RESEARCH

Improved delivery of Mcl-1 and survivin siRNA combination in breast cancer cells with additive siRNA complexes

Tinnabhop Santadkha1,2 · Wanwisa Skolpap1,3 · Remant K.C.2 · Aysha Ansari2 · Cezary Kucharski2 · Teo Atz Dick2 · Hasan Uludağ2,4,5

Received: 27 May 2022 / Accepted: 4 July 2022 / Published online: 14 July 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Summary
This study aimed at investigating the influence of commercial transfection reagents (Prime-Fect, Leu-Fect A, and Leu-Fect C) complexed with different siRNAs (CDC20, HSP90, Mcl-1 and Survivin) in MDA-MB-436 breast cancer cells and the impact of incorporating an anionic additive, Trans-Booster, into siRNA formulations for improving in vitro gene silencing and delivery efficiency. Gene silencing was quantitatively analyzed by real-time RT-PCR while cell proliferation and siRNA uptake were evaluated by the MTT assay and flow cytometry, respectively. Amongst the investigated siRNAs and transfection reagents, Mcl-1/Prime-Fect complexes showed the highest inhibition of cell viability and the most effective siRNA delivery. The effect of various formulations on transfection efficiency showed that the additive with 1:1 ratio with siRNA was optimal achieving the lowest cell viability compared to untreated cells and negative control siRNA treatment (p < 0.05). Furthermore, the combination of Mcl-1 and survivin siRNA suppressed the growth of MDA-MB-436 cells more effectively than treatment with the single siRNAs and resulted in cell viability as low as ~20% (vs. non-treated cells). This aligned well with the induction of apoptosis as analyzed by flow cytometry, which revealed higher apoptotic cells with the combination treatment group. We conclude that commercial transfection reagents formulated with Mcl-1/Survivin siRNA combination could serve as a potent anti-proliferation agent in the treatment of breast cancers.

Keywords Gene therapy · Mcl-1 siRNA · Survivin siRNA · Breast cancer · Prime-Fect · Trans-booster

Introduction
Breast cancer ranks first in cancer incidence among women with more than two million new breast cancer cases in 2020 with an increasing trend [1]. In majority of the cases, development of breast cancer involves estrogen, progesterone and epidermal growth factor receptors, while another sizable fraction of 10–15% [2] involves triple negative cases. Either surgery or surgery in combination with radiotherapy is performed in local therapy of breast cancers and, if necessary, this is followed by systematic treatment with pharmacological agents. Optimal treatment strategy for each breast cancer patient is based on tumor subtype, stage of cancer and patient preferences [3]. Trends in development of novel cancer therapeutics have shifted from conventional to more specific targeted methods. Conventional targeted treatment strategies focus on the use of monoclonal antibodies [4] to seek out target cells that display the corresponding receptor proteins on their surface, for delivering small molecular inhibitors. While this strategy leverages the presence of cell surface proteins to achieve targeted therapy, it possesses little capacity to target intracellular proteins [4]—a limitation of the conventional delivery method [5].

Targeting oncogenes in malignant cells by inhibiting oncoprotein synthesis is a relatively new approach for molecular targeting. Small interfering RNA (siRNA) is a promising intervention in this regard since it can overcome the limitation of small molecular pharmacological agents
by targeting previously undruggable cancer drivers at will. siRNA implements its gene silencing activity by incorporating itself into the RISC (RNA-induced silencing complex), which then binds to the target messenger RNA (mRNA) in order to degrade it, resulting in attenuated protein synthesis [6]. The relatively larger microRNA (miRNA) can also order to degrade it, resulting in attenuated protein synthesis. However, the anionic nature of siRNA makes it impermeable to cell membranes, and hence it requires a carrier for cell entry. Several carriers such as liposomal nanoparticles, cationic lipids, and inorganic and organic polymers have been explored for this purpose. Cationic carriers are often utilized to deliver siRNA as they can form nanoscale complexes with siRNA via electrostatic interaction, prevent enzymatic degradation of siRNA and aid in cellular uptake [7]. Neutral and anionic carriers usually require addition of other components to form a more stable interaction with siRNA and undertake effective delivery [8].

Polyethyleneimine (PEI) is a cationic polymer widely used in non-viral gene delivery. PEI can condense the anionic nucleic acid and has high cellular uptake efficiency resulting in inhibition of gene expression both in vivo and in vitro [9, 10]. In addition to its high stability and low cost, PEI is able to form siRNA polyplexes at the nano-scale range, which is highly suitable for cell uptake [10]. PEI displays low toxicity when used at low molecular weights (MW < 5 kDa), but it is not effective in its native state. The facile chemistry of PEI allows for chemical manipulation to enhance its stability and transfection efficiency while retaining its ability to form complexes with nucleic acids. Numerous strategies have been explored over the years for this purpose. Inclusion of hydrophilic polymers such as polyethylene glycol (PEG), polyhydroxypropylmethacrylamide (pHPMA) and polyvinylpyrrolidone into PEI complexes, resulted in higher transfection efficiency [11]. The tertiary pDNA/PEI complex coated with poly(γ-glutamic acid) (γ-PGA) can convert its cationic surface charge to anionic charge without changing the particle size and lowering the cytotoxicity of cationic PEI [11]. A low MW PEI (4.7 kDa) when modified with C16 and C18 chains modified by ethylene oxide (PEG) units (Cn-EO) for stability [12], during the self-assembly process, allowed enhanced interaction between PEI and plasma membrane, resulting in better cellular uptake [8]. In contrast, the complexation of anionic siRNA with anionic polymers such as hyaluronic acid (HA) can prevent excretion via the glomerular capillary wall owing to repulsive force formation between the anionic species [13]. However, inclusion of anionic γ-PGA into chitosan/siRNA complexes has shown to facilitate intracellular release of siRNA, and consequently improve gene silencing efficacy [14]. Parmar et. al. (2018) investigated the effect of incorporating different polyanions into complex assembly on siRNA delivery efficiency [15], and demonstrated that hyaluronic acid-formulated additive polyplexes lead to the highest transfection and silencing activity with target siRNA in breast cancer cells [15].

Mcl-1, a member of the anti-apoptotic BCL-2 family, is necessary for cell survival and is responsible for resistance to chemotherapy induced apoptosis via the mitochondrial apoptotic pathway. Mcl-1 is highly expressed in various cancer cells, making it an attractive target for tumor treatment [16]. An elevated level of Mcl-1 expression is observed in breast cancer cases owing to its short half-life [17]. In MDA-MB-435 cells, Mcl-1 silencing was effectively achieved with siRNA complexed with cholesteryl-substituted poly(ethylene oxide)-block-poly(e-caprolactone-grafted-spermine) (PEOb-P(CL-g-SP)) micelles [18]. Thapa et. al. (2019) developed a protocol for identification of synergistic combinations of Mcl-1 targeting siRNA and chemotherapy drugs using siRNA libraries to overcome the limitations of single agent therapies [19]. Pengnam et. al. (2021) reported the synergistic effect of Mcl-1 and survivin siRNAs in MCF-7 breast cancer cells, where the cell viability was compromised to a greater extent with the Mcl-1/survivin siRNA combination compared to single siRNA treatment groups.

