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ORIGINAL ARTICLE

Polymeric delivery of siRNA for dual silencing of Mcl-1 and P-glycoprotein and apoptosis induction in drug-resistant breast cancer cells

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Enhanced survival mechanisms of malignant cells in combination with elevated levels of drug transporters can sustain an undesirable resistance against drug therapy. Short interfering RNA (siRNA) delivery against targets involved in aberrant mechanisms is a promising approach and we hypothesize that simultaneous silencing of multiple targets could prove more advantageous than common approach to silence individual targets. To explore this approach, we targeted anti-apoptotic proteins myeloid cell leukemia 1 (Mcl-1) and survivin along with the efflux pump P-glycoprotein (P-gp) in drug-resistant breast cancer cells. Polymeric siRNA delivery was employed for this purpose by using small polyethylenimine (PEI) substituted with lipids. While silencing Mcl-1 caused ~90% cell death in wild-type cells, this effect was less significant in P-gp over-expressing cells. An additive effect for Mcl-1 and P-gp silencing was evident in the latter cells, where simultaneous silencing of these targets created a significantly higher effect compared with silencing each individual target. Prolonged exposure of wild-type cells to doxorubicin (DOX) resulted in upregulation of P-gp, breast cancer resistance protein, survivin and Mcl-1. Dual silencing of P-gp and Mcl-1 again resulted in an additive effect in resistance-induced cells, which displayed an increased dependency on Mcl-1 for survival. Cytotoxic effect of DOX was also enhanced in resistance-induced cells after silencing Mcl-1. We conclude that polymer-mediated siRNA delivery can silence multiple targets simultaneously and reverse drug resistance.

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Keywords: lipophilic polymers; Mcl-1; multidrug resistance; P-glycoprotein; siRNA delivery

INTRODUCTION

Drug resistance is a major cause of failure of chemotherapy in cancer, where the malignant cells frequently become resistant to multiple drugs with unrelated molecular structures. The overexpression of membrane efflux proteins, such as P-glycoprotein (P-gp; *MDR1*), which deports different drugs out of cells, has a major role in drug resistance.[1] However, P-gp inhibition by conventional medicinal agents has shown little benefit in overcoming drug resistance in clinical studies,[2,3] which is indicative of involvement of additional or alternative mechanisms in alleviating cytotoxic effects of the drugs. Several studies suggested a shift in the intracellular balance between pro-apoptotic and anti-apoptotic proteins contributing to drug resistance, including drug resistance in breast cancer cells.[4] Inhibitors of apoptosis proteins, including survivin[5] and Bcl-2 protein family such as Bcl-2, Bcl-XL and myeloid cell leukemia 1 (Mcl-1),[6] are among the anti-apoptotic proteins overexpressed in malignant cells and have been shown to correlate with multidrug resistance.[7,8] The anti-apoptotic mechanisms have been previously linked to drug transporter activity; P-gp overexpressing LR73 fibroblasts, in addition to displaying drug resistance, were shown to resist apoptosis arising from serum starvation.[9] P-gp was suggested to interfere with Fas- or tumor necrosis factor-α-induced cell death mediated by the caspases.[10,11] Co-expression of P-gp and Bcl-x[12] and a drug-independent role for P-gp to inhibit apoptosis were shown *in vitro* in acute myeloid leukemia cells.[13]

To eliminate drug resistance in malignant cells, it is necessary to silence the expression of aberrant molecules responsible for drug resistance. Specific silencing of proteins could be achieved via short interfering RNA (siRNA), but the highly labile and anionic siRNA is ineffective on its own as siRNA binding to cell-surface membranes and subsequent internalization is not possible. Although efficient RNA interference could be achieved with viral vectors, the clinical drawbacks associated with viral vectors makes non-viral approaches to siRNA delivery more desirable. Cationic polymers can assemble the anionic siRNA into nanoparticles via ionic interactions with the siRNA and offer safe alternatives for clinical use. We recently employed a low molecular weight (2 kDa) polyethylenimine (PEI) to deliver siRNA against mediators of drug resistance after substituting lipid moieties on the polymer. Lipid substitution enhanced the assembly (ζ-potential) and hydrophobicity of the nanoparticles, allowing efficient silencing of drug transporters P-gp[14] and breast cancer resistance protein (BCRP),[15] and the anti-apoptotic protein surviving[16] in different cell lines. Among the different lipophilic groups, linoleic acid (LA)-modified PEI was most effective in silencing individual targets and sensitizing the cells to drug therapy. Given the induction of multiple mechanisms for drug resistance, however, it is likely that such a 'mono' therapy will not be beneficial in obviating the drug

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resistance. A more effective approach will involve delivering siRNAs to target multiple targets responsible for the complementary mechanisms of drug resistance.

This study focused on developing a more comprehensive approach to overcoming drug resistance in breast cancer. We hypothesized that silencing complementary targets involved in drug resistance and cell survival will lead to a therapeutic effect superior to silencing individual proteins alone, which was considered an additive effect. We further hypothesized that polymeric delivery of siRNA is an effective approach to silence complementary targets. To test these hypotheses, we employed the drug efflux protein P-gp and the anti-apoptotic proteins survivin and Mcl-1 as targets, whose expressions are linked to adverse outcomes in clinical therapy.[17,18] Breast cancer cells modified for P-gp overexpression or adopted to growth in the presence of doxorubicin (DOX) were employed as drug-resistant models in this study.

MATERIALS AND METHODS

Materials and cell lines

The sources of all reagents used are provided in the Supplementary Information. The binding efficiency of the lipid-substituted polymers to siRNA and the properties of the resulting nanoparticles were reported previously[14] and will not be repeated here. Wild-type (MDA-MB-435wild-type (WT)) and P-gp overexpressing human breast cancer cells (MDA-MB-435multi-drug resistance (MDR)) were kindly provided by Dr Robert Clarke (Georgetown University, Washington, DC) and cultured as described before.[14] Drug-resistant cells were developed through exposure to DOX *via* two different methods: (a) gradual dose increase and (b) shock exposure. In the gradual method, MDA-MB-435WT cells were exposed to increasing doses of DOX, starting from 0.2 $\mu g\,ml^{-1}$ ($\sim$20% of the inhibitory concentration for 50% cell death), and continuing with 0.5, 0.75, 1.0, 1.5 and 2.0 $\mu g\,ml^{-1}$. Cells were exposed to each concentration for three passages, and frozen at the end of each stage. These cells were labeled with 'G' for 'gradual' and the last employed DOX concentration: for example, 2G: cells exposed to gradually increasing DOX concentration up to 2 $\mu g\,ml^{-1}$. Samples of 0.75G, 1.5G and 2G cells were maintained in medium containing 0.2 $\mu g\,ml^{-1}$ DOX for ongoing experiments. In the 'shock' method, MDA-MB-435WT cells were exposed to either 0.5, 1.0 or 2.0 $\mu g\,ml^{-1}$ DOX (equivalent to 0.5 to 2 $\times$ inhibitory concentration for 50% cell death), and surviving cells were maintained in medium containing 0.2 $\mu g\,ml^{-1}$ DOX. These cells were designated as 0.5S, 1S and 2S cells, respectively.

