Enhancing Chemotherapy by RNA Interference

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ABSTRACT
Small interfering RNA (siRNA) has shown tremendous potential for treating human diseases in the past decades. siRNA can selectively silence a pathological pathway through the targeting and degradation of a specific mRNA, significantly reducing the off-target side effects of anticancer drugs. However, the poor pharmacokinetics of RNA significantly restricted the clinical use of RNAi technology. In this review, we examine in-depth the siRNA therapeutics currently in preclinical and clinical trials, multiple challenges faced in siRNA therapy, feasibility of siRNA treatment with anticancer drugs in combined with siRNA in nanoparticles or modified to be parental drugs, sequential therapy of siRNA treatment prior to drug treatment with siRNA and drugs loaded in nanoparticles. We focused on the combinatorial activation of apoptosis by different pathways, namely Bcl-2, survivin, and Pgp protein. Taken together, this review would serve to establish the pathway of effective and efficient combination therapy of siRNA and drugs as a new strategy.

Keywords
chemotherapy, chemotherapeutic enhancement, combination therapy, RNA interference.

Chemotherapy—challenges and current strategies
A single cell mutation is what it takes to initiate cancerous growth. Yet, tumor mass does not develop in a matter of days. Cancer cells develop the ability to escape from the homeostatic control system due to the accumulation of mutation, leading to selective genetic and epigenetic changes that enhance the thriving, proliferation, and avoidance of apoptosis pathways. Chemotherapy for the treatment of cancer was introduced in clinical more than five decades ago and remains the first choice for physicians treating localized primary tumors. Nevertheless, using it for treatment of other epithelial tumors such as breast, colon, and lung cancer is not plausible. Theoretically, the ideal scenario is for a chemotherapeutic drug to target neoplastic cells specifically, thus reducing tumor burden without causing side effects or collateral damage to normal cells. In reality, chemotherapeutics is hampered by the rapid development of drug resistance, poor pharmacokinetics of chemotherapeutic agents, and the systemic toxicity attribute to lack of specificity [1].

Since Matsumura and Maeda discovered the enhanced permeation and retention (EPR) effect, the development of nanoparticles has led a revolution in anticancer drug delivery systems [2]. In general, the design of nanoparticle-based chemotherapeutic is usually with a chemotherapeutic agent encapsulated in, complexed with or attached to, a well-designed nanoparticle [3]. The superiority of nanoparticles over conventional chemotherapeutics includes enhanced pharmacokinetics ability, augmented tumor accumulation attribute to the EPR effect, and increased tumor targeting due to the tumor-specific targeting moiety (i.e., antibody, peptide, small molecule) of nanoparticles. Nanoparticles usually range from <10 nm to approximately 500 nm and thus cannot extravasate from normal vasculatures. As a result, nanoparticle retention in normal tissues, such as skin, spleen, muscle, lung, heart and kidney, is lower compared with chemotherapeutics. Moreover, multiple therapeutic or diagnostic reagents could be incorporated into some nanoparticles to achieve synergistic therapy as well as real-time monitoring [4]. Successful clinical application of
multiple nanoparticle-based therapeutics, such as Taxol®, Doxil®, and Abraxane®, provide promising prospect of nanomedicine [5].

**Overview of current strategies to enhance chemotherapy**

Ingenious strategies have been developed to enhance conventional therapy. Combining two or more drugs with different mechanism has been proven to show synergistic effect on cancer regression. Sharma et al. sensitized leukemic T cells with noncytotoxic imidazole before treating them with camptothecin [10]. Khawli et al. generated a full-length human antibody fusion protein (NHS76/PEP2) and injected it into mice 2 hours before suboptimal doses of various anticancer drugs were given. Interestingly, after NHS76/PEP2 pretreatment, tumors resistant to specific single chemotherapeutics recovered its sensitivity [13]. In another study, Checinska et al. modeled a small molecule Smac mimic that is capable of increasing cisplatin-induced apoptosis by enhancing caspase 3 activity [14]. Another simple yet intriguing way is to add certain known pathway inhibitors to transiently suppressing but not totally inhibiting cell cycle progression or inducing apoptosis. When MEK, PI3K, and mTOR inhibitors were added to hematopoietic progenitor cells, the results demonstrated the enhanced effectiveness of these inhibitors in combination with the chemotherapeutic drug DOX, as illustrated by Abrams et al [15]. Tosso et al. highlighted a new area of enhanced chemotherapy by introducing pro-senescence therapy. Recent studies suggest that senescent cancer cells could activate innate as well as adaptive immune response by secreting immune modulators, which accelerate tumorigenesis in neighboring cells during cancer cell clearance. Because of this conflict, their research on the Jak/Stat3 pathway focused on answering these queries. The combination treatment with docetaxel and JAK2 inhibitor reversed the senescence-associated secretory phenotype (SASP) and enhanced the senescence induced by docetaxel, thus improving anti-tumor immune response. Their data demonstrated that pharmacological treatments is capable of evoking immune surveillance of senescent tumor cells [16]. Furthermore, by alternating electrical current stimulation, chemotherapy efficacy has shown to be enhanced, and it is seen to provide a new way to overcome MDR in tumors [17]. However, tumor cells resistant to one chemotherapeutic agent are normally resistant to others, due to evasion of drug-induced apoptosis, activation of DNA repair, decreased drug uptake, increased drug efflux, etc. [18, 19]. Moreover, combination therapy usually exhibits enhanced toxicity, thus reducing the compliance of the patient. In a study published in 2011, Jung and Shin hypothesized that combined with siRNA treatment, the occurrence of chemoresistance may be significantly reduced. The results demonstrated that combination therapy, with its synergistic effect, is significantly superior as compared to therapy with either siRNA or drugs alone in in vivo experiments [20].

**Strength of RNAi**

RNAi could be mediated by artificially introduced small interfering RNAs (siRNAs). Usually, in the case of the introduction of long double-stranded RNA (dsRNAs), siRNAs are

**Challenges in chemotherapy**

In 2012, Kratz [6], in his review “Finding the optimal balance: challenges of improving conventional cancer chemotherapy using suitable combinations with nano-sized drug delivery systems,” clearly depicted the fine line that we are treading in cancer therapy. He made several points: there is a need to improve current conventional cancer chemotherapy; the improvement should integrate the use of suitable nano-sized drug delivery systems; finding the optimal balance is key. Conventional chemotherapy is usually accompanied by vomiting, nausea, hair loss, loss of appetite, and a compromised immune system, among many other symptoms. Since most anticancer drugs target fast-growing cells (notably, the same kind of cells comprising our hair follicles and keratin), it is also our responsibility to enhance patient compliance and improve the specificity of a drug to reduce its off-target effect.

After repetitive rounds of chemotherapy, tumor cells usually become nonresponsive to the drug through development of drug resistance. Multidrug resistance (MDR) is one of the overriding concerns in cancer therapeutics, as it requires increased drug doses or administration of multiple drugs, which creates a positive feedback loop mechanism [7–9]. Cancer is known to relapse, and most of the time, it recurs with a mutation that makes the tumor more malignant and metastasize faster. Emerging evidence has recently suggested that cancer stem cells (also called tumor-initiating cells) are the original source that sustains the malignancy, including tumorigenicity and chemotherapy resistance, of ovarian as well as other human cancers [10]. Due to the extraordinary tumor-initiating capability of the cancer stem cells, a small amount of these cells could lead to a relapse of cancer even when primary lesion has been eliminated. Hence, cancer stem cells remain a valuable target for cancer therapeutic strategy development.

