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Review

Nanoparticle-based combination therapy toward overcoming drug resistance in cancer

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ABSTRACT

The use of multiple therapeutic agents in combination has become the primary strategy to treat drug resistant cancers. However, administration of combinatorial regimens is limited by the varying pharmacokinetics of different drugs, which results in inconsistent drug uptake and suboptimal drug combination at the tumor sites. Conventional combination strategies in aim to maximize therapeutic efficacy based on maximum tolerated dose does not account for the therapeutic synergism that is sensitive to both dosing and scheduling of multiple drugs. In the present review, we will discuss the development of multidrug-loaded nanoparticles against drug resistant cancers. Nanoparticle-based combination therapy against experimental multidrug resistant (MDR) cancer models will be summarized. In addition, we will highlight the recent advances in nanoparticle-based combination strategies against clinical cancer drug resistance, including co-encapsulation of drugs with different physicochemical properties, ratiometric control over drug loading, and temporal sequencing on drug release. These emerging strategies promise novel and better tailored combinatorial regimens for clinical cancer treatment.

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Contents

1. Introduction

The long-standing challenge in cancer drug resistance and the urgent need for novel combination therapy are highlighted in a recent perspective by Woodcock et al., who liken the complexity of

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cancer biology to webs of interconnected routes with multiple redundancies [1]. Very tellingly, this analogy points out the inadequacy of single-drug therapy, whose one-dimensional action mechanism often activates and strengthens the alternative pathways, prompting the emergence of chemoresistance mutations and tumor relapse. In aim to increase treatment efficacy, combination chemotherapy has long been adopted as the standard of care against many cancer types. It is generally acknowledged that through the proper drug combination the treatment can promote synergistic actions, improve target selectivity, and deter the development of cancer drug resistance [2].

Despite being a clinical standard, current combination approach through the cocktail administration leaves plenty of room for improvements. While *in vitro* cellular studies have generated many leads for combinatorial regimens, their clinical results are often met with little improvement in efficacy and at times higher toxicity [3,4]. One major factor that separates *in vitro* success from impressive clinical outcomes is the varying pharmacokinetics among different drugs. Upon systemic administration, drugs undergo distinctive physiological fates and non-uniform distribution. Predicting and controlling the therapeutic mixtures that reach the diseased cells and tissues therefore become a major clinical challenge. The common approach based on maximum tolerated dose fails to take into account the intricate pharmacologic interactions that are sensitive to both dosing and sequencing of combinatorial drugs. One strategy toward more effective combination therapies thus is devising a better scheme for precise and controlled delivery of multiple therapeutic agents.

Advances in nanotechnology have opened up unprecedented opportunities in controlled drug delivery and novel combination strategies. Nanoscale particles between 10 and 200 nm in diameters have shown more favorable pharmacokinetic profiles as compared to small-molecule drugs; these drug-loaded nanoparticles exhibit prolonged systemic circulation lifetime, sustained drug release kinetics, and better tumor accumulations through both passive and active mechanisms [5–8]. Recently, nanocarriers are gaining increasing attention for their ability to co-encapsulate multiple therapeutic agents and to synchronize their delivery to the diseased cells. Various nanoparticle platforms such as liposomes, polymeric micelles, dendrimers, and mesoporous silica particles have been used to carry broad classes of therapeutics including cytotoxic agents, chemosensitizers, small interference RNA (siRNA), and antiangiogenic agents. In this review, we will cover several nanoparticulate systems that have been used for co-encapsulation and co-delivery of multiple drugs. We will then summarize nanoparticle-based combination strategies to overcome the experimental models of multidrug resistance (MDR) in cancer. Lastly, in light of the complexity in clinical cancer drug resistance, we will offer insights on emerging features in nanoparticle drug delivery that promise broader applicability and better design for combination therapy. These features include co-encapsulating hydrophobic and hydrophilic drugs, precise and ratiometric control over drug loading, and sequenced drug release.