Methods

Protein silencing with siRNA. Cells were grown in 24-well plates at $\sim$20% confluency ($\sim 1.5 \times 10^5$ cells) with 300 μl of medium. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA and specific siRNA with polymer:siRNA weight ratios of 2:1, 4:1 or 8:1 (corresponding to 54 nM siRNA, with 1.5, 3.0 and 6.0 $\mu g\,ml^{-1}$ polymer in culture medium) and added to the wells in triplicate. The plates were incubated at 37 °C for 48 h (for P-gp silencing) or 72 h (for survivin or Mcl-1 silencing). To assess P-gp levels, the medium was removed and 10 μl FITC-labeled P-gp antibody was added to each well, and plates were incubated at room temperature for 45 min. The cells were washed with Hank's balanced salt solution (HBSS) (3 $\times$), trypsinized and fixed. P-gp silencing was quantified by a Beckman Coulter QUANTA SC (Brea, CA, USA) flow cytometer (FL1) as described in Aliabadi *et al.*[14] To analyze survivin levels, the cells were trypsinized, fixed and transferred to test tubes. Cells were permeabilized with a 0.05% Triton X100 (in HBSS) and incubated with fluorescein-conjugated anti-survivin monoclonal antibody for 45 min at room temperature. After washing with the permeabilizing solution (2 $\times$), cells were resuspended in HBSS and analyzed in flow cytometry.[16]

For multiple protein silencing, polymer/siRNA complexes were prepared as a single polyplex formulation with a mixture of two siRNAs, each giving a final concentration of 54 nM in medium (preparation of two different polyplex formulations and simultaneous treatment with these formulations was studied as well, the results of which showed no significant difference; data not shown). As the optimum polymer:siRNA ratio was different for the selected targets (2:1 for survivin and 8:1 for P-gp),[14,16] a polymer:siRNA ratio of 4:1 was selected for multiple silencing experiments. All possible combinations involving survivin (S), P-gp (P), Mcl-1 (M) and control (C) siRNAs were included in these experiments.

Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR). Cells were exposed to siRNAs in 6-well plates and washed with HBSS at indicated time points before treatment with RLT buffer. The cell lysates were then passed through a shredder and total RNA was isolated by using the RNeasy Mini Kit. The extracted RNA was quantified by spectrophotometry and samples were frozen at -20 °C until analysis. To synthesize the complementary DNA (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 $\times$), dithiothreitol (0.1 M) and RNAout RNase inhibitor (1.8 U μl^{-1}) 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. For amplification, 100 ng of the synthesized cDNA was mixed with 10 $\times$ ThermPol Buffer, dNTP mix (5 mM), forward and reverse primers (3 μM each), and Taq polymerase (5 U μl^{-1}). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (for RT-PCR) and β-actin (for qPCR) were also amplified as controls. For the RT-PCR, the samples were run on a 0.5% agarose gel and quantified by densitometry. Real-time PCR was performed on an ABI 7500 HT (Grand Island, NY, USA) with human β-actin (forward: 5′-CCACCCC ACTTCTCTCTAAGGA-3′; reverse: 5′-AATTTACACGAAAGCAATGCTATCA-3′) as the endogenous housekeeping gene and specific Mcl-1 (forward: 5′-CC TTTGTGGCTAAACACTTGAAG-3′; reverse: 5′-CGAGAACGTCTGTGATACTTT CTG-3′), P-gp (forward: 5′-CCTAATGCCGAACACATTGGA-3′; reverse: 5′-TCC AGGCTCAGTCCCTGAAG-3′) and survivin (forward: 5′-CCCCTCGGGCCA ACTG-3′; reverse: 5′-CAGTTTGGCTTGCTGGTCTCT-3′) primers. All the primers were tested to assure equal efficiency (with a slope <0.1 for the ΔC_T vs cDNA dilution graph), and a template concentration of 10 ng μl^{-1} was determined as the optimal concentration based on the standard curves. Analysis was performed by calculating ΔC_T, $\Delta\Delta C_T$ and relative quantity compared with the 'no treatment' group.

Cellular accumulation of DOX. The cells in 24-well plates (500 μl per well) and at $\sim$20% confluency ($\sim 1.5 \times 10^5$ cells) were incubated with 2 $\mu g\,ml^{-1}$ DOX ($\sim$20% of the regular dose used throughout the study for cytotoxic effect) for 24 h, trypsinized and suspended in 3.7% formalin (in HBSS). DOX accumulation was quantified with a Beckman Coulter flow cytometer (FL-2 channel) to determine the mean fluorescence intensity (equivalent to intracellular DOX concentration) and the percentage of DOX-positive cells.[14]

Evaluation of cell viability. The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay in 24-well plates. The cells were exposed to polymer/siRNA complexes or desired DOX concentrations (stock solution of 1 $mg\,ml^{-1}$ in isotonic NaCl) alone or in combination with siRNA treatment (after 48 h exposure to siRNA complexes). The cells were incubated for 24 h after DOX addition and then 40 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (5 $mg\,ml^{-1}$ in HBSS) was added to each well. After 2 h of incubation at 37 °C, the medium was removed, 500 μl of DMSO was added to dissolve the crystals and optical density of the wells was measured at 570 nm. The results were normalized to untreated cells (taken as 100% viability).

Assessment of apoptosis. Level of induced apoptosis in treated cells was evaluated using the FlowTACS Apoptosis Kit (Trevigen; Gaithersburg, MD, USA) (based on free 3′-OH modification of terminal deoxynucleotidyl transferase) and fluorescent microscopy. Level of apoptosis was quantified by the flow cytometer (FL1) for percentage of cells positive for apoptosis and the mean fluorescence in total cell population. In order to ensure the labeling reaction and the validity of the test, a positive control was included in each set of experiments, where the 'No Treatment' cells were treated with a nuclease solution to create DNA fragmentation. Calibration was performed by gating against a cell population treated with normal saline, identified as 'No Treatment' (1–2% of the total population). For microscopic imaging, a combination of acridine orange/ethidium bromide was used. Dyes were added to the cells in 6-well plates simultaneously (5 $\mu g\,ml^{-1}$), plates were centrifuged at 600 r.p.m. for 5 min and cells were observed under a fluorescent microscope. While acridine orange penetrates all cells and stains the nucleus green, ethidium bromide stains the cells (red) only after membrane integrity is lost.[19]

Statistics. The data were presented as mean ± s.d. and analyzed for statistical significance by unpaired Students *t*-test (assuming unequal variance; $\alpha = 0.05$). 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 with silencing of each individual target.

RESULTS

We have previously reported physiochemical properties of polymer/siRNA complexes formed with a library of lipid-substituted low molecular weight PEIs, as well as the siRNA delivery efficiency using different polymer/siRNA weight ratios in the MDA-MB-435 cells.[14] We also showed that LA-substituted PEI (PEI-LA) was effective in silencing P-gp in MDA-MB-435MDR cells, but caprylic acid-substituted PEI (PEI-CA) was more effective in silencing survivin in MDA-MB-231 cells. To identify the optimal polymer for siRNA delivery, initial studies explored P-gp silencing in MDA-MB-435MDR cells and survivin silencing in MDA-MB-435WT and MDR cells (Supplementary Figure 1). After 48 h exposure to polymer/siRNA complexes, P-gp silencing obtained by both PEI-LA and PEI-CA was equivalent (~40%). The survivin silencing was also achieved with PEI-CA and PEI-LA in both MDA-MB-435WT and MDR cells. While PEI-CA was more efficient in survivin silencing in MDR cells (~48% silencing), PEI-LA was equally efficient in both cell lines (37–38%). A preliminary screening of a library of lipid-substituted PEIs for Mcl-1 silencing showed PEI-LA to be the most effective polymer in this regard (data not shown). Therefore, as the silencing accomplished with the two polymers was generally comparable for survivin silencing, we employed only PEI-LA for the rest of the studies.

