Carbonate apatite-facilitated intracellular delivery of c-ROS1 small interfering RNA sensitises MCF-7 breast cancer cells to cisplatin and paclitaxel

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Abstract

Introduction
Breast cancer is one of the leading causes of deaths worldwide with chemotherapy being the only treatment option currently available for advanced stage breast cancer producing severe toxic effects on normal cells. Small interfering RNA (siRNA), a powerful tool to selectively silence gene expression could be harnessed in combination with traditional chemotherapy drugs for effectively treating breast cancer with minimal side effects. However, the limitation of the naked siRNA in penetrating the plasma membrane and its sensitiveness to nuclease-mediated cleavage pose the major challenges to the proper exploitation of siRNA technology in therapeutic intervention. The aim of this study was to discuss how carbonate apatite-facilitated intracellular delivery of c-ROS1 siRNA sensitises MCF-7 breast cancer cells to cisplatin and paclitaxel.

Materials and methods
Carbonate apatite particles were used to deliver c-ROS1 siRNA in the presence of anti-cancer drugs to MCF-7 cells whereas the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and Western blot were performed to assess the cell viability and detect the phosphorylation levels of mitogen-activated protein kinase and AKT1, respectively.

Results
Intracellular delivery of the siRNA targeting c-ROS1 gene transcript in MCF cells which constitutively express the gene, dose-dependently enhanced chemosensitivity to cisplatin and paclitaxel, while demonstrating no significant enhancement in cell death with doxorubicin irrespective of its doses following intracellular delivery of the siRNAs.

Discussion
pH-sensitive carbonate apatite has recently been developed as an efficient device to deliver siRNA into the mammalian cells by virtue of its high affinity interaction with siRNA, the desirable size distribution of the resulting siRNA-apatite complex for effective cellular endocytosis and the ability of the internalised siRNA to escape the endosomes resulting in the effective knockdown of the target gene, such as cyclin B1 or ABCB1 transporter gene. The synergistic effect of c-ROS1 siRNA and paclitaxel on cell death as reported in this paper could be correlated with the inhibition of ERK 1/2 phosphorylation in mitogen-activated protein kinase pathway.

Conclusion
Thus, ROS1 has evolved as a potential target for gene knockdown in cisplatin- and paclitaxel-based chemotherapy of human breast cancer.

Reference

vector-mediated intracellular delivery of the small interfering RNAs (siRNAs) for targeted cleavage of the gene transcripts of those pathways, could be the potential treatment strategy that might additionally render cancer cells extremely sensitive to cytotoxic chemotherapy drugs. On the other hand, conventionally used anti-cancer drugs induce apoptosis of cancer cells by interfering with the major cellular functions which might have some sort of cross-talks with the protein components of cell proliferation/survival pathways. Consequently, selective knockdown of the genes encoding the enzymes of those pathways could not only slow down the growth of cancer cells, but also sensitize them to chemotherapy drugs.

RNA interference, a process for specific silencing of mRNA expression could be harnessed to develop new drugs against the therapeutic genes of interests in two different routes: 1) cytoplasmic delivery of short interfering RNA for precisely breaking down the target mRNA and 2) nuclear delivery of gene expression cassettes transcribing a short hairpin RNA (shRNA) which upon further processing by cellular machinery is converted into siRNA in the cytoplasm. However, siRNA being a synthetic RNA duplex of 21–23 nucleotides, is much more advantageous than shRNA due to the difficulty in construction of a shRNA expression system by recombinant DNA technology, and the requirement of the plasmid-based expression system to overcome the nuclear barrier for shRNA expression. siRNA in the cell cytoplasm incorporates into a multiprotein RNA-induced silencing complex (RISC) prior to being unwound into single-stranded RNAs by Argonaute 2, a multifunctional protein of RISC, forming antisense strand-associated RISC in order to guide and selectively degrade the complementary mRNA. However, the strong anionic phosphate backbone with resultant electrostatic repulsion from the anionic cell membrane is a major barrier to the passive diffusion of siRNA across the plasma membrane. The hydrophobic lipid bilayer could pose an hydrophobic barrier to the hydrophilic siRNA. In addition, naked siRNA could be degraded by the plasma nucleases and subjected to renal clearance due to its small size before going to the target site in vivo.