To further establish the feasibility of using Mcl-1 as a therapeutic target in breast cancer, this study explored several commercial delivery agents for siRNA delivery and gene silencing efficiency. Transfection reagents were derived from the low MW PEI (1.2 kDa) after optimal balance of hydrophobic (lipidic) groups were chemically conjugated to the PEI [20]. Several formulations of the commercial reagents were explored for the feasibility of retarding the growth of breast cancer cells using specific siRNAs.

### Materials and methods

#### Materials

The transfection reagents Prime-Fect, Leu-Fect A, Leu-Fect C and Trans-Booster (will be referred as ‘additive’ from hereon) were purchased from RJH Bioscience Inc. (Edmonton, AB, Canada). Lipofectamine™ 2000 transfection reagent was purchased from ThermoFisher. Hank’s Balanced Salt Solution (HBSS), Dulbecco’s Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium were purchased from Gibco (USA), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI is a cell culture medium that is commonly used in the basic research [21] to reduce risk of polyplex degradation [22]. Matrigel was obtained from Corning (Oneonta, NY). SensiFAST™ cDNA synthesis kit and SensiFAST™ SYBR Hi-ROX kit were
purchased from Bioline (ON, CA). Mcl-1 siRNA (siMcl-1), CDC20 siRNA (siCDC20), HSP90 siRNA (siHSP90) and Survivin siRNA (siSur) were used in this study. siMcl-1 (5'-CGC CCAUUCAUUUUU[A[dT][dT]]-3') was purchased from Sigma-Aldrich (St. Louis, MO). siSur (Cat. No.HSC.RNAI. N001012271.12.1), siCDC20 (5'-GGAUCAAGAGGGCA ACUACUUUGC-3'), siHSP90 (5'-AUGUGAUACCUUGA AUUGGAUACA-3'), the negative control scrambled siRNA (siNC; Cat. no. 308480258), 6-carboxyfluorescein (FAM)-labeled scrambled siRNA, primers for Mcl-1 (forward: 5'-CCT TTGTTGCTAAACCTTGAA-3'; reverse: 5'-CGAGAA CGTCCTGTGATACTTCTG-3'), Survivin (forward: 5'-TGA GAAAGGCCAGACTTG-3'; reverse: 5'-ATGTTCCCT TATGCGGTCTG-3') and Beta-Actin (forward: 5'-CCACC CACTTCTCTCAAGGA-3'; reverse: 5'-AATTACCGAA GCAATGCTATC-3') were purchased from IDT Technologies (Coralville, IA).

Cell culture

MDA-MB-436 breast cancer cell line was cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin under a humidified atmosphere with 5% CO₂ at 37 °C. When cells were ~80% confluent, the spent medium was removed followed by rinsing the monolayer once with sterile 1X HBSS (pH 7.4). Cell monolayers were incubated at 37 °C for 3 min with 1 mL of 0.25% trypsin/EDTA to promote cell dissociation. The suspended cells were centrifuged at 600 rpm for 5 min and resuspended in 10 mL of fresh medium after removing the supernatant. The cells were sub-cultured at a split ratio of 1:9 for subsequent passage and remaining cells were seeded into 96-well plates for transfection experiments.

siRNA complex preparation

The Trans-Booster additive was added to siRNA solution that was dissolved in RPMI at a 1:1 siRNA:additive ratio (w/w) with a final siRNA concentration of 40 or 80 nM in the tissue culture medium. The 1:1 siRNA:additive ratio (w/w) was chosen since this 1:1 ratio gave the highest cellular uptake in a previous study [23]. The desired transfection reagents were then added at a 1:5 or 1:10 (w/w) transfection reagent:siRNA ratio and the mixture was then incubated for 30 min at room temperature to allow for optimal complexation [15]. Table 1 summarizes preparation of typical complexes.

Characterization of siRNA complexes

The ζ-potential of siRNA complexes was measured by LITESIZER 500 (Anton Paar, Austria). siMcl-1 and siNC were formulated into various complexes with siRNA:PrimeFect:additive ratios of 1:10:0, 1:10:1, 1:5:0, and 1:5:1 (w/w/w) and yielding a final concentration of 80 nM siRNA in 20 μL RPMI. The complexes were then incubated for 30 min at room temperature. Prior to the size and ζ-potential measurements, the prepared complexes were further diluted with RPMI to a final volume of 1 mL before each measurement. The instrument was set at a 175° back scatter and 25 °C.

Analysis of the morphology of the polyplexes was performed using a 200kv JEOL 2100 Transmission Electron Microscope (TEM). Samples were prepared over a carbon-coated grid immediately after a glow discharge treatment under vacuum by adding a droplet of the polyplex suspension for 5 min and blotting it out using filter paper. After that, the samples were negatively stained using a 2% uranyl acetate solution for 30 s.

siRNA binding by SYBR green dye exclusion assay

The SYBR Green II stain was used to test the ability of the transfection reagents to bind siRNA. In black 96-well plates, 200 μL of SYBR Green II (1X) was added to each well, followed by 4 μL of 0.025 μg/μL siNC. Different volumes of 0.005 μg/μL of the transfection reagent (diluted from 1 mg/mL stock solution) were added to wells to achieve transfection reagent:siRNA ratios ranging from 1:1 to 70:1 (w/w). The plate was incubated for 30 min in the dark at room temperature and the fluorescence of the samples was then measured with a multiwell plate reader (Thermo Ascent; λ ex 485 nm, λ em 527 nm) to determine the amount of free siRNA. The percentage of bound siRNA vs. transfection reagent:siRNA ratio (w/w) was plotted to compare the relative siRNA binding efficiency of different transfection reagents.

| siRNA Conc, siRNA/Transfection reagent/additive | Transfection volume (µL) | RPMI (µL) | siRNA (0.14 μg/µL) | Trans-Booster (0.14 µg/µL) | Transfection reagent (1 µg/µL) |
|-----------------------------------------------|--------------------------|-----------|-------------------|---------------------------|-------------------------------|
| 80 nM, 1:10:0                                 | 15                       | 11.83     | 1.32              | 0                         | 1.85                          |
| 80 nM, 1:10:1                                 | 15                       | 8.66      | 1.32              | 1.32                      | 3.7                           |
| 40 nM, 1:10:0                                 | 15                       | 13.42     | 0.66              | 0                         | 0.92                          |
| 40 nM, 1:10:1                                 | 15                       | 11.83     | 0.66              | 0.66                      | 1.85                          |
Cell viability assay

