} and 1-200 nM of fluorescein siRNA and centrifugation at 15000 rpm for 3 min, the resulting pellet was washed 3 times with the same medium and dissolved in 100 μL of 10 mM EDTA-PBS. The dissolved particle solution was taken to an assay plate and quantified for the fluorescence intensity by a fluorescence microplate reader. Free fluorescein-labeled siRNA (1pM to 200 nM) in PBS was quantified and plotted to make the calibration curve with the help of which the actual siRNA loading was quantified.

Intracellular localization of siRNAs with apatite nanoparticles

HeLa cells were seeded at 15,000 cells per cm^2 on 0.1% (w/v) collagen-coated glass coverslips (24×24 mm) on the day before intracellular delivery. Fluorescein siRNA/apatite nanoparticles were added onto HeLa cells in the same manner as mentioned above. After adding into the cells and removal of residual complexes at 1, 2 or 4 h, endosomes and lysosomes were stained with LysoTracker™ Red DND-99 (Molecular Probes) according to the manufacturer's protocol and the cells were fixed with formaldehyde solution. Additionally, nuclei were stained with 4', 6-diamino-2-phenylindole (DAPI) and observed with A1 confocal laser scanning microscope (Nikon, Tokyo, Japan).

Gene silencing

HeLa cells expressing GFP and luciferase were generated by transfection with plasmid DNA encoding GFP and luciferase using the apatite and the lipofectamine 2000 (according to the manufacturer's instructions). Briefly, cells were seeded on a 24-well tissue culture plate at a density of 0.5×10^5 cells per well and incubated overnight in DMEM. On the day of transfection, the medium was removed and replaced with fresh media without serum. Apatite/luciferase plasmid or lipofectamine/luciferase plasmid complexes were added onto the cells in each well and incubated for 4 h. Media were then removed and the cells were washed with PBS, followed by replenishing fresh media containing serum. The cells were incubated for 24 h at 37 °C under CO2 atmosphere. In the next day, 1ml of apatite or lipofectamine suspension prepared with 10~100 nM of luciferase siRNA (as described before) was added to each well and incubated at 37°C under a 5% CO_2 atmosphere for 24 h. Luciferase gene expression was monitored by using a commercial kit (Promega) and photon counting (TD-20/20 Luminometer, USA). Each transfection experiment was done in triplicate and transfection efficiency was expressed as mean light units per mg of cell protein.

In case of GFP plasmid co-transfection, 2 μg of plasmid DNA and 100pM-100nM of GFP siRNA were used to prepare the DNA/siRNA complexes of apatite and lipofectamine, that were subsequently added onto the cells with either 10% FBS (for apatite complexes) or no FBS (for lipofectamine complexes) for 4 hr incubation. The medium was replaced by fresh DMEM with 10% FBS and incubated for 72 h.

In case of HeLa cells stably expressing GFP, the apatite/GFP siRNA or the lipofectamine/GFP siRNA particle suspensions produced in the same way as described above, were added onto the cells and incubated for 72h. The cells were lysed using the lysis buffer (NP40) and the intracellular fluorescein intensity was determined using a microplate reader (DTX 880, Multimode Detector by BECKMAN COULTER). In case of cyclin B1 siRNA delivery, apatite nanoparticles with cyclin B1 siRNA were incubated with HeLa cells continuously for 48 h either in presence or absence of cisplatin, doxorubicin and paclitaxel and the cellular toxicity level was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability assessment with MTT assay

30 μL of MTT solution (5 mg/mL) was added to each well after the specified periods of cell incubation (as mentioned in section 2.10), followed by incubation for 4 h at 37°C. 0.5 mL of DMSO was added after removal of media. After resolving crystals and incubating for 5 min at 37°C, absorbance was measured in a microplate reader at 570 nm with a reference wavelength of 630 nm. Cell viabilities were normalized to the absorbance of non-treated cells. MTT assay was also performed in a similar way after 48 h consecutive incubation of HeLa cells with cyclin B1 siRNA/apatite complexes.

