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REVIEW

Nanotechnology-based combination therapy for overcoming multidrug-resistant cancer

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ABSTRACT Multidrug resistance (MDR) is a major obstacle to successful cancer treatment and is crucial to cancer metastasis and relapse. Combination therapy is an effective strategy for overcoming MDR. However, the different pharmacokinetic (PK) profiles of combined drugs often undermine the combination effect *in vivo*, especially when greatly different physicochemical properties (e.g., those of macromolecules and small drugs) combine. To address this issue, nanotechnology-based codelivery techniques have been actively explored. They possess great advantages for tumor targeting, controlled drug release, and identical drug PK profiles. Thus, a powerful tool for combination therapy is provided, and the translation from *in vitro* to *in vivo* is facilitated. In this review, we present a summary of various combination strategies for overcoming MDR and the nanotechnology-based combination therapy.

KEYWORDS Drug delivery; nanotechnology; multidrug resistance; combination therapy; cancer therapy

Introduction

Cancer is one of the leading causes of global death. According to the WHO, up to 8.2 million people had died from malignant tumors in 2012, accounting for 22% of all noncommunicable disease-related deaths[1]. As a fatal threat to health, the long-lasting battle between human and cancer can be dated back to 160 years ago, and the "weapons" had been evolving with time, i.e., from the original caustics[2,3] to various synthetic/natural cytotoxins[4], later the inhibitors targeting specific intracellular pathways with less side effect[5], and molecular tools that can silence the carcinogenic genes had been developed and applied in cancer treatment[6]. To date, the total number of antitumor drugs marketed can be counted in hundreds, which have benefited numerous patients. The medication can produce significant therapeutic responses and lead to remission in lipid cancers, such as acute myeloid leukemia and lymphoma, as well as solid tumors[7,8]. However, they might serve as a "selective stress" that can induce the proliferation of the therapy-resistant cancer cells and finally reshape the cancer geometry[9,10]. As a result, tumor sensitivity to therapeutics gradually decreases and multidrug resistance (MDR) eventually develops[10]. The emerged MDR plays a crucial role in tumor metastasis and relapse[11], accounting for approximately 10%–90% of the clinical recurrences (varying among different types of cancer) during the following three years after the initial remission[12]. Of note, MDR accounts for over 90% of chemotherapy failures in patients with metastatic cancer[13].

MDR can develop via different mechanisms, including increased drug efflux mediated by the overexpressed MDR-related transporters, increased DNA repair capacity, dysfunctional apoptosis, or activation of prosurvival pathways[10,14] (**Figure 1**). Furthermore, the genetic heterogeneity also represents an important factor, which contributed to tumor's MDR against clinical medications[4,15,16]. Considering the complexity of MDR-inducing mechanisms, a combination of two or more drugs targeting different oncogenetic pathways is useful for overcoming MDR and improving the therapeutic index. Drug combination can be screened according to the mechanisms involved in MDR development. For example, chemotherapeutics may be combined with P-glycoprotein (P-gp) inhibitors, tyrosine kinase inhibitors (TKIs), or

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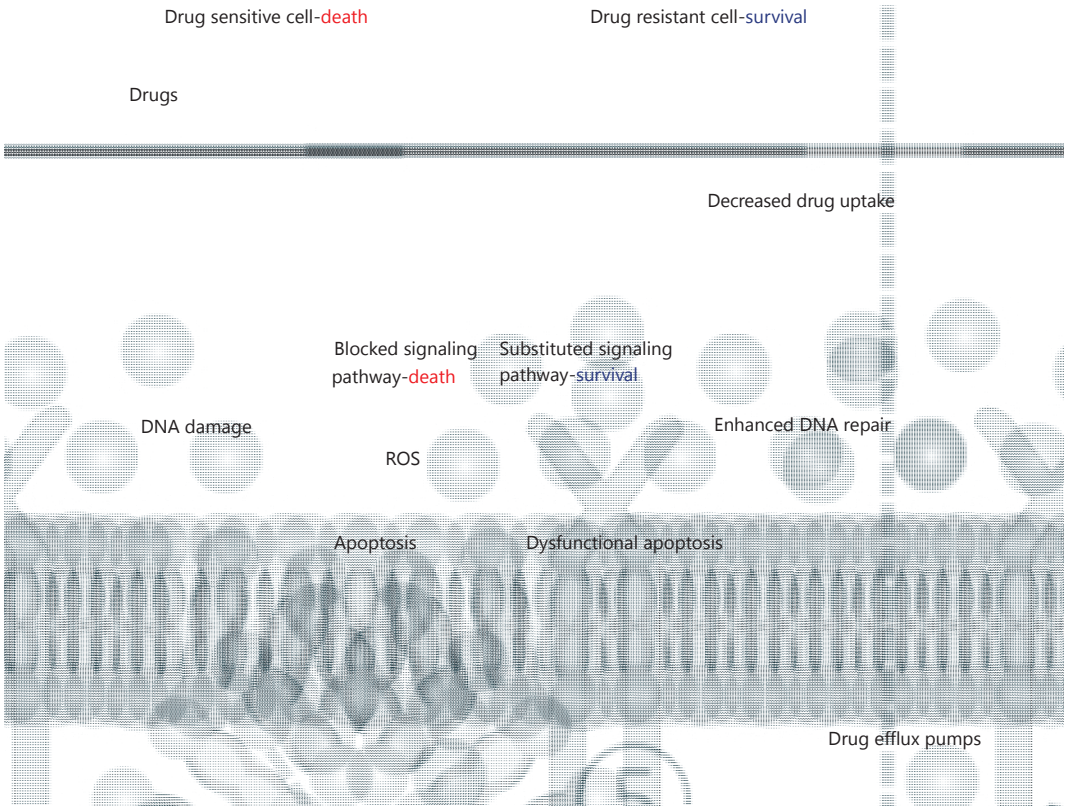

Figure 1 Various mechanisms involved in tumor MDR. The major mechanisms include activated substituted signaling pathway, increased DNA repair capacity, dysfunctional apoptosis, drug efflux mediated by the MDR-related transporters, and decreased drug uptake.

proapoptotic agents to enhance the cytotoxicity[17].

A sharp increase of studies focusing on cancer combination therapy has been observed during the past 30 years, showing more than 35,000 relating works published during the recent 10 years in 2007–2016 (**Figure 2A**). Enormous efforts have been placed on screening the optimized combination formulations and exploring the related molecular mechanisms. Several aspects as follows must also be addressed in achieving an effective therapeutic response *in vivo* when applying the combination regimens screened from the *in vitro* tests.

(a) Pharmacokinetic (PK) diversity. Given that the PK behavior varies among different drugs, an effective dose ratio of the combined drugs, optimized by the cell-based tests, is hard to realize the optimal antitumor responses *in vivo* following systemic administration[18].