A number of existing non-viral vectors are available for intracellular delivery of siRNA with limitations in proper condensation, cellular uptake and endosomal escape of siRNA, leading to a decrease in overall silencing potency of the delivered siRNA. Usually, a non-viral vector with a cationic backbone electrostatically binds with an anionic siRNA to form a stable complex, thereby protecting it from nuclease-assisted degradation, enabling it to cross the plasma membrane through endocytosis and finally facilitating its endosomal escape.

Receptor tyrosine kinases (RTKs) which usually act as regulators for normal cellular processes, also play key roles in initiation and progression of a number of cancers. Out of 58 human RTKs, ROS kinase encoded by c-ROS1 gene is one of the remaining RTKs whose ligands are yet to be identified. The region of chromosome 6 carrying the human c-ROS gene undergoes nonrandom rearrangement in acute lymphoplastic leukemia, malignant melanoma and ovarian carcinoma. Overexpression of ROS was found in primary human gliomas, malignant gliomas, non-small cell lung cancer and fibroadenoma of the breast. An activated form of human c-ROS gene which probably arose due to the loss of a segment representing a putative extracellular domain of the enzyme, was isolated from the DNA of the human breast carcinoma cell line, MCF-7 using a tumorigenicity assay. Thus, silencing of ROS1 gene expression could be an attractive option for the treatment of breast cancer.

We have recently established pH-sensitive carbonate apatite as a potential tool to efficiently deliver siRNA across the cell membrane and knockdown the gene expression of cyclin B1 and ABCG2/ABCB1, thus inducing apoptosis of cervical and breast cancer cells, respectively, in combination with anti-cancer drugs. Here, we report that carbonate apatite-mediated delivery of the siRNA targeting c-ROS1 gene transcript in MCF-7, a human breast cancer cell line constitutively expressing ROS1 RTK led to a significant increase in chemosensitivity to cisplatin and paclitaxel while the synergistic effect of ROS1 siRNA and paclitaxel correlated with the inhibition of ERK1/2 phosphorylation in mitogen-activated protein kinase (MAPK) pathway.

Materials and methods
The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Reagents
Dulbecco’s modified Eagle medium (DMEM) was purchased from BioWhittaker (Walkersville, USA), DMEM powder, foetal bovine serum (FBS) and trypsin-ethylene diamine tetra acetate were obtained from Gibco BRL (California, USA). Calcium chloride dehydrate, 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-diammine platinum (II) dichloride are from Sigma Aldrich (St. Louis, MO, USA). Both doxorubicin and cis-diammine platinum (II) dichloride were dissolved in distilled water and 2 mM stock solutions were prepared whereas
pacilitaxel was dissolved in DMSO and 10 mM stock solution was prepared.

siRNA design and sequence

The validated anti-ROS1 siRNA was purchased from Qiagen (California, US) with the target sequence of 5’-AAGGTAATTGCTCTAACTTTA-3’. siRNA was supplied in lyophilised form and upon delivery, the siRNA (1 nmol) was reconstituted with RNase-free water to obtain a stock solution of 20 μM. The siRNA solution was then allocated into multiple reaction tubes for storage as repeated thawing might affect the silencing efficiency of siRNAs. The siRNAs were stored at -20°C as recommended by Qiagen.

Cell culture and seeding

MCF-7 cells were grown in 25 cm² culture flask in DMEM supplemented with 10% heat-inactivated FBS in a humidified atmosphere containing 5% CO₂ at 37°C. Exponentially growing MCF-7 cells were trypsinised and following addition of fresh medium, the cell suspension was centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. Fresh medium was added to resuspend the pellet and the cells were counted using haemocytometer. Appropriate dilutions were made using culture medium to produce a cell suspension with concentration 5.0 × 10⁶ cells/mL. One mL of the prepared cell suspension was subsequently added into each of the wells in 24-well plate and allowed to attach for overnight incubation at 37°C and 5% CO₂ before siRNA transfection.

Generation of target siRNA/ carbonate apatite complexes and transfection of MCF-7 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 with the pH subsequently adjusted to 7.4 using 0.1 M hydrochloric acid. The prepared DMEM solution was filtered using a 0.2 μm syringe filter in the laminar flow hood, followed by transferring 1 mL of the filtered medium into 1.5 mL microcentrifuge tubes. 4 μL of 1 M calcium chloride was then added into the microcentrifuge tubes, followed by addition of siRNA (10 nM) and/or drug (8 or 40 nM) and incubation at 37°C for 30 min. After the incubation, 10% FBS was added into each microcentrifuge tube. Culture medium from the wells seeded one day before was aspirated and replaced with 1 mL of the prepared medium containing siRNA-loaded carbonate apatite nanoparticles in presence or absence of free drugs. Plates were then incubated at 37°C and 5% CO₂ for two consecutive days.