Results and Discussion

Assessment of binding affinity of siRNA to carbonate apatite

Since siRNA is negatively charged owing to to its phosphate backbone while the carbonate apatite particles are positively charged due to the presence of calcium ions in the apatite structure, it was presumed that siRNA would bind to the apatite by ionic interactions. As shown in Figure 1, the binding affinity of siRNA to the apatite particles is increased almost propotionately with increasing concentrations of the available siRNA (1 to 100 nM) in the medium. A maximum binding of 50% was achieved when the initial siRNA concentration was 100 nM. No further increase in the degree of siRNA binding was observed by increasing

siRNA concentration to 200 nM, suggesting that the anion-binding sites (Ca^{2+}-rich domains) are saturated by complexation with siRNA molecules .

Cellular uptake and endosomal escapes of siRNA carried by apatite nanoparticles

Efficient delivery of siRNA to the cytosol of target cells depends on both the endocytosis of the non-viral vectors across the plasma membrane and their subsequent escape from endosomal/lysosomal compartments. A confocal microscopic analysis was performed on HeLa cells to observe the cellular localization of fluorescein siRNA in a time dependent manner from 1 to 4h after the delivery of fluorescein siRNA/apatite complexes and staining of the endosomes/lysosomes with LysoTracker™ Red. As shown in Figure 2, most of the fluorescein siRNAs were co-localized in stained endosomes and/or lysosomes after 1h incubation of HeLa cells with siRNA/apatite complexes.

However, after 2h most of the siRNAs escaped the endocytic vesicles and following 4 h, only a few dots of fluorescein siRNAs were still associated with the stained endo-lysosomes suggesting that the endosomal escape of siRNA was almost completed after 4 hr incubation. The high dissolution rate of carbonate apatite particles might contribute to the destabilization of endosomes releasing siRNA to the cytoplasm, since v-ATPase-driven excessive proton transfer into the endosomes to dissolve the particles could cause passive chloride influx and create osmotic pressure across the endosomal membrane, leading to endosome swelling and rupture.[12-15]

Estimation of gene silencing efficacy

The final effect of siRNA on silencing gene expression depends on the integrity of the siRNA after release from the endosomes as well as from the particles. The efficiency of siRNA delivery was first assessed by cotransfection of GFP gene and siRNA in HeLa cells. The anti-GFP siRNAs were transfected at varied concentrations (from 100 pM to 100nM) along with GFP plasmids using apatite and silencing efficiency was compared with lipofectamine 2000 (Figure 3). At 100 pmol of siRNA, more than 80% silencing was achieved with apatite particles- a value significantly higher than that of lipofectamine 2000. With increasing siRNA concentration to 1 nM, the silencing efficiency for the apatite became almost 100% whereas to achieve the similar level of efficacy, lipofectamine required 10 time more concentration of the siRNA (i.e., 10 nM) (Figure 3a and b). We also investigated the silencing effects on transiently expressed reporter gene by sequentially trasfecting HeLa cells with luciferase gene (to express the luciferase mRNA and protein) and then, with luciferase siRNA to cleave the expressed mRNA. With 10 and 100 nM of siRNA concentrations, almost 72% and 80% silencing were achieved, respectively, with carbonate apatite nanoparticles, that is highly comparable with lipofectamine 2000 (Figure 3c).