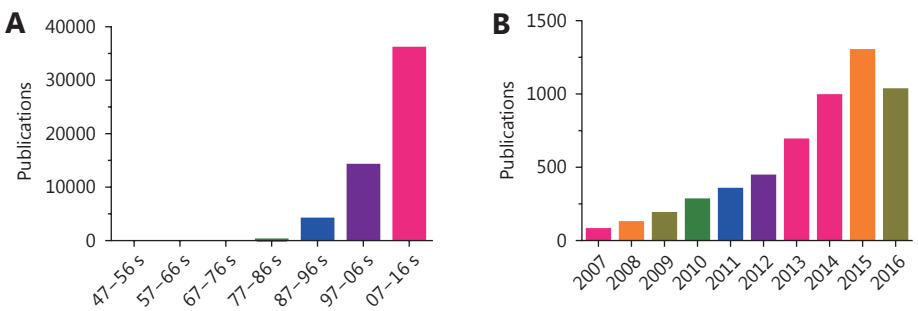

Figure 2 Summary of articles published on "combination therapy of cancer" (A) and "nanotechnology-mediated combination therapy of cancer" (B). We summarized the retrieval number of the articles in the "Web of Science" database from 1947 to 2016 with the search terms "combination therapy and cancer" and 2007 to 2016 with the search terms "nano* and combination therapy and cancer".

(b) Physiological variety. Drugs must undergo a series of PK processes before the arrival on their molecular targets for taking pharmacological action[19]. The physiological diversity in these processes (i.e., enzyme degradation, gastrointestinal absorption, serum protein binding, blood to tumor perfusion, and intracellular delivery) can lead to variation in the drug availability to the tumor cells.

(c) Dose regime. Sequential dosing is needed in certain circumstances when the chemical toxins are combined with a chemosensitizer, for instance. A sensitizer should be preadministrated to reverse the resistance of the tumor cells. The PK differences between the combined drugs, as well as dose timing, often make the treatments poorly predictable with combination regimentation[20].

Nanotechnology-based codelivery

Nanotechnology-based drug delivery is a groundbreaking strategy that has changed the landscape of pharmacotherapy. The nanomaterials (NMs) have been extensively applied for PK improvement and site-specific delivery and thus provided a useful delivery tool for combination therapy[21,22]. First, by taking advantage of NMs, the encapsulated drugs can be simultaneously delivered. Thus, NMs help maintain a relatively identical *in vivo* fate and the dose ratio of the combined drugs. Second, NMs can preferentially accumulate at the tumor site via enhanced permeability and retention (EPR) effect and active targeting mechanisms. In addition, NMs can release the encapsulated drugs in a controlled manner. Thus, NMs are helpful in increasing tumor drug concentration and improving therapeutic efficacy. Third, NMs are resistant to drug efflux mediated by MDR transporters because of the size-exclusion effect, and thus the drugs can retain an effective intracellular concentration[23]. Fourth, the NMs can alter the drug distribution in organelles (e.g., nuclear targeting) and thereby increase the drug concentration in the targeting organelles and enhance the efficacy[24]. Fifth, some NMs also bear the bioactivity of inhibiting the proliferation of neoplasm cells (such as silver nanoparticles) and can display synergistic effect with the delivered drugs[25].

In addition, the druggability can be improved by the nanotechnology. The development of cancer biology and drug discovery has identified thousands of therapeutics, many of which unfortunately failed to further develop into clinical drugs because of unfavorable druggability, such as poor solubility, low-membrane permeability, and instability in biological fluids. Meanwhile, the PK variations among different drugs would result in unwanted toxicity and variable therapeutic effects[26,27]. On this account, NMs have been demonstrated with the capacities of improving drug solubility and stability, as well as promoting the penetration through various biological barriers. Importantly, the NMs can help synchronize the delivery of the coencapsulated drugs and enhance the synergistic effect of the combined drugs.

Given the unique advantages of nanotechnology-based codelivery and its promising applications in anti-MDR cancer therapy, developing the combination strategies for overcoming MDR, such as the rational designs of NMs for optimal combined formulation with different drugs and the patterns of NM-mediated combination therapy, should be considered.

Targeting delivery

NMs can preferentially accumulate at the tumor site via EPR effect and active targeting mechanisms and subsequently release the encapsulated drugs in a controlled manner, thus providing the benefits of increasing the tumor drug concentration and the therapeutic efficacy. In general, the delivery efficiency of the encapsulated drugs to the target organs can be optimized by adjusting the physiochemical features of nanovehicles, such as shape, size, the surface hydrophilicity–hydrophobicity, and zeta potentials[19]. Further modification with targeting ligands is able to improve the delivery efficiency[21]. The targeting delivery is important for both promoting therapeutic effect and reducing adverse effect[21,22,28]. Specifically, NMs may preferentially enter the different subcellular compartments, and it is useful to deliver the specific drugs into certain organelles (e.g., nuclear targeting) and further enhance the therapeutic responses[25].

Drug ratio maintenance

The optimal antitumor efficacy can be obtained *in vitro* by facilely optimizing the concentrations and molar ratio of the coadministrated drugs. However, its predictive *in vivo* application is difficult because of the various PK profiles that thereby result in the submaximal concentrations and a non-optimum molar ratio of combined drugs. Notably, the altering molar ratio of combined drugs might even cause antagonistic effect, providing a challenge during combination therapy[29]. Nanotechnology facilitates the combination treatment by codelivery of the encapsulated drugs to the tumor, with a relatively identical *in vivo* fate and dose ratio of the combined drugs[28]. For example, the combination use of rinotecan and cisplatin displays an antagonistic region

between the molar ratios of 1:2 to 4:1, and beyond this ratio range, the two drugs yield synergistic effect[30]. By coencapsulating the two drugs into liposomes, the fixed drug ratio can be maintained for more than 24 h. However, for the liposomal formulations, several concerns of drug premature release in blood are observed, leading to unwanted effects. To precisely control the drug ratio and *in vivo* release behavior, the phosphorous lipids can be crosslinked by intermolecular disulfide bonds. By using this cross-linked multilamellar liposome vesicle (cMLV), the drug ratio of the coencapsulated doxorubicin (DOX) and paclitaxel (PTX) was precisely maintained for more than 24 h, maximizing the therapeutic effect while minimizing the systemic toxicity[31].

The ratio control of sunitinib and curcumin can be achieved by using the bovine serum albumin (BSA)-coated superparamagnetic iron oxide nanoparticles (SPIOs) in a xenograft tumor-bearing mouse model[32]. *In vitro* formulation screening results showed that the optimal drug ratio of sunitinib to curcumin was approximately 0.5. Deviation from this ratio would lead to the increased combination index value and compromise the synergistic effect. By coencapsulating the two drugs in the protein layers, the sunitinib/curcumin ratio was maintained in an optimal range. Meanwhile, the nanoformulated drugs manifested a significantly increased tumor accumulation (29.8-fold for sunitinib and 8.4-fold for curcumin compared with free drugs), showing the dual benefits of SPIO-mediated combination therapy (**Figure 3**).