Mcl-1 silencing and cell viability

Three different polymer:siRNA ratios were evaluated to investigate the effect of Mcl-1 silencing on cell viability. In MDA-MB-435WT cells treated with Mcl-1 siRNA (Figure 1a–i), cell viability progressively decreased with increasing polymer:siRNA ratio and >90% cell death was observed at the ratio of 8:1. A similar pattern was seen in the MDA-MB-435MDR cells (Figure 1a-ii), where the highest cell death was achieved with the ratio of 8:1; however, the level of cell viability was higher in comparison with MDA-MB-435WT cells. Increasing the polymer:siRNA ratio increased the toxicity of siRNA complexes as cell viability exposed to control siRNA was slightly decreased in both cell types (Figure 1a). The dose-response of siRNA-mediated Mcl-1 silencing was then explored (Figure 1b). There was no effect of Mcl-1 siRNA treatment until 5 nM siRNA, but a gradual decrease in cell viability was obtained at higher concentrations, with MDA-MB-435MDR cells displaying less sensitivity to Mcl-1 silencing. A microscopic evaluation of the effect of Mcl-1 silencing (54 nM) in both cell lines confirmed apoptosis induction, based on ethidium bromide penetration into the cells and fragmentation of nucleus stained with acridine orange (Figure 1c).

Combinational silencing of P-gp, Mcl-1 and survivin

Combinational silencing of the chosen targets was then attempted in MDA-MB-435MDR cells (Figure 2). Both RT-PCR and qPCR were performed to evaluate the silencing efficiency at the messenger RNA (mRNA) level. In RT-PCR, a similar silencing pattern for all three proteins was obtained at 12–48-h time points. While survivin mRNA was slightly decreased after 24 and 48 h in SC group, a significant drop in survivin mRNA levels was clearly observed when a mixture of survivin and P-gp siRNAs was used (Supplementary Figure 2i). Unlike survivin, Mcl-1 mRNA levels were significantly reduced in all three time points when the cells were treated with Mcl-1 siRNA (MC group) and with a mixture of Mcl-1 and P-gp siRNAs (MP group) (Supplementary Figure 2ii). The levels of P-gp mRNA also followed a similar pattern to that of Mcl-1 (Supplementary Figure 2iii). Based on these results, we selected the 48-h time point for the qPCR experiment with the same treatment groups. A similar pattern was observed in the qPCR study: while a 38% decrease in the survivin mRNA was achieved with SC group (compared with NT group), double silencing with SP group resulted in a 52% drop; Mcl-1 silencing with MC group accomplished ~50% decrease in Mcl-1 mRNA, MP group treatment showed ~80% difference from the NT group; and finally, while the P-gp mRNA level was not significantly different in PC and SP groups (48% and 51%, respectively), the P-gp expression level showed a 56% drop in MP group, which was significantly lower than the PC group (Figure 2a).

The effects of P-gp, Mcl-1 and survivin silencing on cell viability are summarized in Figure 2b. Silencing of P-gp (PC group) caused a significant drop in cell viability with 10 μg ml^{-1} DOX, but not without the DOX treatment. Silencing Mcl-1 and survivin separately (MC and SC groups) caused ~65 and ~55% drop in cell viability, respectively, but DOX treatment did not change cell viabilities for these groups. The double silencing of Mcl-1 and survivin produced similar cell viabilities to Mcl-1 silencing alone with no additional effects. However, when P-gp was silenced simultaneously with survivin or Mcl-1 (SP and MP groups), cell viabilities were further decreased as compared with survivin or Mcl-1 silencing alone (SC and MC groups). No further changes in cell viability were noted with MP and SP groups after addition of 10 μg ml^{-1} DOX, as cell viability after the siRNA treatment alone was already very low.

A significant increase in the level of apoptosis for DOX-treated cells was evident after P-gp silencing (Figure 2c), both presented as apoptosis level (mean fluorescence; panel i) and percentage of apoptotic cells (panel ii). A more significant apoptotic response was also observed for simultaneous silencing of (i) Mcl-1 and P-gp (MP group) compared with single silencing of Mcl-1 (68.5 vs 40.9% for percentage apoptotic cells), and (ii) survivin and P-gp (SP group) as compared with survivin silencing alone (37.3% vs 32.9% for percentage of apoptotic cells), even without addition of DOX. Microscopic evaluation of apoptosis for MC and MP groups confirmed the results observed with the flow cytometry method (Figure 2d).

Changes in induced drug-resistant cells

The DOX-exposed 0.75G and 1S cells had a DOX inhibitory concentration for 50% cell death that was equivalent to P-gp overexpressing MDA-MB-435MDR cells (>10 μg ml^{-1} DOX), and significantly higher than the MDA-MB-435WT cells (Figure 3a). The relative DOX accumulation (evaluated using a lower dose of DOX than the dose used for cytotoxic effect; Figure 3b) also followed a similar pattern; while the MDA-MB-435WT cells had significant DOX accumulation, the 1S and 0.75G cells showed limited DOX accumulation that was comparable to MDA-MB-435MDR cells. Changes in P-gp, BCRP, survivin and Mcl-1 expression were then investigated in developed cell lines (0.2G, 0.5G, 0.5S and 1S). The P-gp mRNA levels increased as a result of exposure to DOX (Figure 3c), where the gradual and shock DOX exposure resulted in relatively similar increases in P-gp mRNA. The levels of BCRP mRNA showed a similar increase, where the increase at 1 μg ml^{-1} DOX was more significant than the P-gp (~10-fold vs WT; Figure 3c). An increase was also apparent for the Mcl-1 mRNA, which was matched with an increase in survivin mRNA levels at 1 μg ml^{-1} DOX exposure.

Effect of P-gp, survivin and Mcl-1 silencing in induced drug-resistant cells

All siRNA treatments containing P-gp (PC, MP and SP) caused a significant increase in the DOX accumulation in MDA-MB-435MDR cells, in line with functional activity for P-gp. However, silencing