Effect of cyclin B1 gene silencing on cell viability

In order to evaluate cyclin B1 gene (CCNB1) as a potential target in cancer therapeutics, we employed carbonate apatite as an intracellular carrier of anti-cyclin B1 siRNAs, since expression of cyclin B1 is essentially needed during

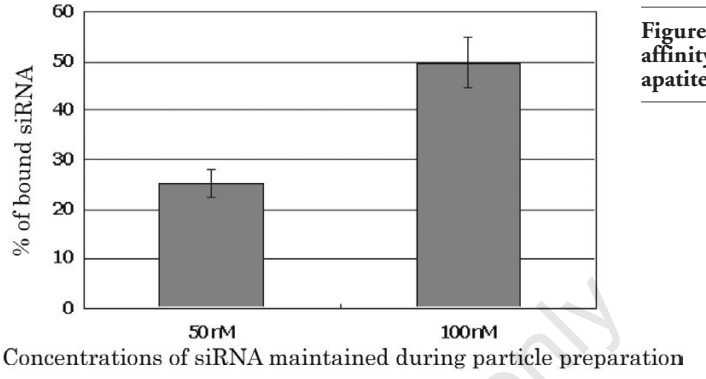

Figure 1. Binding affinity of siRNA to apatite particles.

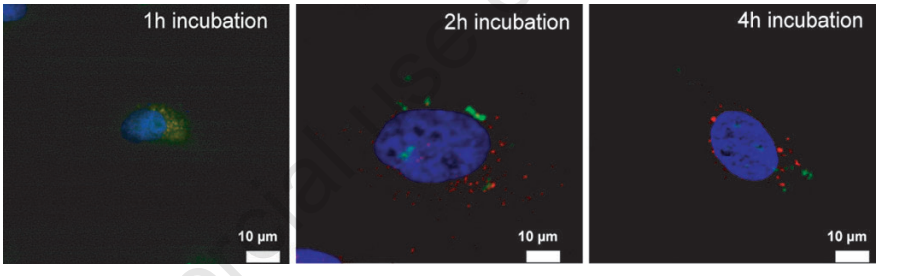

Figure 2. Endosomal escape of fluorescein siRNAs following endocytosis by apatite particles.

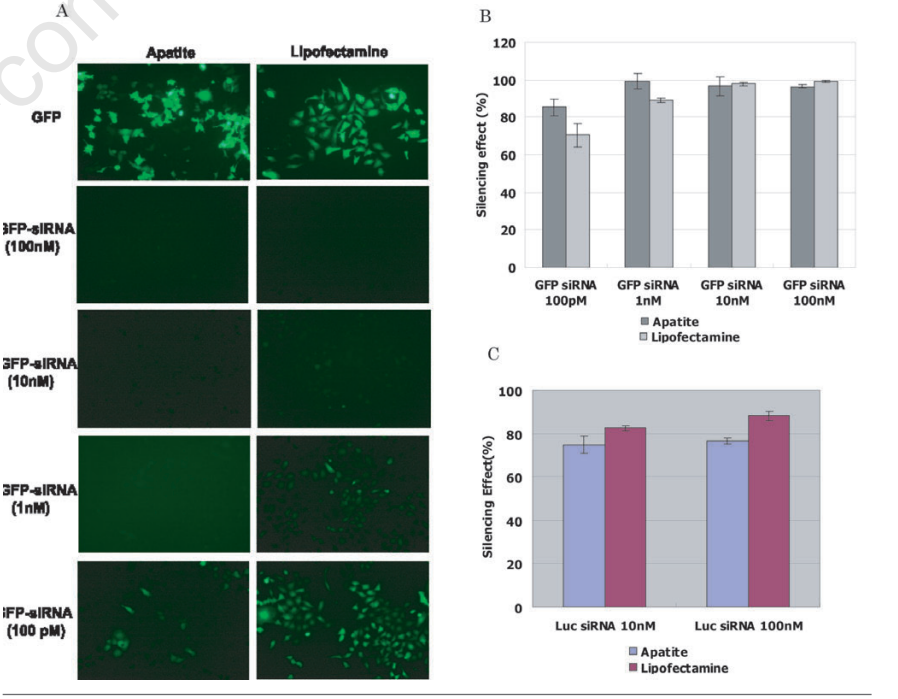

Figure 3. Silencing of GFP and luciferase expression, respectively, by siGFP and anti-luciferase siRNA.