Emmenegger and Kerbel described the deadly relationship between chemotherapy and the emergence of cancer stem cells [11]. Originally published in *Cell*, Gilbert demonstrated that cancer therapy can acutely alter a tumor’s surrounding tissue and organ environment to promote cancer cell survival and thus facilitate metastatic tumor growth and drug resistance. These were potentially relevant factors in seeking enhancement in chemotherapy. They studied a mouse model of Burkitt’s lymphoma, a cancer of the lymphatic system. When doxorubicin (DOX) was used to treat the cancer cells, most of the tumor cells responded to it. However, a small portion of cells survived, and finally, they were detected in the thymus gland. The expression of several genes in the thymus was changed by DOX, with two being significantly upregulated (IL-6 and Timp-1), both of which are associated with chemoresistance [12]. Treatments of anticancer drugs in the long run would create an opportunity for the cells to adapt and overexpress anti-apoptotic genes that, in turn, increase resistance to cancer treatment.
referred to as a short 21- to 23-nucleotide (nt) duplex with a symmetrical 2-nt overhang at the 3′-terminus, 5′-phosphate, and 3′-hydroxyl group formed when the protein dicer binds to dsRNA. After formation of double-stranded siRNA, it would then be involved into the RNA-induced silencing complex (RISC) [21], a nuclease-containing multiprotein complex that is activated when the siRNA duplex loses one of its strands (sense strand) due to helicase activity [22]. Due to the sequence-specifically targeting ability of the now single-stranded siRNA, the RISC complex would success- fully target specific mRNA hence mediate its cleavage. The cleavage of mRNA ensures that the protein concerned will not be expressed and is thus downregulated after siRNA therapy. The superiorities of siRNA compared with small molecule was summarized in Table 1. This strategy is not only applicable to cancer, but also to target hard-to-target diseases, with reduced nonspecific off-target effects. The application of RNAi technology in cancer treatment could turn the tables around for this devastating disease. Compared to conventional chemotherapy, RNAi has high mRNA selectivity and accuracy and negligible side effects.

### Barriers in siRNA delivery system

When siRNA was proven to be able to target any mRNA, i.e., has the ability to silence all possible genes, it sounded too good to be true. In fact, the problem does not lie with siRNA itself; the critical issue is delivery. The nagging question is how to deliver a negatively charged, hydrophilic, easily degradable, fragile biological molecule in vivo systems where multiple barriers must be overcome each step of the way. It is evident that after intravenous injection, siRNA availability is greatly reduced after encountering nucleases. The next barrier, at the tissue level (mucus layers, endothelial cells and extracellular matrices), also represents a massive hurdle for siRNA therapy. siRNAs are known for their high sensitivity to metabolic degradation, and their small size almost guarantees rapid renal clearance. Given the nature of the anionic charge of siRNA, the ability to penetrate or cross through a cell membrane is poor. Considering all these hurdles and barriers, to realize siRNA-based therapies, all these questions need to be addressed.

### Development of siRNA delivery system

Nanoparticle-based RNAi has been explored in-depth since Craig and Fire first reported the phenomenon of RNA interference [23]. However, RNAs should be carefully protected, as it is easily degraded in RNase within minutes of exposure [24, 25]. In addition, RNAi must be able to overcome various barriers to exert its effect in desired specific areas, such as siRNA in the cytoplasm and small hairpin RNA (shRNA) in the nucleus [26–28]. siRNA is usually encapsulated in lipid-based or polymeric nanoparticles [29, 30], viral-based delivery system (mostly of shRNA) [31], complexed with condensation agent [32, 33], adsorbed onto a nanoparticle surface [34], and conjugated with [35, 36] or modified to increase siRNA stability [37, 38]. RNAi is also captivating, in the sense that it could be exploited to silence almost any pathway in various cell functions. It could target MDR (MDR1 or Pgp family protein) to obliterate resistance to chemotherapy [39, 40] or elevate the effect of chemotherapy and radiotherapy by silencing certain repair enzymes [41, 42].

In the past decade, clinical evaluation of siRNA-based drugs has progressed, albeit slowly. Although systemic administration in the field of RNAi field holds great promise and vast progress has been made using various nonhuman primate models [43–48], many strategies exploring RNAi payload delivery were conducted but subsequently ended because of safety issues [49]. All of these reports came with virus-based encapsulation of siRNA, leading scientists to rethink the use of a virus as a packaging material for nanomedicine therapy [50]. Since then, tremendous efforts have been made to refine and advance these local strategies and adjust them for systemic administration [51]. As seen in Figure 1, PubMed data for “siRNA and chemotherapy” showed a steady increase in the number of publications from 2000 to 2020. However, a slight decline in publications in 2017 and 2018 indicated possible saturation of this field, probably due to disappointing results of clinical trials or the

### Table 1 General Comparison Between siRNA and Small Molecule

<table>
<thead>
<tr>
<th></th>
<th>siRNA</th>
<th>Small Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>High, sequence-driven</td>
<td>Low-medium, conformation-driven</td>
</tr>
<tr>
<td>Potency</td>
<td>Typically pM</td>
<td>Varies</td>
</tr>
<tr>
<td>Number of accessible targets</td>
<td>&gt;1000</td>
<td>500–1000</td>
</tr>
<tr>
<td>Number of potential leads and backups</td>
<td>&gt;10–100, depending on length of target</td>
<td>&lt;2–3</td>
</tr>
<tr>
<td>Speed to lead molecule</td>
<td>4–8 weeks</td>
<td>2–4 years</td>
</tr>
<tr>
<td>Species cross-reactivity</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Manufacturing</td>
<td>Common, rapid, scalable</td>
<td>Varies, can be complex</td>
</tr>
</tbody>
</table>

![Figure 1](https://example.com/figure1.png)  
Figure 1 Number of publications found in PubMed regarding siRNA and chemotherapy from 2000 to 2020.
Table 2  Current Status of Clinical Trials for RNAi Therapy in Cancer. Information Obtained from http://clinicaltrials.gov (Updated until 2019) Current Status of Clinical Trials for RNAi Therapy in Cancer

<table>
<thead>
<tr>
<th>Company/institution</th>
<th>Formulation</th>
<th>Nanoparticle/Strategy Used</th>
<th>Treatment</th>
<th>Phase</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnylam Pharma (Tekmira)</td>
<td>ALN-VSP02</td>
<td>SNALP</td>
<td>Liver cancer</td>
<td>Phase I (2009-2011)</td>
<td>Well-tolerated, antitumor activity, antitumor activity</td>
</tr>
<tr>
<td>Tekmira/Alnylam</td>
<td>TKM-PLK-001 (TKM-080301)</td>
<td>SNALP</td>
<td>Cancer</td>
<td>Phase II (ongoing), Phase III (2010-2015)</td>
<td>On-going study</td>
</tr>
<tr>
<td>Alnylam/Calando</td>
<td>CALAA01</td>
<td>Cycloextrim-based nanoparticle</td>
<td>Cancer</td>
<td>Phase I (2008-2012)</td>
<td>Terminated</td>
</tr>
<tr>
<td>Silence Therapeutics</td>
<td>Atu027-I-2</td>
<td>Combination therapy with gemcitabine</td>
<td>Advanced or metastatic pancreatic cancer</td>
<td>Phase Ib/IIa (2013-2015)</td>
<td>On-going study</td>
</tr>
<tr>
<td>Duke University (Durham, NC)</td>
<td>NCT00672542</td>
<td>Mature DCs transfected with RNAs encoding melanoma antigens MART1</td>
<td>Metastatic melanoma</td>
<td>Phase I (2008-2014)</td>
<td>DC-based immunotherapy is enhanced</td>
</tr>
<tr>
<td>Comprehensive cancer center of wake forest university</td>
<td>APN401</td>
<td>siRNA-transfected peripheral blood mononuclear cells</td>
<td>Melanoma, kidney, pancreatic cancer or other solid tumors that are metastatic or cannot be removed by surgery</td>
<td>Phase I (2014-2016)</td>
<td>On-going study</td>
</tr>
<tr>
<td>Dicerna Pharmaceuticals, Inc.</td>
<td>DCR-MYC</td>
<td>Lipid nanoparticle</td>
<td>Hepatocellular carcinoma</td>
<td>Phase Ib-II (2014-2016)</td>
<td>On-going study</td>
</tr>
<tr>
<td>MD Anderson Cancer Center</td>
<td>siRNA-EphA2-DOPC</td>
<td>Liposome</td>
<td>Advanced cancer</td>
<td>Phase I (2015-2020)</td>
<td>Just approved, not yet recruiting</td>
</tr>
</tbody>
</table>

emergence of new RNA techniques such as CRISPR-Cas9 and other noncoding RNAs. Out of the many clinical trials run in NIH, only a certain portion was dedicated to cancer research and therapeutics, as summarized below in Table 2.