MTT Assay was used to assess the effect of siMcl-1 treatment in MDA-MB-436 cells. The treatment was carried out in complete medium (DMEM/10% FBS/Pen-Strep) in triplicates in 96-well plates. 6 × 10^3 cells in a volume of 150 µL were seeded in wells and incubated for 24 h at 37 °C in 5% CO₂ atmosphere. The siRNA complexes were prepared as described above and 15 µL of siRNA polyplexes were added to the wells to give a final siRNA concentration of 40 to 80 nM. The cells were typically incubated for 3 or 6 days after polyplex treatment. At the desired timepoint, MTT solution (5 mg/mL) was added to the cells so as to attain a final concentration of 1 mg/mL in each well. The plates were incubated for an additional 2 h at 37 °C. The residual MTT medium was removed, and the formazan crystals formed were dissolved with DMSO. The absorbance (450 nm) was measured using a microplate reader (Molecular Devices, Spectramax 250) with pure DMSO serving as a blank solution. Percent cell viability was calculated with respect to the absorbance of non-treated cells. Analysis of Variance (one-way ANOVA) was applied to determine statistically significant differences among the study groups, with a confidence interval of 95% (p-value < 0.05) (GraphPad, version 8.0, San Diego, CA). Tukey’s post hoc test was also performed for comparing all possible pairs of means in all groups.

Analysis of apoptosis

MDA-MB-436 cells were seeded (8 × 10^4 cells/well) in 24-well plates for 24 h before being treated with 40 nM and 80 nM siNC, siMcl-1, siSur and siMcl-1/siSur complexes for 72 h. The cells were washed once with HBSS, then collected and transferred to 1.5 mL centrifuge tubes after Accutase® digestion, washed (×2) with 1X binding buffer, and centrifuged for 5 min at 900 rpm. Cells were then resuspended in 300 µL of binding buffer and transferred to 5 mL polystyrene round-bottom tubes. Each sample was incubated with 2.5 µL of FITC-Annexin V and 2.5 µL of Propidium Iodine (Apoptosis kit from BD Biosciences) for 15 min before fluorescence measurement by flow cytometry.

Real-time RT-qPCR for gene silencing

MDA-MB-436 cells (3.2 × 10^5 cells/well) were seeded in triplicates in 12 well plates and incubated for 24 h at 37 °C in 5% CO₂. The cells were treated with siRNA complexes in complete media for 3 days. After washing (×2) cells with HBSS, 0.5 mL TRIzol reagent was added to isolate total RNA. The lysates were incubated at room temperature for 5 min, followed by addition of 100 µL chloroform, and centrifuged for 15 min at 8900 rpm at 4 °C. The upper phase was transferred to a new tube and mixed with 250 µL of isopropanol. The mixture was centrifuged for 15 min at 8900 rpm at 4 °C [24]. The total RNA pellet was stored in 1 mL of 75% ethanol overnight at -20 °C and then centrifuged at 7500 rcf for 5 min. The pellet was then air-dried for 30 min, resuspended in nuclease-free water, and then placed in a water bath at 60 °C for 10 min. One µg of extracted RNA was converted into cDNA by using the SensiFAST™ cDNA Synthesis kit as per the instructions of the manufacturer (Meridian Bioscience, OH, USA). For real-time RT-qPCR analysis, human Beta-actin was used as an endogenous housekeeping gene. All RT-qPCR reactions were performed by the reaction mixtures incubated at 95 °C for 10 min followed by 40 amplification cycles at 95 °C for 15 s, 65 °C for 1 min using StepOnePlus (Applied Biosystems, CA, USA) thermal cycler. The fold change was calculated by the ΔΔCt method. Significant differences among study groups were analyzed using one-way ANOVA (GraphPad, v8.0; San Diego, USA), followed by Tukey’s multiple comparison tests with significance level set at p < 0.05.

Microarray analysis was used to further inspect human apoptotic gene expression (RT2 Profiler™ PCR Array Human Apoptosis; PAHS-0122ZC) in treated cells. RNA extraction from treated cells and conversion of extracted RNA to cDNA was carried out as mentioned above. The RT2 Profiler™ PCR ArrayHuman Apoptosis plate was loaded with the SensiFAST™ SYBR Hi-ROX mastermix and cDNA template (5 ng/µL). Using a StepOnePlus (Applied Biosystems, CA, USA) thermal cycler, real time RT-qPCR was performed as outlined above. The ΔΔCt method was used to calculate the fold change.

Cellular uptake of siRNA polyplexes

To quantify siRNA uptake, MDA-MB-436 cells were transfected with 30 nM FAM-labeled siRNA at siRNA:reagent:additive ratios of 1:10:0 and 1:10:1 (w/w/w). After 24 h of transfection, cells were trypsinized and fixed with 3.7% formaldehyde. The mean fluorescence in cells and FAM-positive cell population was measured using BD Accuri C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ). In addition, the mean fluorescence of the recovered cell population and the percentage of cells showing FAM-fluorescence were determined after gating a representative portion of nontreated cells for auto-fluorescence (typically set at 1% of total population) [25].

A fluorescence microscopy analysis of MDA-MB-436 cells transfected with FAM-labeled siRNA complexes was performed after 24 h and 72 h of cell uptake. Cells were washed (×2) with HBSS, fixed with 3.7% formaldehyde, and stained with DAPI (Brunschwig Chemie, Amsterdam, the Netherlands) to visualize the nuclear border using the fluorescent microscope model FSX100 Bio-imaging navigator (Olympus America, Center Valley, PA).
Mcl-1 and survivin silencing in vivo

15 NCG male mice, aged 6–8 weeks, were purchased from Charles River Labs (Quebec City, Canada) and used in this study. The animal study was carried out in compliance with the Canadian Council on Animal Care guidelines, with approval from the University of Alberta’s Animal Care and Use Committee (AB, Canada). The MDA-MB-436 cell line was injected subcutaneously (2 x 10^6 cells in 50 µL of PBS and 50 µL of Matrigel) to generate tumor xenografts, and the mice were treated after the tumors reached a size of ≥ 50 mm^3. Mice were treated via intravenous (tail-vein) injection of siNC and 1:1 combination of siMcl-1 and siSur polyplexes. The siRNA dose was 20 µg, formulated with 20 µg of Trans-Booster additive and 200 µg of Prime-Fect (giving a siRNA:Trans-Booster:Prime-Fect ratio (w/w) of 1:1:10) in 100 µL of RPMI media. The siRNA polyplexes were administered every third day and mice were observed daily. Tumor size was measured using a calliper on days 0, 3, 6 and 9 and the mean/standard error of relative tumor volume was determined in each group using the formula: 0.5LW^2 (the tumor’s long diameter is L, and its short diameter is W.) The level of gene knockdown in the tumor was measured using real-time RT-qPCR. RNA from the tumors was extracted using the TRIzol method and converted to cDNA using the SensiFAST™ cDNA Synthesis kit. The real time RT-qPCR reactions were performed as outlined above. The ΔΔCt technique was used to calculate the fold change.

