Knockdown of $ROS1$ gene sensitizes breast tumor growth to doxorubicin in a syngeneic mouse model

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Abstract. Treatment of breast cancer, the second leading cause of female deaths worldwide, with classical drugs is often accompanied by treatment failure and relapse of disease condition. Development of chemoresistance and drug toxicity compels compromising the drug concentration below the threshold level with the consequence of therapeutic inefficacy. Moreover, amplification and over-activation of proto-oncogenes in tumor cells make the treatment more challenging. The oncogene, $ROS1$ which is highly expressed in diverse types of cancers including breast carcinoma, functions as a survival protein aiding cancer progression. Thus we speculated that selective silencing of $ROS1$ gene by carrier-mediated delivery of siRNA might sensitize the cancer cells to the classical drugs at a relatively low concentration. In this investigation we showed that intracellular delivery of c-$ROS1$-targeting siRNA using pH-sensitive inorganic nanoparticles of carbonate apatite sensitizes mouse breast cancer cells (4T1) to doxorubicin, but not to cisplatin or paclitaxel, with the highest enhancement in chemosensitivity obtained at 40 nM of the drug concentration. Although intravenous administrations of $ROS1$-loaded nanoparticles reduced growth of the tumor, a further substantial effect on growth retardation was noted when the mice were treated with the siRNA- and Dox-bound particles, thus suggesting that silencing of $ROS1$ gene could sensitize the mouse breast cancer cells both in vitro and in vivo to doxorubicin as a result of synergistic effect of the gene knockdown and the drug action, eventually preventing activation of the survival pathway protein, AKT1. Our findings therefore provide valuable insight into the potential cross-talk between the pathways of $ROS1$ and doxorubicin for future development of effective therapeutics for breast cancer.

Introduction

The overpowering growth of cancer cells in a tumor is associated with development of profound complexity in tumor microenvironment with a consequence of developing chemoresistance and metastasis that leads to treatment failure, progressive morbidity and mortality. Breast cancer is the second leading cause of death among women worldwide with chemo-/radiotherapy being the most preferred strategy to combat the disease after surgical removal of the cancerous tissues. A large number of chemical drugs with dissimilar structures and mode of actions are used to treat solid tumors like breast cancer. The updated list of the FDA-approved drugs for treating breast cancer consists of at least 24 drugs with different formulations for combinatorial therapy. Among them, three of the most widely used anticancer drugs: doxorubicin, cisplatin and paclitaxel from three different families were used separately in this study. All of the three drugs enter into cells via passive or carrier-mediated diffusion and act as substrates for the ATP binding cassette multidrug transporters that extrude the drugs out of the cells. The drugs are known to exert many adverse effects principally caused by off-target toxicity of anti-cancer drugs affecting particularly highly dividing healthy cells of the body.

Although the above drugs are currently used in different combinations with other drugs for better treatment outcome, non-specific distribution all over the body causing inadequate drug concentration in the tumor microenvironment diminishes the chemo-effectiveness. Toxicity to healthy tissues compromises the doses and frequencies of treatment resulting in therapeutic failure. Moreover, intrinsic and acquiring multi-drug resistance (MDR) by genetic and epigenetic modifications of cancer cells nullifies the effects of many therapies. In tumor cells, many oncogenes overexpressed or amplified, are involved in cell survival and proliferation abetting cancer progression and minimizing therapeutic activity of the drugs. Thus knocking these genes down using specific siRNA would
sensitize cancer cells to conventional drugs by reducing the effective threshold concentration of drugs inside the cells.

The proto-oncogene **ROS1** encoding for a type-I integral membrane protein with tyrosine kinase activity having roles in growth and differentiation, is highly expressed in a variety of tumor cells (1-5). The tumorigenicity assay of human breast cancer cells identified an activated form of **ROS1** gene encoding for the extracellular domain as a result of a loss of segment from the gene (6,7), thus making it a potential molecular target to be silenced for breast cancer treatment.