the onset of mitosis and thus, knock-down of the gene might result in apoptosis of cancer cells.[16] The apatite-siRNA complexes made using 10 nM of pre-validated anti-cyclin B1 siRNA were used for transfection of HeLa cells for a consecutive period of 48 h prior to the assessment of cell viability by MTT assay. As shown in Figure 4, knockdown of cyclin B1 gene expression caused more than 25% of the cells to be killed in comparison to the nanoparticle-treated cells, suggesting that cyclin B1 is potentially a vital target for cancer therapy.

Influences of cyclin B1 gene knock-down on cisplatin-induced cell toxicity

Cisplatin is one of the most effective anti-cancer drugs for solid tumors, including ovarian, testicular, cervical, and small cell lung cancers.[17,18] Treatment of HeLa cells with 1 μM of cisplatin for 2 consecutive days caused 8% of cell death compared with particles only (Figure 5) while 32% of the cells were killed for the treatment where both apatite/siRNA complexes and cisplatin were incubated together with the cells for the same period of time. Since the combination of siRNA and cisplatin produced much better effects in cancer cell killing than either siRNA or drug only, we suggest that 1 μM of cisplatin might induce expression of more cyclin B1 than the basal level[19] in HeLa cells and therefore, silencing of cyclin B1 expression significantly perturbed cell growth by the dual effects of gene knockdown and drug action. On the contrary, the combined treatment with apatite/anti-cyclin B1 siRNA complex and a lower dose of cisplatin (200 nM) resulted in no further decrease in cell viability compared to siRNA or drug alone, suggesting that the concentration of the drug might be a determining factor in influencing the expression of cyclin B1.19,20

Influences of cyclin B1 gene knock-down on doxorubicin-induced cell toxicity

Doxorubicin is another chemotherapy drug widely used for the treatment of a variety of cancers including cervical cancer.[21,22] Doxorubicin which killed 50% of the cells at 1 μM concentration of the drug (Figure 6) seems to be more potent than cisplatin which killed only 8% of the cells at the same dose (Figure 5)

following continuous 2 day incubation with HeLa cells. Silencing of cyclin B1 gene following intracellular delivery of apatite/anti-cyclin B1 siRNA, sensitized the cells to doxorubicin similarly like cisplatin at that particular concentration (1 μM) killing more than 53% of the cells due to the synergistic effect of the drug and the gene knockdown. This could be due to the induction of cyclin B1 accumulation by doxorubicin stimulating the synthesis and inhibiting the degradation of cyclin B1[23] and consequently, its knockdown synergistically enhanced the cancer cell apoptosis in presence of doxorubicin.

The combined treatment with apatite/anti-cyclin B1 siRNA complex and a lower dose of doxorubicin (200 nM) demonstrated no further decrease in cell viability compared to siRNA or drug alone, suggesting that the lower concentration of doxorubicin might not have increased intracellular cyclin B1 levels or induced senescence sensitizing the cells to apoptosis.[24]

Influences of cyclin B1 gene knock-down 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).[25,26] As shown in Figure 7, 1 μM paclitaxel when incubated with HeLa cells continuously for 2 days, caused 73%

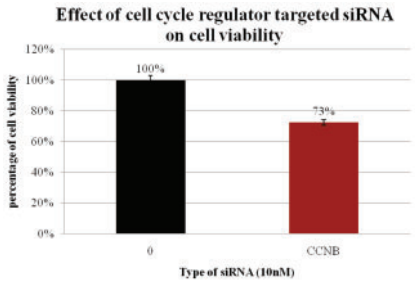

Figure 4. Cell viability assessment following 48 h incubation of cyclin B1 siRNA/apatite complexes with HeLa cells.