Results

Effect of siMcl-1 treatment on cell viability

The siRNA binding affinity of transfection reagents is summarized in Fig. 1A. All reagents displayed a similar binding pattern with siRNA, with Leu-Fect A displaying a more complete binding at higher reagent:siRNA ratios. The effect of transfection reagent:siRNA ratio and Trans-Booster additive on the complex size was evaluated (Fig. 1B). Increasing the reagent:siRNA ratio resulted in smaller complexes with Prime-Fect (225 vs. 306 nm), but did not alter the size of polyplexes for other transfection reagents used in complexation process. The typical size distribution is displayed in Fig. 1C. The presence of the additive resulted in a slight increase in size of Leu-Fect A and Leu-Fect C complexes (Fig. 1A). With increasing transfection reagent:siRNA ratio, the zeta-potential increased, but it dropped significantly when the additive was introduced in Prime-Fect, Leu-Fect A and Leu-Fect C polyplexes (Fig. 1D).

The TEM images of siRNA complexes with Prime-Fect are shown in Fig. 2. The complexes were prepared at Prime-Fect:siRNA ratios of 5 and 10, with and without the additive. In the absence of the additive, a more heterogeneous complex structures were evident, where smaller, presumably single particles were present as well as apparently aggregated particles. The agglomerates were constituted in some cases by more than 10 particles of variable sizes in a close-packed conformation, measuring up to 1 µm (upper right portion of Fig. 2A). It is likely that the agglomerated particles were a result of the TEM processing and especially the drying process. In the presence of the additive (1:5:1 and 1:10:1 samples), more uniform particle sizes and shapes were evident where individual spherical particles were evident without any signs of agglomerated particles. There was, however, some variation in particle sizes even with the additive.

The siRNA mediated inhibition of cell growth was then investigated in MDA-MB-436 cells by employing three different siRNAs (siCDC20, siHSP90, and siMcl-1), which were promising targets in past studies from the authors’ group. The transfection reagent Prime-Fect was used for siRNA delivery as well as different siRNA:transfection reagent ratios (1:5 and 1:7.5) and siRNA concentrations (40, 60 and 80 nM). Among the three siRNAs, only Mcl-1 was effective in this cell model with ~35% inhibition (compared to non-treated group) in cell viability at the highest siRNA concentration (80 nM) (Fig. 3A). No growth inhibition was observed in the siCDC20 and siHSP90 treated groups. With the Trans-Booster additive (Fig. 3B and C), the growth inhibition with siMcl-1 became more pronounced, giving significant differences between the siNC and siMcl-1 at 1:10:1 siRNA:transfection reagent:additive formulation. Different ratios of formulation were explored to identify the optimal ratio, but 1:10:1 appeared to be optimal in inhibiting cell viability with siMcl-1 (Fig. 1S), so that ratio was used for the rest of this study. The efficiency of commonly used transfection reagent Lipofectamine 2000 (Fig. 3C) was then examined for siMcl-1 delivery. Lipofectamine generally showed higher toxicity than the complexes formed with Prime-Fect, despite the lower transfection reagent ratio used (1:2, ratio recommended by the manufacturer), and clear inhibition of cell growth with the specific siRNA was only evident at a single siRNA concentration (60 nM). When the cell seeding density was increased from 1,500 to 6,000 cells/well (Fig. 3D), the non-specific toxicity of siNC was decreased from ~55% to ~15% inhibition without altering the response to siMcl-1. With prolonged treatment (6 days), maximum cell death was observed after transfection was carried out at the formulation ratio of 1:10:1 with 80 nM of siMcl-1 (Fig. 3E). Cell viability was ~35% on day 6 vs. ~50% on day 3 after siRNA transfection. With complexes at 1:5:1 and 1:10:1 ratios (siRNA/transfection reagent/additive), the Prime-Fect transfection reagent showed lower toxicity than other transfection reagents after 3 and 6 days of treatment.
Combination of Mcl-1 and survivin siRNAs

Treatment with a combination of siMcl-1 and siSur was then explored to determine if it was more effective than treatment with a single siRNA (Fig. 4). The combination siRNA treatment resulted in a strong MDA-MB-436 cell growth inhibition (~80% decrease in cell viability), which was significantly lower than single siRNA treatments. The siMcl-1/siSur combination was evaluated in an additional breast cancer cell model, MDA-MB-231 cells. The results showed a similar trend, although the response was relatively lower than the MDA-MB-436 cells. To investigate the effect of siRNA treatments on target mRNA levels, MDA-MB-436 cells were treated with siNC and siMcl-1 s at 60 and 80 nM siRNA and target transcript levels were quantified after 3 days of treatment (Fig. 4C). The siMcl-1 treated cells displayed significant Mcl-1 gene silencing (60% and 70% silencing at 60 and 80 nM respectively), as compared to the siNC treated cells, which did not show any silencing effect. Gene silencing was higher at the 80 nM siRNA concentration (40% and 26% in 1:5:1 and 1:10:1 groups, respectively). Treatment with combination of siMcl-1 and siSur (Fig. 3D) led to ~60% Mcl-1 and ~75% survivin gene silencing.

Although silencing of target mRNAs was clearly observed in the combination treatment, a trend of overexpression of non-target gene (survivin in the case of siMcl-1 treatment alone, and vice versa) was noted. This is not surprising given the equivalent roles of these two anti-apoptotic proteins and the possibility of compensating for reduced availability of one functional protein with a similar protein. A microarray of human anti-apoptotic genes was utilized to screen for other expression patterns after the siMcl-1/siSur treatment in combination (Fig. 4E; see Fig. 2S for raw data). In the microarray assay, both Mcl-1 and survivin mRNA levels were found to be suppressed to ~40% and ~17%, respectively. Additionally, other genes including BCL2L10, CASP14, CD40, CIDEA, LTA, and PYCARD, were downregulated by
more than 80%. The mRNA levels of BIRC3, HRK, and BCL2A1 genes were observed to be upregulated by 9.9-, 5.3-, and 5.3-fold, respectively, possibly indicating their involvement to enhance cell survival when Mcl-1/survivin are suppressed.