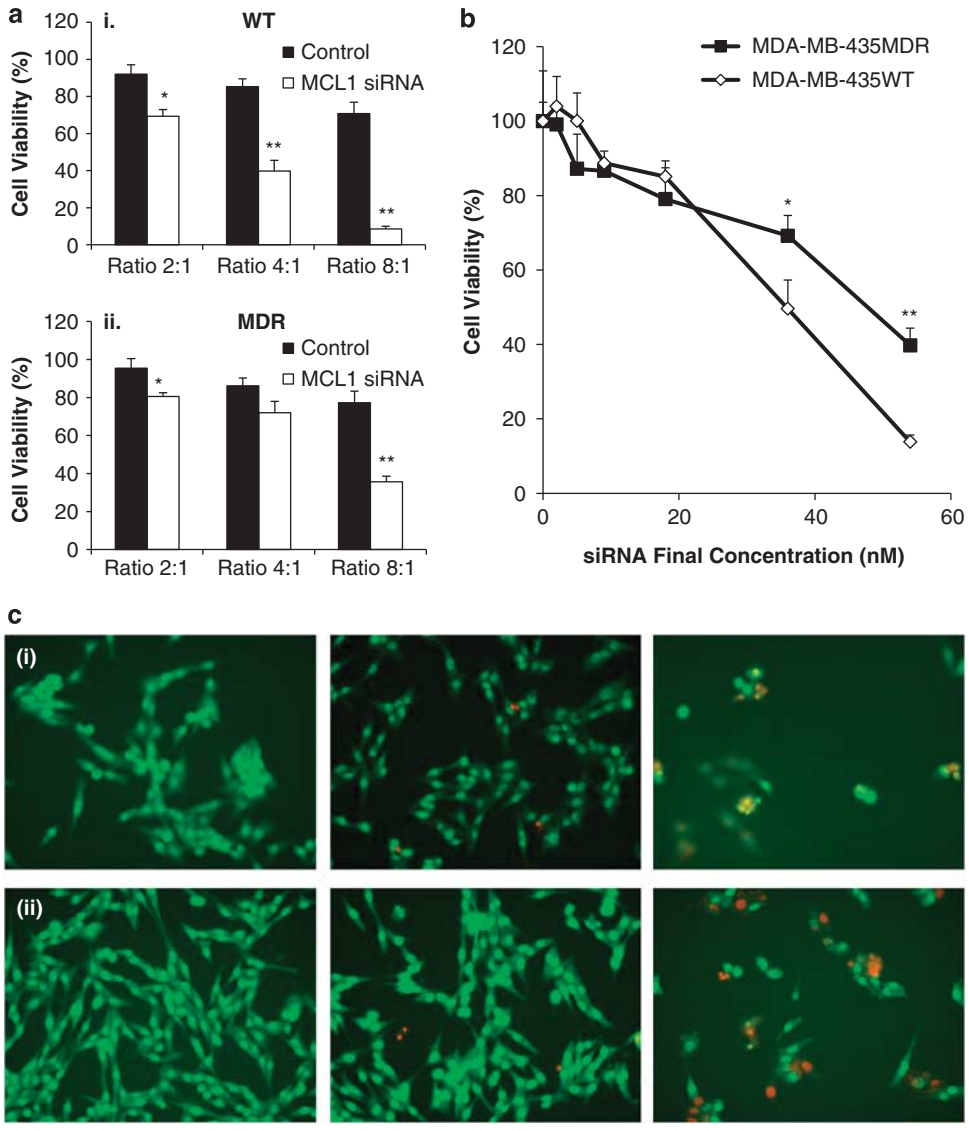

Figure 1. Mcl-1 silencing and cell viability. (**a**) Cell viability after Mcl-1 silencing by polymer/short interfering RNA (siRNA) complexes at different weight ratios (2:1, 4:1 and 8:1) in MDA-MB-435wild-type (WT) (i) and MDA-MB-435multi-drug resistance (MDR) (ii) cells. The viability was assessed after 72-h exposure to 54 nM siRNA complexes. The cells were treated with scrambled siRNA (black bars) or Mcl-1-specific siRNA (white bars). The viability was expressed as a percentage of non-treated cells. Significant difference between specific and scrambled siRNA treatment in each group is represented by * ($P<0.05$) and ** ($P<0.005$). (**b**) Cell viability after Mcl-1 silencing by different concentration of siRNA in MDA-MB-435WT and MDA-MB-435MDR cells. The cell viability was assessed after 72-h exposure to siRNA complexes (weight ratio of 8:1) and expressed as a percentage of non-treated cells. Significant differences between MDA-MB-435WT and MDA-MB-435MDR cells are indicated with * ($P<0.05$) and ** ($P<0.005$). (**c**) Microscopic images of MDA-MB-435WT (i) and MDA-MB-435MDR (ii) cells treated with saline (left), 54 nM scrambled siRNA complexes (middle) or Mcl-1 siRNA (right). Cells are stained with acridine orange/ethidium bromide.

survivin and Mcl-1 alone, or in combination (SC, MC and MS groups, respectively), had no significant effect on the DOX accumulation levels in the MDA-MB-435MDR cells (Figure 4a). A similar increase in DOX accumulation was observed in induced drug-resistant cells after P-gp silencing. While the 0.75G cells behaved similarly to MDA-MB-435MDR cells, two differences were observed with the 2G and 2S cells: (1) increase in DOX accumulation was slightly higher when Mcl-1 was silenced along with P-gp, and (2) silencing survivin and Mcl-1 produced an increase in DOX accumulation, both as single silencing (2.7–2.6-fold increase in 2G cells and 1.3–1.4-fold increase in 2S cells, compared with CC group) and double silencing (2.4-fold and 1.5-fold increase for MS group in 2G and 2S cells, respectively; Figure 4a).

The effect of Mcl-1 silencing on viability of induced drug-resistant cells is shown in Figure 4b. As before, MDA-MD-435WT cells gave lower viability at 36 and 54 nM siRNA as compared with MDA-MD-435MDR cells. The loss of cell viability in induced drug-resistant cells was similar or more significant than MDA-MD-435WT cells, with 2S cells showing the most sensitivity (Figure 4b). A lower concentration range (18 and 36 nM) of Mcl-1 siRNA was then evaluated in combination with DOX treatment to investigate the effect of Mcl-1 silencing on the sensitivity against the drug responsible for the resistance. Unlike MDA-MD-435WT cells, DOX treatment had no significant effect on the viability of all induced drug-resistant cells (Figure 4c). After Mcl-1 silencing, the loss of cell viability for the 36 nM groups in 0.75G, 2G and 2S cells was more significant than the MDA-MB-435WT and MDR cells. A further drop in cell viability was observed in 2G and 2S cells after DOX treatment (Figure 4c).

Finally, double silencing of Mcl-1 and P-gp was investigated in 0.75G and 2S cells in comparison with MDA-MB-435MDR cells