NM-mediated MDR inhibition

Aside from the functions of improving the PK behavior, some NMs can function as P-gp inhibitors and restore the antineoplastic activities of the loading drugs in the MDR cells. The NMs may reverse MDR effect via different ways. For example, inorganic silver nanoparticles are resistant to drug efflux because of the size-exclusion effect[23]. Many amphiphilic copolymers can inhibit the activities of overexpressed P-gp pumps. For example, Pluronic P123/F127, methoxy poly (ethylene glycol)–poly (lactide) copolymer (mPEG-PLA), and TPGS 1000 (D-R-tocopheryl polyethylene glycol 1000 succinate) can inhibit the P-gp by ATP depletion[33-35]. However, surface modification using TPGS does not induce ATP depletion-associated P-gp inhibition. Instead, the anti-MDR effect of TPGS-modified PLA nanoparticles can be attributed to the increased drug uptake and intracellular protection from enzyme degradation[36,37]. Liposomal drugs have been reported with effects on altering the raft compositions in the resistant cells and decreasing the lipid raft-associated P-gp and the DOX-loaded liposomes, thus impaired the transport and ATPase activity of P-gp[38].

Despite the various mechanisms involved in P-gp inhibition, NMs may restore tumor's sensitivity by simply enhancing the cellular uptake of the payload drugs. For example, Liu and co-workers used a cMLV for codelivery of DOX and PTX into the drug-resistant 4T1 cells[39]. The results showed that neither single drug-loaded cMLV nor soluble drug mixtures can inhibit P-gp expression, whereas the dual drug-loaded cMLV can efficiently suppress P-gp expression and cell proliferation. The enhanced uptake mediated by the cMLV yielded the sufficiently high intracellular concentration of both drugs and their coaction thus inhibited the P-gp function of 4T1 cells.

Tailing drug release to achieve the optimal synergistic effect

The synergy of combined drugs for certain instances requires the drugs to be given in a sequential manner. For example, sequential treatment with erlotinib and DOX can rewire the apoptotic signaling pathway and increase antineoplastic effects on the triple-negative BT-20 cells[20]. Similar trends can be found in a combination use of chemotherapeutics with RNAi agents. Given that the target depletion effect of RNAi generally occurs at 48 h after the RNAi treatment, the coloaded RNAi agent and antitumor drugs should be released sequentially[40]. Of note, sequential or simultaneous dosing regimentation depends on the specified synergistic patterns of the combined drugs. For example, sequential application of verapamil (P-gp inhibitor) and vincristine (antitumor agent) in treating MCF7/adverse drug reaction (ADR) cancer did not yield superior antitumor effect compared with the simultaneous administration[41]. However, the sequential application of verapamil and vincristine displayed higher efficacy than the reverse sequential application (i.e., vincristine→verapamil). As known, MDR can be effectively reversed by P-gp inhibitors, such as verapamil, tariquidar, zosuquidar, and laniquidar[42], and combination of cytotoxins with MDR inhibitors may significantly increase intracellular drug retention and enhanced cytotoxicity[43,44]. The results indicated that small molecular P-gp inhibitors can lead to immediate inactivation of the drug efflux pump. Thus, sequential application of chemodrugs with P-gp inhibitors is not needed.

For combination therapy of the vascular disruptive combretastatin A4 (CA4) with chemodrugs, however, sequential release pattern is optimal[45]. The number of blood

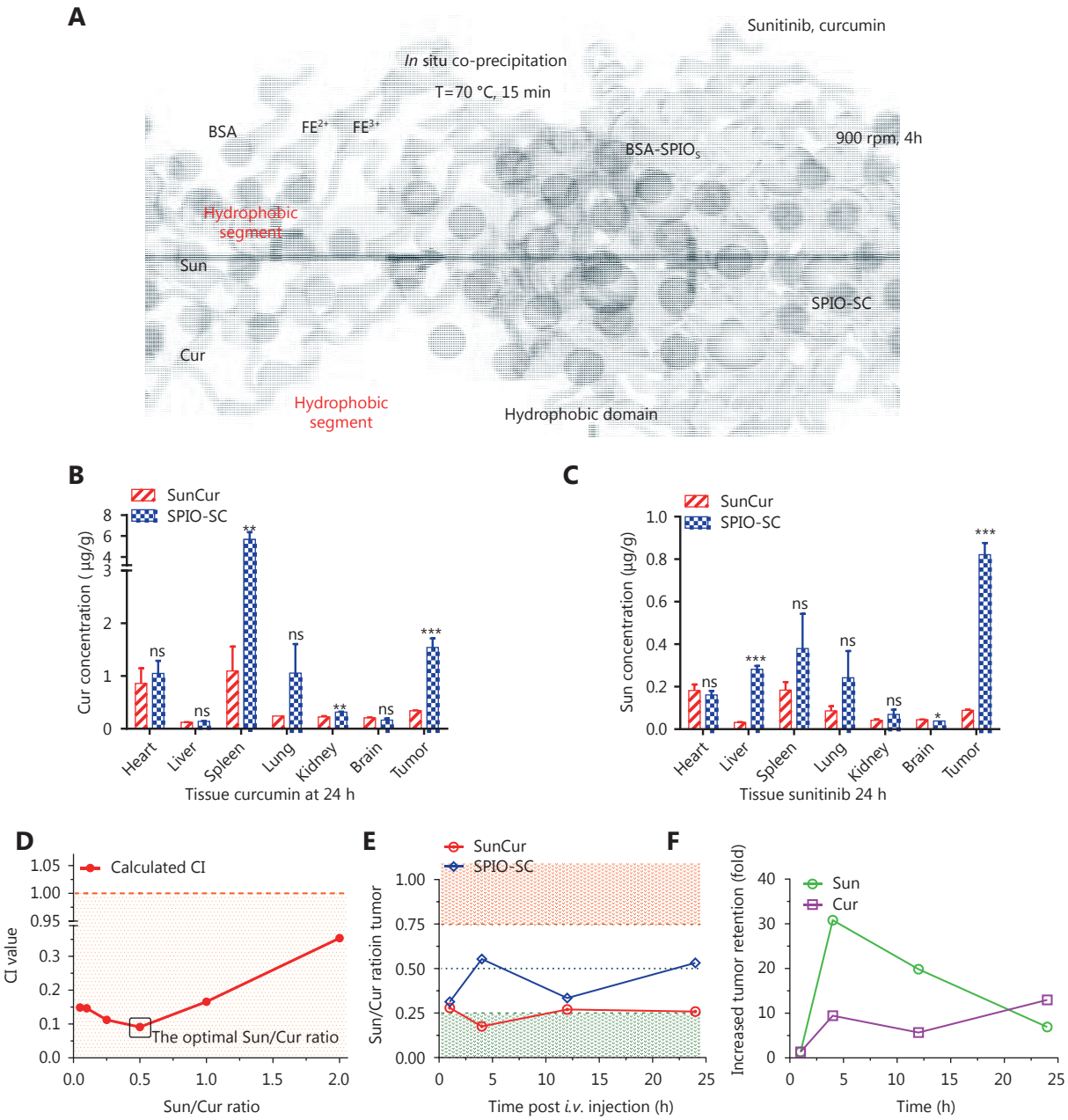

Figure 3 Ratio controlled delivery of sunitinib and curcumin by BSA-stabilized SPIOs. (A) Preparation scheme of the BSA-stabilized SPIOs and subsequent encapsulation of sunitinib and curcumin (SPIO-SC). (B) Sunitinib distribution in the major organs. (C) Distribution of curcumin in the major organs. (D) Combination index (CI) value of sunitinib and curcumin at different Sun/Cur ratios. CI > 1, antagonistic effect; CI = 1, additivity; CI < 1, synergistic effect. (E) Dynamic variation of the SunCur ratio in the tumor over time post administration. (F) Increased drug retention of SPIO-SC in the tumor compared with SunCur. Reprinted with permission from Ref. 32.