**Pro-apoptotic effect of siRNA treatment**

The Annexin-V/PI apoptosis assay was used to quantify the effect of single and combination siRNA treatments on inducing apoptosis. The percentage of total apoptotic population in the Mcl-1 and survivin (Fig. 5C) siRNA treated groups was greater than the siNC treated group, which was evident in both early and late apoptotic populations (Fig. 5A-B). The siRNA combination treated group harboured ~25% late apoptotic population and ~30% total apoptotic population, which were significantly greater than the single siRNA treated groups (Fig. 5C).

**Cellular uptake of siRNA complexes**

The MDA-MB-436 cells were transfected with FAM-labeled siRNA and uptake was assessed after 1 and 3 days by measuring siRNA positive cell population and mean fluorescence intensity per cell (Fig. 6). The FAM-positive population was ≥80% on day 1 for all formulations with the polymer, but FAM-positive cells dramatically decreased in groups transfected with siRNA:transfection reagent ratio (1:5) on day 3 (Fig. 6Ai). Presumably, the higher amount of polymer with the 1:10 complex treated groups could better protect the siRNA from degradation. Based on mean FAM fluorescence intensity/cell, the additive polyplexes showed a higher uptake at both siRNA:polymer ratios (Fig. 6Aii). Despite the decrease in siRNA levels on day 3, better cellular uptake and FAM-positive population for the 1:10:1 formulation was evident compared to the lower ratio (1:5:1) formulation.

The uptake of siRNA complexes was confirmed using fluorescence cell imaging. From Fig. 6B, a higher uptake in the additive complex group was evident as compared to additive-free polyplexes for both the 1:5:1 and 1:10:1 formulations, in line with the flow cytometry data in Fig. 6A. At day 3 (Fig. 6Bii), the siRNA uptake was less than day 1, but the uptake demonstrated by the additive polyplexes was still higher than the additive-free groups.
Animal study

Tumor volumes were measured on days 3, 6 and 9 after RPMI (no treatment), siNC and siMcl-1/siSur combination injections every third day. Although tumor growth was observed up to tumor sizes of 50 mm³, there was no exponential growth observed in this model. Upon indicated treatments, reduction in tumor volume was not observed since the cell line (MDA-MB-436) utilized for generating tumor xenografts might not have been compatible with the specific strain of mice used. However, both Mcl-1 and survivin gene knockdown was observed (Fig. 7A and B) in the tumors treated with the siRNA combination compared to those treated with siNC and no treatment groups. Approximately 38% Mcl-1 silencing and 31% survivin silencing was seen in combination siRNA treated tumors compared to non-treated tumors.

Discussion

Nucleic acid-based approaches can form the foundation of future therapeutic agents with a greater specificity for inhibiting cancer cell proliferation than the traditional targeted therapies [26]. The siRNA therapy can be used to target specific cells if the aberrant mRNA sequence is known; however, an effective siRNA carrier must be developed to improve extracellular siRNA stability and siRNA uptake in target cells [27]. The transfection reagents evaluated here are based on lipid modification of low MW PEIs to improve
**Fig. 4** Effect of the combination treatment with siMcl-1 and siSur. Percentage cell viability after combination treatment in MDA-MB-436 cells (A) and MDA-MB-231 cells (B). C) Changes in Mcl-1 mRNA levels (mean ± S.D.) at siRNA concentrations of 60 nM and 80 nM, and siRNA:transfection reagent:additive weight ratios of 1:5:1 and 1:10:1. D) Changes in Mcl-1 and survivin mRNA levels (mean ± S.D.) after combination treatment. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ and (****) $p < 0.0001$ when compared with the control. ($n=3$). E) Heat map for gene expression changes after analysis with human anti-apoptotic gene microarray PCR of cells treated with siMcl-1 and siSur combination in MDA-MB-436 cells.
cell delivery; those transfections reagents were employed to deliver the siRNA where the conditions for siRNA delivery were optimized in vitro by utilizing mainly the anti-apoptotic protein Mcl-1 as the target. The MDA-MB-436 breast cancer cells were utilized as our model for TNBC, which is the most aggressive form of breast cancer with the highest mortality rate [28].

The results show that supplementing the complexes with the anionic Trans-Booster can reduce the size of complexes assembled with Prime-Fect, the most effective delivery agent among those explored in this study. It is likely that the increased anionic charge of the complexes minimized the interactions among the complexes to yield fewer aggregates and smaller complexes. The reduced surface charge,

Fig. 5  FITC-Annexin-V/PI apoptosis assay in MDA-MB-436 cells. MDA-MB-436 cells were treated with siRNAs (40 nM) targeting Mcl-1 and survivin. After 72 h of treatment, apoptosis assay was conducted. A) Representative scatter plots (Annexin vs. PI) for different treatment groups. B) Percentage cell population in early and late apoptotic stages. C) Total percentage of cells undergoing apoptosis.
as indicated by the zeta-potential, can be expected to hamper the interaction between cationic complexes and cell membranes [29]. But contrary to this expectation, the Trans-Booster incorporated complexes exhibited higher cellular uptake as shown in Fig. 6. The more negatively charged complexes may also be beneficial in lowering the cytotoxicity of complexes [30], as well as protecting the siRNA from enzymatic degradation. Moreover, the additive complexes may

**Fig. 6** Cellular uptake of siRNA polyplexes. A) Quantification of cellular uptake by flow cytometry after treatment with 30 nM of FAM-labeled siRNA polyplexes; (i) Percentage of FAM-labeled siRNA positive cell population and (ii) Mean fluorescence intensity of FAM-labeled siRNA/cell post 1 and 3 days of treatment (n = 2). B) Representative images of FAM-labeled siRNA polyplexes in cells captured by fluorescence microscopy (i) 1 day and (ii) 3 days after transfection. Green: FAM-labeled siRNAs. Blue: cell nuclei stained by DAPI.
also improve the availability of siRNA intracellularly due to
due to better siRNA release [14] from complexes, which is
consistent with the results of Prabhakar et al. mesoporous
silica nanoparticles modified with PEI and anionic dithi-
othreitol charges with a lower zeta-potential had a higher
DNA release than nanoparticles with a higher zeta-potential
[31].

The morphology of the polyplexes was studied with
TEM. At siRNA:PRIME-Fect ratio of 1:5, particles were
found either as single spheres or agglomerates, which
explains the increased size obtained by the DLS. Increas-
ing the siRNA/PRIME-Fect ratio from 5 to 10 generated
less agglomeration and smaller particle sizes. The presence
of the additive resulted in a well-dispersed sample with no
presence of agglomerates, probably due to the shifting of
zeta potential to more negative values. Larger particles in
this sample showed a darker region at their center (Fig. 2E).
Figure 2F shows a scheme of changes in agglomeration as a
consequence of polyplex composition. In short, the increase
in polycation concentration reduces the agglomerate size by
reducing the number of particles in agglomerates. For both
polycation compositions, the presence of additive avoided
the formation of agglomerates.