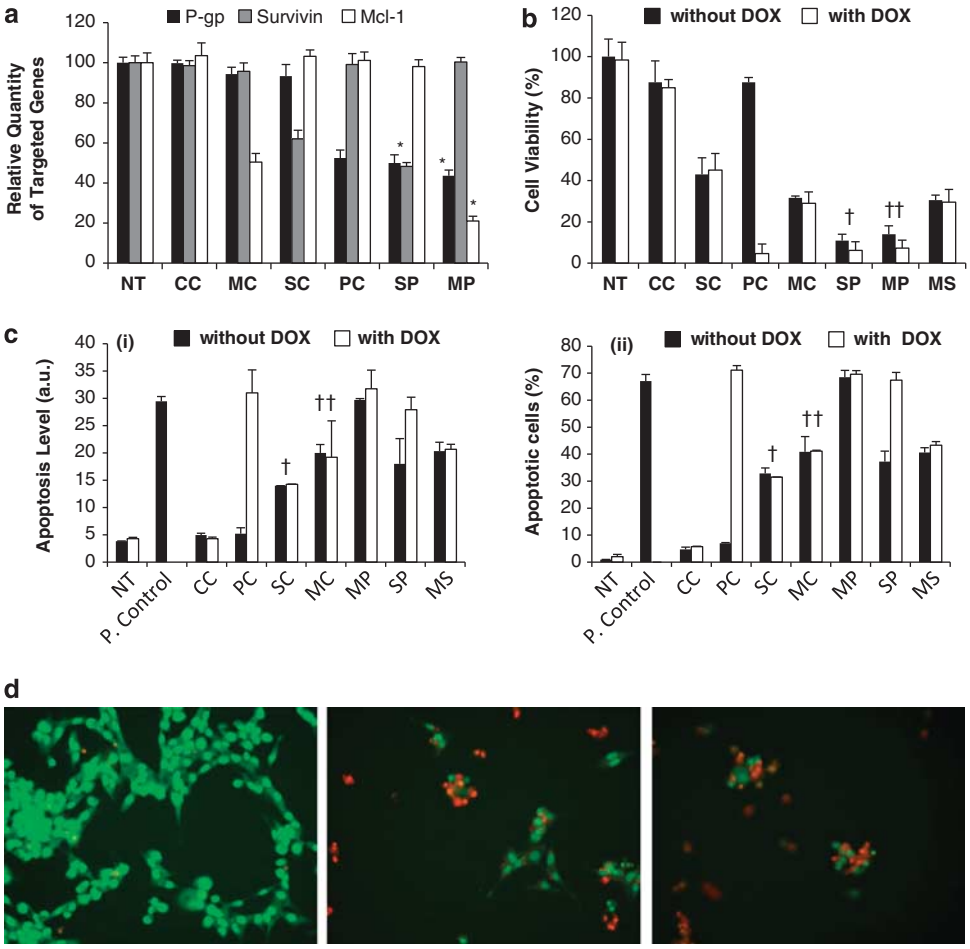

Figure 2. Combinational silencing of P-glycoprotein (P-gp), survivin and Mcl-1 in MDA-MB-435MDR cells. (**a**) Changes in mRNA levels after double silencing analyzed with qPCR. The mRNA levels were evaluated after 48 h for survivin, Mcl-1 and P-gp after exposure to a cocktail of siRNAs (polymer:siRNA ratio of 4:1, 36 nM siRNA for each protein). Combinations for control (C), P-gp (P), survivin (S) and Mcl-1 (M) were used in pairs. Beneficial effect of combinational silencing was observed for the three proteins as compared with single silencing (SP vs SC, MP vs MC and MP vs PC; asterisks represent significant difference between double- and single-silenced groups). (**b**) Viability of MDA-MB-435MDR cells after double silencing. Cell viability was determined after 72 h of exposure to the siRNA complexes shown in **a**. Lower cell viability was evident with a combination of siRNAs targeting P-gp and survivin (vs survivin alone; $P<0.01$) or P-gp and Mcl-1 (vs Mcl-1 alone; $P<0.01$) (†: significant difference between SC and SP; ††: significant difference between MC and MP). (**c**) Assessment of apoptosis. The level of apoptosis was indicated by mean fluorescence from flow cytometry (i) or apoptotic cell population (presented as percentage of apoptotic cells) (ii). Combination of Mcl-1 and P-gp, or survivin and P-gp, produced a higher level of apoptosis compared with single silencing (†: significant difference between SC and SP; ††: significant difference between MC and MP; $P<0.05$). The 'P. Control' group is the positive control with the nuclease-treated cells to validate the labeling reaction. (**d**) Microscopic images of cells treated with polymer complexes with scrambled siRNA (CC; left), scrambled and Mcl-1 siRNAs (MC; middle), and P-gp and Mcl-1 siRNAs (PM; right), and stained with acridine orange/ethidium bromide.

(Figure 4d). As before, silencing P-gp alone (PC group) resulted in sensitizing the cells to DOX with a significant drop in cell viability. Silencing Mcl-1 alone (MC group) caused a drop in cell viability compared with untreated cells and cells treated with control siRNA (NT and CC groups, respectively) independent of DOX treatment, and adding DOX to the treatment did not change the response significantly. This effect was more significant with the 2S cells, where $<10\%$ cell viability was observed with Mcl-1 silencing. Simultaneous silencing of P-gp and Mcl-1 showed an additional effect on cell viability in MDA-MB-435MDR cells as compared with Mcl-1 silencing alone (MP vs MC group, respectively). The same phenomena could not be assessed for the 2S cells owing to almost complete loss of viability with single Mcl-1 silencing.

DISCUSSION

Overexpression of anti-apoptotic proteins contributes to drug resistance in breast cancer cells. To explore non-viral silencing of anti-apoptotic proteins as a strategy to overcome drug resistance, silencing survivin,[20,21] Mcl-1[22–25] and Bcl-2[26] has been reported by employing siRNA delivery against the individual targets. Mcl-1, the focus of this study, was initially identified as an immediate-early gene expressed during PMA-induced differentiation of ML-1 myeloid leukemia cells.[27] Mcl-1 is primarily localized to outer mitochondrial membrane and promotes cell survival by inhibiting mitochondrial cytochrome c release via suppression of pro-apoptotic activities of Bim, Bax and Bak.[28] Expression of Mcl-1 is frequently elevated in malignancies,[29,30] and high Mcl-1 expression was associated with resistance to drug or radiation therapy.[31] Mcl-1 silencing can sensitize malignant cells to effects of proteasome inhibitors.[23] Clinically employed signal transduction inhibitors NVP-BEZ235[32] and sorafenib,[18] and cyclin-dependent kinase inhibitor roscovitine[33] led to downregulation of Mcl-1, whose silencing was sufficient to mimic the drug effects in some cases. A recent preclinical study in acute myeloid leukemia model also demonstrated tumor suppression *via* anti-Mcl-1 short-hairpin RNA.[34] The present study along with these studies collectively emphasized the promise of this target for therapeutic purposes.