Activation of a receptor tyrosine kinase (RTK) triggers phosphatidylinositol 3-kinase (PI3K) survival pathway stimulating numerous downstream proteins and thereby, regulating key signaling pathways in many cellular processes, such as differentiation, cell survival, transformation, growth, proliferation, apoptosis, motility and cytoskeletal rearrangement. Oncogenic alterations of this pathway were reported in different cancers including breast cancer, offering it as a valuable therapeutic target (8-19). Furthermore, mitogen activated protein kinase (MAPK) pathway also plays a pivotal role in transducing signals in response to extracellular cues, modulating numerous physiological processes, i.e., cell proliferation, growth, differentiation, stress response, immune response, cell migration and apoptosis (20). The activation of MAPK pathway is involved in cancer growth and metastasis in many cancers including breast cancer (21-23). There is fine-tuned cross-talk between the survival and mitogenic pathways, thus endowing cells with capabilities for processing and decoding signals from the multiple receptors activated by different growth factors (24). Stimulation of the cells expressing chimeric receptors that contain the ROS1 kinase domain fused to the ligand binding domain of EGFR, with the corresponding growth factors was eventually found to activate various combinations of PI3K/AKT and MAPK/ERK signaling components, indicating the plausible roles of ROS1 in these pathways (25). Therefore, the level of these pathway proteins could be altered by silencing **ROS1** expression in cancer cells through delivery of anti-ROS1 siRNA.

However, a suitable nano-carrier to transport siRNA and drugs together inside tumor cells and subsequently release them in cytoplasm is one of the utmost needs for achieving the optimal therapeutic outcomes. The recently developed pH-sensitive inorganic nanoparticles (NP) of carbonate apatite with high binding affinity for nucleic acid therapeutics and conventional drugs have the potential for combined intracellular delivery of the diverse drugs via endocytosis and their fast release at endosomal acidic pH (26-31). Moreover, this carrier has been used successfully to deliver classical anti-cancer drugs and siRNA for in vitro study.

### Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM), calcium chloride dehydrate (CaCl₂·2H₂O), sodium bicarbonate (NaHCO₃), thiazolyb blue tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), Nonidet P-40 (NP-40), didithio- reitol (DTT), protease inhibitor cocktail, phosphatase inhibitor, bovine serum albumin (BSA), sodium chloride (NaCl), Trit base and the anti-cancer drugs doxorubicin hydrochloride (Dox), cis-diammine platinum (II) dichloride (Cis) and paclitaxel (Pax) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dox and Cis were dissolved in miliQ water (2 mM stock) and Pax was dissolved in DMSO (10 mM stock). DMEM powder, fetal bovine serum (FBS), trypsin-ethylenediamine tetraacetate (trypsin-EDTA) and penicillin-streptomycin were obtained from Gibco BRL (Carlsbad, CA, USA). Functionally validated siRNA (1 nmol) against **ROS1** gene with target sequence of 5'-AAGGTAATTGCTCTAACTTTA-3' and AllStars Negative Control siRNA in lyophilized forms were procured from Qiagen (Valencia, CA, USA). The siRNAs were reconstituted in RNAse-free water to obtain 20 µM stock solution. Bradford assay kit, nitricellulose membrane (0.45 µm), Tween-20, Pierce ECL western blot detection reagents were from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All the four different monoclonal IgG primary antibodies raised in rabbit for phospho-p42/44, total p42/44, phopho-ERK and total AKT, and the horseradish peroxidase conjugated secondary goat anti-rabbit IgG antibody were from Cell Signaling Technology, Inc. (Beverly, MA, USA).

**Preparation of carbonate apatite NP and complexing with drugs and siRNA for in vitro study.** Synthesis of carbonate apatite NP and their complexing with drugs in presence or absence of **ROS1** siRNA were accomplished according to the protocol described previously (30). Briefly, particles were prepared by mixing 4 mM (4 µl of 1 M stock) of exogenous calcium chloride (CaCl₂) with 1 ml of the freshly prepared DMEM, pH 7.4, followed by incubation at 37°C for 30 min. For complexing of NP with drugs, 4 mM of CaCl₂, and 8 or 40 nM of one of the three drugs (Dox, Cis or Pax) were added with or without 10 nM of **ROS1** siRNA (NP-drug/NP-siRNA-drug) to 1 ml of DMEM media prior to 30 min incubation at 37°C. siRNA-loaded NP (NP-siRNA) was prepared in the same manner, except that no drug was added. Free drugs were maintained in DMEM media without any exogenous CaCl₂ and incubated for 30 min. In another set of experiment, NPs were formed in the presence of 1 µM to 10 nM of negative siRNA. Further growth of particles following the incubation period was prevented by adding 10% FBS. Finally, the cells were treated with the different formulations for a consecutive period of 48 h prior to cell viability assessment by MTT assay and detection of downstream signaling proteins by western blotting.