By now, the majority of the gene therapy clinical trials are focused on cancer therapy and have accounted for more than half (64.4%) of total trials. Various malignant cancers have been included into the gene therapy clinic trials, for example, solid tumors like myeloma, liver, pancreatic, skin, gastrointestinal, lung, urological, gynecological, neurological, and pediatric tumors, and also hematological malignances. Despite various other cancer suppressor gene such as BRCA-1, Fus-1 and endostatin, p53 is the most wildly investigated among cancer suppressor genes in clinical trials. The gene therapy with p53 transfer was used to enhance chemotherapy or radiotherapy. Gene-directed enzyme pro-drug therapy is another type of gene therapy. By specifically delivering and expressing certain enzymes that catalyze pro-drugs into cytotoxic reagent in the targeting site, the side effect of chemotherapy would be significantly reduced. For example, the nontoxic pro-drug ganciclovir could be converted into cytotoxic triphosphate ganciclovir by herpes simplex virus thymidine kinase. The first in-human systemic administration trial engaging the use of both siRNA and drug for advanced cancer therapy has been recently approved for phase I clinical trial by the FDA and is scheduled to run from 2015 to 2020.

RNAi strategies for enhancing chemotherapy

In the past decade, the application of RNAi technology in cancer research, as in other fields, has obtained crucial progress. RNAi technology has become a routine tool to investigate the function of certain gene by eliminating it. High-throughput RNAi screening has emerged as a second wave of functional genomic technologies [52]. This HTS RNAi screening method was first demonstrated by Gonczy and Fraser in separate back-to-back studies [53, 54]. Researchers have been screening for novel molecular targets by dissection of cancer-associated pathways or oncogenes with RNAi and dissecting cancer-associated phenotypes. RNAi can be used to target any RNA identified in the genome, enabling the rational design of comprehensive RNAi libraries. After targeting a certain mRNA, the same siRNA will be able to remove individual splice variants. For optimum results, one can also simultaneously target 2-3 mRNAs in a siRNA cocktail delivery. The most important point is that RNAi brings a loss-of-function analysis to many well-defined cell culture systems and can be used in eukaryotes for which classical genetic tools are lacking, enabling cross-species comparison [55]. Not only that, RNAi screening provides a high-throughput way of simultaneously detecting the function of numerous genes, thus deepening the understandings of the composition and function of the complex, intertwined signal transduction networks in each cell [56].

Combination of siRNA and drug to enhance chemotherapy efficacy

Combining siRNA with chemo/radiotherapy not only significantly resensitized the cancer cells but also reduced side effects by significantly lowering the dosage of chemo/radiotherapeutics. From the clinical point of view, the effect of different treatments is equal to the sum of the effects obtained by individual treatments; thus, these treatments are considered to be additive. Otherwise, if the combined effect is considerably higher than those of individual treatments, these treatments are considered to be synergistic. There is also a chance, although uncommon, that the combination is antagonistic in nature [20, 57, 58]. Co-delivery therapy consists of putting both anticancer drugs and siRNA in the same nanoparticles and delivering them simultaneously. More often but not, this strategy is used to reverse drug resistance in cancer therapy [59, 60]. Simultaneous delivery of the siRNA and drug proved to have synergistic effects when used together, given that the mechanistic pathway of both are related, or the encapsulation efficacy of drug and siRNA can be improved by charge or affinity interactions. The opposite may also be true in the sense that the drug and siRNA can act on different anticancer pathways, which might result in better tumor cell retardation. Sequential therapy is undoubtedly especially useful in treating cells with MDR [61–63]. This treatment allows one to deliver siRNA to knockdown-specific genes (in this case, from the Pgp family) and sensitize the cells to the normal dose of drugs.

Activation of apoptosis to enhance chemotherapy efficacy

Cancer cells can develop drug resistance after long-term chemotherapy. The combination of different types of anticancer drugs can inhibit or block drug resistance. However, a wide variety of cancer cells are resistant to many drugs, which is known as MDR [64]. The blockage of apoptosis is one of the main factors that contribute to the development of MDR in cancer [65]. Apoptosis is a process of programmed cell death that is regulated by many different gene products. Mutations in some of them, including B-cell lymphoma 2 (Bcl-2) protein and survivin to activate cellular anti-apoptotic pathway, have been identified as the cause for the development of MDR in cancer [66]. Therefore, downregulation of the cellular anti-apoptotic protein expression by RNAi can enhance the sensitivity of cancer cells to anticancer drugs, thereby resulting in improved therapeutic efficacy. Cancer cells can develop drug resistance after long-term chemotherapy. The combination of different types of anticancer drugs can inhibit or block drug resistance. Nevertheless, many cancer cells developed MDR [64]. One of the mechanisms of the development of MDR is the blockage of apoptosis in cancer cells [65]. Mutations in apoptosis regulatory genes, including B-cell lymphoma 2 (Bcl-2) protein and survivin to activate cellular anti-apoptotic pathway, have been proven to contribute to the development of MDR in cancer [66]. Therefore, downregulation of the cellular anti-apoptotic
protein expression by RNAi is expected capable to resensitize chemoresistant cancer cells, thereby resulting in improved therapeutic efficacy.

**Activation of apoptosis by knockdown of Bcl-2 protein expression**

The B-cell lymphoma 2 (Bcl-2) family of proteins, including both anti-apoptotic (e.g., Bcl-2, Bcl-xL) and pro-apoptotic (e.g., Bax) proteins, are a class of gene products that regulate apoptosis [66, 67] (see Figure 2). For example, the pro-apoptotic proteins act as a mitochondrial gateway to induce the release of cytochrome c to trigger programmed cell death. However, frequent administration of anticancer drugs, including DOX, 5-fluorouracil (5-FU), paclitaxel (PTX), camptothecin (CPT), mitoxantrone (MTO), and cisplatin, usually induces the overexpression of anti-apoptotic proteins in cancer cells and inhibits apoptosis [68].

Knockdown of anti-apoptotic protein expression by RNAi can activate the apoptotic signal and effectively enhance chemotherapy efficacy. It has been shown that Bcl-2 siRNA-mediated RNAi technique could enhance the drug sensitivity of cancer cells and thus significantly improve therapeutic efficacy [60, 69]. Nakamura et al. [70] used Lipofectamine 2000/Bcl-2 siRNA lipoplexes to downregulate the Bcl-2 expression in DLD-1 human colorectal cancer cell line and found that these treated cells showed improved chemosensitivity to anticancer drug 5-FU. Their in vivo results revealed that the combination therapy with Bcl-2 siRNA (oral administration of PEG-coated Lipofectamine 2000/Bcl-2 siRNA lipoplexes) and 5-FU (intravenous administration of Tegafur) presented significantly enhanced tumor-inhibiting effect compared with monotherapy by either Tegafur or free Bcl-2 siRNA. Moreover, the co-administration of 5-Fu and Bcl-2 siRNA showed a good synergistic effect, i.e., administration of 5-Fu improved intratumoral Bcl-2 siRNA targeting and administration of Bcl-2 siRNA enhanced tumor chemosensitivity. To track the siRNA/drug co-delivery system in cancer cells in real time, Bae et al. recently developed a new type of quantum dot (QD)-incorporating solid lipid nanoparticles (SLNs) to co-deliver Bcl-2 siRNA and PTX to achieve both synergistic therapy and in situ real-time imaging [71]. To construct this optically traceable co-delivery system, a lipid mixture of cholesteryl oleate (41 wt%), cationic 3-[N-(N',N'-dimethylaminoethane)-carbamoyl]-cholesterol hydrochloride (28 wt%), cholesterol (10 wt%), triglyceride (3 wt%), l-α-dioleoyl phosphatidylethanolamine (DOPE, 14 wt%), and distearoyl phosphoethanolamine polyethylene glycol (DSPE-PEG, 0.1 wt%) were utilized to design a natural low-density lipoprotein (LDL) mimic. After mixing the above LDL mimic with cadmium selenide/zinc sulfide (CdSe/ZnS) QDs and PTX, core-shell SLNs could be formed with QDs and PTX embedded in the core and cationic surface to further complex Bcl-2 siRNA. These newly developed SLN/siRNA complexes were found to effectively deliver both Bcl-2 siRNA and PTX into A549 human lung carcinoma cells and exhibited synergistic anticancer activity.