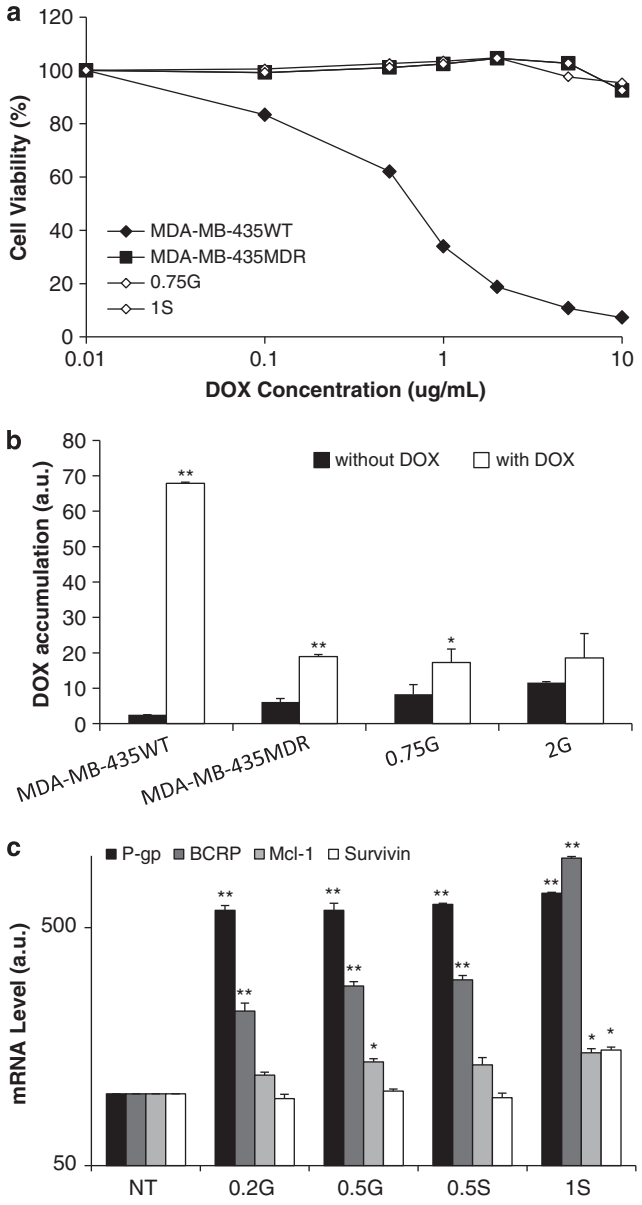

Figure 3. Resistance induction in MDA-MB-435WT cells by doxorubicin (DOX) exposure. (**a**) Cell viability as a function of DOX concentration for MDA-MB-435WT and MDR cells, and the developed cells 0.75G and 1S. Note the similarity in drug response between the P-gp-overexpressing MDA-MB-435MDR cells and the induced cells. (**b**) DOX accumulation in the MDA-MB-435WT and MDR cells, and the developed cells 0.75G and 1S after 24-h exposure to saline (without DOX) as a negative control, or DOX (with DOX). Note the reduced DOX accumulation in MDA-MB-435MDR cells and induced cells. Significant difference between with and without DOX treatment in each group is represented by * ($P<0.05$) and ** ($P<0.005$). (**c**) Changes in P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), myeloid cell leukemia 1 (Mcl-1) and survivin messenger RNA (mRNA) levels for induced drug-resistant cells. Significantly different groups are indicated with * ($P<0.05$) and ** ($P<0.005$). Significant increases in expression of all proteins were observed for the cells exposed to the highest DOX concentration (1S).

We previously reported successful delivery of siRNA using lipid-substituted PEIs,[14–16] where the hydrophobic moieties on PEI enhanced the cellular uptake of polymer/siRNA complexes dramatically as compared with native PEI, without a significant increase in polymer toxicity. These polymers were now found to be successful in Mcl-1 silencing in wild-type and drug-resistant cells. The loss of viability was significantly higher in wild-type cells, which indicated resistance of P-gp-overexpressing cells to apoptosis induction owing to Mcl-1 silencing. Involvement of P-gp in innate resistance of tumors has led to studies exploring anti-apoptosis roles for P-gp.[35] We observed lower levels of targeted mRNAs in dual silencing groups as compared with single silencing groups, indicating a beneficial effect of P-gp silencing on Mcl-1 and survivin silencing (and vice versa). The more effective silencing with dual siRNA delivery was reflected in more potent cell death after Mcl-1 and P-gp siRNA delivery. The same effect was not observed for dual silencing of survivin and P-gp, indicating a stronger functional dependence between Mcl-1 and P-gp. Nevertheless, increased cell death observed with dual silencing should enable more potent siRNA therapy, ultimately helping to reduce siRNA and/or drug doses needed in clinical studies. This approach provides considerable specificity toward cancer cells owing to overexpression of selected targets in these cells. We have recently performed siRNA screenings with an apoptosis library (which includes both survivin and Mcl-1) on the MDA-MB-435WT cells, as well as human skin fibroblast cells (representing a non-cancer cell line). While both Mcl-1 and survivin silencing caused cell death in the breast cancer cell line and were hits in the screening, no significant drop in cell viability was observed in the skin fibroblast cells as a result of silencing these proteins, which provided additional evidence for the specificity of this strategy for cancer cells (data not shown).

As P-gp overexpression by viral means (that is, in the case of MDA-MB-435 cells) might not be representative of clinical scenario, drug resistance was induced in a more clinically relevant manner. Levels of mRNA for P-gp, BCRP, survivin and Mcl-1 showed a significant jump at the earliest stage of induction ($0.2\,\mu g\,ml^{-1}$) compared with the naïve cells; the next significant increase was not observed until $1\,\mu g\,ml^{-1}$ DOX, which indicated the importance of treatment dose on the subsequent adaptation. While overexpressions of DOX efflux proteins P-gp and BCRP were expected, increases in Mcl-1 and survivin are also noteworthy, as it indicates the possibility of crosstalk with the transporters. In dual silencing experiments, Mcl-1 and survivin silencing did not have any significant effect on DOX accumulation in the MDR cells or 0.75G cells, unlike the P-gp silencing. The cells conditioned at higher DOX levels reacted differently; a beneficial effect in DOX accumulation was evident for simultaneous silencing of P-gp and survivin or Mcl-1. The viability studies also showed that cells conditioned at high DOX concentrations responded better to single silencing of Mcl-1 (even better than WT cells in some cases; Figure 4). This outcome was indicative of increased reliance of induced drug-resistant cells on Mcl-1, which may prove to be ideal for sensitizing malignant cells.

A common pathway for expression of P-gp and anti-apoptotic proteins was recently elucidated. PI3K/Akt has been shown to regulate survivin and Mcl-1 expression in breast cancer cells[32,36] and Akt pathway might block apoptosis via increased survivin levels.[37] Akt pathway may be also involved in overexpression of Mcl-1;[38] Akt enhances the transcriptional induction of Mcl-1 and blocks inhibition of translation of several proteins, including Mcl-1, by 4EBP1.[39] Furthermore, the PI3K/Akt pathway has been shown to modulate P-gp expression.[40] More than one pathway are likely to have a role in P-gp expression, which could include translocation of nuclear factor kappa B[41] and nitric oxide produced by NO synthase,[42] both of which have been linked to PI3K/Akt pathway.[43,44] Consistent with these connections, Ji *et al.* employed short-hairpin RNA for RNA interference (under selection pressures) and observed an additive effect of simultaneous silencing of P-gp and Mcl-1 in reversing drug