vessels decreased in 72 h after applying CA4. The subsequent use of antineoplasmic agents also enhanced the EPR effect of tumor and yielded an increased tumor uptake and improved therapeutic response. Inspired by this effect, several kinds of nanovehicles including liposome, poly (ethylene glycol)-block-poly (D,L-lactic acid) (PEG-b-PLA) micelle, and mesoporous silica nanoparticles (MSNs), have been developed for sequential delivery of CA4 and antineoplastic drugs. In the liposomal formulation, hydrophobic CA4 and hydrophilic DOX•HCl were separately encapsulated into the

lipid bilayer and inner aqueous phase of RGD liposomes. The release of aqueous DOX in the inner phase was confined by the lipid bilayer because of the hydrophilic differences, and a differential release of DOX and CA4 from the liposome was achieved[46]. In the micellar formulation, DOX or PTX was covalently conjugated with the hydrophobic terminal of the copolymeric PEG-b-PLA. Hydrophobic CA4 was also incorporated into the micellar cores. On this account, the release kinetics of the payloads was dependent on the binding pattern between the micelles and the encapsulated drugs. The release of CA4 in the hydrophobic cores of micelles was relatively faster than the polymer-conjugated DOX or PTX[47,48]. Another interesting application was the coencapsulation in the MSNs. The hydrophilic CA4P (combretastatin phosphate) and DOX were encapsulated by MSNs via physical adsorption. CA4, due to the difference in surface charge, was quickly released from the negatively charged MSNs because of electrostatic repulsion, whereas positively charged DOX was released in a slow pattern[49].

In addition, the benefit of simultaneous application of a chemodrug and a sensitizer was also demonstrated in treating cancer stem cells. Sun et al.[50] reported that simultaneous delivery of a differentiation agent all-trans-retinoic acid (ATRA) with a chemodrug DOX in the same nanovehicle can efficiently induce cancer stem cell (CSC) differentiation and tumor suppression. In general, CSC is a promising target for cancer therapy[51]. Being self-renewal and resistant to drug interference, CSC is considered as a major mechanism responsible for MDR. In this case, combining chemical drugs with the differential stimuli agents can drive CSC into differentiation and restore sensitivity to chemotherapy[52]. As reported, the key to avoiding CSC enrichment was simultaneously delivering ATRA and DOX using the same carriers[50].

However, the principle of sequential release control during combination use of chemotherapeutics with their sensitizers is hard to achieve when multiple dosing is needed because the steady-state plasma drug concentration (plateau concentration) of both drugs after multiple administrations will attenuate the benefits from sequential regimentation[53].

Methods to coencapsulate drugs with different physiochemical properties

Drugs can be encapsulated into NMs via different methods, such as adsorption, conjugation or encapsulation. Surface adsorption of drugs into the NMs is advantageous for the facile preparation. In addition, the drug release pattern is typically characterized by a rapid and burst release kinetics. In comparison, encapsulation inside the NMs may synchronize the drug release in response to NM degradation[54]. Thereby, the release rate is tunable by strategically selecting the materials with varying degradation rates. Furthermore, drugs can be covalently conjugated to the NMs via the cleavable bonds/linkers, by which the drug release is dependent on the cleavage of the bonds/linkers[55-57].

Nanotechnology-based combination therapy

Combination of cytotoxins/cytotoxins

In general, the cytotoxic drugs can be categorized into four groups, namely, cell cycle-independent alkylating agent (e.g., cisplatin, tetrazine, and alkylate nucleoids), cell cycle-dependent antimetabolites (e.g., methotrexate, pemetrexed, and gemcitabine), cell-cycle dependent antimicrotubule agents (e.g., PTX and vincristine), and topoisomerase inhibitors (e.g., DOX and etoposide). Combination application of two or more chemical toxins with different pharmacological actions may synergistically decrease cell viability and is the most widely used strategy in clinical practice. Chemical drugs are diverse in solubility, hydrophobicity, and PK behavior, providing difficulty to maintain the *in vivo* molecular ratio of the combined drugs in the tumor. Furthermore, most small-molecular-weight chemotherapeutics are the substrates of the MDR transporters[58], which can significantly attenuate their antitumor effect. Overall, the nanovehicles should be carefully designed to take advantage of the merits of all combined drugs and avoid unfavorable metabolisms.

A large amount of functional NMs have been developed, bearing various properties in structure, physicochemical characteristics, as well as the loading pattern of drug payloads, enabling the modulation of release behavior of the encapsulated drugs.

(a) Stimulus-responsive NMs. Applications of "smart" NMs can facilitate the encapsulated drugs to be released in a stimulus-sensitive manner. For example, the MSNs are advantageous for drug loading but suffer from nonspecific leakage and burst release of the encapsulated drugs. To overcome this problem, the pH-responsive polymers, such as methacrylic-type ionic liquid terpolymer[59], or thermo-sensitive poly (N-isopropylacrylamide) (PNIPAM)[60] can be grafted into the surface of the MSNs and serve as the switchable "gate keepers", enabling the encapsulated drugs to be released in response to the tumor microenvironment (TME). For example, a polyelectrolyte multilayer-modified MSN was constructed via coating poly (allylamine

hydrochloride) and poly (styrene sulfonate) into the surface of MSNs layer by layer with the DOX encapsulated into the pore of the MSNs under pH 2[61]. This system can respond to low pH and release the DOX at pH 5.0 but without drug release at pH 7.4. Chen et al.[62] modified the azide into the SiO_2 surface and synthesized the PNIPAM via the reversible addition-fragmentation transfer polymerization of N-isopropylacrylamide monomer. Then, a thermos-responsive nanoparticle system was constructed by grafting PNIPAM to the SiO_2 surface based on the azide-alkyne cycloaddition.

(b) Hybrid NMs. Coadministration of two NMs loading with different drugs (one in each) has been explored for achieving synchronic delivery. However, even encapsulated by the same kind of NMs, the optimum synergistic effect may not be guaranteed if the two drugs are separately loaded[47]. This challenge can be addressed by using the hybrid NMs—the different drug loading nanoparticles can be crosslinked together or using the core–shell-structured nanoparticles. Depending on the structure of the hybrid NMs, the payloads may be simultaneously released from the hybrid NMs or be sequentially released from a core–shell-structured NM[63]. For example, for sequential delivery of DOX and PTX, the DOX-loaded mesoporous nanoparticles were first prepared, then the surface was coated with polylactic-co-glycolic acid as PTX-loading layer[64].

Combination of cytotoxins and molecularly targeted agents

The mutations in the cancer cell signal pathways provide the pathological basis for targeted therapies by applying the inhibitors to selectively block the mutated signal pathways that are crucial in tumorigenesis. Therefore, the molecularly targeted agents display the improved therapeutic efficacy but reduced systemic toxicity compared with the chemotherapeutics. However, cancer cells also develop drug resistance to these molecularly targeted agents via the mechanisms including upregulation of the therapeutic target, activating the alternative compensatory survival signaling pathways, and inactivating the cell death signaling pathways[65]. Novel combination strategies should be designed to disturb these mechanisms to reverse the resistance to the targeted drugs.