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

Following two days of siRNA transfection, the fraction of viable MCF-7 cells was determined using the MTT assay. Briefly, 50 μL of MTT (5 mg/mL in phosphate buffered saline) was added aseptically into each of the wells in the siRNA transfected-plate, followed by incubation at 37°C and 5% CO₂ for 4 h. After the incubation, medium containing MTT was aspirated and the purple formazan crystals at the bottom of each well were dissolved by mixing with 300 μL of DMSO solution. Absorbance of the resulting formazan solution was then determined spectrophotometrically 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 (SD) of three independent experiments.

Data analysis

The cell viability in the treated wells was expressed as a percentage and was calculated using the absorbance values obtained from MTT assay by using the following formula:

\[
\text{% cell viability} = \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100\%
\]

Subsequently, the percentage enhancement of chemosensitivity in siRNA-treated cells was determined using the formula below:

\[
\text{% enhancement of chemosensitivity} = \frac{U - T}{U} \times 100\%
\]

Where U is the percentage of cell death in siRNA-treated cells in the presence of drug and T is the percentage of cell death in the presence of drug only.

Western blotting

MCF-7 cells were incubated with ROS1 siRNA-loaded nanoparticles in presence of 40 nM pacilitaxel for a consecutive period of 48 h and washed with pre-chilled phosphate buffered saline prior to lysis. The cell lysates were then centrifuged at 15,000 rpm for 10 min at 4°C using a refrigerated centrifuge (Sartorius Stedim Biotech, Germany) and protein concentration of the cell lysate was estimated using the Bradford assay with absorbance being measured in a microplate reader (Bio-Rad, USA). The protein extracts were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (7.5% gel) at 200 V for 40 min and the resolved proteins were electro-transferred for 12 min at 18 V to nitrocellulose membranes (Thermo Scientific, USA) using Western blotting semi-dry transfer unit (Hoefer, San Francisco, USA). The membranes were blocked for 1 h at room temperature with Tris buffered saline with Tween 20 (TBST) containing 5% bovine serum albumin (Merck, USA). The membranes were then incubated with 1:1000 primary antibody (Cell Signalling Technology, USA) in TBS-T with 5% bovine serum albumin.
overnight at 4°C with gentle shaking. Membranes were washed twice with TBST for 10 min each and further incubated with 1:5000 secondary antibody (Thermo Fisher Scientific, USA) for 1 h at room temperature. After the incubation with secondary antibody, the membrane was washed two times for 10 min each with TBST to remove any unbound secondary antibody. The membrane was incubated with Pierce ECL Western Blotting Detection Reagent (Thermo Fisher Scientific, USA) at room temperature for five minutes. Excess detection reagent was drained off and the membrane was exposed to an X-ray film (Thermo Scientific, USA) where the X-ray film was placed on top of the membrane for an appropriate period of time in an X-ray film cassette (Amersham, USA). Membranes were also tested for equal loading.

**Statistical analysis**

Statistical analysis was done using the SPSS statistical package (version 17.0 for Windows). LSD post-hoc test for one-way Analysis of Variance (ANOVA) was used to analyse and compare the significant difference between treated and non-treated samples. Data are presented as mean ± SD with P < 0.05 being considered as statistically significant.

**Results**

Optimisation of carbonate apatite formulation based on particle growth and resulting cytotoxicity

Different formulations of carbonate apatite were made with different concentrations of calcium chloride (Ca²⁺) in 1 mL DMEM containing fixed amounts of inorganic phosphate and bicarbonate in order to find out the best formulation for intracellular delivery of the target siRNA (Figure 1). The turbidity value at 4 mM of Ca²⁺ corresponds to that of the particle sample used in the earlier study considering its desirable particle size distribution for effective endocytosis and insignificant level of cytotoxicity 30–32. The images taken 24 h after incubation of MCF-7 cells with the particles formulated with various concentrations of Ca²⁺ (Figure 1-B) demonstrate that the aggregated particles formed with 1 mM Ca²⁺ were enormously low (Figure 1) with the number of particles being gradually increased with increasing concentrations of Ca²⁺ (Figure 1). When the concentration of Ca²⁺ reached 6 mM,