2. Nanoparticulate systems for combinatorial drug delivery

Nanoparticulate systems such as liposomes, polymeric micelles, and polymer–drug conjugates have led to about two dozen clinically approved therapeutic products [6]. Herein, we highlight the nanocarriers that have been demonstrated to carry two or more types of therapeutic payloads. While these systems share the common aim in promoting synergism through controlled combinatorial drug delivery, each platform has its unique strength and characteristics. The different particle structures, materials, and preparation processes are emphasized here to provide design considerations toward developing combinatorial therapeutics.

2.1. Liposomes

Liposomes are spherical vesicles consisting of amphiphilic phospholipid bilayers. Phosphatidylcholine and phosphatidylethanolamine are the common building blocks for liposomal preparation whereas cholesterol is a frequent additive that serves to modify the rigidity of the lipid membranes. Liposomes are typically prepared by rehydrating lipid films to form multilamellar vesicles (MLV), which subsequently undergo mechanical extrusions to form unilamellar vesicles [9]. The resulting structure contains a lipid bilayer and an inner aqueous core, which are capable of carrying lipophilic and hydrophilic drugs, respectively.

Liposomal drug loading can be accomplished either through active extrusion or through passive diffusion. In the active extrusion approach, drugs are suspended along with the phospholipids in aqueous solution. The resulting mixture of MLV and drugs are then extruded through membrane with defined pore size to form drug-loaded liposomes. In the passive diffusion approach, liposomes are first prepared and then mixed with solubilized drugs. These drug molecules then enter the liposomes by diffusing through the lipid bilayers. Multidrug-loaded liposomes can be prepared using either of the loading schemes followed by filtration of unloaded drugs. For instance, in preparing CPX-351, a combinatorial liposome for leukemia treatment, cytarabine is hydrated and extruded with the lipid components yielding cytarabine-loaded liposomes. These liposomes are then incubated with daunorubicin to achieve dual-drug encapsulation [10]. Currently liposomes are the only nanoparticle-based combinatorial drug delivery platform that has entered clinical trials.

2.2. Polymeric nanoparticles

In contrast to liposomal vehicles that carry drug cargoes in their aqueous cavity, polymeric nanoparticles contain a solid, polymer-filled core that is better suited for water-insoluble drug payloads. The solid structure also gives polymeric nanoparticles higher stability, more sustained and controllable drug release profiles, and more uniform size distribution. Polymeric nanoparticles are typically prepared through the self-assembly of amphiphilic diblock copolymers. A variety of polymers have been used to prepare polymeric nanoparticles, including biodegradable synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) and natural polymers such as polysaccharides and polypeptides [11–13]. In general, drug encapsulation into polymeric nanoparticles is achieved by mixing the drugs with the polymer solution. As the polymers self-assemble into particles, they physically entrap the drug compounds. Multiple hydrophobic therapeutic compounds have been loaded simultaneously through this physical entrapment approach. Other encapsulation schemes have taken advantage of the synthesis flexibility in the polymeric building blocks. Through drug–polymer conjugations and particle functionalization, more advanced combinatorial drug encapsulation schemes have been developed to extend compatibility to hydrophilic drugs [14–16], precisely controlled drug loading ratios [17], and fine tuned drug release sequence and kinetics [18,19].

2.3. Polymer–drug conjugates

Covalently attaching therapeutic agents to water-soluble polymers is another approach that improves the drugs' systemic circulation lifetime and reduces their exposure to normal tissues. Many low-molecular-weight anticancer drugs such as paclitaxel, doxorubicin (DOX), and camptothecin have shown improved pharmacokinetic profiles and clinical efficacy following polymer

conjugation [20]. Polyethylene glycol, poly (L-glutamic acid), and N-(2-hydroxypropyl)methacrylamide (HPMA) are examples of polymers that have been accepted into clinical practice [21]. Owing to the multivalent functional groups on these polymers, recent research efforts have synthesized conjugates with a combination of drugs to the polymer chains and demonstrated cooperative efficacy [22,23]. Since drugs need to be detached from the polymer conjugates to take effects, it is possible to modify the drug release kinetics through the use of environment- or enzyme-sensitive linkers [22].