For example, the combination of cytotoxins and epidermal growth factor receptor (EGFR)-TKIs has been commonly used. The EGFR–tyrosine kinase pathway is a clinical therapeutic target for several types of cancers[66]. The pathway can be blocked by antiEGFR antibodies (e.g., cetuximab), or by small molecular drugs (e.g., erlotinib and sunitinib) to inhibit the intracellular-mutated tyrosine kinase. The combination with cytotoxins may synergistically inhibit the proliferation of tumor cells. Furthermore, certain TKIs may help rewire the apoptotic pathways and sensitize cells to cytotoxins[67], or inhibit P-gp activity of tumor cells, thus increasing the efficacy of cytotoxic drugs[68].

(a) Combination of cytotoxins/TKIs. Nanotechnology can help increase the tumor drug accumulation and overcome acquired drug resistance. For example, lapatinib, an inhibitor of EGFR and HER-2, has been approved by the FDA to treat HER-2 positive refractory breast cancers combined with various therapeutic agents, such as capecitabine, anthracyclines, taxanes, and trastuzumab. Lapatinib has also been reported with the ability to inhibit the activity of drug efflux transporters. Based on the amphiphilic poly (ethylene glycol)-block-poly (2-methyl-2-benzoxycarbonylpropylene carbonate) polymers, we developed the DOX micelles and lapatinib micelles, simultaneously, to codeliver DOX and lapatinib in treating multidrug-resistant breast cancer[69]. The results of fluorescence microscopy and flow cytometry showed that coadministration of the DOX micelles and lapatinib micelles can enhance DOX uptake in the MCF-7/ADR cells overexpressing drug efflux transporters but not MCF-7 cells low-expressing drug efflux transporters. Coinciding with the uptake result, codelivery of DOX micelles and lapatinib micelles significantly improved the *in vitro* antitumor efficacy in the MCF-7/ADR cells. For *in vivo* treatment, lapatinib can restore the sensitivity of breast tumor to DOX and reduce the systemic toxicity of DOX (**Figure 4**).

(b) Combination of cytotoxin/antivasculature agents. Combination of cytotoxic drugs with antivasculature drugs may be synergistically effective. Zhang et al.[46,47] have developed two independent systems (RGD-liposomes and PEG-b-PLA mixed micelles) for codelivery of the cytotoxic DOX with the antivasculature agent combretastatin A4 (CA4). In both systems, CA4 was observed to be released from the NMs much faster than DOX, which can destroy the vascular walls and facilitate the extraversion of post released DOX, thus increasing the therapeutic DOX efficacy.

Combination of cytotoxins and sensitizing agents

(a) Combination of cytotoxins with MDR inhibitors. MDR inhibitors, such as verapamil, tariquidar, zosuquidar, and laniquidar[42], are commonly used as chemosensitizers for blocking the drug efflux transporters and thus restoring the sensitivity of tumor cells to chemotherapeutics. The

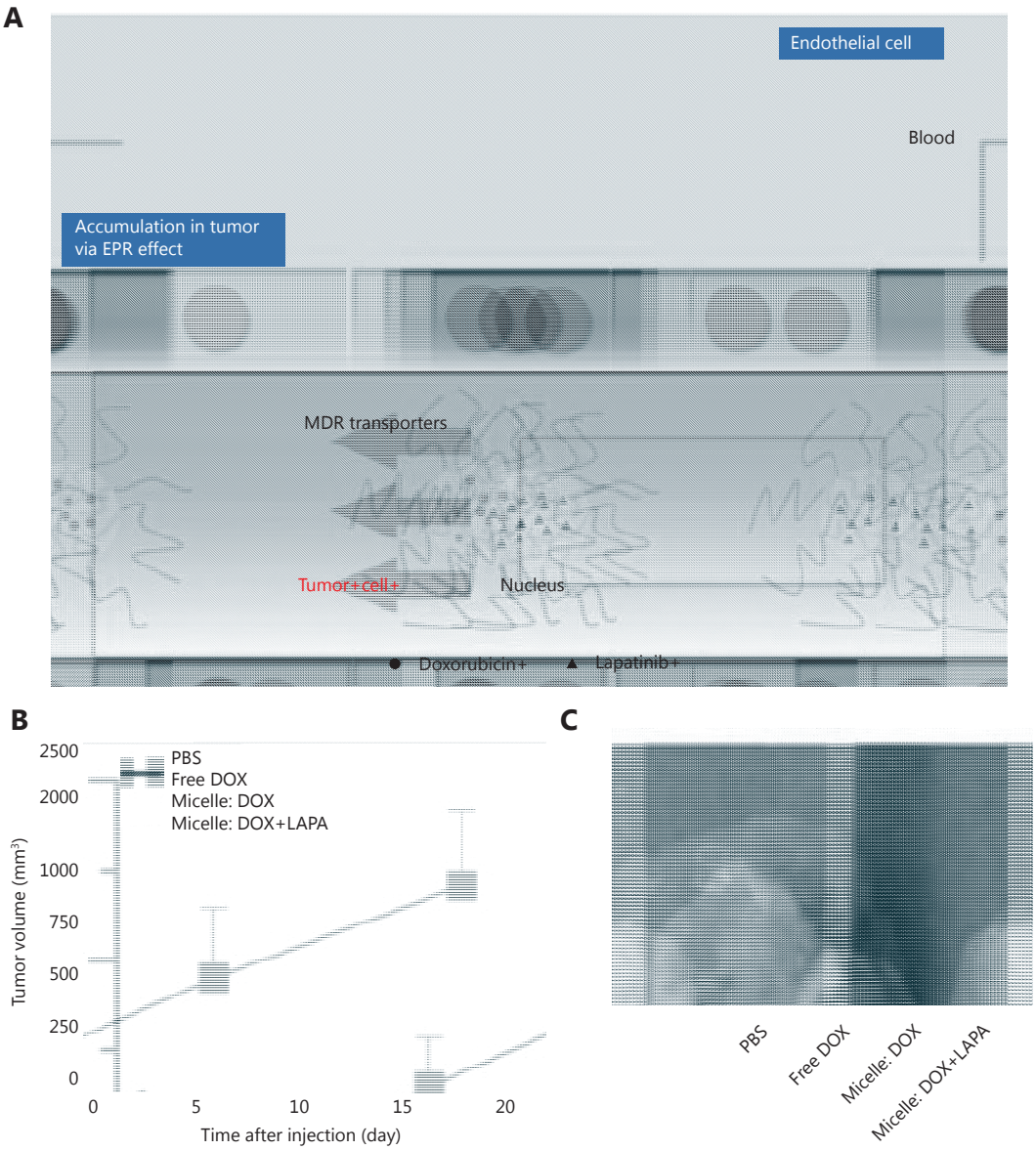

Figure 4 Codelivery DOX and lapatinib (LAPA) by polymer-based micelles. (A) Scheme of codelivery DOX and LAPA by polymer-based micelles in treating resistant breast tumor. (B) Improved therapeutic efficacy of coadministration of DOX micelles and LAPA micelles in xenograft MCF-7/ADR tumor-bearing mice model, and (C) the tumor images. Reprinted with permission from Ref. 69.

combination of cytotoxins with MDR inhibitors may significantly increase intracellular drug retention and improve tumor killing effect[43,44]. NMs can improve the poor selectivity and low affinity of these inhibitors and achieve satisfactory synergistic results[57,70]. Qin et al.[71] encapsulated DOX and verapamil into the hydrogel nanoparticles. Coadministration of DOX-NPs and verapamil-NPs can significantly improve the uptake and DOX cytotoxicity in NCI/ADR-RES cells.