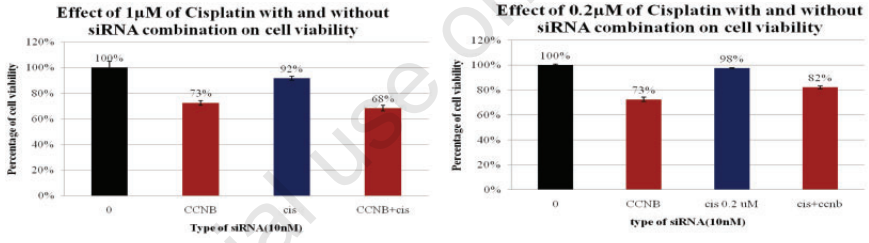

Figure 5. Effects of silencing cyclin B1 expression on viability of cancer cells under higher (left side) and lower doses (right side) of cisplatin.

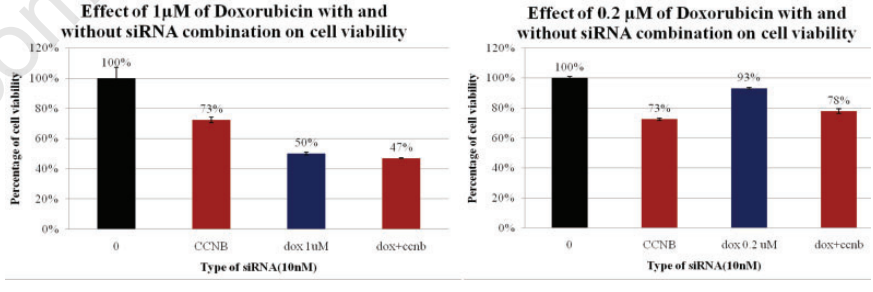

Figure 6. Effects of silencing cyclin B1 expression on viability of cancer cells under higher (left side) and lower doses (right side) of doxorubicin.

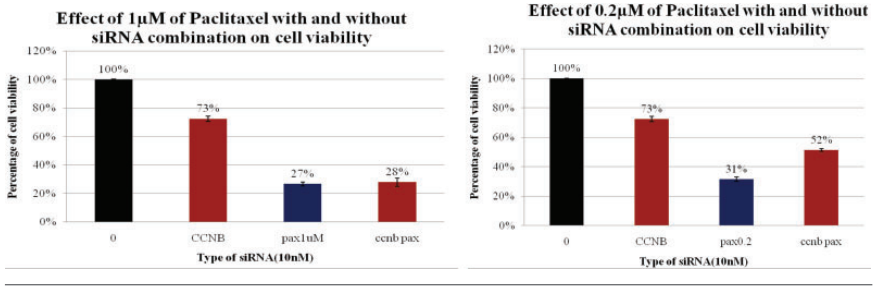

Figure 7. Effects of silencing cyclin B1 expression on viability of cancer cells under higher (left side) and lower doses (right side) of paclitaxel.

of the cells to death, indicating that paclitaxel was most effective among the drugs used in the study. Treatment with both anti-cyclin B1 siRNA and paclitaxel killed 72% of the cells, whcih was higher than the effectiveness of the siRNA alone, but almost similar to that of paclitaxel alone which killed 73% of the cells. Although paclitaxel might increase cellular cyclin B1 level,[27] the hight cytoxicity of paclitaxel had the diminating role over the gene-knockdown in killing the cancer cells. Similar findings were obtained in presence of 0.2 μM doxorubicin which killed almost same number of cells as 1 μM of the drug.

We have developed an intracellular siRNA delivery system based on pH sensitive carbonate apatite nanoparticles taking the advantages of endocytosis-mediated siRNA entry and its subsequent escape from the endocytic vesicle. The interesting effects of carbonate apatite-facilitated konckdown of cyclin B1 in combination with three popular anti-cancer drugs in inducing apoptosis of cervical cancer cells would obviously inspire more studies to be carried out in other cancer cell lines and various animal models of cancers .

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