**Maintenance of mouse breast cancer cell line and cell viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium...**
bromide (MTT). Mouse breast cancer cell line, 4T1 was cultured in 75 cm² tissue culture flasks and maintained in DMEM media (pH 7.4) supplemented with 10% FBS and 1% penicillin and streptomycin antibiotic, in a 37°C incubator humidified with 5% CO₂. One day before the treatment, cells from exponential growth phase were seeded on a 24-well plate with each of the wells allowed to have approximately 5x10⁴ cells. After 24 h, the cells were treated with media only (untreated), NP, free drug(s), NP-drug(s), NP-siRNA or NP-siRNA-drug(s) for two days. After completing the incubation, 50 μl of MTT (5 mg/ml in PBS) was added to each well and the plate was left in the incubator for 4 h for formation of formazan crystals by metabolically active cells. Dimethyl sulfoxide (DMSO) (300 μl) was added by replacing the media and plates were agitated on built-in plate shaker for 20 sec to dissolve the dark blue crystals. For control, cells were kept untreated in media containing no exogenous CaCl₂. Formazan quantification in the form of optical density (OD) was performed at 595 nm wavelength with 630 nm of reference wavelength using a microplate reader. The cell viability for treated cells was calculated using the following equation:

\[
\text{(% of cell viability (CV) } = \frac{\text{OD treated} - \text{OD reference}}{\text{OD untreated} - \text{OD reference}} \times 100
\]

Each experiment was done in triplicate and expressed in graphs as mean ± SD of % of cell viability.

**Formulation of particles for in vivo study.** Particles were formed by mixing of 4 µl of 1 M CaCl₂ in 100 µl of freshly prepared bicarbonate (44 mM) DMEM media and incubating at 37°C for 30 min. For complexing with Dox and siRNA (NP-siRNA-Dox), 0.34 mg/kg of Dox and 50 nM siRNA (20 or 40 nM siRNA was used to prepare complexes for intratumor injection for downstream protein expression study) were used before incubation. NP-Dox and NP-siRNA were prepared in the same way in the absence of siRNA and Dox, respectively. The control and sample solutions (100 µl) were intravenously injected into mice.

**Effects of drug-/siRNA-loaded NP on tumor regression in 4T1-induced breast cancer model.** Twenty-four female Balb/c mice, aged 6–8 weeks, weighing 15-20 g were purchased and maintained in 12:12 light: dark condition by providing them ad libitum chow and water. All experiments were done in accordance with the regulations imposed by Monash University Animal Welfare Committee. Approximately 1x10⁴ 4T1 cells (in 100 µl PBS) were injected subcutaneously on the mammary pad of mice (considered as day 1) and the mice were checked regularly for outgrowth of tumor by touching the area of injection. When the volume of the outgrowth reached an average 13.20±2.51 mm³, mice were grouped in different assemblies (6 mice per group) randomly and treated intravenously (tail-vein) at the right or left caudal vein. The second dose was administered after 3 days from the 1st dose. The gross body weights of mice were monitored and the lengths and widths of the tumors were measured using the vernier caliper in mm scale for 30 days while the mice were monitored for their activities.

The volume of the tumor was calculated using the following formula:

\[
\text{Tumor volume (mm}^3\text{)} = 1/2 (\text{Length} \times \text{Width}^2)
\]

The data are presented here as the mean ± SD of tumor volume from each group.

Separate sets of mice were treated with 100 μl of free/ NP-Dox (0.34 mg/kg), NP-siRNA (20 or 40 nM) and NP-Dox-siRNA intratumorally when the tumor volume reached 75 mm³, with two doses per week over a period of 14 days. After treatment, the mice were sacrificed and tumors were excised and processed for western blot analysis.

**Western blot analysis for PI3K and MAPK pathways in vitro and in vivo.** Cells treated with NP-Dox, NP-Dox-siRNA and NP-Dox-siRNA for two consecutive days were washed in pre-chilled PBS and lysed in lysis buffer (1% NP40, 9.3 mM DTT in PBS, 1:1000 protease inhibitor and 1:100 phosphatase inhibitor) pipetting up and down. The cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C to remove cellular debris. The protein extracts were collected from supernatant and subjected to Quick Start Bradford Protein Assay (according to instructions provided by the manufacturer; Bio-Rad Laboratories, Inc.) for estimating the total proteins. Moreover, the excited tumor tissues from treated mice were lysed in lysis buffer using tissue homogenizer (Omni International, Kennesaw, GA, USA) and centrifuged for 15 min at 15,000 rpm at 4°C to sediment the cellular and tissue debris. Tumor tissue lysate from each group of mice were pooled and protein content was measured by Bradford assay. All the samples were aliquoted and stored at -80°C for further study.