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**Figure 1**: A. Measurement of turbidity at 320 nm as an indicator of particle growth which was induced by addition of different concentrations of Ca²⁺ to a 1 mL bicarbonate-buffered solution (pH 7.4) followed by incubation at 37°C for 30 min. The data are presented as mean ± SD of triplicates. B. Images taken 24 h following incubation of MCF-7 cells with the carbonate apatite particles generated by addition of (a) 1 mM Ca²⁺; (b) 2 mM Ca²⁺; (c) 3 mM Ca²⁺; (d) 4 mM Ca²⁺; (e) 5 mM Ca²⁺; and (f) 6 mM of Ca²⁺ to 1 mL bicarbonate-buffered medium (pH 7.4) and incubation at 37°C for 30 min. *' indicates significant difference from the control value with ANOVA test and P < 0.05 compared with untreated cells.
the cells appeared to be surrounded by and overloaded with the particles (Figure 1-B, f), indicating that higher number of the particles could lead to potential cytotoxicity.

In addition to the turbidity measurement and observation of the aggregated particles under optical microscope, quantitative study on cytotoxicity of the particles was vital prior to their application in siRNA transfection studies. The MTT assay was conducted to assess the potential toxicity in MCF-7 breast cancer cells. As shown in Figure 2, a decreasing trend in cell viability was observed for the particles formed with increasing concentrations of Ca\(^{2+}\) while the lower concentrations of Ca\(^{2+}\) exhibited relatively lower cytotoxicity.

Co-delivery of ROS1 and chemotherapy drugs into MCF-7 cells
Since c-ROS1 gene which encodes RTK is over-expressed in human breast cancer 27–29 indicating the potential importance of ROS1 as atherapeutic target, Qiagen-validated siRNA targeting ROS1 gene was intracellularly delivered in presence or absence of the widely used anti-cancer drugs—doxorubicin, paclitaxel and cisplatin 33–35, following complexation of the target siRNA (10nM) with carbonate apatite particles and incubating the complexes with MCF-7 cells for 48 h. The MTT assay was subsequently used to determine the cytotoxicity.

**Influences of c-ROS1 gene knockdown on doxorubicin-induced cell toxicity**
As a popular anti-cancer agent in the treatment of metastatic breast cancer\(^{33–35}\), doxorubicin was investigated for any possible synergistic role it might play together with knockdown of c-ROS1 gene transcript on accelerating the death of MCF-7 cells. Validated specific siRNA (10 mM) against c-ROS1 mRNA was added together with Ca\(^{2+}\) (4 mM) to the bicarbonate-buffered DMEM prior to the incubation at 37°C for 30 min to form carbonate apatite/siRNA complexes. As shown in Figure 3, while doxorubicin showed a prominent cytotoxicity effect even at a very low concentration (8 nM) of the drug killing almost 20% of the total cells following a consecutive period of 48 h incubation, incubation of the cells with c-ROS1 siRNA-loaded carbonate apatite particles for the same period of time resulted in more than 30% of the cells killed in comparison to the untreated cells, suggesting that ROS1 RTK might be actively involved in initiating the signalling cascade responsible for proliferation or survival of MCF-7 cells. A similar level of cytotoxicity was observed with the particles prepared in presence of the drug (8 nM). The higher cytotoxicity of the nanoparticles in presence of the chemotherapy drug compared to the free one could be attributed to the cellular uptake processes of free and apparently formed nanoparticle-bound drugs with the former being passively translocated across the plasma membrane while the latter being likely to be internalised through endocytosis.

**Influences of ROS1 gene knockdown on cisplatin-induced cell toxicity**
Cisplatin is one of the most effective anti-cancer drugs clinically used for the treatment of malignant breast cancers 33–35. Treatment of MCF-7 cells either with 8 or 40 nM of cisplatin in presence or absence of the particles for a consecutive period of twodays led to much fewer number of the cells being killed (Figure 4) compared with the same doses of doxorubicin (Figure 3). However, when both apatite/c-ROS1 siRNA complexes and cisplatin either at 8 or 40 nM were incubated together with the cells for the same period of time, a significant drop in cell viability was noticed compared with the cells being killed alone (Figure 4), indicating that silencing of c-ROS1 gene sensitised MCF-7 cells to cisplatin irrespective of the doses (low or high) by killing more than 40% of the cells in each case.

