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Effective response of doxorubicin-sensitive and -resistant breast cancer cells to combinational siRNA therapy

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ABSTRACT

Chemotherapy is an effective approach to curb uncontrolled proliferation of malignant cells. However, most drugs rapidly lose their efficacy as a result of resistance development. We explored the potential of combinational siRNA silencing to prevent growth of drug-resistant breast cancer cells independent of chemotherapy. Resistance was induced in two breast cancer lines by chronic exposure to doxorubicin. Microarray analysis of apoptosis-related proteins showed Bcl2, survivin, NF B, and Mcl1 to be prominently up-regulated in drug-resistant cells. Human siRNA libraries against apoptosis-related proteins and kinases were screened using lipid-substituted polymers as non-viral carrier, and siRNAs were selected to diminish cell growth without affecting growth of skin fibroblasts. Surprisingly, the selected siRNAs led to similar responses in wild-type and drug-resistant cells, despite their phenotypic differences. Promising kinase siRNAs were co-delivered with anti-apoptotic Mcl-1 siRNA and Ribosomal Protein S6 Kinase (RPS6KA5) was found the most promising candidate for simultaneous silencing with Mcl-1. In both MDA435 wild type (WT) and MDA435 resistant (R) xenografts in nude mice, double silencing of Mcl-1/RPS6KA5 also led to improved inhibition of tumor growth in the absence of chemotherapy. We conclude that combinational silencing of well-selected targets could be a feasible therapeutic strategy in the absence of drug therapy and could provide a new avenue for therapy of drug-resistant breast cancers.

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1. Introduction

While chemotherapy is still the dominant approach in malignancies, molecularly-targeted drugs in particular have been found effective in specific malignancies where aberrant mechanisms are specifically modulated by these drugs. However, even the most effective drugs lose their efficacy in advanced cancers as a result of resistance development [1–3]. The inherent plasticity of the transformed cells and diverse mechanisms of drug resistance enable malignant cells to mount an effective response against the drugs [4]. Molecular changes in drug resistant cells are diverse and include over-expression of drug transporters [5], and changes in expression of various kinases and proteins involved in cell survival and apoptosis [6–8]. Coordinated integration of these distinct mechanisms further enhances the resiliency of cells, where the conventional drugs cannot overcome the orchestrated and effectively-executed survival mechanisms [6]. An alternative approach to treatment of chemoresistant tumors could be based on RNAi by using small interfering RNA (siRNA). The siRNA molecules are short (~21 bp) double-stranded oligonucleotides that can bind and prevent translation of a specific mRNA, thereby reducing intracellular abundance of target proteins [9]. With an appropriate delivery system, siRNA treatment was shown to improve therapeutic responses to chemotherapeutic agents in drug-resistant cells, but this was possible with *a priori* selection of targets known to contribute to the drug resistance [10–13]. The siRNA therapy has the potential to control unchecked cell proliferation independent of chemotherapy, but this requires a systematic approach to identify and silence critical targets in drug-resistant cells. Here, we present such an approach to identify potent siRNAs and deliver them using non-viral carriers either alone or in combination to prevent tumor growth. An integrated approach was taken to identify candidate targets with crucial roles in drug resistance and/or in enhanced cell proliferation and survival for siRNA silencing. We hypothesized that combinational silencing of selected proteins could be a novel strategy for treatment of breast cancer cells, even after resistance development to conventional chemotherapy. Our results show that both wild-type

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(WT) and drug-resistant (R) breast cancer cells were similarly responsive to siRNA therapy, whose potency could be enhanced by careful selection of siRNA combinations.

2. Methods

2.1. Materials and cell lines

The sources of cell culture reagents and polymer synthesis ingredients were provided previously [14]. Ultrapure agarose, Taq DNA polymerase, M-MLV reverse transcriptase and RNaseOUT ribonuclease inhibitor were purchased from Invitrogen (Grand Island, NY). The Silencer Human Kinase siRNA Library (AM80010V3), scrambled (control) siRNA (AM4635) and all the siRNAs against the selected kinases were supplied by Ambion (Burlington, Ontario). AllStars Negative siRNA Fluorescein (1027290), the siRNA versus Mcl-1 (SI02781205), RNAlater™, RNeasy Mini Kit and RDD buffer were purchased from Qiagen (Mississauga, ON). ThermoPol Buffer was provided by New England Biolabs (Ipswich, MA). The siGENOME Human Apoptosis siRNA library (G-003905) was supplied by Dharmacon (Waltham, MA). The primers used for the RT-PCR reactions (***SI Appendix:*** **Fig. 8S**) were designed using Primer Express 3.0.1 software and synthesized by the IDT Technologies (Coralville, Iowa). MDA435 cells were kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC) and cultured as described before [14]. MDA231 cells were a generous gift from Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, AB). The cells were cultured in low glucose DMEM (with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37 °C and 5% CO_2, and trypsined (0.05% Trypsin/EDTA) regularly for passage (1:10 dilution).

2.2. Polymer synthesis and characterization

The synthesis of linoleic acid and caprylic acid substituted 2 kDa polyethylenimine (PEI) was described elsewhere [14–16]. The purified polymers were analyzed by ^{1}H-NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shift of lipids (δ ~0.8 ppm; —CH3) and PEI (δ ~2.5 – 2.8 ppm; NH–CH2–CH2–NH–) was integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on the synthesized polymer (***SI Appendix:*** **Table 2S**). The binding efficiency, of the lipid-substituted 2 kDa PEI to siRNA and the properties of the resulting nanoparticles (including size, ζ-potential, stability in serum, and cytotoxicity) were reported previously [14].

2.3. Resistance induction in MDA435 and MDA231 cells

The cells were exposed to DOX, starting from 0.2 and 0.05 μg/mL (~20% of the IC_{50}), and continuing with gradually increasing concentrations up to 2.0 μg/mL. Cells were exposed to each concentration for 3 passages or a week (whichever longer), and frozen at the end of each stage. Cells exposed to the highest DOX concentration (2.0 μg/mL) were maintained in medium containing 0.2 μg/mL DOX for ongoing experiments. Induction and maintenance of resistance were confirmed periodically by evaluating the IC_{50} of DOX in WT and drug-resistant cells via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay.

2.4. Evaluation of viable cell number

The viable cell numbers were evaluated by the MTT assay. After the treatment period (72 h for siRNA silencing and library screening, and 24 h for DOX exposure), 20 μL of MTT solution (5 mg/mL in HBSS) per each 100 μL of medium was added to each well. Plates were incubated at 37 °C for 90 min, after which the medium was removed, and DMSO was added to dissolve the crystals. The optical density of the wells was measured at 570 nm. The results were normalized to untreated cells (taken as 100%) after discounting the reading for the blank (untreated cells in the plate with no MTT solution added, dissolved in DMSO).