To sensitize tumor cells to the cytotoxic drugs, the cells/tumor need to be pretreated with the sensitizing agents. To achieve this process, NMs with sequential release behavior should be designed and utilized for the combined delivery of toxins and sensitizing drugs[67]. However, pretreatment may not be a requisite for all sensitizing agents.

(b) Combination of cytotoxins with immune regulators. TME plays an important role in cancer development, MDR, and metastasis. Therefore, remodeling TME is a potential target for overcoming MDR. We developed a mannosylated albumin nanoparticle system for codelivery of the cytotoxic agents disulfiram/copper complex and the M2 macrophage modulator regorafenib[72]. Given that the albumin-binding

protein (e.g., SPARC) pathway and mannose receptor (MR) were highly expressed in both the drug-resistant colon tumor cells and M2 macrophages, such system can achieve dual targeting to both cell-membrane receptors and both cells. The "one-stone-two-bird" delivery strategy can significantly enhance the delivery efficiency and treatment efficacy against the drug-resistant colon cancer both *in vitro* and *in vivo*.

Combination of cytotoxins and peptides (or proteins)

With the advances of biotechnology, proteins and peptides have been widely investigated in cancer therapy because of their high specificity and efficacy[73-75].

(a) Cytotoxin/therapeutic antibodies. Therapeutic antibodies (e.g., cetuximab, rituximab, and trastuzumab) represent an important class of protein/peptide drugs capable of inhibition cell proliferation by selectively binding to their membrane receptors[76]. The combination with antiserum or trastuzumab (HER2 antibody) markedly benefit the therapeutic effect of cytotoxins[77,78]. Of note, antibody-drug conjugates have shown significant therapeutic effects in clinical cancer treatment and attracted worldwide attention. This area has been specially reviewed by several articles[79,80].

(b) Cytotoxin/apoptotic peptide. Certain intrinsic or extrinsic peptide/protein toxins are capable of inducing apoptosis of cancer cells, which may synergize cytotoxicity of chemotherapeutics. For example, N7 peptide of second mitochondria- derived activator (Smac N7) can bind with inhibitor of apoptosis and activate proapoptotic pathway[81-83]. When combined with cytotoxic PTX, N7 peptide may effectively promote PTX-induced toxicity, indicating a synergistic effect existed between Smac N7 and PTX[84].

By rational design, chimeric peptides with unique physiochemical properties can serve as a potent therapeutic agent and as novel functional NMs for drug loading. The apoptotic peptide AVPI was a small hydrophobic peptide with poor membrane permeability. To address this problem, we designed a novel chimeric peptide containing AVPI and a cell penetrating peptide R8, wherein the added R8 peptide not only improved the water solubility of AVPI and its membrane permeability but also simultaneously acted as the DNA binding site[85,86]. The chimeric peptide and p53 DNA would self-assemble into nanoparticles through the electrostatic interaction between R8 and DNA, thus forming a codelivery system of the therapeutic AVPI and p53 DNA. The prepared cell-penetrating AVPIR8/p53 DNA nanocomplex can significantly increase the sensitivity of the resistant MCF-7/ADR cells to DOX. The *in vivo* therapeutic results showed that coadministration of the AVPIR8/p53 DNA nanocomplex with additional mixing with DOX can effectively inhibit the tumor growth with a reduced DOX dose and produce less side effect (**Figure 5**). Similarly, Li et al.[82] designed a novel amphiphilic peptide derivative with its hydrophilic part composed of Smac N7 peptide and a cell penetrating peptide and hydrophobic part being composed of four aliphatic tails. The peptide derivative can self-assemble into micelles and encapsulate DOX for combination therapy.

(c) Cytotoxins/protein toxins. Some protein toxins (e.g., recombinant trichosanthin) have been reported with the ability to kill the MDR cancer cells, as well as reverse the resistance of MDR cancer cells to chemotherapeutics. However, immunogenicity, enzymatic degradation, and poor membrane permeability are challenges for the application of such protein drugs[87]. Unlike small molecular drugs, macromolecular protein drugs that actually are in a nanosized scale can passively accumulate in the tumor site via EPR effect[88]. PEGylation and ligand modification further extend the blood circulation and tumor targeting[89]. However, PEGylation can reduce the membrane permeability of proteins. We previously developed a PEGylated, matrix metallopeptidase 2 (MMP2)-activatable cell-penetrating protein toxin trichosanthin (TCS) (termed rTLM-PEG) based on a recombinant intein-mediated site-specific conjugation method[90]. The protein system can dePEGylate in the TME via the enzymatic activation of matrix metalloproteinase to the substrate peptide and thus release the cell penetrating TCS. Such protein delivery system was further investigated for overcoming the drug-resistant lung cancer in combination with the PTX liposomes[91]. TCS effectively restored the sensitivity of A549/T cancer cells to PTX (**Figure 6B**). The mechanisms involved the inhibition of the caspase 9 phosphorylation and promotion of the caspase 3-dependent apoptosis (**Figure 6C**). TCS coadministration with PTX liposomes entirely arrested the tumor growth on A549/T tumor-bearing mouse model (**Figure 6D**).

Combination of chemotherapeutics and nucleic acid drugs

Tumorigenesis involves multiple genetic mutations[92]. The therapeutic stress drives further mutations and aggravates the changes of tumor geometry, which is partially accounted for acquired resistance to cancer therapy[93]. Various molecular tools, such as small interfering RNA, short hairpin RNA, and plasmid DNA, enable the precise regulation of the expression of a specific gene because of the development of molecular