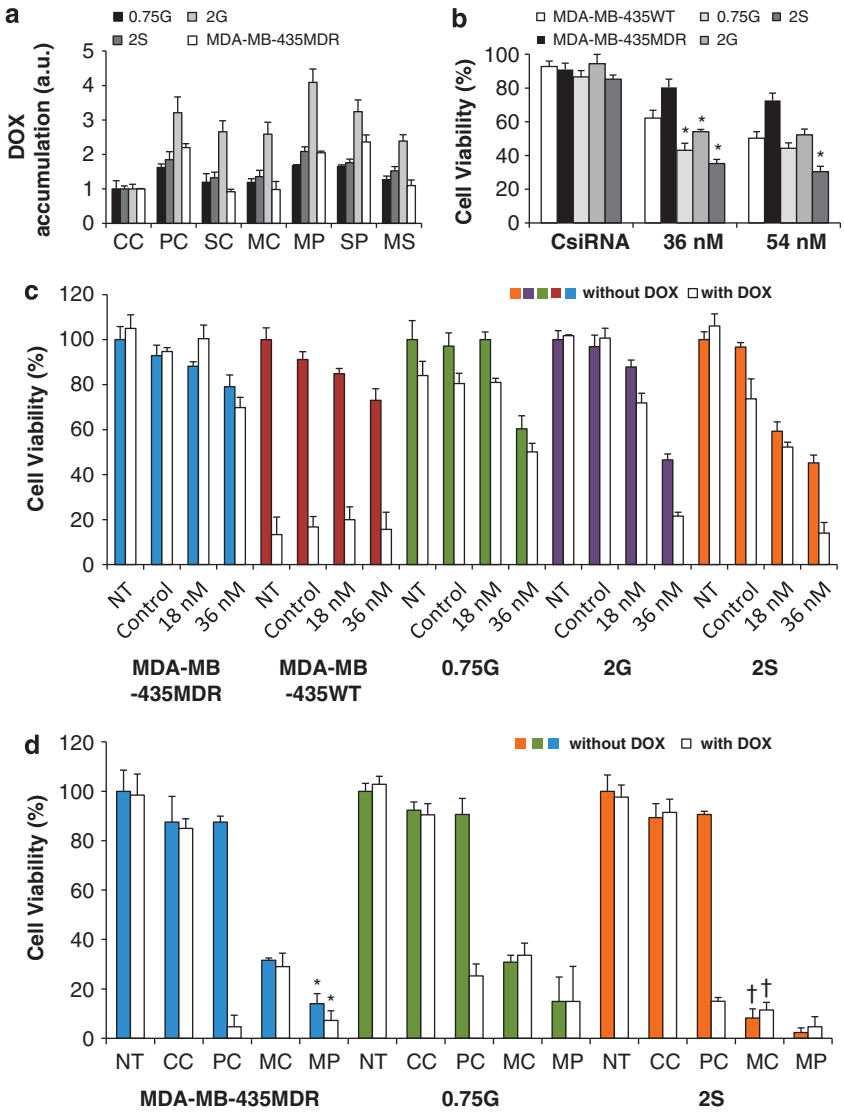

Figure 4. Combinational silencing of P-gp, survivin and Mcl-1 in induced drug-resistant cells. (**a**) Doxorubicin (DOX) accumulation after 72 h treatment with combinations Mcl-1 (M), P-gp (P) and survivin (S) siRNA, and 24-h exposure to DOX. (**b**) Viability of MDA-MB-435WT, MDA-MB-435MDR and induced drug-resistant 0.75G, 2G and 2S cells after Mcl-1 silencing. The cells were treated with 36 and 54 nM Mcl-1 complexes (polymer/siRNA ratio of 4:1) for 72 h, after which viability was assessed. While the cells treated with control siRNA showed a minimal response, the response of induced drug-resistant cells was equivalent or more significant than the MDA-MB-435WT cells. Asterisks represent significant difference compared with the corresponding treatment group in the MDA-MB-435 WT cells. (**c**) Viability of MDA-MB-435WT, MDA-MB-435MDR and induced drug-resistant 0.75G, 2G and 2S cells after Mcl-1 silencing and DOX treatment. The cells were treated with 18 and 36 nM Mcl-1 complexes (polymer/siRNA ratio of 4:1) for 72 h, after which cells were treated with DOX (5 $\mu g\,ml^{-1}$) for 24 h and cell viability was assessed. While MDA-MB-435WT displayed the expected DOX effect, a further decrease in cell viability was seen in 2G and 2S cells after DOX treatment. (**d**) Viability of MDA-MB-435MDR and induced drug-resistant 0.75G and 2S cells after combinational treatment with control (C), P-gp (P) and Mcl-1 (M) siRNA. The cells were treated with 54-nM-specific siRNAs (polymer/siRNA ratio of 4:1) for 72 h, after which cells were treated with DOX (10 $\mu g\,ml^{-1}$) for 24 h and cell viability was assessed. The DOX treatment was most effective with P-gp silencing, Mcl-1 silencing alone or in combination with P-gp. Asterisks represent significant difference compared with the corresponding MC and MP treatment groups in the same cell line. † represents significant difference between the cell viability for the MC treatment group MDA-MB-435 MDR and induced resistant cells.

resistance in leukemia cells.[45] Our results in breast cancer cells based on a more clinically acceptable approach to RNA interference (that is, polymer-mediated siRNA delivery) are consistent with this observation. In addition to the direct effect of Mcl-1 silencing, we observed that Mcl-1 silencing in 2G and 2S cells further sensitized the cells to the cytotoxic effect of DOX. It appears that Mcl-1 silencing not only induced cell death in resistance-induced cells, but also sensitized the cells to the cytotoxic effect of the agent used for resistance induction. Simultaneous silencing of P-gp and Mcl-1 or survivin showed a similar trend. The increased dependence on Mcl-1 against drug toxicity may be the weak point of drug resistance-induced cells in sensitizing them against the drug therapy.

CONCLUSION

In conclusion, we report effective silencing of anti-apoptotic protein Mcl-1 via siRNA delivery using lipid-modified cationic carriers. Mcl-1 silencing caused significant cell death in breast cancer MDA-MB-435 cells, with a higher efficiency in wild-type

cells as compared with P-gp-overexpressing cells. Simultaneous silencing of P-gp and Mcl-1 or survivin enhanced silencing of targeted proteins at the mRNA level and led to elevated cell death. Wild-type cells that were rendered drug resistant by DOX exposure showed overexpression of P-gp, BCRP, Mcl-1 and survivin, and they became more susceptible to loss of viability after Mcl-1 silencing. Combinational silencing of anti-apoptotic and efflux proteins by polymeric siRNA delivery could overcome drug resistance and eradicate tumor cells more efficiently than previously pursued single-targeting strategies in breast cancer cells.

ABBREVIATIONS

BCRP, breast cancer resistance protein; CA, caprylic acid; DOX, doxorubicin; HBSS, Hank's balanced salt solution; LA, linoleic acid; Mcl-1, myeloid cell leukemia 1; MDR, multi-drug resistance; PEI, polyethylenimine; P-gp, P-glycoprotein; RT-PCR, reverse transcription PCR; siRNA, short-interfering RNA; WT, wild-type