2.5. Cellular uptake of siRNA

The polymer/fluorescein-labeled siRNA complexes were added to the cells in 48-well plates in triplicate (36 nM siRNA and 2 μg/mL polymer; polymer:siRNA weight/weight ratio of 4:1). Complexes formed with non-labeled scrambled siRNA were used as negative control. After incubation at 37 °C for 24 h, cells were washed with HBSS (×3), trypsinized, and fixed in 3.7% formaldehyde solution. The siRNA uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel. The percentage of fluorescence-positive cells and mean fluorescence in cell population were determined. Calibration was performed by gating the negative control (cells treated with non-labeled siRNA) to 1–2% of the total cell population.

2.6. Silencing with siRNA

For validation studies, cells were seeded in different plate formats at ~20% confluency (~1.5×10^5 cells/mL) and treated with desired siRNA complexes after 24 h. The plates were incubated at 37 °C for 72 h, before evaluation of silencing efficiency. For combinatorial silencing, polymer/siRNA complexes were prepared as a single polyplex formulation with a mixture of two siRNAs, each contributing half of the final concentration. For comparison purposes, the single target silencings included in these studies were performed with a mixture of scrambled siRNA and the target-specific siRNA.

2.7. siRNA library screenings

Using a PerkinElmer Janus Automated Workstation, cells were seeded in Greiner Bio-One 96-well cell culture plates (90 μL medium per well). After 24 hours, 1.0 μM dilution plate sets were prepared from the 96-well 5.0 μM plates of the Ambion Silencer Human Kinase and Dharmacon siGENOME Human Apoptosis siRNA libraries. Transfections were performed in triplicate by first spotting the plates with 16.2 μL aliquots from either the Kinase or Apoptosis 1.0 μM dilution plate sets (54 nM final siRNA concentration). For the combination of kinase library screen with Mcl-1 silencing, the polypropylene plates received aliquots of the 1.0 μM Kinase library dilution plates as well as aliquots of 1.0 μM MCL-1 siRNA to give a 27nM final concentration of each siRNA. A polymer/saline mixture was added to the siRNA seeded plates and incubated for 30 min at room temperature for complex formation. Finally, 10 μL of the complex solution was added to cells in triplicate. Treated cells were then incubated at 37 °C for 72 h, before the final evaluation with MTT assay. To ensure the efficiency of siRNA silencing using the selected delivery system, Kinesin spindle protein (KSP) and Mcl-1 silencing siRNAs were selected as positive control in apoptosis and kinase libraries, respectively.

2.8. Microarray analysis and q-PCR

Microarray analysis was performed using an apoptosis-related array from Qiagen (RT^2 Profiler™ PCR Array Human Apoptosis; PAHS-012ZC). Cells were lysed with RLT buffer and passed through a shredder, and total RNA was isolated by using the RNeasy Mini Kit (Qiagen). To synthesize the cDNA, 0.5 μg total RNA was reverse transcribed by using random hexamer primer and dNTP mix, and heated at 65 °C for 5 min. Synthesis buffer (5×), DTT (0.1 M), and RNAout RNase inhibitor (1.8 U/μL) were added and the solutions were incubated at 37 °C for 2 min. MMLV RT enzyme was added to the solutions and incubated at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 15 min for cDNA synthesis. Real-time PCR was performed on an ABI 7500 HT with human β-actin as the endogenous housekeeping gene. All the primers

were tested to assure equal efficiency (with a slope < 0.1 for the $\triangle C_T$ vs. cDNA dilution graph; ***SI Appendix:* Fig. 8S B**), and a template concentration of 10 ng/μL was determined as the optimal concentration based on the standard curves. Analysis was performed by calculating $\triangle C_T$, $\triangle\triangle C_T$, and Relative Quantity of mRNA (RQ) using endogenous gene and "no treatment" group as reference points.

2.9. Animal studies

All experiments were performed in accordance with the University of Alberta guidelines for the care and use of laboratory animals. 4–6 weeks old female NCR nu/nu nude mice (Taconic Farms) were kept in a bio-containment unit and ~2 million wild type MDA435 (MDA435WT) or resistant version (MDA435R) cells were injected subcutaneously into the right flank of the mice. Tumor growth was monitored every 48–72 h and volume measurements were performed by a digital caliper. Tumor induction was considered complete when all animals developed a tumor of 50–100 mm^3 (length × $width^2$ × 0.4). Tumor-bearing animals were treated with intratumoral (three injections, 72 h apart, 1.5 μg each siRNA/mouse [~0.06 mg/kg/day], and polymer:siRNA w/w ratio of 8:1) or intraperitoneal (four injections, 72 h apart, 10 μg each siRNA/mouse [~0.5 mg/kg/day], and same polymer:siRNA ratio) injections of PEI-LA2.1/siRNA complexes. Tumor volume was monitored every 72 h, and any mouse with tumors larger than 1000 mm^3 was euthanized for humane considerations. At the end of treatment period, tumors were collected, weighed, and stored in RNAlater® in −20 °C until q-PCR analysis. In the intraperitoneal treatment study, blood samples were also collected via heart puncture for analysis of functional markers of kidney (serum creatinine and urea) and liver (alanine aminotransferase and Gamma-glutamyl transpeptidase), which was performed in the laboratories of University of Alberta Hospital.

2.10. Statistics

The data were presented as mean ± SD and analysed for statistical significance by unpaired Students *t*-test (assuming unequal variance; $\alpha = 0.05$). Pearson's correlation coefficient was calculated where indicated and its significance was determined by the *t*-test. Percentage of Control (POC) was calculated in siRNA library screenings as the percentage of cell growth compared to cells treated with scrambled siRNA. The *z* values were calculated by the following equation:

$$z = \frac{xi - \mu}{s}$$

where x_i is the percentage of the cell growth (compared to "no treatment" cells) for each well, μ is the average of all x_i in the whole plate, and s is the standard deviation for the calculated μ. For inter-screening comparisons for the same library (presented in ***SI Appendix:* Fig. 2S** for apoptosis library, and ***SI Appendix:* Fig. 5S** for the kinase library), the outliers were singled out by selecting the responses with $-1.96 < z < 1.96$. The combinational silencing was considered beneficial and to create an additive effect, when a significant increase was observed in the silencing effect with a combination of siRNAs compared to silencing of each individual target.

3. Results

3.1. Regulation of apoptosis-related genes

Drug resistance was induced in two human breast cancer cell lines, MDA-MB-231 (MDA231) and MDA-MB-435 (MDA435), by exposure to gradually increasing concentrations of doxorubicin (DOX: 0.1 to 2 μg/mL). The resistance induction was confirmed by >50-fold increase in IC_{50} (i.e., concentration for 50% cell death) of DOX in both cell lines: 0.3 vs. ~52 μg/mL for MDA231 cells and 0.7 vs. ~58 μg/mL for MDA435 cells (Fig. 1a). A PCR microarray was used for analysis of mRNA levels of 84 apoptosis-related proteins in wild-type (WT) and drug-resistant (R) cells (Fig. 1b and ***SI Appendix:* Fig. 1S**). Different members of caspase and TNFR families, and FAS were among down-regulated proteins in resistant cells, with a more dominant loss of caspase family in MDA231 cells and TNFR family in MDA435 cells. Proteins expressed preferentially in chemoresistant cells included anti-apoptotic proteins Bcl2, survivin, NF B, and Mcl-1, among which Mcl-1 showed the most significant up-regulation (5.2-fold and 4.5-fold in MDA231 and MDA435 cells, respectively). Among the 84 proteins, 48 proteins displayed similar changes in both cell types, expression of 18 proteins did not change in at least one cell type, and 18 proteins displayed opposite changes in expression (Fig. 1c and ***SI Appendix:* Table 1S**).