2.4. Dendrimers

Dendrimers are hyperbranched polymers that are characterized by a central inner core surrounded by layers of repeating units and an outermost layer of multivalent functional groups. The tree-like globular morphology initiates from the core and branches outward in a symmetrical fashion. While the outer functional groups can be coupled with charged polar molecules through electrostatic interactions, the hydrophobic pockets spanning the inside of dendrimers favor the encapsulation of uncharged, non-polar molecules [24]. Despite that dendrimers are in a relatively nascent stage as a drug delivery platform, their unique structural attribute has drawn interest for the concurrent delivery of hydrophobic and hydrophilic therapeutic agents [25].

2.5. Other nanoparticles

Other nanoparticles that have been demonstrated to deliver therapeutic cargoes in combination include oil nanoemulsions [26], mesoporous silica nanoparticles [27], and iron oxide nanoparticles [28,29]. For the oil emulsion, hydrophobic drug mixture is homogenized along with the oil and loaded in the oil phase of the resulting nanoemulsions. In the case of the mesoporous nanoparticles and the iron oxide particles, the inorganic cores are functionalized with additional polymeric matrices to carry multiple drug payloads. A graphical illustration of the different multidrug-loaded nanoparticle platforms can be found in Fig. 1.

3. Combinatorial nanoparticles against multidrug resistance in cancer

Many combinatorial nanoparticle formulations have been successful in reversing MDR in *in vitro* and *in vivo* cancer models through co-delivering combinations of chemosensitizing agents and chemotherapy agents [30,31]. Among the many cellular mutations that diminish the effectiveness of anticancer drugs, the over-expression of multidrug transporters and the altered apoptosis are the two underlying mechanisms through which cancer cells acquire resistance to multiple structurally and mechanistically different drugs [1,32]. In transporter-dependent MDR, an upregulated level of transmembrane drug efflux pumps under the ATP-binding cassette (ABC) superfamily actively export drugs to reduce their effective intracellular concentration. These transporters act on a variety of anticancer drugs. For instance, P-glycoprotein (P-gp), an ATP-driven pump over-expressed in liver, ovarian, pancreatic, and gastrointestinal cancers [33–39], can readily pump out DOX, vinblastine, and paclitaxel. In apoptotic-pathway-dependent MDR, pro-survival mutations such as the deregulation of BCL2 and nuclear factor kappa B (NF-κB) enable cancer cells to tolerate drug-inflicted injuries and significantly decrease their apoptotic response. To this date, a number of chemosensitizers have been developed to inhibit drug-efflux pumps and to restore the proper apoptotic signaling. In addition, emergence of siRNA has made possible the silencing of MDR-related genes. Nanoparticles that combine these MDR modulators and cytotoxic drugs have the ability to sensitize drug resistant cancer cells to the chemotherapeutic payloads. Herein we highlight different combinatorial nanoparticle formulations against the effect of MDR in cancer.

3.1. Combination of efflux pump inhibitors with chemotherapeutics

Compared to small-molecule drugs which diffuse through cellular membrane and are susceptible to transmembrane multi-drug transporter, nanoparticles carry a high dose of drug payloads that can overwhelm the drug efflux kinetics. In addition, since nanoparticles enter cells through endocytosis, they shuttle the