To identify an effective target, siRNAs against the targets
CDC20, HSP70 and Mcl-1, known mediators that are highly
expressed [30, 32, 33] and implicated in breast cancer malign-
nancy, were screened. These three targets were selected
since they are involved in different pathways for control-
ing cell growth. The transcription factor CDC20 activates
the Anaphase-Promoting Complex (APC) during mitosis,
leading to chromatid separation [34]. HSP90 is a protein
that stabilizes the cells against heat stress and helps with
folding of a number of proteins required for tumor growth
[35]. Mcl-1, a member of the BCL-2 family gene, blocks
cytochrome C secretion from mitochondria to prevent induc-
tion of cell apoptosis [36]. Mcl-1 was found to be the most
potent among the investigated targets and was hence chosen
as a candidate for inhibition of malignant cell proliferation.

The data in Fig. 3B and C show that higher inhibition
of cell growth can be achieved with higher transfection
reagent:siRNA ratio. This was due to better intracellular
delivery of the siRNA cargo at higher ratio, as seen in the
flow cytometry-based uptake results. The optimal ratio was
1:10 and higher ratios led to undesired toxicity on the cells
(data not shown). The efficiency of siRNA delivery with
the selected transfection reagents was also compared with
Lipofectamine, a commonly used commercial gene carrier.
Lipofectamine was observed to be equivalent to Prime-
Fect in efficacy, but displayed more toxicity even when it
was used in low amounts. Wang et. al. had also reported a
high toxicity of Lipofectamine in Huh-7 liver cancer cells,
SHSY5Y neuroblastoma cells and MCF-7 breast cancer cells
[37]. These studies were conducted with the optimal cell
seeding density in multiwell plates where too low a cell den-
sity resulted in higher toxicity than desired, while too high
a cell density hampered Mcl-1 gene silencing. Beyond the
transfection reagent:siRNA ratio, another critical variable
was the inclusion of Trans-Booster in the complexes. Such
additive complexes led to robust inhibition of cell growth (as
much as 60% vs. non-treated cells with siMcl-1) while the
conventional additive-free complexes showed minimal inhi-
bition of cell growth (<15% inhibition). This was evident
when using complexes with different ratios (1:5 and 1:10)
as well as concentration of siRNA. The enhanced ability of
additive complexes in reducing cell viability may be a direct
consequence of higher cellular uptake seen with them com-
pared to additive-free complexes. The cell killing activity
of additive complexes improved with increase in duration

Fig. 7 In vivo siMcl-1 and siSur
activity in NCG mice-bearing
MDA-MB-436 tumor xenografts treated with intravenous
injections of Prime-fect/siRNA/additive polyplexes. A) Relative
tumor volume of MDA-MB-436
dxenografts after combination
siRNA treatment. B) Mcl-1
gene expression in tumors and
C) Survivin gene expression in
tumors quantified by RT-qPCR.
of transfection (6 vs. 3 days post transfection), reflecting a delayed action by the reduced Mcl-1 levels. The gene silencing ability of siMcl-1 was confirmed by RT-qPCR, where siRNA dose and formulation ratio significantly affected the mRNA levels of the target gene; the gene silencing Mcl-1 mRNA level can be increased by increasing siRNA dose and transfection reagent:siRNA ratio.

siSur was also examined in our study since it is an anti-apoptotic gene that is widely expressed in malignant cells [38], and has been shown to be a viable target in the management of breast cancer [39]. The combination of siMcl-1 and siSur showed an additive effect in inhibiting cell viability in both MDA-MB-436 and MDA-MB-231 cells. At a lower siRNA concentration of 40 nM in MDA-MB-231 cells, a synergistic activity was observed where the ineffective siMcl-1 improved the cell killing activity when delivered in combination with siSur (Fig. 4). The additive effect of Mcl-1 and siSur was also confirmed by analysis of apoptotic cell population after treatment (Fig. 5). The effect of siMcl-1 and its combination with siSur has not been previously reported with MDA-MB-436 cells. The synergistic effects of siRNA and chemotherapy drugs are routinely reported in the literature [38–40]. In the microarray PCR assay, other genes such as AKT1, an anti-apoptagy gene, were down-regulated since the down-regulation of survivin and Mcl-1 can induce autophagy in cells. Norouzi et. al. for example, used doxorubicin in combination with siSur in PEI-modified silk fibroin nanoparticles in 4T1 cells and discovered a synergistic effect between the small molecular drug and siRNA [40]. The siMcl-1 and siSur combination was recently investigated by Pengnam et al. [41], but they showed no effective response (i.e., cell killing) with siMcl-1 and siSur in MDA-MB-231 cells using a delivery system of cationic niosomes (PCN-B). The positive results reported in this study were presumably due to better siRNA transfection efficiency achieved by the specific transfection reagents employed.

An in vivo study was carried out in NCG mice with MDA-MB-436 xenografted tumors to assess siRNA polyplex activity. The NCG mice were able to establish human MDA-MB-436 tumors, but the xenografts in our hands did not grow as expected (i.e., exponentially) as in the other conventional xenograft models (Fig. 3S). After systemic (IV) administration of siRNA combination targeting Mcl-1 and survivin genes, reduced levels of Mcl-1 and survivin mRNA were noted, although the difference did not reach statistically significant levels (p ~0.78, p ~0.34 for Mcl-1 and survivin vs. Negative control, respectively). Our preliminary result suggests that NCG mice were unsuitable as an immuno-compromised host for preclinical studies of MDA-MB-436 tumors. Future studies will be designed to employ a more established mouse strain for breast cancer grafting, such as the nude mouse or NOD/SCID mouse that were previously used to this end [42, 43]. siSur and siMcl-1 individually (not in combination) were previously used in various xenograft models (4T1, SKBr-3, MCF7, MDA-MB-468, and MDA-MB-435) in mice successfully, leading to significant tumor reduction [44–47]. The effect(s) of anti-apoptotic protein silencing should be better revealed in an animal model with exponentially growing tumors.