It is not known if induction of drug-resistance alters siRNA delivery efficiency to the cells. Using lipid-substituted 2 kDa polyethylenimine (PEI2) [17–19] and FAM-labeled siRNA, we investigated siRNA delivery to WT and drug-resistant cells. The polymers had different substitution levels of linoleic acid (LA) and caprylic acid (CA; ***SI Appendix:* Table 2S**), two lipids previously found to be most efficacious for siRNA delivery [14,17,20]. The results (Fig. 1d) indicated that LA-substitution was more efficient in delivering siRNA to both MDA435 and MDA231 cells. The optimal delivery was achieved at 1.4-2.1 LA/PEI2 for MDA435 cells, while increasing LA substitutions resulted in increasing siRNA delivery in MDA231 cells. More importantly, siRNA delivery in drug-resistant cells was equal to or even higher than the WT cells for most of the evaluated polymers.

3.2. Screening siRNA libraries for effective targets

Given the significant changes in apoptosis-related proteins with induction of drug resistance, a library of 446 apoptosis-related siRNAs (***SI Appendix:* Table 3S**) were screened with MDA435 cells to identify targets, silencing of which would retard cell growth in the absence of drugs. We used human skin fibroblasts as a control to eliminate siRNAs affecting normal cells (siRNA delivery with 2.1 LA substituted PEI2). The ability of siRNAs to retard growth was significantly lower in skin fibroblasts (80–100% viable cell number, as a percentage of control siRNA; no toxicity was observed for the carrier), while growth suppression was readily seen in MDA435 cells with numerous siRNAs (Fig. 2ai). Using the lowest level of viable cell number in fibroblasts as a cut-off, 316 siRNAs were found to retard growth in MDA435 cells, while 381 siRNAs were effective in MDA435R cells (Fig. 2aii). The correlation in the viable cell number of treated MDA435 and MDA435R cells is summarized in Fig. 2b, where a significant correlation was evident ($r = 0.68$, $p < 0.0001$). Among the targets investigated, 97.2% of siRNAs led to equipotent response in both WT and drug-resistant cells ($-1.96 < z < 1.96$), while WT cells displayed greater sensitivity to a small set of siRNAs ($n = 12$, ***SI Appendix:* Fig. 2S**). The critical targets that retarded cell growth in at least one of the 3 cell types are summarized in Fig. 2c. Among the effective siRNAs were BIRC7, NF B and Mcl-1 siRNAs, whose targets were over-expressed in both MDA435R and MDA231R cells.

We next explored the possibility of enhancing siRNA-mediated cell death with dual silencing, with the purpose of identifying more potent target combinations. We chose to combine Mcl-1 siRNA (i.e., most up-regulated target after drug resistance and an effective siRNA in library screen) with siRNAs from a kinase library composed of 719 siRNAs (***SI Appendix:* Table 4S**). As in apoptosis library, MDA435 cells were generally more responsive to kinase silencing than the fibroblasts (***SI Appendix:* Fig. 3S**) and the response of MDA231 cells to kinase siRNAs was relatively less as well (Fig. 3ai). Combining Mcl-1 siRNA with the kinase siRNAs did not affect viable cell number in many cases ($n = 681$ siRNAs; 94.7% of library; $-1.96 < z < 1.96$), but some kinase siRNAs decreased ($n = 19$) or increased ($n = 19$) viable cell number when combined with the Mcl-1 siRNA (Fig. 3aii, ***SI Appendix:* Fig. 4S**). Combining kinase siRNAs with Mcl-1 led to ~2-fold impact on viable cell number