The protein extracts were subjected to SDS-PAGE (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 Fisher Scientific, Waltham, MA, USA) using western blotting semi-dry transfer unit (Hoefer, San Francisco, CA, USA). The membranes were blocked for 1 h at room temperature with TBST (10X buffer; 200 mM Tris base, 1.5 M NaCl in MiliQ water; 0.1% Tween-20 was added and pH was adjusted to 7.5 for 1X buffer)-containing 5% bovine serum albumin (BSA). The membranes were then incubated with one of the four primary antibodies in TBST with 5% BSA overnight at 4°C with gentle shaking. Membranes were washed twice with TBST for 10 min each and further incubated with secondary antibody (1:5000) for 1 h at room temperature. Then the membrane was washed two times for 10 min each with TBST to remove any non-bound secondary antibody. The membrane was incubated with Pierce ECL Western Blotting Detection Reagent (Bio-Rad Laboratories, Inc.) at room temperature for 5 min. Excess detection reagent was drained off and the membrane was exposed to X-ray film placed on top of the membrane for an appropriate time in an X-ray film cassette (Amersham, Piscataway, NJ, USA). Membranes were also tested for equal loading.

**Statistical analysis.** Statistical analysis was done using the SPSS (version 17 for Windows) for in vivo (tumor regression study) data. LSD post-hoc test for one way ANOVA was used.
to analyze and compare the significant difference between different treatment groups. Data were considered statistically significant at p<0.05.

Results

Influences of particle-bound drugs and ROS1 siRNA on cell viability. Fig. 1A depicts the viability of mouse breast cancer (4T1) cells treated with different concentrations of ‘Allstars Negative Control siRNA’ (1 pM to 10 nM) originally used to complex with carbonate apatite NP with apparently no alteration in cell viability when incubated for two consecutive days. The influences of the NP loaded with siRNA against c-ROS1 gene and Dox (NP-siRNA-Dox) on cell viability is shown in Fig. 1B. Treatment of 4T1 cells with the NP formed with 4 mM of exogenous CaCl$_2$ and fixed concentrations of endogenous inorganic phosphate (0.9 mM) and bicarbonate (44 mM) showed 92.84±6.61% of viability compared to the untreated cells and therefore the same conditions were maintained for forming complexes with drugs and siRNA in the subsequent in vitro studies. The NP-siRNA treatment rendered the cells less viable reducing the viability to 75.6±2.61% compared to the untreated cells and therefore the same conditions were maintained for forming complexes with drugs and siRNA in the subsequent in vitro studies. The NP-siRNA treatment rendered the cells less viable reducing the viability to 75.6±2.61% compared to the untreated cells whereas the NP-Dox formulation was found slightly less viable than free Dox at both 8 and 40 nM of drug concentrations. Interestingly, the NP-siRNA-Dox complex formed at 40 nM of Dox markedly reduced the cell viability to 30.93±1.88% compared to the untreated cells. On the contrary, the NP-siRNA-Dox complex formed at 8 nM of the drug did not result in a decrease in cell viability (Fig. 1B).

As shown in Fig. 1C, NP-Cis treatment abated cell viability compared to free Cis predominantly at lower concentration of Cis (8 nM). However, the NP-siRNA-Cis treatment was not accompanied by significant cytotoxicity regardless of the concentrations of the drug used. Moreover, although NP-Pax showed more cytotoxicity than free Pax at both low (8 nM) and high concentration (40 nM) of the drug, treatment of the cells with NP-siRNA-Pax did not demonstrate any significant effect (Fig. 1D).

Figure 1. Effect of NP-bound siRNA and different drug treatments on 4T1 cell viability. Cells treated with (A) NP-bound negative control siRNA from 1 pM to 10 nM; (B) NP-bound Dox, ROS1 siRNA, Dox+ROS1 siRNA (C) NP-bound Cis, ROS1 siRNA, Cis + ROS1 siRNA and (D) NP-bound Pax, ROS1 siRNA, Pax + ROS1 siRNA. Values are presented as the mean ± SD of % of cell viability compared to untreated cells for triplicate samples.