Conclusions

The incorporation of Trans-Booster additive resulted in improved cellular uptake of siRNA, effective self-assembly of complexes and enhanced inhibition of breast cancer cell growth with the use of commercial transfection reagent Prime-Fect. Using optimal formulations, the siRNA treatment achieved ~65% decrease in cell viability compared to the no treatment group. The combination of siMcl-1 and siSur augmented the effect of the single Mcl-1 or siSur delivery alone, where the growth inhibition with the combination siRNA treatment reached ~80% after three days of treatment. This study provides evidence for the feasibility of exploring different target siRNA combinations, relevant in breast cancer malignancy, both in vitro and in vivo, with an optimized siRNA delivery system that is commercially available.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10637-022-01282-y.

Acknowledgements This work was financially supported by the Royal Golden Jubilee (RGJ) Ph.D. program National Research Council of Thailand (NRCT) [grant number PHD/0027/2560]. The authors would like to acknowledge all Uludağ lab members at the University of Alberta that contributed to the completion of this study. We thank Edmonton Civic Employees – Charitable Assistance Fund (ECE-CAF) for equipment and Natural Sciences and Engineering Council of Canada (NSERC) for operating funds at the Uludağ Lab.

Author contribution T.S. performed the main part of experiments and wrote the main manuscript, H.U. and W.S. designed the experiment and proof-read the main manuscript. A.A. assisted in the experiments for Fig. 4 and edited the manuscript, C.K. and R.K.C. prepared Fig. 6 and conducted all in vivo studies, T.D. prepared Fig. 2. All authors read and approved the final manuscript.

Funding The research was supported by the Royal Golden Jubilee (RGJ) Ph.D. program National Research Council of Thailand (NRCT) [grant number PHD/0027/2560], Edmonton Civic Employees – Charitable Assistance Fund (ECE-CAF) and Natural Sciences and Engineering Research Council of Canada (NSERC).

Availability of data and materials All the data presented, and the materials used in this study are available on request from the corresponding author.
Declarations

Consent for publication All the authors agree to the publication of this manuscript.

Competing interests H.U. and R.K.C. are share holders in RHJ Biosciences Inc and hold intellectual property rights on the described materials. Other authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, et al (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71:209–249. https://doi.org/10.3322/caac.21660
2. Cronin PA, Gemignani ML (2018) 14 - Breast Diseases. In: DiSaia PJ, Creasman WT, Mannel RS, McMeekin DS, Mutch DG (eds) Clinical Gynecologic Oncology (Ninth Edition). Elsevier, pp 320–352.e326
3. Waks AG, Winer EP (2019) Breast Cancer Treatment: A Review. JAMA 321:288–300. https://doi.org/10.1001/jama.2018.19323
4. Ngamcherdtrakul W, Yantasee W (2019) siRNA therapeutics for breast cancer: recent efforts in targeting metastasis, drug resistance, and immune evasion. Transl Res 214:105–120. https://doi.org/10.1016/j.trsl.2019.08.005
5. Hu B, Zhong L, Weng Y et al (2020) Therapeutic siRNA: state of the art. Signal Transduct Target Ther 5:101. https://doi.org/10.1038/s41992-020-0207-x
6. Santhekadur PK, Kumar DP (2020) RISC assembly and post-transcriptional gene regulation in Hepatocellular Carcinoma. Genes Dis 7:199–204. https://doi.org/10.1016/j.gendis.2019.09.009
7. Wang H, Zhang S, Lv J et al (2021) Design of polymers for siRNA delivery: Recent progress and challenges. VIEW 2:20200026. https://doi.org/10.1002/viw.20200026
8. Mohseni M, Kucharski C, KC RB (2021) Therapeutic delivery of siRNA with polymeric carriers to down-regulate STAT3A expression in high-risk B-cell acute lymphoblastic leukemia (B-ALL). PLoS ONE 16:e0251719. https://doi.org/10.1371/journal.pone.0251719
9. Zakeri A, MaJ K, Beheshtkhoo N et al (2018) Polyethylenimine-saccharides for siRNA Delivery: Nanocarriers Based on Chitosan, Hyaluronic Acid, and Their Derivatives. Molecules 24:148897
10. Prabhakar N, Zhang J, Desai D et al (2016) Stimuli-responsive hybrid nanocarriers developed by controllable integration of hyperbranched PEI with mesoporous silica nanoparticles for sustained intracellular siRNA delivery. Int J Pharm 529:120033. https://doi.org/10.1016/j.ijpharm.2020.120033
11. Zakeri A, Mahdipoor P et al (2015) Targeting Cell Cycle Proteins in Breast Cancer Cells with siRNA by Using Lipid-Substituted Polyethylenimines. Front Bioeng Biotechnol 3. https://doi.org/10.3389/fbioe.2015.00014
12. Parmar MB, Aliabadi HM, Mahdipoor P et al (2015) Targeting of unmodified siRNA and relevance to clinical use. Oligonucleotides 25:345–354. https://doi.org/10.1089/oli.2014.0149
13. Parmar MB, Sundaram DN, KC RB (2018) Combinational siRNA delivery using hyaluronic acid modified amphiphilic polypelexes against cell cycle and phosphatase proteins to inhibit growth and migration of triple-negative breast cancer cells. Acta Biomater 66:294–309. https://doi.org/10.1016/j.actbio.2017.11.036
14. Mohseni M, Kucharski C, KC RB (2021) Therapeutic delivery of siRNA with polymeric carriers to down-regulate STAT3A expression in high-risk B-cell acute lymphoblastic leukemia (B-ALL). PLoS ONE 16:e0251719. https://doi.org/10.1371/journal.pone.0251719
15. Parmar MB, Aliabadi HM, Mahdipoor P et al (2015) Targeting 