Figure 2  Bcl-2 pathway when inducing apoptosis. Doxorubicin (extrinsic pathway) can also trigger apoptosis in this manner. However, prolonged treatment of doxorubicin or other anticancer drugs often leads to resistance as the cells overexpress antiapoptotic drug to counteract the drug.
by triggering caspase cascade and finally leading to cell apoptosis. In addition, by detecting the fluorescence from the embedded QDs, SLNs provide an efficient tool for real-time in situ imaging of intracellular translocation investigation.

Besides lipid-based nanocarriers, synthetic cationic polymers are another class of important carriers for the co-administration of Bcl-2 siRNA and chemotherapeutic reagents. The representative one is poly(ethylene amine) (PEI)-based polymer, which is widely introduced for siRNA delivery due to its high complexation of siRNA and strong “proton sponge” effect, which facilitates endosomal escape [72]. Low-molecular-weight PEI (Mw = 2 kDa) was conjugated to poly(ɛ-caprolactone) (PCL) by Cao et al. [73] to obtain block copolymer PCL-ɛ-PEI to co-deliver Bcl-2 siRNA and DOX. In addition, to improve the accumulation of both Bcl-2 siRNA and DOX in tumor, folic acid (FA), which is the ligand of folate receptors overexpressed on epithelial tumors [74], was conjugated to another block copolymer, poly(ethylene glycol)-b-poly(glutamic acid) (PEG-b-PGA). The cationic PEI-b-PCL nanocarriers preloaded with DOX and Bcl-2 siRNA were then coated with FA-PEG-b-PGA according to electrostatic interaction for targeted co-administration of Bcl-2 siRNA and DOX to human hepatic cancer cell lines (Bel-7402 cells). This co-administration system was able to downregulate Bcl-2 protein expression. Only 5% of the cells were alive after being treated by the FA-functionalized Bcl-2 siRNA/DOX-loaded nanocarriers, which is 12-fold lower than cells incubated with the carriers loading scrambled siRNA and DOX. Subsequent work showed that the FA-functionalized Bcl-2 siRNA/DOX-loaded nanocarriers can significantly inhibit glioma growth, indicating a good synergistic effect on apoptosis and highlighting the importance of targeted delivery for cancer therapy [75]. Another example of the polymeric delivery system is the polypeptide cationic micelles for Bcl-2 siRNA and docetaxel co-administration. Zheng and coworkers prepared a triblock copolymer poly(ethylene glycol)-b-poly(t-lysine)-b-poly(t-leucine) (PEG-b-PLL-b-PLLeu) [76]. The hydrophobic PLLeu segment could entrap docetaxel into the core of nanocarriers, while the cationic PLL segment located on the surface of the MSNs. When incubated with HeLa cells and trigger the cells apoptosis. Compared to free DOX, the anticancer efficacy was increased by 132-fold. This extremely large difference is mainly attributed to the following two reasons: Bcl-2 siRNA significantly suppresses Bcl-2 mRNA and thus efficiently overcomes drug resistance and the perinuclear location of DOX provides an additional benefit that possibly reduces pump resistance and thus further improves drug effectiveness.

Stimuli-responsive nanocarriers play a key role in cancer treatment. In the past decade, a vast amount of attention has been focused on the construction of conditional releasing end-capped MSNs for drugs or siRNA delivery, which is responsive to tumor microenvironments, for example, weakly acidic pH [81], overexpressed enzymes [82] and chemicals [83]. These end-capped MSNs behave “zero premature release” before arriving at the target site and only releases their cargo upon the tumor microenvironment stimuli. With the advantage of preventing leakage beyond the target site, the on-command releasing nanocarriers become excellent alternatives to traditional drug or siRNA delivery systems [78, 79]. Based on the fact that glutathione S-transferase (GSH) can specifically break disulfide bonds and generally shows 100- to 1000-fold higher concentration in the cytoplasm (approximately 2-10 μM) than that in extracellular fluids (approximately 2-20 μM) [84, 85]. Ma et al. [86] recently introduced disulfide bonds to the surface of MSNs to construct redox-responsive MSNs for co-administration of Bcl-2 siRNA and DOX. To construct this redox-responsive co-delivery system, the surface of MSNs were functionalized with thiol groups (MSNs-SH), forming disulfide bonds with cysteamine to yield amino-modified MSNs (MSNs-NH₂). After reacting with adamanate-1-carboxylic acid (AD-COOH) and being capped by ethylenediamine-modified β-cyclodextrin (β-CID) via host-guest interaction, redox-responsive MSNs can be obtained with DOX blocked inside the pores and Bcl-2 siRNA located on the surface of the MSNs. When incubating human cervix carcinoma cell lines (HeLa cells) with the DOX/Bcl-2-loaded redox-responsive MSNs, the intracellular GSH could efficiently break the disulfide bond, resulting in rapid release of Bcl-2 siRNA in cytoplasm then knockdown of Bcl-2 expression. With this downregulated Bcl-2 expression, the co-administration system showed a much stronger cytotoxicity compared
to the redox-responsive MSNs loading DOX alone. The half maximal inhibitory concentration (IC$_{50}$) dose was 17.5 µg/mL, which is >2-fold lower than that of MSNs loading DOX alone (IC$_{50} = 37.2$ µg/mL). When replacing the Bcl-2 siRNA with green fluorescent protein (GFP) siRNA and then injecting GFP siRNA/DOX-loaded redox-responsive MSNs into the brain ventricle of transgenic zebrafish larvae, GFP expression could be observed in the choroid plexus. The result shows that the delivered DOX could efficiently inhibit the development of choroid plexus, presenting significant reduction of GFP expression in the choroid plexus.

 Activation of apoptosis by knockdown of survivin protein expression

Survivin is one of the well-known apoptosis inhibitors (IAPs). It negatively regulates apoptosis by inhibiting caspase activation, leading to negative regulation of apoptosis or programmed cell death [87]. As an apoptosis inhibitor, survivin is enriched in various cancer cells characterized by chemoresistance, facilitated recurrence, and shorter patient survival [88]. It has been proven that interference with survivin pathways facilitates the enhancement of chemotherapy efficacy, thereby resulting in an enhanced apoptosis and decreased tumor growth [89]. Wang et al. [90] reported a nanoemulsion system for co-administration of survivin siRNA and two types of chemotherapeutic reagents (DOX and PTX). This nanoemulsion system was constructed from a synthetic amphiphilic block copolymer, methoxy poly(ethylene glycol)-b-poly(lactide-co-glycolide) (mPEG-PLGA) and cationic ε-polysilane (EPL). In this co-delivery system, PTX and DOX were separately encapsulated into the hydrophilic shell or hydrophobic core of the nanoemulsion, while survivin siRNA was bound on the surface via electrostatic interaction with EPL. Through the synergistic effect of the above three therapeutic agents (PTX binds to β-tubulin subunits, thus disrupting disintegration of microtubules; DOX inserts to the anthracycline portion and inhibits DNA topoisomerase II activity, thus damaging DNA; survivin siRNA downregulates survivin expression, thus activating apoptosis), this co-delivery system exhibited excellent anticancer property and effective inhibition of tumor metastasis in B16-F10 melanoma-bearing mice at a low administration dose of PAX (17.24 µmol/kg), DOX (8.62 µmol/kg), and survivin siRNA (1.5 mg/kg).