Figure 2. Effects of co-delivery of c-ROS1 siRNA and Dox on AKT and MAPK activation in 4T1 cells. After two days of consecutive treatment, cell lysates were subjected to western blot for detection of (A) phospho- and total AKT or (B) phosphor-p44/42 MAPK (ERK1/2).
Effects of co-treatment of c-ROS1 siRNA and Dox on AKT and MAPK activation. Since the NP complexes of ROS1 siRNA and Dox (40 nM) exhibited a synergistic effect on cytotoxicity in 4T1 cells (Fig. 1B), western blotting was performed to assess the activation status of MAPK or PI-3 kinase pathway by observing the intensity of the phosphorylated form of MAPK or AKT, respectively, following incubation of the cells with the NP-siRNA-Dox complexes for two consecutive days (Fig. 2). As shown in Fig. 2A, treatment of the cells with siRNA-loaded NP (NP-siRNA) reduced the intensity of phosphorylated AKT (Phospho-AKT) (Ser473) compared to the untreated or NP-treated cells, although free Dox and NP-Dox did not change it. Surprisingly, the combined delivery of siRNA and Dox (NP-siRNA-Dox) caused the disappearance of phospho-AKT in the treated cells, while the total AKT level remained unchanged. The combined treatment also decreased the level of phosphorylated ERK1 (42 kDa) protein in MAPK pathway, whereas the effect of free Dox, NP-Dox or NP-siRNA was not so significant (Fig. 2B).

Effects of NP-facilitate combined delivery of c-ROS1 siRNA and Dox on tumor regression. As delivery of siRNA against c-ROS1 gene induces the highest chemosensitivity of 4T1 cells to Dox (Fig. 1B), NP-siRNA-Dox complexes were intravenously administered in mice bearing 4T1-induced breast tumors to confirm antitumor activity of the drug in vivo. Fig. 3 showed the influences of the NP loaded with Dox, siRNA or both on tumor progression over a period of time. The average body weight of mice showed no observable variance with time among the different groups during tumor development and treatment period (data not shown). The tumor was palpable at day 6 to 9 after subcutaneous inoculation of the tumor cells. NP-Dox treated mice showed similar tumor outgrowth with time as the NP-treated mice, whereas NP-siRNA treatment showed relatively slow development of tumors in mice compared to the NP alone, although the difference was not significant except for day 27 (p=0.011). When the mice were treated with Dox- and siRNA-embedded particles (NP-siRNA-Dox), the tumor volumes were observed to drastically decline particularly after the 2nd treatment on day 17 compared to other groups.

Effects of co-delivery of ROS1 siRNA and Dox on AKT and MAPK activation in the tumor. In an attempt to correlate the chemosensitizing effect of ROS1 gene knockdown on substantial tumor regression, to the activation of PI-3 kinase or MAP kinase pathway, western blot analysis was carried out in the tumor lysates, following intratumoral delivery of free Dox, NP, NP-Dox, NP-siRNA and NP-Dox-siRNA. However, unlike the in vitro scenario, the combined delivery of siRNA and Dox in vivo did not successfully block the phosphorylation of ERK.

Figure 3. Effects of co-delivery of c-ROS1 siRNA and Dox on regression of the 4T1-induced breast tumor. Six mice per group were treated intravenously with NP, NP-Dox (0.34 mg/kg), NP-siRNA (50 nM) and NP-siRNA-Dox and data are presented as the mean ± SD of tumor volume. Values are significant when *p<0.05 compared to NP group and #p<0.05 compared to NP-siRNA treated group.

Figure 4. Effects of co-delivery of c-ROS1 siRNA and Dox on AKT and MAPK activation in tumor tissues. After 14 days of intratumor treatment, tumor tissue lysates were subjected to western blotting for detection of (A) phospho- and total p44/42 MAPK (ERK1/2) or (B) phospho- and total AKT.
protein in MAPK pathway (Fig. 4A). On the contrary, the same treatment was found to have an effect on reducing the phosphorylated AKT1 (Ser473) level particularly at higher siRNA concentration (40 nM) compared to NP-siRNA (Fig. 4B), which is consistent with the effect observed in vitro.