Cancer Cells. Biomacromol 19:4193–4206. https://doi.org/10.1021/acs.biomac.8b00918
16. Zhu M, Zhang Y-M (2017) Function of myeloid cell leukemia-1 and its regulative relations with hepatocellular carcinoma. Hepatoma Res 3:129–140. https://doi.org/10.20517/2394-5079.2017.14
17. Campbell KJ, Dhayade S, Ferrari N et al (2018) MCL-1 is a prognostic indicator and drug target in breast cancer. Cell Death and Dis 9:19. https://doi.org/10.1038/s41419-017-0035-2
18. Garg SM, Falamarzian A, Vakili MR et al (2016) Polymeric micelles for MCL-1 gene silencing in breast tumors following systemic administration. Nanomedicine (Lond) 11:2319–2339. https://doi.org/10.2217/nmn-2016-0178
19. Thapa B, Remant KC, Uludag H (2019) siRNA Library Screening to Identify Complementary Therapeutic Pairs in Triple-Negative Breast Cancer Cells. Methods Mol Biol 1974:1–19. https://doi.org/10.1007/978-1-4939-9220-1_1
20. Phianwong S, Thapa B, KC RB et al (2020) Enabling Combinatorial siRNA Delivery against Apoptosis-Related Proteins with Linoleic Acid and α-Linoleic Acid Substituted Low Molecular Weight Polyethylenimines. Pharm Res 37:46. https://doi.org/10.1007/s11095-020-2770-9
21. Yao T, Asayama Y (2017) Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol 16:99–117. https://doi.org/10.1002/rmb2.12024
22. Hickerson RP, Vlassov AV, Wang Q et al (2008) Stability study of unmodified siRNA and relevance to clinical use. Oligonucleotides 18:345–354. https://doi.org/10.1089/oli.2008.0149
23. Parmar MB, Sundaram DN, KC RB (2018) Combinational siRNA delivery using hyaluronic acid modified amphiphilic polypelexes against cell cycle and phosphatase proteins to inhibit growth and migration of triple-negative breast cancer cells. Acta Biomater 66:294–309. https://doi.org/10.1016/j.actbio.2017.11.036
24. Rio DC, Ares M Jr, Hannon GJ et al (2010) Purification of RNA using TRIZol (TRI reagent). Cold Spring Harb Protoc 2010.pdb.prot5439. https://doi.org/10.1101/pdb.prot5439
25. Parmar MB, Aliabadi HM, Mahdipoor P et al (2015) Targeting Cell Cycle Proteins in Breast Cancer Cells with siRNA by Using Lipid-Substituted Polyethylenimines. Front Bioeng Biotechnol 3. https://doi.org/10.3389/fbioe.2015.00014
26. Slastnikova TA, Ulasov AV, Rosenkranz AA et al (2018) Targeted Intracellular Delivery of Antibodies: The State of the Art. Front Pharmacol 9. https://doi.org/10.3389/fphar.2018.01208
27. Song H, Hart SL, Du Z (2021) Assembly strategy of liposome and polymer systems for siRNA delivery. Int J Pharm 592:120033. https://doi.org/10.1016/j.ijpharm.2020.120033
28. Qin J-J, Yan L, Zhang J et al (2019) STAT3 as a potential therapeutic target in triple-negative breast cancer: a systematic review. J Exp Clin Cancer Res 38:195. https://doi.org/10.1186/s13046-019-1206-z
29. Zhou Z, Zhang M, Liu Y et al (2018) Reversible Covalent Cross-Linked Polycations with Enhanced Stability and ATP-Responsive Behavior for Improved siRNA Delivery. Biomacromol 19:3776–3787. https://doi.org/10.1021/acs.biomac.8b00922
30. Li H, Liu L, Chang H et al (2018) Downregulation of MCL-1 and upregulation of PUMA using mTOR inhibitors enhance antitumor efficacy of BH3 mimetics in triple-negative breast cancer. Cell Death and Dis 9:137. https://doi.org/10.1038/s41419-017-0169-2
31. Prabhakar N, Zhang J, Desai D et al (2016) Stimuli-responsive hybrid nanocarriers developed by controllable integration of hyperbranched PEI with mesoporous silica nanoparticles for sustained intracellular siRNA delivery. Int J Nanomedicine 11:6591–6608. https://doi.org/10.2147/ijnm.S120611
32. Karra H, Repo H, Ahonen J et al (2014) Cdc20 and securin overexpression predict short-term breast cancer survival. Br J Cancer 110:2905–2913
33. Halaci SO, Halaci B, Altundag K (2013) The significance of heat shock proteins in breast cancer therapy. Med Oncol 30:575
34. Curtis NL, Ruda GF, Brennan P et al (2020) Deregulation of Chromosome Segregation and Cancer. Annu Rev Cancer Biol 4:257–278. https://doi.org/10.1146/annurev-cancerbio-030419-033541
35. Ali MMU, Roe SM, Vaughan CK et al (2006) Crystal structure of an Hsp90–nucleotide–p23/Sba1 closed chaperone complex. Nature 440:1013–1017. https://doi.org/10.1038/nature04716
36. Michels J, Johnson PWM, Packham G (2005) Mcl-1. Int J Biochem Cell Biol 37:267–271. https://doi.org/10.1016/j.biocel.2004.04.007
37. Wang T, Larcher LM, Ma L et al (2018) Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides. Molecules (Basel, Switzerland) 23:2564. https://doi.org/10.3390/molecules23102564
38. Zaffaroni N, Daidone MG (2002) Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions. Drug Resist Updat 5:65–72. https://doi.org/10.1016/s1368-7646(02)00049-3
39. Jha K, Shukla M, Pandey M (2012) Survivin expression and targeting in breast cancer. Surg Oncol 21:125–131. https://doi.org/10.1016/j.suronc.2011.01.001
40. Norouzi P, Motasadizadeh H, Atyabi F et al (2021) Combination Therapy of Breast Cancer by Codelivery of Doxorubicin and Survivin siRNA Using Polyehtylenimine Modified Silk Fibroin Nanoparticles. ACS Biomater Sci Eng 7:1074–1087. https://doi.org/10.1021/acsbiomaterials.0c01511
41. Pengnam S, Planwong S, Patrojanaasophon P et al (2021) Synergistic Effect of Doxorubicin and siRNA-Mediated Silencing of Mcl-1 Using Cationic Niosomes against 3D MCF-7 Spheroids. Pharmaceutics 13. https://doi.org/10.3390/pharmaceutics13040550
42. Thapa B, Kc R, Liu X et al (2022) TRAIL Therapy for Breast Cancer Treatment by Employing Lipopolymer mRNA Delivery. GEN Biotechnology 1:101–112. https://doi.org/10.1089/genbio.2021.0007
43. Aliabadi HM, Maranchuk R, Kucharski C et al (2013) Effective response of doxorubicin-sensitive and -resistant breast cancer cells to combinational siRNA therapy. J Controll Release 172:219–228. https://doi.org/10.1016/j.jconrel.2013.08.012
44. Ueda A, Oikawa K, Fujita K et al (2019) Therapeutic potential of PLK1 inhibition in triple-negative breast cancer. Lab Invest 99:1275–1286. https://doi.org/10.1038/s41374-019-0247-4
45. Domínguez-Gómez G, Díaz-Chávez J, Chávez-Blanco A et al (2015) Nicotinamide sensitizes human breast cancer cells to the cytotoxic effects of radiation and cisplatin. Oncol Rep 33:721–728. https://doi.org/10.3892/or.2014.3661
46. Zong B, Sun L, Peng Y et al (2021) HORMAD1 promotes docetaxel resistance in triple negative breast cancer by enhancing DNA damage tolerance. Oncol Rep 46. https://doi.org/10.3892/or.2021.8089
47. Janghorban M, Farrell AS, Allen-Petersen BL et al (2014) Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer. Proc Natl Acad Sci USA 111:9157–9162. https://doi.org/10.1073/pnas.1317630111

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.