Another strategy to suppress survivin expression is using shRNA survivin. shRNA is a tight hairpin structured artificial RNA widely used in RNAi technology due to its stable target gene-silencing ability. Compared to siRNA, shRNA shows unique superiority owing to its multiple targeting capability within the same dose, long-lasting performance, fewer off-target effects, and inducible application [91]. Hu et al. employed host-guest interaction to construct supramolecular micelles for co-administration of PTX and survivin shRNA [92]. They used β-cyclodextrin (β-CD) and low-molecular-weight PEI ($M_c = 600$) to prepare cationic PEI-β-CD, which can self-assemble into supramolecular micelles through the host-guest interaction with 2-amineadamantane-conjugated PAX (Ada-PTX) with a low critical micelle concentration (~0.02 mg/mL). Simultaneously, the positively charged surface of these supramolecular micelles could complex survivin shRNA to form an anticancer drug and shRNA co-delivery system. When using this co-administration system to treat human ovarian cancer cell lines (SKOV3 cells), a 70% reduction in survivin mRNA level could be achieved after 24 h of treatment. With this downregulated survivin, as many as 82.93% of SKOV3 cells are apoptotic after 48 h of treatment by the co-delivery system, which is significantly higher than the cells treated by free PTX (36.95%), survivin shRNA-loaded micelles (36.95%), or the mixture of free PTX and survivin shRNA-loaded micelles (51.9%). After intratumoral injection of the survivin shRNA/PTX-loaded micelles in SKOV-3 xenograft nude mice, the synergistic effect of two therapeutic agents showed strong anticancer efficacy and effective inhibition of tumor growth. However, due to its large size (210 nm), which can be easily cleared by blood, this co-delivery system may not be suitable for cancer therapy by intravenous injection. Shen et al. recently reported a novel nanoplatform composed of PEI-conjugated pluronic P85 (P85-PEI) and α-tocopheryl polyethylene glycol 1000 succinate (TPGS) to co-deliver survivin shRNA and PTX for lung cancer therapy [93]. Pluronic triblock polymers, poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), have been widely used to reverse drug resistance as they have outstanding advantages in reducing ATPase activity, inhibiting drug efflux pump, and selectivity with respect to drug resistance cell phenotype [94]. For example, pluronic P85 has been proven to inhibit GST activity, which is an important enzyme in enhancing detoxification. As a result, pluronic P85 could increase the toxicity of anticancer drugs and hence reverse the drug resistance in lung cancer [95, 96]. TPGS, a ramification of polyethylene glycol 1000 and natural vitamin E (α-tocopherol), is a popular choice as cosolvent and absorption accelerator to enhance the stability and encapsulation capability of the resulting drug carriers [97]. Shen et al. [93] employed a thin-film hydration method to prepare PAX loaded P85-PEI/TPGS micelles with a particle size of 150–180 nm. When incubating this co-administration system with PTX-resistant human lung cancer cell lines (A549/T cells), GST activity was efficiently inhibited and survivin expression was significantly suppressed, leading to strong mitosis inhibition and 73.98% of A549/T cells arresting in the G2/M phase. This efficient mitosis inhibition further induced 11.84% early apoptosis and 52.39% late apoptosis, which is significantly higher than the free PTX treatment group (10.91% early apoptosis and 5.51% late apoptosis). Based on the synergistic effect of inhibition of GST activity, suppression of survivin expression, and PTX-mediated chemotherapy, this co-delivery system showed stronger cytotoxicity compared to free PTX; the IC$_{50}$ of PAX in this formulation (0.043 µg/mL) was >350-fold lower than that of free PTX (15.22 µg/mL). In vivo administration of survivin shRNA/PTX-loaded micelles by intravenous injection also demonstrated their high-performance anticancer activity. The volume of the A549/T solid tumor was only 32.47% of the tumor treated by the commercial drug Taxol.
Activation of apoptosis by knockdown of myeloid leukemia cell differentiation protein (Mcl-1) expression

Mcl-1 protein, encoded by Mcl-1 gene, essentially belongs to the Bcl-2 protein family, which normally enhances cell survival by inhibiting apoptosis [98]. Therefore, the knockdown of Mcl-1 protein expression could increase the sensitivity of cancer cells to anticancer therapeutics and hence efficiently enhance chemotherapy efficacy. Based on oligolysine, Shim et al. [99] prepared a range of its lipid derivatives with different numbers of lysine residue and/or lipid moieties and found that trilysinoyl oleylamide-based liposomes (TLOL) exhibited optimal delivery efficacy as well as minor cytotoxicity. Intravenous administration of Mcl-1 siRNA-loaded pegylated TLOL (pTLOL) significantly reduced the Mcl-1 protein expression in KB-xenograft nude mice. However, the tumor growth was only slightly inhibited although the expression of Mcl-1 protein was remarkably reduced. In contrast, co-administration of anticancer therapeutics suberoylanilide hydroxamic acid (SAHA) and Mcl1 siRNA by pTLOL showed significant anticancer effect. The KB cell viability was 2.6-fold lower than that of the cells treated by pTLOL loading Mcl1 siRNA alone. Intravenous administration of this co-delivery system remarkably inhibited tumor growth on KB tumor-bearing nude mice, and the tumor volume was approximately 4-fold smaller than that of mice treated with free SAHA. Recently, Kang et al. [100] synthesized [100] a new N,N′-dioleylglutamide-based cationic liposomes (DGL) for mitogen-activated protein kinase (MEK) inhibitor PD0325901 and Mcl-1 siRNA co-administration. In cancer biology, Raf/MEK/extracellular signal-related kinase (ERK) pathway is an important pathway involved in cell proliferation as well as apoptosis. By using a selective, cell-permeable inhibitor of the MEK/ERK pathway (PD0325901), phospho-ERK1/2 (pERK1/2) levels could be downregulated thus suppressing downstream pathways, leading to the inhibition of the tumor growth [101]. When incubating human epithelial carcinoma cell line (KB cells) with the PD0325901 and Mcl-1 siRNA-loaded DGL, the DGL are capable of co-delivering the PD0325901 and Mcl-1 siRNA into cells, resulting in the inhibition of the expression of both phosphorylated ERK1/2 and Mcl1 proteins. With these two downregulated proteins, <10% KB cells were alive at a PD0325901 concentration of 0.72 µg/mL. In contrast, the cell viability remained unchanged when treating KB cells with free PD0325901 at the same concentration. When intratumoral injection of the Mcl-1 siRNA/PD0325901 loaded DGL to KB-xenograft nude mice, this co-administration system shows considerably inhibition activity in tumor growth, leading to a reduction of tumor size by 79%, much higher than that of mice treated by DGL loading Mcl-1 siRNA (47% reduction in tumor size) or PD0325901 (13% reduction in tumor size) alone.

Differing from the aforementioned work where anticancer drugs are physically encapsulated into the carriers, Chang et al. recently modified MTO with lipidic chains (palmitoleyl group) to create MTO-derived lipids for Mcl-1 siRNA delivery [102]. They synthesized two anticancer drug-derived lipids, monopalmityltes-MTO (mono-Pal-MTO) and dipalmitoleys-MTO (di-Pal-MTO) and found that the mixture of di-Pal-MTO and di-Pal-MTO at a molar ratio of 1:1 can self-assemble into multilayer cationic nanoparticles (md11-Pal-MTO nanoparticles), in which the positive charge of MTO can complex Mcl-1 siRNA within the multilayers. When incubating this co-delivery system with KB cells, there was nearly no Mcl-1 protein expression, leading to an 81% reduction in cell viability compared with a 68% reduction by Lipofectamine 2000-mediated transfection. Intratumoral administration of this co-delivery system to KB tumor-bearing mice remarkably reduced the tumor size by 83.4% compared with control group, which is much higher than that in mice treated by md11-Pal-MTO nanoparticles without Mcl-1 siRNA (53.9% reduction in tumor size) or free MTO (55.4% reduction in tumor size). Yu et al. [103] recently reported a lipid derivative, 1,2-dioleoyl-snglycero-3-ethylphosphocholine (EDOPC), for the co-administration of chemotherapeutic reagents and Mcl-1 siRNA. This new lipid derivative can form cationic lipid nanoparticles, which allow the loading of drugs with poor water solubility, for example, PTX, into the core and complexation of Mcl-1 siRNA onto the surface. The viability of KB cells treated by this co-delivery system decreased to 38%, much lower than that of the cells incubated with free PTX (90%), free Mcl-1 siRNA (90%) or nanoparticles loading Mcl-1 siRNA alone. The in vivo results showed a similar tendency. Compared to the untreated control, there was a 48% decrease in tumor size in the KB xenograft mice model treated by free PTX or nanoparticles loading Mcl-1 siRNA alone, which is considerably lower than that of mice treated by the co-delivery system (88% reduction in the tumor size).