**Figure 2:** Assessment of cytotoxicity of carbonate apatite particles in MCF-7 cells. MCF-7 cells were incubated for a consecutive period of 48 h with the particles formulated with different concentrations of calcium chloride and subjected to assessment for cell viability by MTT assay. The data are presented as mean ± SD of triplicates.


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Influences of c-ROS1 gene knockdown on paclitaxel-induced cell toxicity
Paclitaxel is another chemotherapy drug widely used for the treatment of malignant breast cancer 33–35. As shown in Figure 5, with nanoparticle formulation paclitaxel killed 10% to 20% more cells that cisplatin at 8 and 40 nM of the individual drug concentrations following continuous two-day incubation with MCF-7 cells. However, knockdown of ROS1 gene expression at 8 and 40 nM of paclitaxel killed more than 50% and 70% of the cells following a consecutive two-day incubation of the cells with apatite/c-ROS1 siRNA and paclitaxel, with the level of the chemosensitivity enhancement being 48% and 42% at 8 and 40 nM of paclitaxel, respectively (Table 1).

Phosphorylation of MAP kinase following combined treatment with ROS1-siRNA and paclitaxel
Since siRNA-loaded carbonate apatite particles in combination with 40 nM of paclitaxel caused the highest number of cell deaths as a result of synergistic effects of the treatments in MCF-7 cells (Figure 5), Western blotting was carried out following a consecutive two-day incubation of the cells with siRNA/apatite complexes and paclitaxel (40 nM) in order to detect the phosphorylation levels in MAP kinase(ERK1/2) and AKT1 (Ser 473).

Discussion
As shown in Figure 1-A, increasing trend in absorbance was observed with the increasing concentration of Ca\(^{2+}\) indicating that the turbidity, that is, the growth of the formulated carbonate apatite particles was enhanced when the concentration of Ca\(^{2+}\) increased. The large particles generated particularly at 5 or 6 mM of Ca\(^{2+}\), as observed in Figure 1-B, suggests that they could be potentially toxic to the cells. Finally, the cell

Figure 3: Effects of intracellular c-ROS1 siRNA delivery on enhancement of chemosensitivity to doxorubicin. c-ROS1 siRNA-loaded carbonate apatite particles were generated by exogenous addition of 4 mM calcium chloride and c-ROS1 siRNA in presence or absence of doxorubicin (8 mM or 40 mM) to 1 mL bicarbonate-buffered DMEM (pH7.4), followed by incubation at 37°C for 30 min and supplementation with 10% FBS prior to incubation with MCF-7 cells for a consecutive period of 48 h. MTT assay was then performed with the absorbance being taken at wavelength of 570 nm with reference to 630 nm. The data are presented as mean ± SD of triplicates.

Figure 4: Effects of intracellular c-ROS1 siRNA delivery on enhancement of chemosensitivity to cisplatin. c-ROS1 siRNA-loaded carbonate apatite particles were generated by exogenous addition of 4 mM calcium chloride and c-ROS1 siRNA in presence or absence of cisplatin (8 mM or 40 mM) to 1 mL bicarbonate-buffered DMEM (pH7.4), followed by incubation at 37°C for 30 min and supplementation with 10% FBS prior to incubation with MCF-7 cells for a consecutive period of 48 h. MTT assay was then performed with the absorbance being taken at wavelength of 570 nm with reference to 630 nm. The data are presented as mean ± SD of triplicates.
viability assessment as shown in Figure 2 established the notion.

Thetoxicity level increased proportionally with increasing concentration of Ca\(^{2+}\). From the measured turbidity profile, microscopic images and cytotoxicity assay, particles formulated with 4 mM Ca\(^{2+}\) were found to be most suitable to be used for the subsequent siRNA delivery experiments.