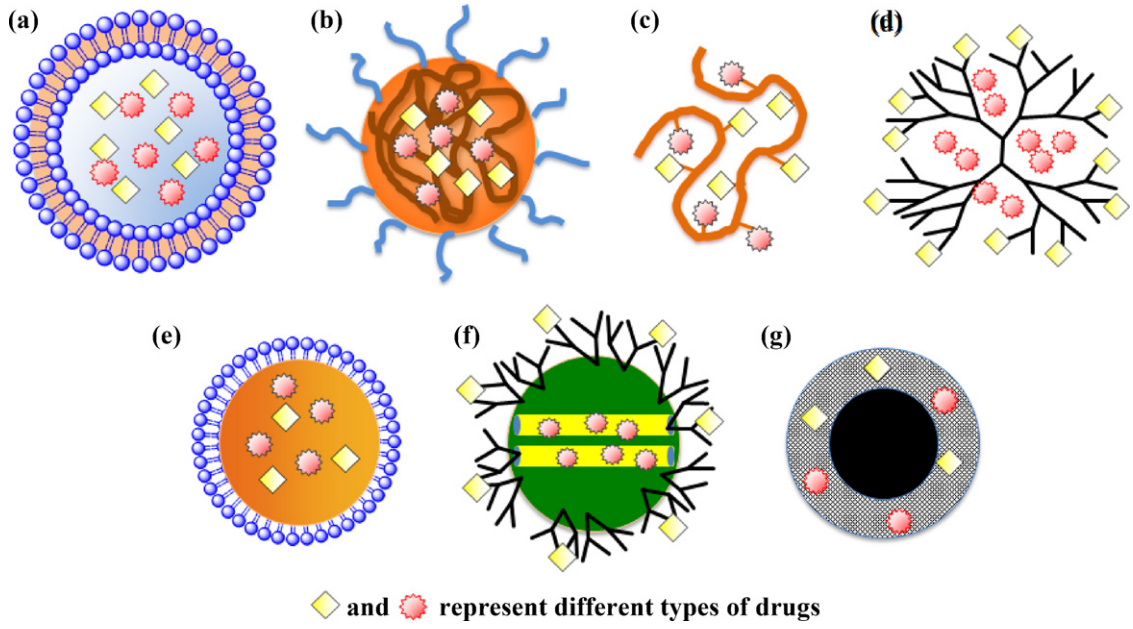

Fig. 1. Schematic illustration of nanoscale drug carriers used for combinatorial drug delivery: (a) liposome, (b) polymeric micelle, (c) polymer–drug conjugate, (d) dendrimer, (e) oil nanoemulsion, (f) mesoporous silica nanoparticle, and (g) iron oxide nanoparticle.

drug cargoes away from the drug-pump-spanning cellular membrane [33–35]. Building upon these inherent advantages, therapeutic nanoparticles containing a combination of cytotoxic drugs and efflux pump inhibitors, such as cyclosporine, verapamil, and tariquidar, have further aided the suppression of MDR effect. These co-encapsulation strategies also address the poor pharmacokinetics and high systemic toxicity frequently associated with these chemosensitizers [40,41].

The first attempt to co-deliver chemosensitizer with chemotherapeutics in a single nanocarrier was a polyalkylcyanoacrylate nanoparticle system loaded with P-gp-inhibiting cyclosporin A (CyA) and DOX [42]. Against a DOX-resistant leukemia cell line (P388), Soma et al. showed that the co-encapsulation of CyA and DOX results in nearly 2-fold increase in toxicity as compared to DOX-only nanoparticles. It should also be noted that the enhanced efficacy was not observed when free CyA was applied with the DOX-only nanoparticles. The finding suggests that nanoparticle-coordinated delivery of two bioactive agents is essential for their cooperative activity.

MDR reversion through the combination of P-gp modulators and cytotoxic drugs has also been achieved with oil nanoemulsions [26], polymeric micelles [43], and liposomes [26,44–46]. A summary of these combinatorial nanoparticles can be found in Table 1. Some of these nanoparticle formulations have shown improved efficacy against drug-resistant tumors in *vivo*. Liang et al., for instance, prepared a stealth liposomal formulation combining vincristine and quinacrine and tested it against a murine model bearing the MDR K562 human chronic myelogenous leukemia. The study showed superior efficacy by the combinatorial liposomal formulation as opposed to the cocktail administration of the free drugs. The quinacrine was found to restore the MDR cells' response to vincristine, as was confirmed by the increased activity of caspase 9 and 3 [46].