**Discussion**

Naked siRNA being anionic in nature is electrostatically repelled by anionic cell membrane and also subjected to degradation by endonucleases present in intracellular acidic vesicles as well as the blood stream, therefore necessitating an efficient carrier to carry and release them to the target site (the cytosol). The pH sensitive inorganic particles of carbonate apatite have been established as smart delivery vehicles to transport siRNAs both in vitro and in animal models for silencing a diverse range of genes (28-30, 33-36). Assessment of cell viability using particle-loaded 'AllStars Negative Control siRNA', a scrambled siRNA that has no target mRNA sequence, demonstrated no apparent cytotoxic effect irrespective of the siRNA concentrations used (Fig. 1A), signifying the potential role of siRNA in gene-specific knockdown and subsequent alteration in cell functionality. The reduction in cell viability upon intracellular delivery of the functionally-validated siRNA targeting ROS1 gene (Fig. 1B) could be due to the knockdown of ROS1 mRNA transcript, thus preventing the production of ROS1 receptor tyrosine kinase and the activation of its possible downstream signaling cascades, such as MAPK or PI-3 kinase pathway. However, NP-assisted delivery of ROS1 siRNA in 4T1 cells resulted in dephosphorylation of AKT, but not MAPK (Fig. 2), suggesting that PI-3 kinase pathway might be a predominant downstream signaling cascade of ROS1 receptor tyrosine kinase in this particular cell line. The absence of dephosphorylated form of AKT1 in tumor lysate upon treatment with NP-siRNA (Fig. 4) could be due to the inefficient knockdown of ROS1 gene owing to the existence of barriers to siRNA delivery in vivo. Similar effect of declining cell viability was observed in MCF-7 cells pre-treated with NP-loaded ROS1 siRNA for 48 h (30). Moreover, ROS1 inhibitor was also found to play an antiproliferative role in lung cancer cells and antitumor activity in lung cancer patients (37,38).

Carbonate apatite particles have been shown in earlier studies to have high binding affinity for structurally different classical drugs and exert more cytotoxic effect and antitumor activity by delivering more drugs in cancer cells through particle-mediated endocytosis in contrast to the free drugs that mainly penetrate the cell membrane via passive diffusion (32,31). However, in this study, NP-Dox treatment did not demonstrate any significant decrease in cell viability compared to free Dox, probably because of the inadequate amount of the drug (8 and 40 nM) used, which was not sufficient enough to effectively kill these aggressive mouse breast cancer cells. However, knockdown of ROS1 gene was found to sensitize the cells to Dox, as evident from the enhanced cytotoxic effect following co-delivery of ROS1 siRNA and Dox with the help of NP (NP-siRNA-Dox). This could be correlated to the substantial decrease in phosphorylated AKT and ERK proteins (Fig. 2) of PI-3 kinase and MAPK pathways, respectively, by the treatment with NP-siRNA-Dox. Despite the apparent inefficacy of ROS1 siRNA to deactivate phosphorylated MAPK, the co-delivery of Dox and ROS1 siRNA suppressed the MAPK activation (Fig. 2), suggesting that Dox and ROS1 gene knockdown might synergize to dephosphorylate MAPK. It was found earlier that inhibition of PI3K/AKT repressed resistance of cells to Dox (39) and activation of MAPK pathway conferred resistance to Dox, as these pathway proteins induce the expression of drug transporter proteins responsible for extruding drugs from inside to outside of the cell and thus, developing drug resistance (40-42). Targeted cleavage of ROS1...
nRNA might not only assist in declining cell proliferation/survival, but also reduce Dox resistance through inactivation of PI-3 kinase and MAPK pathways, thereby rendering the cells more responsive to Dox. Similar findings were observed in vivo with a noticeable and persistent decline in tumor growth following intravenous injection of NP-siRNA-Dox (Fig. 3). However, only the band for phospho-AKT, but not phospho-MAPK disappeared in the tumor treated with NP-siRNA-Dox, which could be explained by the inefficient delivery of the siRNA as well as the drug to the tumor, or the difference in activation level of the two pathways depending on the in vitro and in vivo conditions.

In summary, simultaneous delivery of ROS1 siRNA and Dox using carbonate apatite NPs into 4T1 breast cancer cells and the established tumor in a syngeneic mouse model sensitized the cancer cells, and tumor to Dox (Fig. 5) by silencing the ROS1 gene expression and predominantly downregulating PI-3 kinase pathway, thereby enabling the killing of cancer cells at very low concentrations of the drug. Toxicological and immunological studies in future are needed to confirm the applicability of this combination in breast cancer treatment. This novel approach has extraordinary clinical prospects in tumor therapy to efficiently and selectively destroy cancer cells and circumvent the drug-induced toxicity in normal healthy cells.

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