Assessment of cell viability using ‘AllStars Negative Control siRNA’, a scramble siRNA (designed and synthesised by Qiagen) resulted in no cytotoxic effect irrespective of the siRNA doses used (not shown here), indicating the importance of siRNA specificity in gene knockdown and eventual change in cell functionality. When the cells were incubated with siRNA/apatite complexes in presence of doxorubicin (8 nM), no statistically significant enhancement in cell toxicity was observed compared to siRNA-loaded particles either in presence or absence of the drug, indicating that any cross-talk is unlikely to exist between the pathway of ROS1 signaling and the route of doxorubicin-induced cytotoxicity. An increase in the doxorubicin concentration (40 nM) apparently neither increased the cytotoxicity level nor exerted any synergistic effect with silencing of c-ROS1 gene expression. However, a possible synergistic effect should not be ruled out on the basis that activated Raf/MEK/ERK cascade could confer resistance to doxorubicin (or paclitaxel) by inducing the expression of the drug pump Mdr-1.\(^{36,37}\)

Sensitization of MCF-7 cells to cisplatin following c-ROS1 gene knockdown could be explained by the earlier finding that cisplatin-resistant MCF-7 cells could grow partly due to the increased ERK1 phosphorylation and the high levels of AKT1 kinase activity\(^{38}\), and therefore, silencing of ROS1 RTK expression might interrupt the downstream signaling cascades of MAPK and AKT1, leading to the enhancement of chemosensitivity by 79% and 47% at 8 and 40 nM of cisplatin, respectively (Table 1).

Targeted cleavage of c-ROS1 mRNA might deactivate the Raf/MEK/ERK cascade which would otherwise not only assist in cell proliferation/surival, but also induce resistance to paclitaxel by inducing the expression of Mdr-1.\(^{36,37}\), thereby synergistically enhancing the cancer cell apoptosis in presence of paclitaxel. Moreover, paclitaxel can rapidly activate MAPK pathway\(^{39}\) and so, inactivating the MAPK pathway through knock down of ROS1 could also result in sensitisation of MCF-7 cells to paclitaxel.

As shown in Figure 6(a), phosphorylation of ERK1/2 in MAPK pathway was disappeared in response to the combined treatment of paclitaxel (40 nM) and ROS1 siRNA (10 nM), indicating that the synergistic effect of ROS1 genesilencing and paclitaxel on induction of MCF-7 cell death might be due to their roles in suppressing phosphorylation of ERK1/2. The combined treatment, however, showed no effect on phosphorylation of AKT1 (Ser473) in PI3K pathway as shown in Figure 6(c), suggesting that PI3K pathway might have no role in augmenting the expression of multidrug resistance protein(s), such as Mdr-1.

Conclusion
We have identified c-ROS1 as a highly promising therapeutic target for cancer therapy.

Table 1: Enhancement of chemosensitivity to traditional anti-cancer drugs following knockdown of c-ROS1 gene

<table>
<thead>
<tr>
<th>siRNA</th>
<th>% Enhancement of chemosensitivity</th>
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<tr>
<td>10 nM ROS1 siRNA</td>
<td>38% No effect</td>
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Figure 5: Effects of intracellular c-ROS1 siRNA delivery on enhancement of chemosensitivity to paclitaxel. c-ROS1 siRNA-loaded carbonate apatite particles were generated by exogenous addition of 4mM calciumchloride and c-ROS1 siRNA in presence or absence of paclitaxel (8 nM or 40 nM) to 1 mL bicarbonate-buffered DMEM (pH7.4), followed by incubation at 37°C for 30 min and supplementation with of 10% FBS prior to incubation with MCF-7 cells for a consecutive period of 48 h. MTT assay was then performed with the absorbance being taken at wavelength of 570 nm with reference to 630 nm. The data are presented as mean ± SD of triplicates.

Figure 6: (a) c-ROS1 mRNA knockdown results in decreased expression of AKT1 (Ser473) and (b) ERK1/2 phosphorylation in response to paclitaxel (8 nM) and c-ROS1 siRNA (10 nM) treatment.

References
8. Dickerson EB, Blackburn WH, Smith MH, Kapa LB, Lyon LA, McDonald JF.

Figure 6: Effect of co-treatment of ROS1 siRNA and 40 nM Paxon phosphorylation of (a) MAP kinase and (c) AKT1 in MCF-7 cells. MCF-7 cells were incubated with ROS1 siRNA-loaded nano particles in presence of 40 nM paclitaxel for a consecutive period of 48 h and washed with pre-chilled phosphate buffered saline prior to lysis. The cell lysates were then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and subsequently, Western blot analysis to detect the phosphorylation levels in AKT1 and MAPK to gether with total MAPK (b).