Surface functionalization of nanoparticles with cancer-targeting ligands has been proposed to prevent the MDR modulators from affecting normal tissues. Since multidrug binding proteins play a key role in regulating the exchange of molecules at the intestinal lining and blood brain barrier [47,48], their molecular substrates and inhibitors can greatly affect physiological functions. Against healthy tissues, MDR modulators can disrupt cellular metabolisms and inflict injuries. Cardiotoxicity, nephrotoxicity, and neurotoxicity, for instance, are among the common side effects of these modulator compounds [49]. In aim to reduce systemic exposure of drug-efflux-pump blockers, Wu et al. prepared a transferrin-conjugated liposome containing both DOX and verapamil. In addition to achieving MDR reversal, the ligand-functionalized formulation showed faster accumulation *in vitro* to a drug-resistant leukemia cell line (K562) and demonstrated higher degree of MDR reversal as compared to the non-functionalized liposomal combination [43]. In a different study, Patil et al., using a biotin-conjugated PLGA nanoparticle co-encapsulating paclitaxel and tariquidar, also showed that the nanoparticle functionalized with the breast-cancer-targeting moiety not only improves tumor reduction *in vivo* but also improves the overall survival of the inoculated mice as compared to the non-targeted formulations [50]. The targeted, concurrent delivery of chemosensitizers and chemotherapeutics promises a safer and more effective approach in treating MDR tumors.

3.2. Combinations of pro-apoptotic compounds with chemotherapeutics

In response to MDR associated with the alterations of the apoptosis pathways, therapeutic nanoparticles have been co-encapsulated with compounds that repair the dysfunctional apoptotic signaling. One example of such pro-apoptotic compound is ceramide, which is produced by cells under environmental stress and serves as a key messenger in programmed cell death. Some MDR cancer cells hinder apoptosis initiation by over-expressing glucosylceramide synthase that converts ceramide to its inactive, glycosylated form. To address the ceramide metabolism, a polymeric micelle formulation based on poly(ethylene oxide)-poly(epsilon caprolactone) (PEO-PCL) was prepared to co-deliver exogenous ceramide and paclitaxel [51]. Against a paclitaxel-resistant ovarian cancer cell line (SKOV-3TR), the combinatorial formulation was found to raise paclitaxel sensitivity of the MDR cells to the same level as non-MDR cells. Combination with ceramide showed a 100-fold increase in efficacy as compared to paclitaxel-only nanoparticles. Caspase activity study and western blotting results suggest that the co-delivery of ceramide encouraged programmed cell death in the MDR cells. In a more recent study, the combinatorial formulation of ceramide and paclitaxel was found to show improved efficacy against MDR tumors *in vivo* using a PLGA nanoparticle system [52]. The co-delivery system was shown to increase apoptotic activity and tumor reductions in murine models without significant liver toxicity or reduction in white blood cell count.

Other combinatorial nanoparticle formulations have been prepared to combat drug resistance caused by the over-expression of NF-κB. These nuclear factors have been implicated to the production of anti-apoptotic proteins as well as to paclitaxel resistance [53]. A chitosan-based nanoparticle system was prepared to co-deliver DOX with a NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) [54]. In addition to combination therapy, the chitosan-based nanoparticle also employs active targeting and stimuli-responsive drug release kinetics to maximize DOX retention and sensitivity in the MDR cancer cells. Another example of NF-κB inhibitor, curcumin, which also blocks multidrug transporters, has

Table 1
Selected examples of combinatorial nanoparticle formulations containing chemotherapeutics and chemosensitizers.