Activation of apoptosis by knockdown of other protein expression

Polo-like kinase 1 (Plk1) is a vital protein in cell mitosis, and it is overexpressed in various of tumors. Knockdown of Plk1 expression would lead to mitotic arrest, thus triggering apoptosis [104]. Li et al. [58] recently reported a type of dual sensitive CPT prodrug-based liposome for Plk1 siRNA co-delivery. To construct this co-delivery system, CPT was conjugated with the pH-sensitive zwitterionic polymer poly (carboxybetaine) (PCB) via pH- and esterase-sensitive ester bonds to create dual sensitive prodrug PCB-CPT, which is further mixed with cationic dimethylidodecylammonium bromide (DDAB) to form liposomes for co-administration of Plk1 siRNA. When incubating this dual sensitive co-delivery system with HeLa cells, it can simultaneously deliver CPT and Plk1 siRNA into cells then show nonpermanent controlled release, i.e., after PCB protonation in endosomes/lysosomes, Plk1 siRNA was rapidly released in 4 h, whereas CPT was released continuously due to its responsibility to pH and esterase and finally enriched in nucleus after 12 h. As a result, the co-delivery of CPT and Plk1 siRNA achieved synergistic effect by the remarkable cytotoxicity of CPT along with significant apoptosis induced by Plk1 siRNA. The cell viability decreased to approximately 13.9% when the concentration of Plk1 siRNA is 3 µg/mL, which is 2-fold lower than that of the cells treated by the prodrug liposomes loading nonsense Plk1 siRNA (30%). Intravenous administration of this dual sensitive co-delivery system exhibited
significantly improved tumor accumulation as well as synergistic anti-tumor effect in HeLa xenograft mice model.

As a serine/threonine protein kinase, mitogen-activated protein kinase kinase kinase 7 (MAP3K7) takes part in various vital cell functions including apoptosis and transcription regulation and apoptosis [105]. The inhibition of MAP3K7 expression can also re-sensitize chemoresistant cancer cells. Tang et al. [106] used disulfide bonds as a linkage to conjugate CPT to an oligo-peptide, RRRRRHHHHHC (R5H5). The mixture of CPT produg (CPTtsR5H5) and MAP3K7 siRNA can self-assemble into small-sized liposome-like vesicles (20 nm) at an N/P ratio >10. Due to the presence of histidine residues in the produg, this redox-responsive co-delivery system could efficiently escape from endosomes and rapidly release MAP3K7 siRNA and CPT in cytoplasm. When incubating the MAP3K7 siRNA/CPT-loaded vesicles with MDA-MB-231 cells, the synergistic effect between CPT and MAP3K7 siRNA induced a 49% reduction in the cell’s viability compared to the untreated control, which is >4-fold higher than that of the cells treated by the mixture of commercial Lipofectamine 2000 and MAP3K7 siRNA (12% reduction in cell viability).

c-Myc (Myc), the c-Myc protein, encoded by the c-Myc gene, is a transcription factor involved in multiple important processes in apoptosis, cell cycle progression, and cellular transformation [107]. c-Myc is generally overexpressed in many cancers and capable of induction upregulation of series of genes related to cell proliferation, thus resulting in carcinogenesis [108]. It has been shown that the knockdown of c-Myc expression can increase apoptosis and decrease tumor growth. Chen et al. [109] modified PEGylated LPD (liposome-polycation-DNA) with NGR (asparagine-glycine-arginine) peptide, a ligand that could specifically bind to aminopeptidase N (CD13) overexpressed on cancer cells and tumor vascular endothelium, to achieve tumor targeting. When using this NGR-modified PEGylated LPD (LPD-PEG-NGR) to complex c-Myc siRNA and then incubating it with HT-1080 cells (CD13+) or HT-29 cells (CD13−), as expected, c-Myc was efficiently knocked down in HT-1080 cells but not in HT-29 cells. In contrast, nanocarriers containing a control peptide, LPD-PEG-ARA, are hardly uptaken and only a slight knockdown effect was seen in both HT-29 and HT-1080 cells. Intravenous administration of LPD-PEG-NGR nanoparticles loaded with c-Myc siRNA to HT-1080 xenograft nude mice could effectively suppress the expression of c-Myc, resulting in cancer cell apoptosis and inhibition of tumor growth. When treating tumor-bearing mice with c-Myc siRNA and DOX loaded by LPD-PEG-NGR, the siRNA and DOX could accumulate in tumor tissues and lead to an enhanced therapeutic effect.

Nuclear factor-κB (NF-κB) is an important transcription factor that regulates the expression of many genes in the inhibition of apoptosis in cancer [110]. It has been demonstrated that NF-κB plays a vital role in developing MDR in cancer cells [111]. Therefore, the inhibition of NF-κB expression could also improve the sensitivity of cancer cells to chemotherapy. Zhao et al. [112] recently prepared a spermine-grafted poly-γ-benzyl-L-glutamate (PGS) polyelectrolyte brush for co-delivery of DOX and NF-κB p65 siRNA. Due to the knockdown of NF-κB p65 expression, the cancer cells showed an enhanced sensitivity to DOX. At the same DOX concentration, the apoptosis of the cells treated by the co-delivery system is approximately 2-fold higher than that of the cells treated by the PGS polyelectrolyte brush loading DOX alone.

Increase of drug influx to enhance chemotherapy efficacy

Increased drug efflux is another important mechanism of MDR development in cancer cells. Increased drug efflux is a membrane-bound efflux pump activated by adenosine triphosphate (ATP). These pumps (i.e., Pgp; multidrug-associated protein [MRP]) could actively remove many anti-cancer drugs from the intracellular compartment to decrease intracellular drug concentration and thereby therapeutic efficacy [113–115] (see Figure 3). Since the RNAi technique can silence MRP expression, this could subsequently improve the intracellular concentration of chemotherapeutic reagents, and the combination of the RNAi technique and chemotherapy could effectively improve therapeutic efficacy.

Knockdown of Pgp expression

Pgp, encoded by the MDR1 gene, is an ATP-dependent transporter belonging to the ATP-binding cassette family (ABC). It is the primary MDR-related protein that could be found upregulated in multiple cancers, such as cancer of the breast, brain, liver, pancreas, and ovary [115].