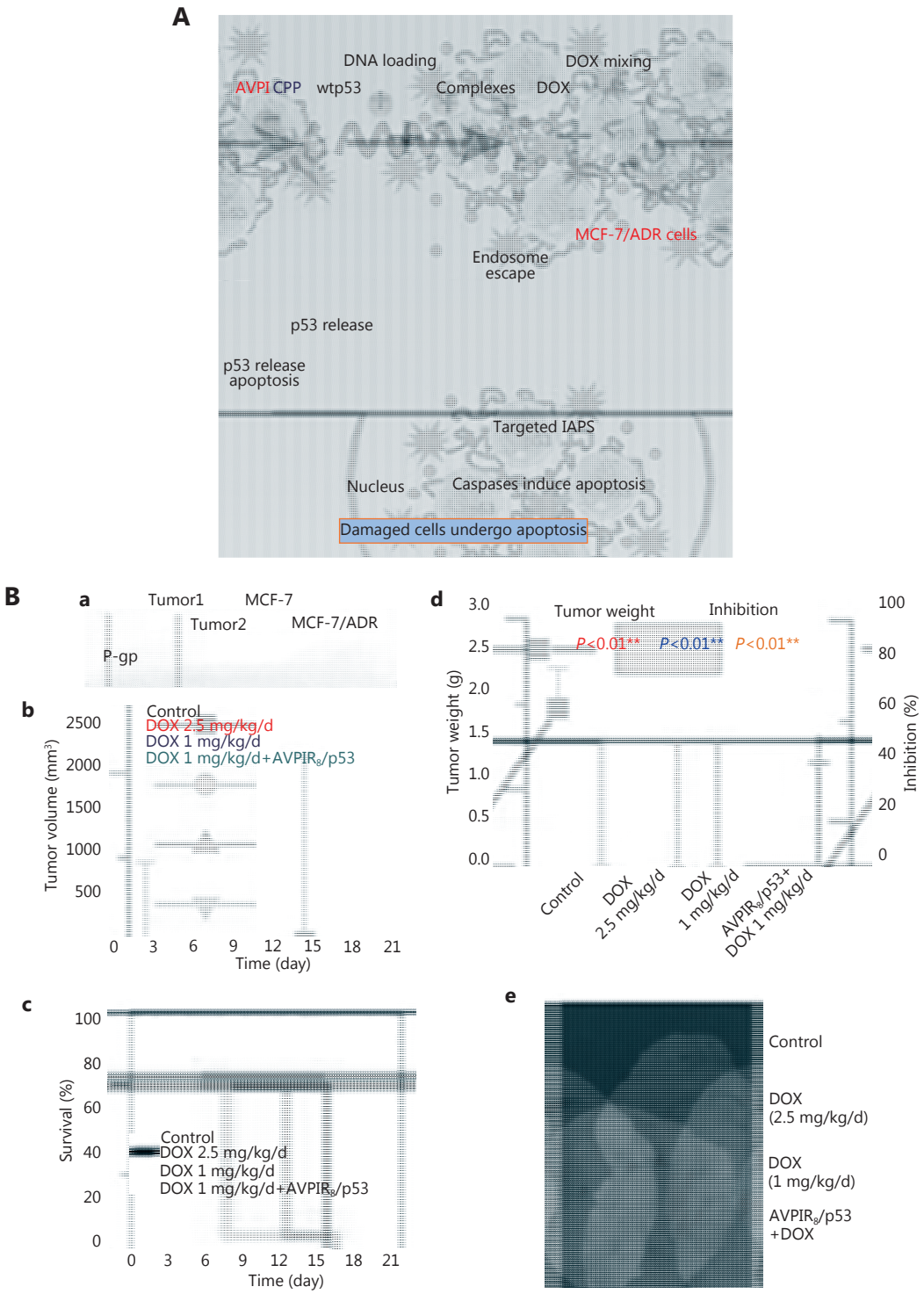

Figure 5 Cell-penetrating AVPIR8/p53 DNA nanocomplex as adjuvant therapy of cytotoxic agent DOX to overcome drug-resistant breast cancer. (A) Scheme of the cell-penetrating AVPIR8/p53 DNA nanocomplex combined with DOX to overcome MDR. (B) Improved therapy effect and reduced side effect of the cell-penetrating AVPIR8/p53 DNA nanocomplex with DOX. Reprinted with permission from Ref. 86.

biotechnology. However, the short half-life in blood, enzymatic degradation, renal clearance, and poor membrane permeability are major obstacles for clinical applications of nucleic acid drugs. Furthermore, single gene therapy may not

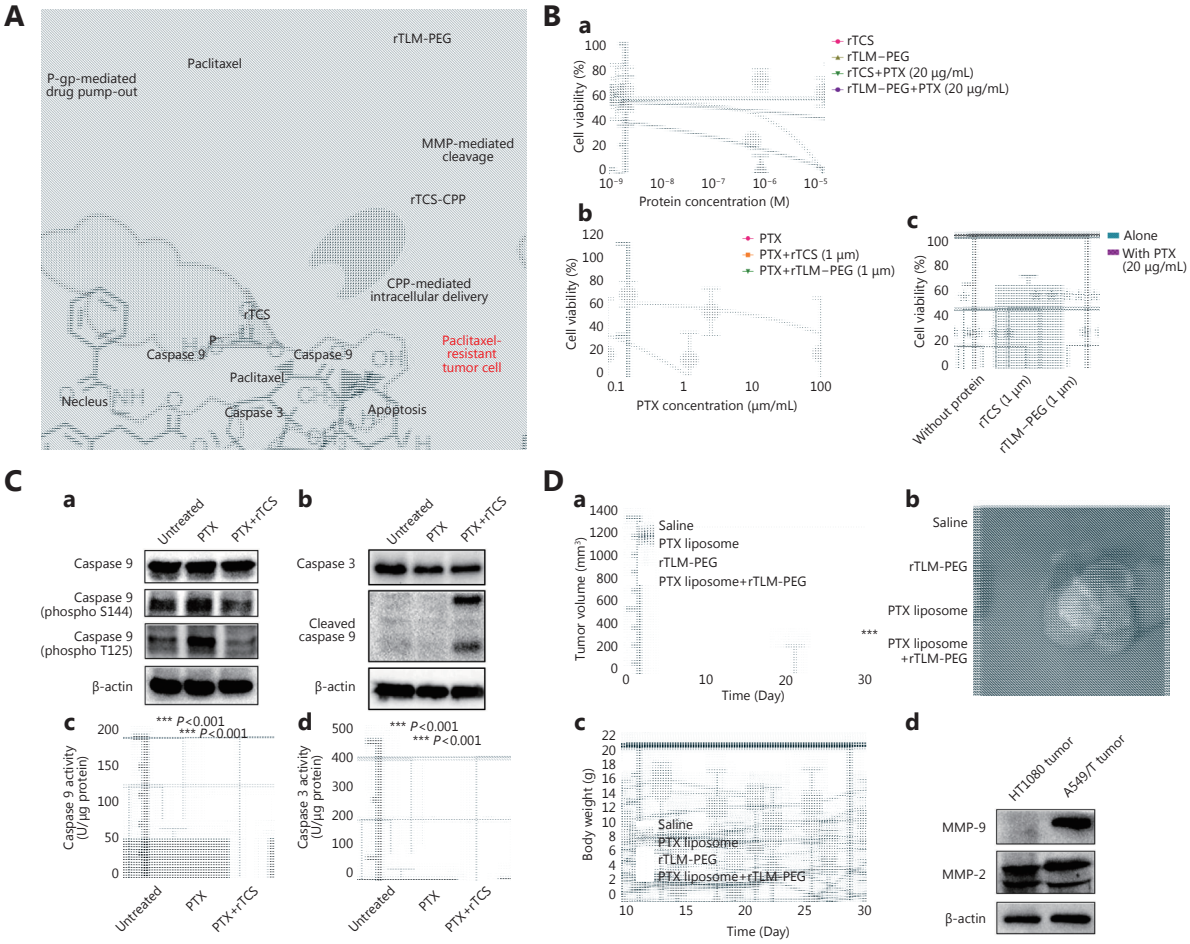

Figure 6 Combination of PEGylated, MMP2-activatable cell-penetrating TCS with PTX liposomes for overcoming the drug-resistant lung cancer. (A) Scheme of the PEGylated, MMP2-activatable cell-penetrating TCS combined with PTX for overcoming drug resistance in A549/T cells. (B) *In vitro* synergistic cytotoxicity of PEGylated, MMP2-activatable cell-penetrating TCS, and PTX. (C) Regulatory effect of PEGylated, MMP2-activatable cell-penetrating TCS and PTX on caspase 9 phosphorylation and caspase 3. (D) Synergistic therapeutic effect of the PEGylated, MMP2-activatable cell-penetrating TCS and PTX liposomes *in vivo*. Reprinted with permission from Ref. 91.

be sufficient to achieve the satisfactory treatment outcomes, leading to MDR[94]. Thus, combination therapy with two or more gene cocktails (e.g., targeting EGFR, MDR-1, bcl-2, and survivin) and cytotoxins can further improve the therapeutic efficacy[95], and the nanotechnology would facilitate the application.