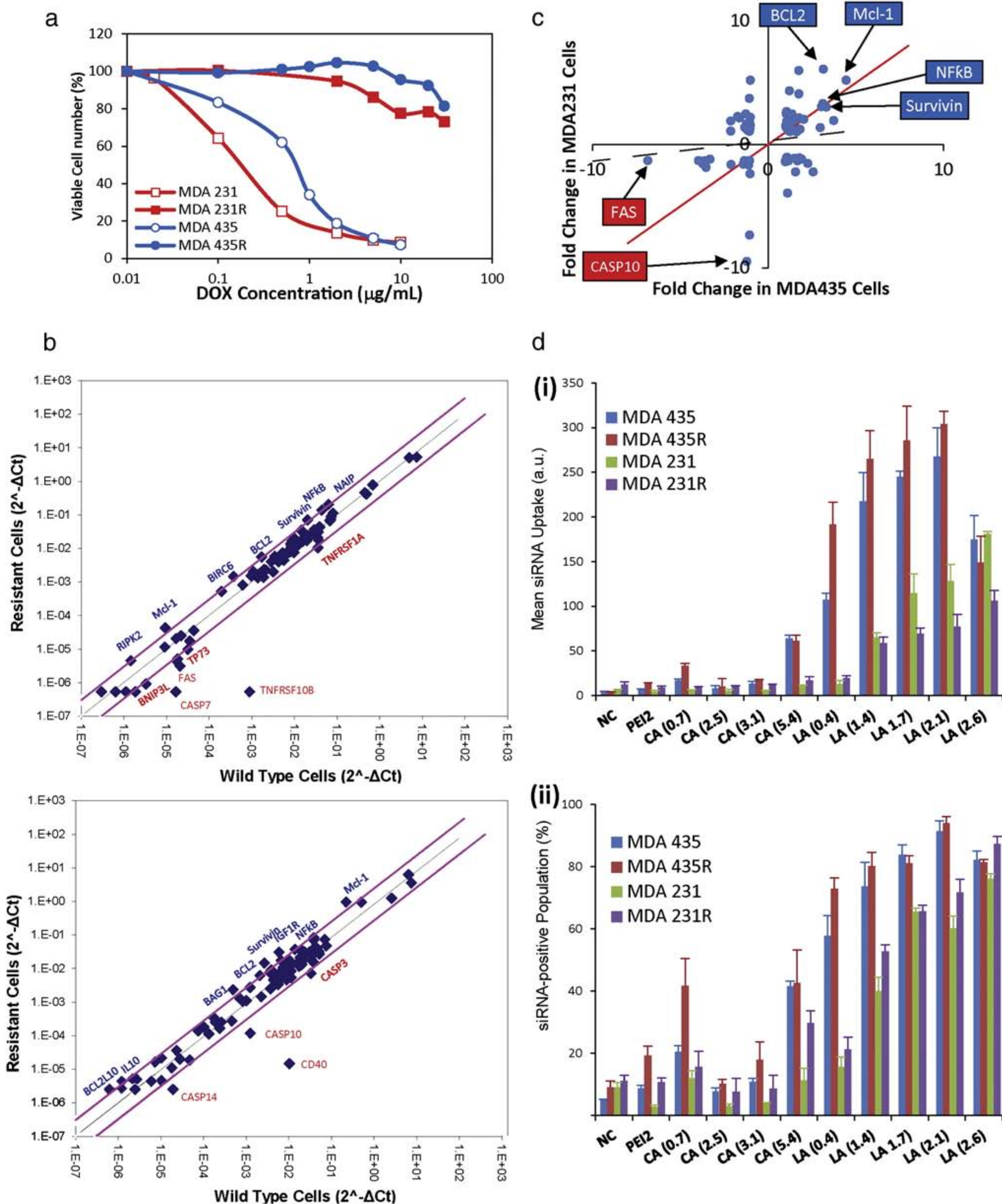


Fig. 1. Drug resistance induction and siRNA delivery to resistant cells. a, Evaluation of viable cell number to determine IC_{50} of DOX in wild-type and resistant MDA435 and MDA231 cells. Both cell lines showed >50-fold increase in IC_{50} as a result of exposure to DOX. **b,** Microarray analysis of apoptosis-related proteins in wild-type and drug-resistant MDA435 (i) and MDA231 (ii) cells. Select proteins are highlighted in the graph. The expression levels for specific proteins included in this analysis are shown in ***SI Appendix:*** **Fig. 1S** and **Table 1S**, respectively. **c,** Correlation for changes in the expression level of evaluated proteins in selected cells. Solid line represents the equal change line, while the dotted line represents the regression line for the actual expression values obtained ($r = 0.55$). **d,** Cellular delivery of FAM-labeled siRNA in breast cancer cells using several lipid-modified PEIs, as the mean fluorescence (i) and the percentage of siRNA-positive cells (ii). The uptake level was higher in LA-substituted PEIs compared to CA-substituted PEIs. The highest delivery was achieved with 2.1 LA/PEI substitution and the efficacy of siRNA delivery was not decreased by the induced drug-resistance.

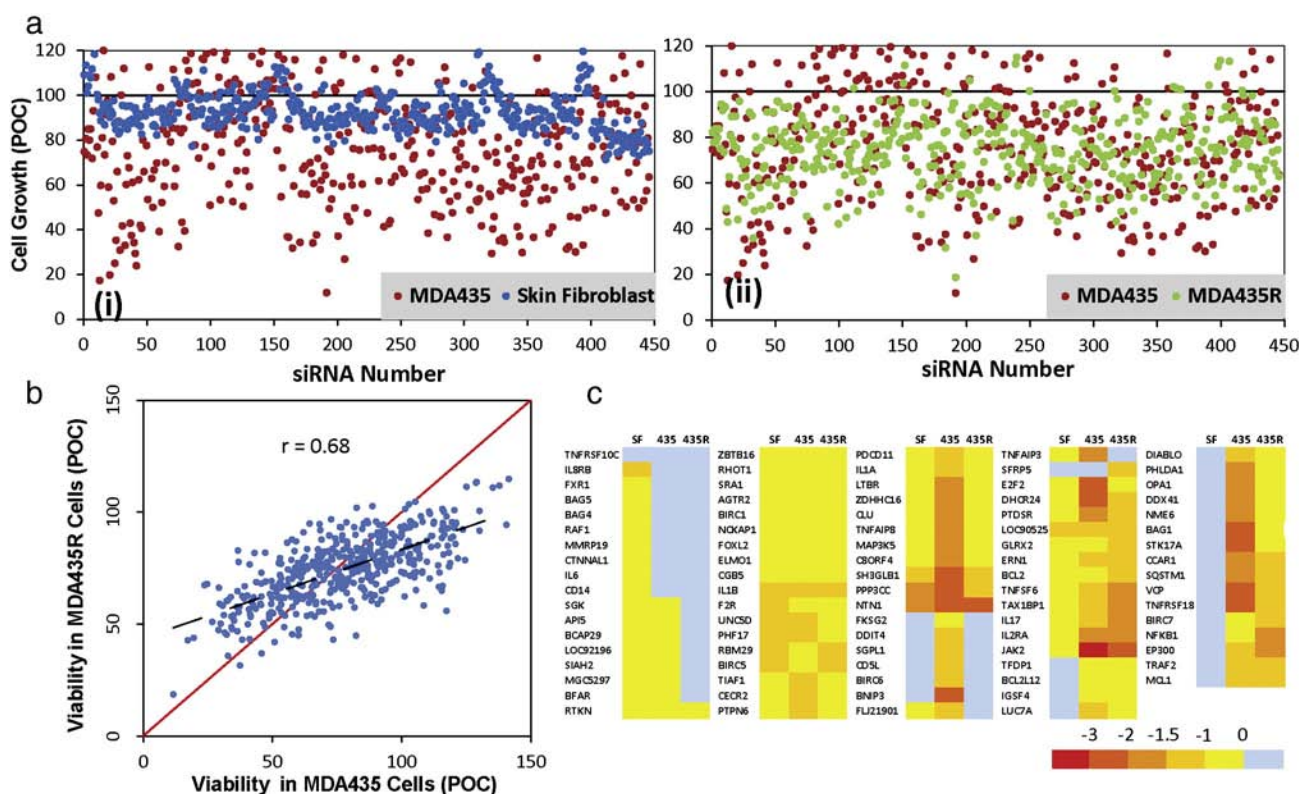


Fig. 2. Apoptosis siRNA library screen. a, The viable cell number, expressed as percentage of control (POC; calculated as percentage of viable cells treated with a specific siRNA compared to scrambled siRNA treated cells), for the 446 siRNAs in the library (***SI Appendix:*** **Table 3S**) in MDA435 and skin fibroblasts (i), and MDA435 and MDA435R cells (ii). The siRNA concentration used was 54 nM. **b,** The correlation between the viable cell number of MDA435 and MDA435R cells after exposure to siRNAs from apoptosis library. A significant correlation in viable cell number was observed and the ratio of MDA435R:MDA435 cell viabilities (***SI Appendix:*** **Fig. 2S**) was not significantly different from 1 for 97.2% of the siRNAs in the library, indicating similar efficacy of siRNA silencing in wild-type and drug-resistant cells. **c,** Heat map for the siRNAs that induced significant cell death ($z < -1$) in at least one cell line. Mcl-1 (among others) significantly reduced viable cell numbers in both wild-type and drug-resistant cells, without showing the same effect in skin fibroblasts.

under best conditions (i.e., ratio of ~0.5 in ***SI Appendix:*** **Fig. 4S**). Among the latter siRNAs, the kinase RPS6KA5 siRNA had no effect on its own, but gave the most additive effect with the Mcl-1 siRNA. The kinase siRNAs that led to significant reductions in cell growth are shown in Fig. 3b. Eight kinases (***SI Appendix:*** **Fig. 5S**) were evaluated further based on the following criteria; (i) only effective in MDA231 cells: *TESK1*, (ii) effective (z-value $= -3$) in MDA435 cells with or without Mcl-1 silencing: *PIK3CB* and *MAP2K3*, (iii) effective in both MDA231 and MDA435 cells with or without Mcl-1 silencing: *STK6, PRKCD, PCTK3* and *PASK*, and (iv) ineffective in MDA435 cells without Mcl-1, but effective in combinational silencing: *RPS6KA5*.