In normal cells, Pgp plays an important role not only in expelling harmful molecules, but also in transporting nutrients and other beneficial substances across the surface and intracellular membranes. In cancer cells, a number of cancer drugs can be recognized by the overexpressed Pgp and then expelled to decrease the intracellular concentration of anticancre drugs. In the past few years, plenty of effort was paid to enhance chemotherapy efficacy by suppressing Pgp expression [115]. Navarro et al. [116] conjugated a glutaryl-modified phospholipid, 1,2-di-oioeyl-sn-glyco-3-phosphoethanolamine (DOPE), to low-molecular-weight PEI (Mw = 1.8K) and used this DOPE-PEI to complex Pgp siRNA for drug-resistant breast cancer cell line (MCF-7/ADR cells) treatment. They found that the DOPE-PEI/siRNA complex can significantly inhibit Pgp expression while increasing the uptake of DOX. When using this DOPE-PEI/siRNA complex to pretreat MCF-7 cells for 4 h, the free DOX showed much stronger cytotoxicity than that of the untreated control. Only approximately 10% of cells were alive after 95 h of treatment, which is over 4-fold higher than that of cells treated by free DOX alone. In their subsequent work [117], they synthesized PEG-modified PEI (PEG-PEI), which was mixed with DOPE-PEI to prepare new Pgp siRNA-loaded nanocarriers. Because of the outer layer of PEG, these nanoparticles showed good stability in NaCl aqueous solution. Intravenous injection of this siRNA delivery system into MCF-7/MDR xenograft nude mice led to a >50% reduction in Pgp expression, which is >2-fold higher than that of the mice treated by free siRNA. With this decreased Pgp expression, the tumor cells became more sensitive to free DOX and there was no increase in tumor size in 40 days. Conversely, without the Pgp siRNA pretreatment, the injection of free DOX did not
inhibit tumor growth and there was approximately 3-fold increase in tumor size. Shen et al. [118] also chose PEI ($M_w = 600$) and prepared PEI-β-CD-cholesterol, which can self-assemble into micelles, with the polycationic PEI-β-CD shell to compress the Pgp siRNA within the hydrophobic core in order to encapsulate DOX. When incubating MCF-7/ADR cells with this co-delivery system, Pgp siRNA can effectively downregulate the Pgp protein expression, thus increasing the intracellular concentration of DOX and resulting in cell apoptosis. By intratumoral administration of the Pgp siRNA/DOX-loaded micelles to the MCF-7/ADR tumor-bearing mice, a synergetic effect has been achieved, as the expression of Pgp was significantly reduced and tumor cell apoptosis was remarkably increased at a small dosage (0.5 mg/kg) of treatment. To track the DOX and Pgp siRNA-loaded nanoparticles in cancer cells in real time, Xiong et al. [119] designed a tumor-targeting and membrane-penetrating nanoplatform for DOX and Pgp siRNA co-administration. To construct this co-delivery system, functional groups are attached to both blocks of the biodegradable block copolymer PEO-b-PCL. With a pH-sensitive hydrazone linkage, the functional groups on PCL block could conjugate DOX and were used to form short polyamines to encapsulate siRNA. After complexation of Pgp siRNA to form micelle-like nanoparticles, the PEO outer shell was functionalized by RGD4C (integrin αvβ3-specific peptide) to improve the tumor-targeting and membrane-penetrating nanoplatform for DOX and Pgp siRNA co-administration. To construct this co-delivery system, functional groups are attached to both blocks of the biodegradable block copolymer PEO-b-PCL. With a pH-sensitive hydrazone linkage, the functional groups on PCL block could conjugate DOX and were used to form short polyamines to encapsulate siRNA. After complexation of Pgp siRNA to form micelle-like nanoparticles, the PEO outer shell was functionalized by RGD4C (integrin αvβ3-specific peptide) to improve the tumor-targeting and membrane-penetrating ability. In vitro experiments showed that this multifunctional co-delivery system can enhance DOX concentration in MDA-MB-435/LCC6 cells compared to the cells incubated with nanoparticles loading DOX and scrambled siRNA, thus leading to a much lower cell viability (30%) than that produced by the DOX and scrambled siRNA co-administration system (60%).

Inorganic nanoparticles have been also used as functional nanoplatforms for the co-administration of Pgp siRNA and anticancer reagents. For example, Meng et al. [120] recently reported the use of PEI-PEG copolymer-modified MSNs for co-administration of DOX and Pgp siRNA in MDR breast cancer cell lines (MCF-7/MDR cells). The DOX was loaded in the porous interior while siRNA was condensed to the surface of MSNs via electrostatic interaction with PEI. Incubating this co-delivery system with MCF-7/MDR cells can induce a 55% reduction of Pgp expression compared to that of the untreated control, leading to 3-fold stronger intracellular DOX accumulation than that of cells incubated with MSNs loading DOX alone. When intravenously administrating this co-delivery system to MCF-7/MDR xenografts, a synergistic therapeutic effect can be achieved. Investigation of different tumor biopsies showed that the expression of Pgp was remarkably reduced and apoptosis was significantly increased at the site where DOX was released.

**Knockdown of multidrug resistant protein (MRP) expression**

MRP is another vital ABC membrane transporter protein related to the development of MDR. The currently reported MRP includes MRP1 and MRP2. MRP1 has been shown to be overexpressed in various cancers. It can bind to negatively charged or biomodified molecules, for example, sulfonation, glycosylation, or other post-translational modifications [121]. The Minko group used a single lipid-based nanocarrier formulated by 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to co-deliver DOX and two types of siRNA (Bcl-2 siRNA and MRP1 siRNA) [122] (see Figure 4). They found that Bcl-2 siRNA or MRP1 siRNA-loaded nanocarriers can...
inhibit the growth of small cell lung carcinoma (SCLC) cancer cells. This is easily understood since Bcl-2 siRNA can knock down Bcl-2 expression to induce apoptosis. For the MRP1 siRNA, its anticancer effect is probably attributed to the fact that downregulated MRP1 protein can prevent cells from expelling drugs, and meanwhile results in accumulation of their own metabolic wastes. When DOX is added to Bcl-2 siRNA or MRP1 siRNA-loaded nanocarriers, the synergistic effect between anticancer drugs and the siRNA-mediated RNAi technique leads to significantly decreased viability of SCLC cancer cells. The IC50 dose of Bcl-2 siRNA/DOX or MRP1 siRNA/DOX-loaded nanocarriers is approximately 40% or 60% less than that of free DOX, implying that the silencing of MRP1 expression is more superior in re-sensitizing cancer cells to chemotherapeutic reagents. Compared to the formulations described above, the treatment of Bcl-2 siRNA/MRP1 siRNA/DOX-encapsulated nanoparticles induced the highest toxicity against cancer cells, and the IC50 dose of this formulation was 80% less than that of free DOX, revealing that the optimal anticancer effect could be achieved by obstructing two drug resistance channels simultaneously while delivering anticancer drugs. However, in vivo use of this ternary system may have significant toxicity and other side effects affecting healthy tissues, due to the absence of cancer-targeting ability. To enhance the specificity of the aforementioned lipid-based ternary nanocarriers to cancer cells, the same group decorated the ternary nanocarriers using a mimic of natural luteinizing hormone-releasing hormone (LHRH), which could specifically target the overexpressed receptors on the plasma membrane of lung cancer cells [123]. This LHRH-modified ternary system is suitable for inhalation therapy for lung cancers. Compared to intravenous injection, inhalation of the HRH-modified ternary delivery system facilitates effective delivery of encapsulated therapeutics into lung cancer cells without damaging normal tissues, thus efficiently decreasing the delivery of the HRH-modified ternary nanocarriers to healthy organs. With these advantages, this HRH-modified ternary delivery system showed enhanced anticancer efficacy but lower toxicity on normal organs compared with free chemotherapeutic reagents or other similar intravenous delivery systems.

Besides conjugation of LHRH to lipid-based nanocarriers, the Minko group also used LHRH to modify MSNs and investigated the combination of siRNA and chemotherapeutic reagents for lung cancer therapy [124]. To construct this co-delivery system, DOX and cisplatin were first encapsulated into the pores of MSNs, and Bcl2 and MRP1 siRNAs were then conjugated to MSNs via disulfide bonds. In addition, hydrophilic PEG was also introduced to the surface of MSNs to improve their stability. When treating human adenocarcinoma cell lines (A549 cells) with Bcl-2 or MRP1 siRNA-loaded MSNs, RT-PCR results suggested a 56% reduction in Bcl-2 mRNA level or 58% reduction in MRP1 mRNA level. With these suppressed Bcl-2 and MRP1 siRNA levels, there was a 75% reduction in the viability of the cells incubated with the MSNs loading DOX, cisplatin, Bcl-2 siRNA, and MRP1 siRNA; however, only a 15% reduction in cell viability was achieved when treating the cells with a combination of free DOX and cisplatin. The IC50 of parental chemotherapeutics was 30-fold higher than that of this co-delivery system.

**Knockdown of the expression of other MRPs**

Besides Pgp and MRP1, the rapid development of high-throughput “omic” techniques, including genomics, proteomics, transcriptomics, and metabolomics, has...
enabled the identification of other MRPs in cancer [125]. For example, Y-box binding protein 1 (YB-1) was recently reported to be an MRP highly expressed in various cancers, including cancer of the prostate, lung, breast, colon, ovary, and bone [126]. YB-1 is a member of the Y-box protein family, which is involved in gene expression regulation by binding to certain DNA and RNA to modulate their transcription or translation [127]. It can activate proliferation-associated genes, such as MDR1, MMP-2, and epidermal growth factor receptor (EGFR) [128], to enhance cancer development [128]. Zeng et al. [129] recently reported a trilayer micelle system based on triblock copolymer poly(ethylene glycol)-b-poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide]-b-poly(ɛ-caprolactone) (PEG-b-PAsp(DET)-b-PCL) for co-administration of YB-1 siRNA and rapamycin (RAP). This trilayer micelle system consists of a hydrophilic PEG shell to optimize pharmacokinetics, an intermediate PAsp(DET) structure to complex YB-1 siRNA, and an hydrophobic PCL core to encapsulate RAP. They found that a PCL core shows superior loading efficacy of RAP; furthermore, the PAsp(DET) compartment could excellently complex YB-1 siRNA with a high transfection rate but only slight cytotoxicity. When incubating this co-delivery system with human PC3 prostate cancer cells, it can significantly suppress YB-1 expression and improve the toxicity of RAP. Approximately 38% of cells were alive after 48 h of incubation, which is much lower than the viability of cells treated by the trilayer micelles loading RAP (60%) or YB-1 siRNA (>90%) alone. With this synergistic effect, intravenous administration of the co-delivery system can efficiently inhibit tumor growth on the PC3 xenograft nude mouse model. An approximately 75% tumor volume reduction could be achieved after treatment by the RAP/YB-1 siRNA co-delivery system.