Nanocarrier platform	Chemotherapeutic	Chemosensitizing agent	Indication	Status[a]	Refs.
Liposome	Topotecan	Amlodipine	Leukemia	*In vivo*	[45]
Liposome	Vincristine	Quinacrine	Leukemia	*In vivo*	[46]
Liposome	Paclitaxel	Tariquidar	Ovarian cancer	*In vitro*	[44]
Liposome (transferrin-conjugated)	Doxorubicin	Verapamil	Leukemia	*In vitro*	[43]
Liposome (cationic)	Doxorubicin	MRP-1 and BCL2 siRNA	Lung cancer	*In vitro*	[65]
Cationic core-shell nanoparticle	Paclitaxel siRNA	BCL2-siRNA	Breast cancer	*In vitro*	[63]
PLGA-PEG nanoparticle	Vincristine	Verapamil	Breast cancer	*In vitro*	[76]
PLGA-PEG-biotin nanoparticle	Paclitaxel	Tariquidar	Various cancer types	*In vivo*	[50]
PLA-PEG-biotin nanoparticle	Paclitaxel	P-gp siRNA	Various cancer types	*In vivo*	[64]
Polyalkylcyanoacrylate nanoparticle	Doxorubicin	Cyclosporine A	Various cancer types	*In vitro*	[42]
Oil nanoemulsion	Paclitaxel	Curcumin	Ovarian cancer	*In vitro*	[26]
Mesoporous silica nanoparticle	Doxorubicin	BCL2-siRNA	Ovarian cancer	*In vitro*	[27]

[a] *In vivo* refers to live animal-based tests and *in vitro* refers to cultured cell-based studies.

also been co-delivered with chemotherapeutics in oil nanoemulsions and in PLGA nanoparticles [26,55]. A summary of combinatorial nanoparticles containing pro-apoptotic compounds and chemotherapeutics can be found in Table 1.

3.3. Combinations of MDR-targeted siRNA with chemotherapeutics

The advancement in siRNA technology and its application to cancer treatment promise highly specific therapeutic options that silence target genes [56]. MDR-targeted siRNA have been constructed to target the likes of MRP1 (a MDR-associated protein) and BCL2 (an anti-apoptotic protein) genes, but their therapeutic application is hindered by the rapid degradation of siRNA molecules in serum, lack of cellular target selectivity, and poor cellular uptake [57]. Several studies have used nanoparticles to deliver siRNA both *in vitro* and *in vivo* and showed enhanced gene transfection through nanoparticle-mediated cellular uptake [58–60]. Such enhancement can be attributed to the improvements in siRNA stability and pharmacokinetic profiles. In investigating the effect of nanoformulations on siRNA delivery, Gao et al. showed that unmodified siRNA are cleared from the blood circulation through plasma degradation and renal excretion within minutes of intravenous injection. In a pegylated liposomal formulation, however, the clearance was much slower, with 30% of the siRNA remaining in the blood after 30 min [61]. More recently, Paolo et al. also demonstrated that a ligand-functionalized liposomal formulation significantly improved the pharmacokinetics of siRNA, retaining approximately 20% of the siRNA content 24 h following the administration. The attachment of targeting ligands to these nanoparticles also offered the opportunity to address the off-targeting issue in siRNA delivery [62]. Even though siRNA as a therapeutic agent is still in the developmental stage, early success in nanoparticle-based siRNA transfection has led many to explore the feasibility of combining chemotherapeutics and siRNA using a variety of nanocarrier platforms (see Table 1).

A poly ((*N*-methyldietheneamine sebacate)-*co*-((cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide) sebacate) (P(MDS-*co*-CES)) nanoparticle formulation was among one of the earlier efforts in demonstrating the potential of combinatorial nanoparticle co-delivering chemotherapeutics and siRNA [63]. The cationic amphiphilic copolymer self-assembled into a core-shell structured nanoparticle with a positive surface charge. The negatively charged, BCL2-targeted siRNA readily formed a complex with the nanocarrier through electrostatic interaction. The resulting paclitaxel- and siRNA-loaded nanoparticle was successful in downregulating the expression of BCL2 and more effective against a breast cancer cell line (MDA-MB-231) *in vitro* as compared to the individual agents and their cocktail mixture. Using another polymeric nanoparticle formulation, Patil et al. later demonstrated the *in vivo* application of paclitaxel-siRNA combination against a mouse model of drug-resistant tumor. An siRNA sequence aiming to knock down the expression of P-gp drug transporter was complexed to a PLGA-PEI (polyethyleneimine) copolymer [64]. It was found that the paclitaxel-siRNA nanoparticle resulted in significantly higher paclitaxel retention in the MDR cancer cells. A targeted formulation of the combinatorial nanoparticle showed observable reduction of the drug resistant tumor xenograft, which had little response to paclitaxel without the MDR gene silencing.