3.3. Validation of siRNA therapy in sensitive and resistant cells

Using these siRNAs, *in vitro* silencing with single or dual delivery was performed to validate the selected targets. The siRNA concentrations used in these studies (18–36 nM) were significantly lower than the screens (54 nM) to better identify the potent siRNAs. With single delivery, MAP2K3 siRNA induced the most significant response in MDA435 and MDA435R cells (Fig. 4a). A significant correlation in siRNA response was evident between the two cell types upon single siRNA delivery (Fig. 4ci). Combinational delivery was then attempted with selected kinases and Mcl-1 siRNA, while further lowering the concentration of individual siRNAs (9 to 27 nM) and formulating the siRNAs with the polymers at 1:2, 1:4 and 1:8 siRNA:polymer ratios. A greater response was observed in this case, with most significant response seen for Mcl-1/RPS6KA5 combination (Fig. 4b). While Mcl-1/MAP2k3 combination was also efficient; however, the significance of the synergistic effect was lower due to the robust effect of MAP2K3 silencing alone. A strong correlation was again evident for combinational silencing between the two cell types (Fig. 4cii). Increasing the siRNA concentration and polymer:siRNA ratio both increased the functional effect obtained (***SI Appendix:*** **Fig. 6S**). The silencing effect with combinatorial delivery was also evaluated at the mRNA level (see ***SI Appendix:*** **Fig. 7S** for primer sequences and PCR validation); relative quantities (RQ) of target mRNAs were ~40% and ~20% for 9 nM/9 nM and 18 nM/18 nM treatment doses, respectively (Fig. 4d, ***SI Appendix:*** **Fig. 8S**). There was no significant effect on target mRNA levels when a particular siRNA was delivered individually or in combination (unlike the additive effect observed on functional activity, inhibition of cell growth). A combinational silencing experiment was also performed in MDA231 and MDA231R cells (Fig. 4e). The obtained response was to a lower extent than the effect on MDA435 cells, which was consistent with the results from the kinase library screen. A strong correlation in the outcome of siRNA treatment was again noted between the MDA231 and MDA231R cells ($r = 0.90$, $p < 0.0001$; ***SI Appendix:*** **Fig. 9S**).

3.4. Combinational siRNA therapy in vivo

We further evaluated our approach by investigating the combinational delivery of siRNAs to breast cancer xenografts in nu/nu mice. We chose to undertake simultaneous delivery of Mcl-1 and RPS6KA5 siRNAs since this combination provided the most additive response *in vitro*, and the siRNA delivery was evaluated both as intratumoral and intraperitoneal treatments. The injected dose of siRNAs was exceptionally low: 1.5 μg/mouse for intratumoral delivery and 10 μg/mouse for intraperitoneal injections. By comparison, investigational siRNAs are usually tested at 5–10 μg doses for intratumoral delivery or at 60 μg for systemic delivery [15]. Volumetric tumor measurements were performed before and during the treatments, and the extracted

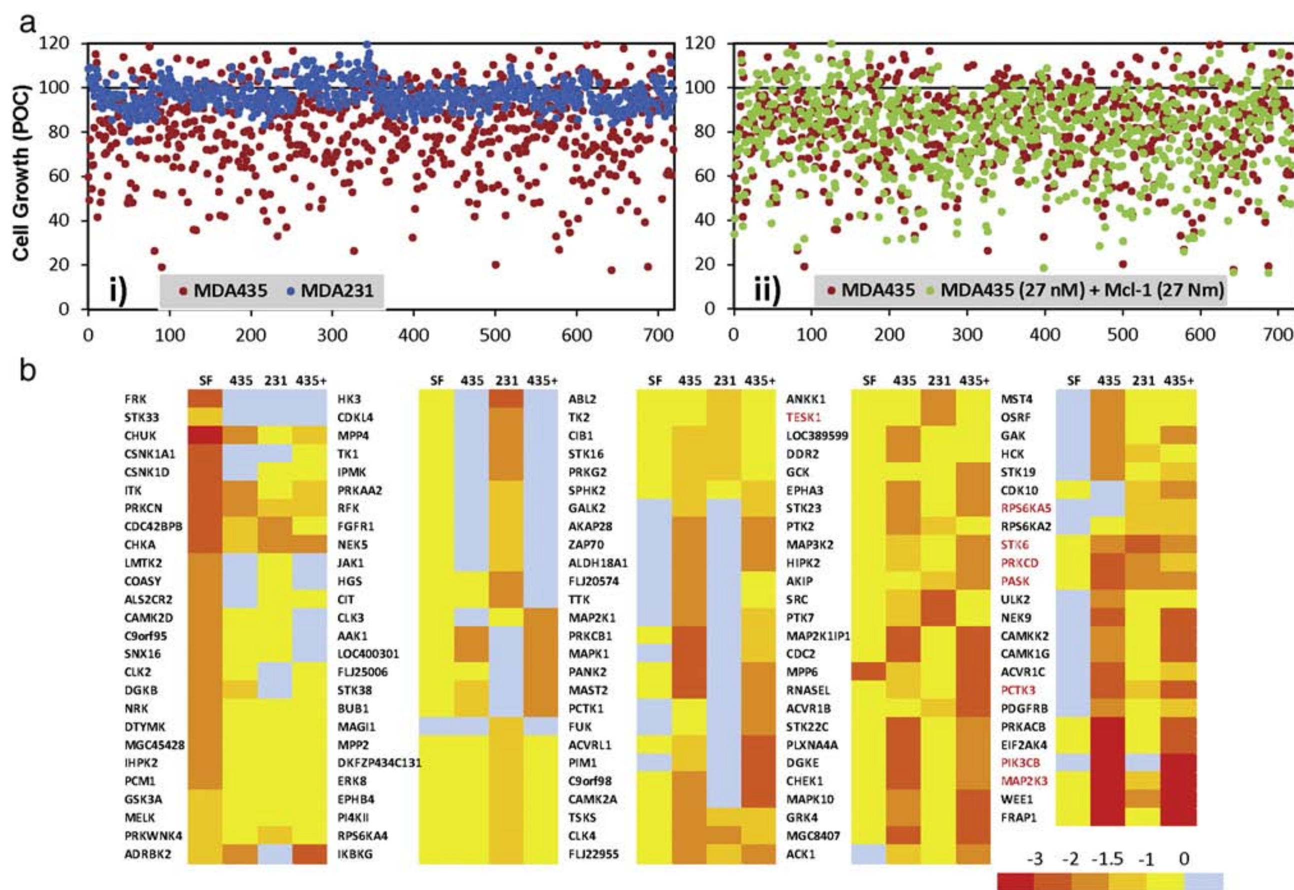


Fig. 3. Kinase siRNA library screen. a, The viable cell number (in POC) after treatment with the 719 siRNAs in the library (***SI Appendix: SI Appendix:*** **Table 4S**) in MDA435 and MDA231 cells (**i**), and MDA435 cells treated without and with Mcl-1 combination (**ii**). The siRNA concentration was either 54 nM for single treatments (**i** and ***SI Appendix:*** **Fig. 3S**), or 27 nM for kinase siRNAs with 27 nM for Mcl-1 siRNA (**ii**). **b,** Heat map for the siRNAs that induced significant cell death ($z < -1$) in at least one screen. Selected siRNAs are ranked from left to right based on the suitability of their effect (with the most appropriate siRNAs on the right column). Eight siRNAs selected for further validation are highlighted with the red font. The cell viabilities obtained for the selected targets and their background information are summarized in ***SI Appendix:*** **Fig. 5S**.