(a) Cytotoxins and *p53* gene combination. *p53* is the "gatekeeper of the genome" and can regulate cell apoptosis through transcription-dependent and transcription-independent pathways by activating the expression of proapoptosis proteins and suppressing the activity of antiapoptosis proteins. Dysfunction of TP53 tumor suppressor is a main mechanism that cancer cells escape apoptosis and become insensitive to drugs[96]. Delivery of wild-type *p53* is a promising strategy to restore cancer cells sensitive to therapeutic agents. *p53* DNA combined with DOX[86,97] and PTX can significantly improve the therapeutic effect[98]. Adenovirus-mediated *p53* gene (Ad-p53) transfection can restore the sensitivity of MCF-7/ADR cells to DOX[99].

(b) Cytotoxins and Bcl-2 siRNA. RNA interference technology has offered a flexible tool for selective silencing tumorigenic genes. siRNA codelivery that knocks down the drug efflux transporters and antiapoptosis genes can restore cancer cells sensitive to chemotherapeutic drugs[100]. By codelivery of DOX with the siRNA targeting the Bcl-2 gene using the folate-targeted nanocarrier, DOX-induced apoptosis in the SKOV-3 cells overexpressing folate receptor was significantly enhanced through a mechanism of downregulating the antiapoptotic protein Bcl-2. In parallel,

the proapoptotic protein Bax was also upregulated[101].

(c) Cytotoxins and MDR siRNA. siRNA coadministration targeting the P-gp mRNA can improve the sensitivity of cancer cells to the chemotherapeutic agents. The codelivery system of antiP-gp siRNA and DOX displayed a synergistic effect in DOX-resistant cell line HepG2/adriamycin and xenograft tumor model[102]. P-gp downregulation can resensitize the DOX-resistance cancer cells to DOX[103]. Nanoparticle-mediated P-gp targeted siRNA and chemodrug PTX can effectively silence the MDR-1 gene and obviously increase the PTX accumulation[104]. Other MDR proteins, such as the major vault protein, can serve as a target with siRNA to downregulate the expression and yield the increased efficacy of the conventional cytotoxins[105].

(d) Cytotoxins and survivin siRNA. Survivin plays an important role in cancer carcinogenesis and angiogenesis[106]. Silencing survivin with small hairpin RNA was found to be in concert with PTX treatment[107]. Torchilin and coworkers demonstrated that the survivin siRNA can sensitize the tumor cells to PTX[108].

Other combinations

Other combinations (e.g., chemotherapy/thermotherapy, chemotherapy/phototherapy, and chemotherapy/radiotherapy) based on the nanotechnology have also been actively investigated. For example, nanographene oxide (NGO) and gold nanostructures can be used as cancer drug carriers and for photothermal ablation of tumor based on their photothermal transition properties. The NGO nanocomposites to deliver DOX showed the high therapeutic efficacy both *in vitro* and *in vivo* via the combination of the photothermal therapy of the NGOs and chemotherapy of DOX[109,110].

Immunotherapy is a promising method in treating cancer, in which dendritic cells (DCs) are the major target. Cancer cells can suppress the maturation and function of DCs, which often results in the tumor immune tolerance. Therefore, immunotherapy is an attractive strategy to promote maturing DCs using immune-stimulatory factors. Chemotherapeutics (e.g., PTX) displayed a "Yin-and-Yang" nature that high-dose chemotherapeutics compromised the functions of immune cells, whereas the low dose promoted and stimulated the DC maturation, thus exhibiting the bidirectional modulation of suppression and activation. We have recently reported the polymeric nanoassembly system for microneedle-assisted codelivery of pTRP-2 vaccine targeting the epidermis DCs and the immunomodulatory low-dose PTX for enhanced cancer immunotherapy[111].

Cytosine-guanosine (CpG) oligodeoxynucleotides is a common immunostimulatory factor in clinical practice for melanoma. Yu Tao and coworkers designed the gold nanorods–CpG–DOX conjugates to achieve combined cancer therapy, including immunotherapy, photothermal therapy, and chemotherapy[112]. Gold nanoparticles can also enhance the tumor radiosensitivity during radiotherapy because of its high absorption capability to X-ray[113-115].

Another interesting application is the combination of the bioactive functional NMs and drugs. Silver nanoparticles (AgNPs) possess potent antitumor activity and thus are used as therapeutic agents[23,25]. Ostad and coworkers revealed the combination effect of AgNPs and tamoxifen, wherein AgNPs combined with tamoxifen can effectively kill the parent and tamoxifen-resistant cells and reduce the tamoxifen doses[116].

Prospects

MDR is a crucial challenge in antitumor therapy. As one of the most important strategies to address this problem, combination therapy has achieved remarkable progress in clinical cancer treatment. However, the conventional combination has been suffering from the varying PKs of different drugs, leading to inconsistent therapeutic responses during *in vitro* to *in vivo* translation. On this account, NMs with their potentials in drug coencapsulation, targeting delivery, controlled release, and PK improvement provide powerful tools for drug combination therapy.

However, several issues need to be addressed in the future development. First, although the nanocarriers can synchronize the PK behavior of the combined drugs, drug ratio in the tumor may also change because of the complexity of *in vivo* process[57,117]. Second, despite that NMs may preferentially accumulate in the tumors, the overall tumor retention is still limited, accounting for merely 1%–5% of the administered dose[118]. Thus, improving the efficacy of the NM-based combination therapy is greatly required. Third, most of NMs are highly entrapped by the RES organs (e.g., the liver, lung, and spleen), imposing potential safety issues. For example, to inhibit the P-gp-mediated drug resistance, cytotoxins were coencapsulated with MDR inhibitors, which can facilitate the drug uptake in the tumors but also increase the risk of hepatotoxicity. Fourth, long-term exposure of combined drugs may also lead to resistance[119]. Therefore, the NMs for combination therapy need to be multivalent and switchable, and drug replacement should be achieved readily. Fifth, nucleic acid drugs represent the effective means to overcome drug resistance in cancer therapy. However, the transport efficiency of nonvirus vectors is still limited. Sixth,

the large-scale production of nanomedicine with a precise ratio control is a challenge. A marketed nanomedicine product with coencapsulation of drugs is still not available. In a word, nanotechnology has provided the convenient tools for combination therapy. However, nanotechnology still needs much improvement for clinical translation.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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