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Abbasi M, Lavasanifar A, Uludag H. Recent attempts at RNAi-mediated P-glycoprotein downregulation for reversal of multidrug resistance in cancer. *Med Res Rev* 2013; **33**: 33–53.
2 Mayer LD, Shabbits JA. The role for liposomal drug delivery in molecular and pharmacological strategies to overcome multidrug resistance. *Cancer Metastasis Rev* 2001; **20**: 87–93.
3 Ferry DR, Traunecker H, Kerr DJ. Clinical trials of P-glycoprotein reversal in solid tumours. *Eur J Cancer* 1996; **32A**: 1070–1081.
4 Moulder S. Intrinsic resistance to chemotherapy in breast cancer. *Womens Health (Lond Engl)* 2010; **6**: 821–830.
5 Jha K, Shukla M, Pandey M. Survivin expression and targeting in breast cancer. *Surg Oncol* 2012; **21**: 125–131.
6 Mitchell C, Yacoub A, Hossein H, Martin AP, Bareford MD, Eulitt P *et al.* Inhibition of MCL-1 in breast cancer cells promotes cell death *in vitro* and *in vivo*. *Cancer Biol Ther* 2010; **10**: 903–917.
7 Booy EP, Henson ES, Gibson SB. Epidermal growth factor regulates Mcl-1 expression through the MAPK-Elk-1 signalling pathway contributing to cell survival in breast cancer. *Oncogene* 2011; **30**: 2367–2378.
8 Shi Z, Liang YJ, Chen ZS, Wang XH, Ding Y, Chen LM *et al.* Overexpression of Survivin and XIAP in MDR cancer cells unrelated to P-glycoprotein. *Oncol Rep* 2007; **17**: 969–976.
9 Robinson LJ, Roberts WK, Ling TT, Lamming D, Sternberg SS, Roepe PD. Human MDR 1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. *Biochemistry* 1997; **36**: 11169–11178.
10 Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proc Natl Acad Sci USA* 1998; **95**: 7024–7029.
11 Johnstone RW, Cretney E, Smyth MJ. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood* 1999; **93**: 1075–1085.
12 Pallis M, Zhu YM, Russell NH. Bcl-x(L) is heterogenously expressed by acute myeloblastic leukaemia cells and is associated with autonomous growth in vitro and with P-glycoprotein expression. *Leukemia* 1997; **11**: 945–949.
13 Pallis M, Russell N. P-glycoprotein expression is associated with resistance to spontaneous in vitro apoptosis in AML. *Leukemia* 1999; **13**: 1468–1469.
14 Aliabadi HM, Landry B, Bahadur RK, Neamnark A, Suwantong O, Uludag H. Impact of lipid substitution on assembly and delivery of siRNA by cationic polymers. *Macromol Biosci* 2011; **11**: 662–672.
15 Aliabadi HM, Landry B, Mahdipoor P, Uludag H. Down-regulation of breast cancer resistance protein (BCRP) by siRNA delivery using lipid-substituted aliphatic polymers. *Eur J Pharm Biopharm* 2011; **81**: 33–47.
16 Aliabadi HM, Landry B, Mahdipoor P, Uludag H. Induction of apoptosis by survivin silencing through siRNA delivery in a human breast cancer cell line. *Mol Pharm* 2011; **8**: 1821–1830.
17 Vegran F, Boidot R, Oudin C, Defrain C, Rebucci M, Lizard-Nacol S. Association of p53 gene alterations with the expression of antiapoptotic survivin splice variants in breast cancer. *Oncogene* 2007; **26**: 290–297.
18 Ding Q, Huo L, Yang JY, Xia W, Wei Y, Liao Y *et al.* Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Res* 2008; **68**: 6109–6117.
19 Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol* 2005; **5**: 12.
20 Ai Z, Yin L, Zhou X, Zhu Y, Zhu D, Yu Y *et al.* Inhibition of survivin reduces cell proliferation and induces apoptosis in human endometrial cancer. *Cancer* 2006; **107**: 746–756.
21 Li QX, Zhao J, Liu JY, Jia LT, Huang HY, Xu YM *et al.* Survivin stable knockdown by siRNA inhibits tumor cell growth and angiogenesis in breast and cervical cancers. *Cancer Biol Ther* 2006; **5**: 860–866.
22 Kang SH, Cho HJ, Shim G, Lee S, Kim SH, Choi HG *et al.* Cationic liposomal co-delivery of small interfering RNA and a MEK inhibitor for enhanced anticancer efficacy. *Pharm Res* 2011; **28**: 3069–3078.
23 Zhou W, Hu J, Tang H, Wang D, Huang X, He C *et al.* Small interfering RNA targeting mcl-1 enhances proteasome inhibitor-induced apoptosis in various solid malignant tumors. *BMC Cancer* 2011; **11**: 485.
24 Shim G, Han SE, Yu YH, Lee S, Lee HY, Kim K *et al.* Trilysinoyl oleylamide-based cationic liposomes for systemic co-delivery of siRNA and an anticancer drug. *J Control Release* 2011; **155**: 60–66.
25 Zhang H, Guttikonda S, Roberts L, Uziel T, Semizarov D, Elmore SW *et al.* Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene* 2011; **30**: 1963–1968.
26 Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, Kashimori I *et al.* Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. *Clin Cancer Res* 2004; **10**: 7721–7726.
27 Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci USA* 1993; **90**: 3516–3520.
28 Inuzuka H, Fukushima H, Shaik S, Liu P, Lau AW, Wei W. Mcl-1 ubiquitination and destruction. *Oncotarget* 2011; **2**: 239–244.
29 Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003; **22**: 8590–8607.
30 Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell Mol Life Sci* 2009; **66**: 1326–1336.
31 Oakes SR, Vaillant F, Lim E, Lee L, Breslin K, Feleppa F *et al.* Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. *Proc Natl Acad Sci USA* 2012; **109**: 2766–2771.
32 Faber AC, Li D, Song Y, Liang MC, Yeap BY, Bronson RT *et al.* Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. *Proc Natl Acad Sci USA* 2009; **106**: 19503–19508.
33 Ortiz-Ferron G, Yerbes R, Eramo A, Lopez-Perez Al, De Maria R, Lopez-Rivas A. Roscovitine sensitizes breast cancer cells to TRAIL-induced apoptosis through a pleiotropic mechanism. *Cell Res* 2008; **18**: 664–676.
34 Glaser SP, Lee EF, Trounson E, Bouillet P, Wei A, Fairlie WD *et al.* Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev* 2012; **26**: 120–125.
35 Mizutani T, Masuda M, Nakai E, Furumiya K, Togawa H, Nakamura Y *et al.* Genuine functions of P-glycoprotein (ABCB1). *Curr Drug Metab* 2008; **9**: 167–174.
36 Asanuma H, Torigoe T, Kamiguchi K, Hirohashi Y, Ohmura T, Hirata K *et al.* Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells. *Cancer Res* 2005; **65**: 11018–11025.
37 Liu F, Liu S, He S, Xie Z, Zu X, Jiang Y. Survivin transcription is associated with P-glycoprotein/MDR1 overexpression in the multidrug resistance of MCF-7 breast cancer cells. *Oncol Rep* 2010; **23**: 1469–1475.
38 Naughton R, Quiney C, Turner SD, Cotter TG. Bcr-Abl-mediated redox regulation of the PI3K/AKT pathway. *Leukemia* 2009; **23**: 1432–1440.
39 Khan WN. B cell receptor and BAFF receptor signaling regulation of B cell homeostasis. *J Immunol* 2009; **183**: 3561–3567.
40 Kuo MT, Liu Z, Wei Y, Lin-Lee YC, Tatebe S, Mills GB *et al.* Induction of human MDR1 gene expression by 2-acetylaminofluorene is mediated by effectors of the

phosphoinositide 3-kinase pathway that activate NF-kappaB signaling. *Oncogene* 2002; **21**: 1945–1954.
41 Heemskerk S, Peters JG, Louisse J, Sagar S, Russel FG, Masereeuw R. Regulation of P-glycoprotein in renal proximal tubule epithelial cells by LPS and TNF-alpha. *J Biomed Biotechnol* 2010; **2010**: 525180.
42 Bauer B, Hartz AM, Miller DS. Tumor necrosis factor alpha and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier. *Mol Pharmacol* 2007; **71**: 667–675.
43 Chhabra A, Verma A, Mehta K. Tissue transglutaminase promotes or suppresses tumors depending on cell context. *Anticancer Res* 2009; **29**: 1909–1919.
44 Muniyappa R, lantorno M, Quon MJ. An integrated view of insulin resistance and endothelial dysfunction. *Endocrinol Metab Clin North Am* 2008; **37**: 685–711.
45 Ji M, Li J, Yu H, Ma D, Ye J, Sun X *et al.* Simultaneous targeting of MCL1 and ABCB1 as a novel strategy to overcome drug resistance in human leukaemia. *Br J Haematol* 2009; **145**: 648–656.

Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)