tumors were weighed for end point evaluation, which confirmed the accuracy of volumetric measurements with a correlation factor of >0.8 at this time point (***SI Appendix:*** **Fig. 10S**). With intratumoral treatments in MDA435WT tumors, no effects of scrambled and RPS6KA5 siRNAs were evident on tumor volumes (Fig. 5ai). Delivery of Mcl-1 siRNA alone was effective in significantly reducing tumor volume after day 16 ($p < 0.05$ vs. scrambled siRNA); however, delivering both Mcl-1 and RPS6KA5 siRNAs showed a more potent response, reaching significance after 13 days ($p < 0.005$ vs. scrambled siRNA). The explanted tumor weights further confirmed this observation (Fig. 5aii). Similar results were obtained with intraperitoneal siRNA delivery to MDA435WT tumors; (i) the tumor growth was retarded with Mcl-1 treatment but did not reach significance on day 17, and (ii) Mcl-1/RPS6KA5 siRNA co-delivery gave significant reduction in tumor volume after day 13 ($p < 0.05$; Fig. 5bi), which was confirmed with the explanted tumor weights (Fig. 5bii). The effect of siRNA treatment was also evident at the xenograft mRNA levels (Fig. 5c). The RQ of target mRNAs was lower in the intratumoral delivery compared to peritoneal delivery, in line with the extent of functional tumor responses observed between the two delivery modes. The siRNA silencing did not affect the weight gain of treated mice (***SI Appendix:*** **Fig. 11S**) and selected serum markers did not show any signs of nephrotoxicity or hepatotoxicity after systemic treatment (***SI Appendix:*** **Table 5S**).

In order to further compare the efficiency of siRNA silencing in MDA435 and MDA435R tumors, the intratumoral injections were repeated for the scrambled siRNA, Mcl-1, and Mcl-1/RPS6KA5 siRNA groups in MDA435 and MDA435R xenografts (Fig. 5d). A similar response was observed in relative volumes in MDA435 tumors as the previous experiment, where the combinational delivery was most potent in retarding the tumor growth. Moreover, MDA435R tumors responded in almost identical way to Mcl-1 silencing alone and the dual Mcl-1/RPS6KA5 silencing (Fig. 5di). The q-PCR analysis of the mRNA levels of the target proteins also confirmed the similar silencing efficiency in the two types of tumors (Fig. 5dii).

4. Discussion

We previously demonstrated the effectiveness of lipid-modified PEIs for delivery of selected siRNAs in *in vitro* models [14,17,20]. In the present study, we report for the first time that siRNA delivery using the lipid-modified PEIs is unaffected by resistance induction due to chronic exposure of cells to an anticancer agent. We identified further targets for single and combinational silencing to impact cell growth in drug sensitive and resistant breast cancer cells. After establishing resistance in two breast cancer cell lines, microarray PCR analysis identified the familiar effectors of apoptosis, namely caspases, FAS, and TNFR receptors, to be down-regulated. Down-regulation of caspase 3 is reported to contribute to drug resistance [16], and a correlation between caspase 9 and caspase 10 levels and sensitivity to DOX-induced apoptosis was observed in 18 breast cancer cell lines [21]. FAS and caspase 3 expressions have been also correlated with DOX resistance in highly proliferative leukemia cells [22] and TNFR death receptors' role in apoptosis has been reported [23]. Mcl-1 was one of the most significantly up-regulated proteins in our study (considering both cell lines), over-