**Other pathways to enhance chemotherapy efficacy**

Besides the activation of apoptosis and suppression of MDR-related proteins expression, there are some other pathways that enhance chemotherapy using the RNAi technique. For example, the cytotoxicity of cisplatin is based on DNA damage. However, there are also pathways responding to the DNA interactions, such as DNA repair pathways and translesion DNA synthesis (TLS) by specialized DNA polymerases [130]. The Rev1/Rev3L/Rev7-dependent error-prone TLS pathway was proven to be vital in mutations induced by cisplatin and improves the repair ability or DNA damage tolerance of cancer cells, leading to undesired MDR [131, 132]. Xu et al. [133] recently reported a poly(lactide-coglycolide)-b-poly(ethylene glycol) (PLGA-PEG) copolymer-based co-delivery system to suppress Rev1/Rev3L expression and thus enhance the therapeutic efficacy of cisplatin. To construct this co-delivery system, cisplatin was modified by decanoic acid to form a hydrophobic prodrug that can be degraded in the cytoplasm. In addition, a positively charged amphiphilic molecule, 1,2-epoxytetradecane-decorated generation 0 of poly(amidoamine) (PAMAM) dendrimer (G0-C14), was prepared to complex Rev1/Rev3L siRNA. By using the double-emulsion method, hollow PLGA-PEG-based nanoparticles could be formed with Rev1/Rev3L siRNA loaded in the hollow core with cisplatin as prodrug in the shell. Incubation of this co-delivery system with human LNCaP prostate cancer cells led to >80% decrease in Rev1/Rev3L mRNA levels. With this downregulated mRNA level, the PLGA-PEG-based co-delivery system showed significantly improved cytotoxicity. At a cisplatin concentration of 0.1-0.2 µM, <40% of LNCaP cells were alive after

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**Figure 5** (A) Chemical structure of PLGA-PEG/G0-C14 nanoparticles developed by Xu et al. This trilayer particle comprises three compartments: an PEG shell, a PLGA/G0-C14 layer, and an amphiphilic core containing siRNA. (B, C) Synthesis of the cisplatin prodrug and the complexation agent G0-C14. (D) Dynamic Light Scattering, DLS analysis of the nanoparticles (cisplatin and siRNA) indicating uniform size distribution. (E) Transmission electron microscopy image of the nanoparticle. Adapted with permission from reference [133].
treatment by the co-delivery system. However, cell viability was >60% when treated by PLGA-PEG nanoparticles loading cisplatin prodrug alone. Intratumoral administration of this co-delivery to LNCaP xenograft SCID-beige mouse models can induce a >60% reduction in Rev1/Rev3L mRNA levels within 48 h. As a result, tumor growth was significantly inhibited and tumor size was >12-fold smaller than that in mice treated by the PLGA-PGE nanoparticles loading cisplatin prodrug alone (see Figure 5).

Suppression of neovascularization by the RNAi technique is another strategy to enhance chemotherapy efficacy. Vascular endothelial growth factor (VEGF) is a crucial factor in tumor angiogenesis. Tumor cells secrete VEGF to promote neovascularization, thus ensuring the oxygen and nutrition supply to the tumor region [134, 135]. VEGF siRNA has been shown to effectively inhibit tumor growth by suppressing angiogenesis. For example, Huang et al. [136] grafted stearic acid (SA) to PEI ($M_w = 10$ kDa) to prepare PEI-SA micelles with cationic shells to load VEGF siRNA and a hydrophobic core to load DOX. This co-delivery system showed excellent stability as well as higher cell permeability rather than naked VEGF siRNA in the existence of serum proteins. Intratumoral administration of this co-delivery system resulted in a 56.7% reduction in tumor size, which is >4-fold higher than that of tumor-bearing mice treated by PEI-SA micelles loading DOX alone (13% reduction in tumor size).

Another strategy for enhanced chemotherapy efficacy is inhibition of cancer cell migration and invasion. Snail and Twist, which belong to the helix-loop-helix and zinc-finger protein families, respectively, are two transcription factors that play predominant role in breast cancer metastasis but have different underlying mechanisms [137, 138]. Snail activates MMPs [139], whereas Twist seems to target other molecules independent from MMP, such as YB-1 [140]. Preventing the metastasis of breast cancer by simultaneously blocking the Snail and Twist signal pathways using the RNAi technique can significantly improve chemotherapy efficacy. Shen et al. [141] synthesized a pH-sensitive nanoplatform for the co-administration of anti-metastasis siRNA (Snail and Twist siRNAs) and PAX for breast cancer therapy. To construct this pH-responsive nanoplatform, pH-sensitive poly (β-amino esters), acrylate-terminated poly[(1,4-butanediol)-diacrylate-b-5-hydroxyamylamine] (PDHA) was conjugated to PEI ($M_w = 1.8$ kDa) to construct the amphiphilic block copolymer (PEI-PDHA) for condensation of Snail and Twist siRNAs. In addition, another copolymer PEG-decorated PDHA (PEG-PDHA) was prepared to cover this nanocarrier by a PEG layer in order to improve the pharmacokinetics as well as to achieve better tumor targeting ability. The obtained PEG-PDHA and PEI-PDHA assembled micelle-like nanoparticles, with PAX being encapsulated into the hydrophobic core and anti-metastasis siRNA being loaded on the hydrophilic shell. In vitro experiments showed that the Snail and Twist siRNA-loaded nanoparticles had a weak wound healing ability, with a healing rate of 26.2%, which is relatively lower than that of the cells treated by nanoparticles loading Snail siRNA (47.7%) or Twist siRNA (35.6%) alone. With inhibited wound healing and invasion rates, the PAX/Snail siRNA/Twist siRNA-loaded nanoparticles showed strong cytotoxicity, and the IC_{50} dose was 54.7-fold lower than that of free PAX, which is much higher than of cells incubated with nanoparticles loading PAX alone (38.2-fold compared to free PAX). Intravenous administration of the PAX/Snail siRNA/Twist siRNA-loaded nanoparticles 4T1 tumor-bearing mice showed superior anticaner efficacy to other formulations, with a tumor-inhibiting rate of 76.5%.

Challenges and Opportunities

In the light of combination therapy with siRNA, hurdles and challenges remain. Substantial optimization still needs to be achieved regarding the dose of siRNA, dosage of drug, delivery vehicles, route(s) of treatment, frequency of treatment, regime of treatment, and difference among cell lines, xenograft models, and orthotopic models. However, as research on siRNA delivery vehicles continues, it becomes possible for siRNA to achieve clinical application. Especially the unique biodistribution profiles and hemodynamic properties nanocarriers could provide during siRNA delivery, due to the obvious difference of their magnetic, biological, optical, electronic, and thermal properties from the substances with larger or smaller size [142]. Although there are no RNAi nanodrugs approved yet, promising candidates are in the pipeline. It is anticipated that with the improvement of the delivery system, the combination therapy of siRNA and chemotherapeutic agent would display increasingly outstanding advantages, for example, increasing the sensitization of cancer cells, reversing MDR, or providing other biological effects by against different molecular targets. By further optimizing the combinatorial library of siRNA and drugs as well as developing the delivery system, we can expect a clearer picture on the most effective treatment method for the eradication of cancer.

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