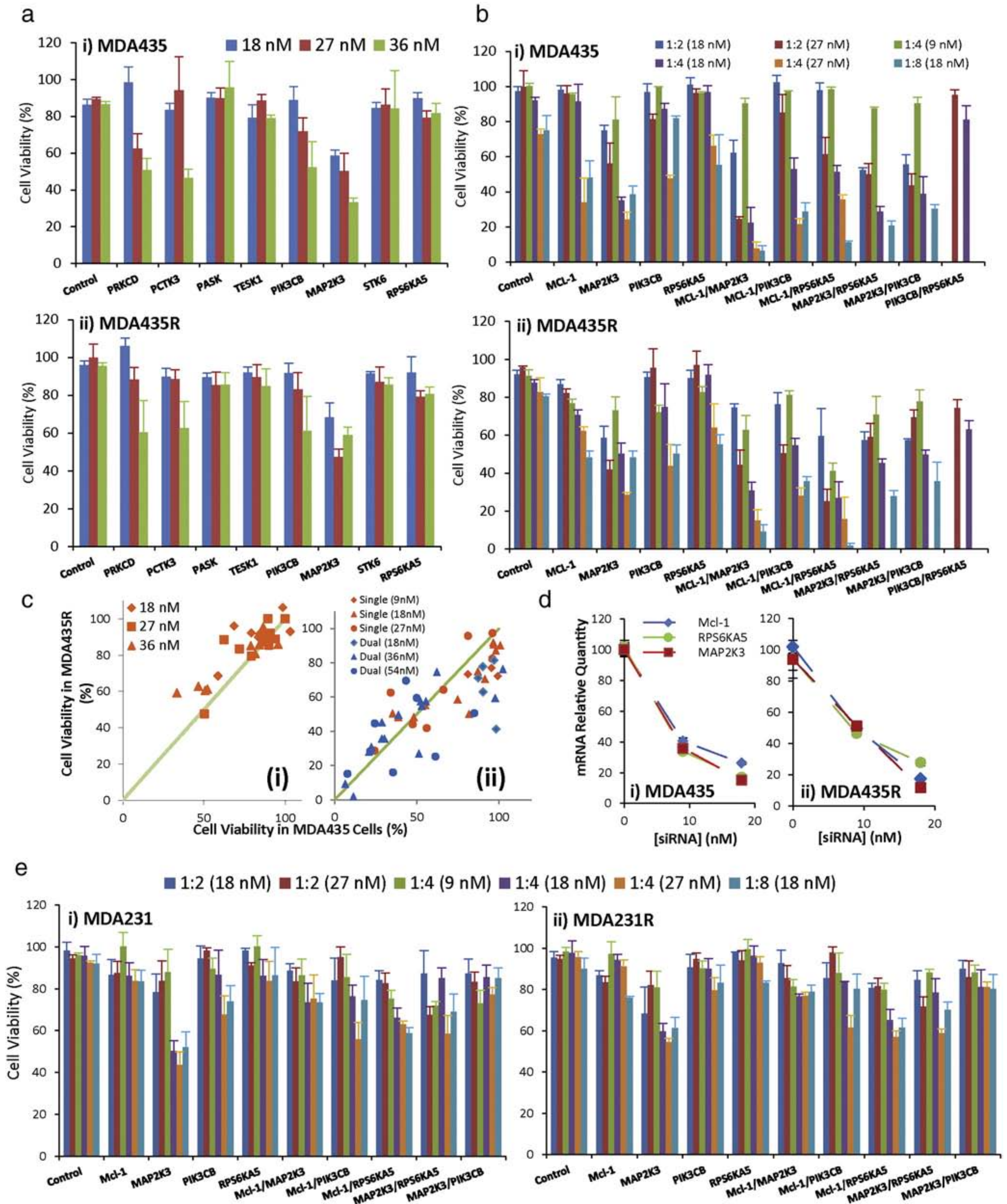
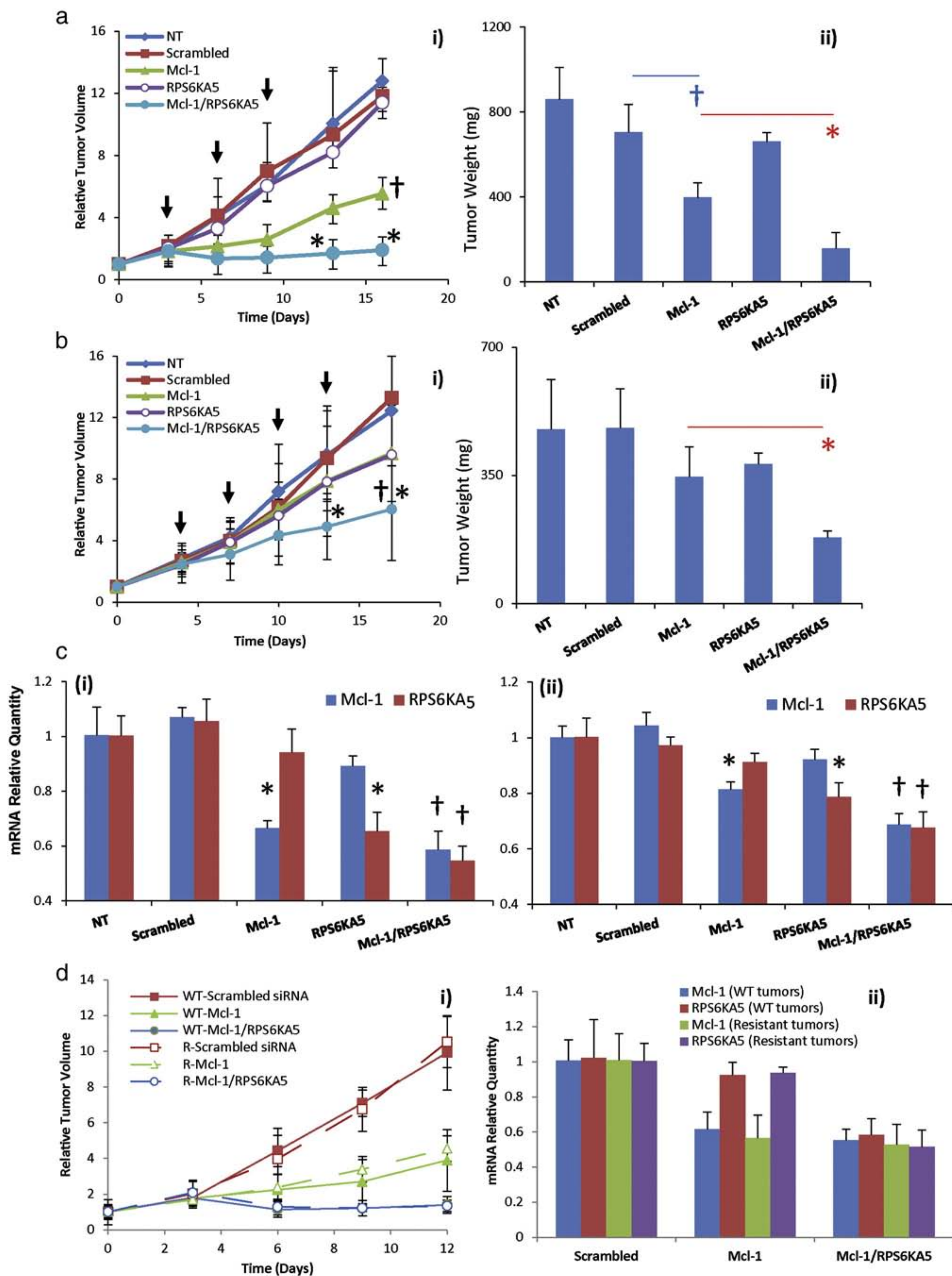


Fig. 4. ***In vitro*** **single and combinational siRNA delivery in wild-type and drug-resistant cells. a,** Validation of single siRNA silencing of selected kinases in wild-type (**i**) and drug-resistant (**ii**) MDA435 cells. Most significant reduction of viable cell number was observed with MAP2K3 siRNA in both cell lines. **b,** Combinational silencing in wild-type and drug-resistant MDA435 cells using different siRNA concentrations (9 to 27 nM for each siRNA) and different siRNA:polymer weight ratios (1:2, 1:4 and 1:8). Combination of Mcl-1 and RPS6KA5 showed the most significant additive effect compared to silencing of individual targets. **c,** Correlations between the silencing effects of individual (**i**) and dual siRNAs (**ii**) in MDA435 and MDA435R cells. A strong correlation between the two cell types was observed in both silencing strategies. The dose–response and the effect of siRNA:polymer ratio are summarized in ***SI Appendix:*** **Fig. 6S**. **d,** mRNA levels of selected targets after dual silencing, as determined by q-PCR (primer information and validation, as well as the complete results including study groups are summarized in and ***SI Appendix:*** **Figs. 7S** and **8S**, respectively). While significant drop in expression level of all selected targets was observed with 9 and 18 nM siRNAs, the overall results followed a similar pattern in both MDA435 (**i**) and MDA435R (**ii**) cells. **e,** Combinational silencing in MDA231 and MDA231R cells using the similar study groups employed for MDA435 cells in part **b**. The overall effect of siRNA silencing on viable cell number was not as significant as the effect observed in MDA435 cells, which was consistent with results from the kinase library screen. The correlation between the silencing effects of dual siRNAs in wild-type and drug-resistant MDA231 cells is summarized in ***SI Appendix:*** **Fig. 9S**.


a
i)
NT
Scrambled
Mcl-1
RPS6KA5
Mcl-1/RPS6KA5
Relative Tumor Volume
Time (Days)
ii)
Tumor Weight (mg)
b
i)
ii)
c
(i)
(ii)
mRNA Relative Quantity
d
i)
WT-Scrambled siRNA
WT-Mcl-1
WT-Mcl-1/RPS6KA5
R-Scrambled siRNA
R-Mcl-1
R-Mcl-1/RPS6KA5
ii)
Mcl-1 (WT tumors)
RPS6KA5 (WT tumors)
Mcl-1 (Resistant tumors)
RPS6KA5 (Resistant tumors)

expression of which was reported in solid tumors [24–26], as well as being implicated in chemoresistance [27]. The differential responses in CD40 and CD40LG observed in our cells (***SI Appendix:*** **Table 1S**), and significant up-regulation of BAG1, IGF1R and IL10 in MDA231 warrant further investigation, since their roles in chemoresistance have not been elucidated.

Employing a siRNA library against apoptosis proteins identified ample targets that were effective in reducing cell growth in the absence of drug treatment. Some differences between the responses of WT and resistant cells were observed against the delivered siRNAs, but the overall siRNA response in resistant cells was similar to the WT cells despite >50-fold increase in IC_{50} values for DOX. This was consistent with the unaltered siRNA delivery observed despite the significant phenotypic changes induced by the drug resistance. BIRC7, NF B and Mcl-1 were among the "hits" in both WT and resistant cells. In addition to their role in chemoresistance [28], inhibitors of apoptosis proteins including BIRC7, could activate NF B [29], which can block apoptosis, enhance proliferation [30] and induce carcinogenesis [31]. A transcription factor-binding site for NF B was even reported in the Mcl-1 promoter region [27], so that this set of functionally-integrated proteins appears to be involved not only in induction of drug resistance, but also in subsequent cellular survival in the absence of drug. On the other hand, kinase library screenings identified not only kinases that were effective in retarding breast cancer cell growth on their own, but also potential targets that could enhance the therapeutic effects of silencing anti-apoptosis proteins. Targeting two distinct mechanisms (namely apoptosis regulators and kinases that ubiquitously regulate cell proliferation and survival) was intended to identify synergistic combinations more powerful than any single target alone.

Among the targets we selected and validated, PI3KCB silencing was attempted before to control glioma cell growth *in vivo* [32] and STK6 silencing induced apoptosis in rhabdoid tumor cells *in vitro* [33]. We were, however, particularly interested in investigating the synergistic effects of selected targets with combinational silencing. Detailed connections among the chosen targets were not explored in this study, but others reported links between the Mcl-1 and AKT pathway [34,35], MAP kinase family, and protein kinase C [36]. Our *in vitro* experiments with selected targets revealed a potential additive effect (even with doses as low as 9-18 nM) for Mcl-1 and RPS6KA5 simultaneous silencing. Involvement of ribosomal protein S6 kinase family in regulation of cell growth [37], as well as resistance to tamoxifen therapy in hormone-sensitive breast tumors [38], has been reported in literature. No direct connection between RPS6KA5 and Mcl-1 is reported in the literature; however, Anjum & Blenis have reviewed a wide range of substrates for the family of RPS kinases that included cAMP response element-binding protein (CREB; transcription factor linked to cell survival), NF B, eIF4B, MYC, and c-FOS [39] which are all linked (directly or through other signaling factors) to increase in cell survival and/or proliferation, including Mcl-1. While silencing RPS6KA5 individually did not show a significant impact on the cell survival in the cell lines selected for this study, the significant improvement of Mcl-1 silencing when combined with RPS6KA5 silencing confirmed that combining carefully selected siRNAs in this study provided potent siRNA cocktails that were effective at low concentrations despite induction of drug resistance in the targeted cells.

In vivo results corresponded to the results in cell culture in showing functional delivery to be possible with the non-viral delivery approach using lipid-modified PEIs. The efficacy of siRNA therapy was expectedly lower with systemic administration as compared to intratumoral treatment; however, combinational silencing of Mcl-1 and RPS6KA5 still retarded the tumor growth during the treatment period. While tumor suppression with Mcl-1 siRNA has been reported in BALB/c nu/nu xenografts of human epithelial carcinoma KB cells [40], combinatorial siRNA delivery *in vivo* has not been reported before. The changes in animal weights and selected physiological markers after siRNA therapy indicated no obvious adverse effects, confirming the safety of the siRNAs and selected delivery system in the mouse model employed. The low dose of siRNA employed here (e.g., 0.06 mg/kg/day vs. 0.14 mg/kg/day Mcl-1 siRNA in Chang et al. report) [40] and the correspondingly low amount of polymeric carrier needed for effective delivery were likely contributing factors to the lack of adverse events. Finally, similar results obtained in tumors induced using wild type and resistant cells are further evidence that that this therapeutic strategy based on siRNA silencing might not be affected by previous exposure to traditional anticancer agents such as doxorubicin.

5. Conclusion

We conclude that siRNA silencing could be an effective therapeutic strategy, independent of chemotherapy and especially in breast cancers resistant to conventional drugs. A significant potential use of combinational silencing was identified after careful selection of targets. While combinational drug strategies have been the mainstay of clinical cancer chemotherapy, combinational delivery of siRNA is yet to be explored. This approach may not only lead to more effective treatment strategies, but also help to better understand connections among different mechanisms of drug resistance and cell survival.

Conflict of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.08.012.

Fig. 5. ***In vivo*** **siRNA therapy. a,** MDA435WT xenografts in nu/nu nude mice were established and then treated with 3 intratumoral injections (72 h apart) of siRNA formulations (1.5 μg each siRNA/mouse per injection or ~0.06 mg/kg/day; $n = 5$–6). Changes in tumor volumes (Relative Tumor Volume = tumor volume at any time point [mm^3]/initial tumor volume [mm^3]) (**i**) and weight of extracted tumor mass at the end of treatment period (**ii**) indicated significant retardation of tumor growth with Mcl-1 siRNA (compared to scrambled siRNA; †), and Mcl-1/RPS6KA5 dual siRNAs (compared to Mcl-1 siRNA; *). The correlation between the volume and explanted tumor weights at the end of treatment period is in ***SI Appendix:*** **Fig. 10S**). **b,** A similar trend was observed when the same MDA435WT xenografts were treated with four intraperitoneal injections (72 h apart) of the same siRNA formulations (10 μg each siRNA/mouse per injection or ~0.5 mg/kg/day; $n = 5$–6). However, Mcl-1 silencing alone was not significantly different from the scrambled siRNA delivery during the study period, and Mcl-1/RPS6KA5 dual silencing achieved significant tumor retardation only compared to scrambled siRNA group (*). Black arrows mark the injection day in both treatment strategies. **c,** Targeted mRNA levels were determined in both intratumoral (**i**) and intraperitoneal treated (**ii**) tumors by using q-PCR. A significant drop in targeted mRNAs was observed in all treatment groups; however, the level of target silencing was more significant in intratumoral treatment. **d,** head-to-head comparison of *in vivo* siRNA silencing efficiency in wild type and resistant cells; while a similar trend was observed in the response of MDA435WT cells, the similar response of wild type and resistant cells, analyzed by tumor volume analysis (i) and qPCR (ii), further indicated that siRNA silencing of the selected targets is not affected by development of DOX resistance in MDA435 cells.

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