Liposomes, dendrimers, and silica nanoparticles are among other nanoparticle formulations used to deliver combinations of siRNA and chemotherapy drugs. Saad et al. prepared a cationic liposome using positively charged 1,2-dioleoyl-3-trimethylammonium-propane for the co-delivery of MRP1 and BCL2 siRNA in combination with DOX [65]. The formulation suppressed MDR effects by silencing both the drug efflux activity and anti-apoptotic signaling. Generation 3 dendrimers with silsesquioxane cubic core and poly(L-lysine) have been prepared to deliver siRNA-DOX mixture [66]. The dendrimer system, which is also conjugated to an RGD-based tumor-targeting peptide, showed excellent gene silencing and growth inhibition in glioblastoma cells (U87). Inorganic nanoparticles can also be complexed with siRNA upon functionalization with a positively charged outer shell. Mesoporous silica nanoparticles, for instance, have been used to co-deliver MDR-targeted siRNA and DOX following surface coating with PEI [67] and with amine-terminated dendrimers [27]. Both of these formulations showed resensitization of MDR cancer cells to DOX *in vitro*.

4. Combination strategies against clinical cancer drug resistance

While the upregulation of multidrug transporters and altered apoptosis pathways remain the major targets of interest in experimental MDR cancer models, clinical occurrence of cancer drug resistance is usually caused by more complicated and less defined mechanisms. Combinatorial nanoparticles containing multiple cytotoxic drugs with different mechanisms of action are therefore commonly adopted in aim to target the multi-faceted nature of clinical cancer drug resistance. In this section we highlight the developments that lead to more sophisticated combinatorial nanoparticle designs and enable higher level of

Table 2
Selected examples of combinatorial nanoparticle formulations containing multiple cytotoxic drugs.

Nanocarrier platform	Drug combinations	Indication	Significance	Status	Refs.
Liposome (CPX-351)	5:1 cytarabine and daunorubicin	Acute myeloid leukemia	Ratiometric drug loading with synergistic ratio maintained *in vivo*	Phase II	[77]
Liposome (CPX-1)	1:1 irinotecan and floxuridine	Colorectal cancer	Ratiometric drug loading with synergistic ratio maintained *in vivo*	Phase II	[73,78]
PLGA-PEG nanoparticle (aptamer-conjugated)	Cisplatin and docetaxel	Prostate cancer	Hydrophobic and hydrophilic drugs are co-encapsulated with differential drug release	*In vitro*	[15]
PLGA-PEG nanoparticle (aptamer-conjugated)	Doxorubicin and docetaxel	Prostate cancer	Hydrophobic and hydrophilic drugs are co-encapsulated	*In vitro*	[16]
Lipid-polymer hybrid nanoparticle	Doxorubicin and camptothecin	Pancreatic cancer	Adjustable ratiometric drug loading	*In vitro*	[17]
Lipid-coated PLGA nanocell	Combretastatin and doxorubicin	Lung carcinoma and melanoma	Temporally sequenced drug release achieves higher efficacy	*In vivo*	[18]
PLGA-PEG nanoparticle	Combretaastatin and paclitaxel	Lung carcinoma and melanoma	Temporally sequenced drug release achieves higher efficacy	*In vivo*	[19]
HPMA-Gem-Dox	Gemcitabine and doxorubicin	Prostate cancer and various cancer types	Enzyme-labile drug linkers enables differential drug release	*In vivo*	[22]

tuning in the drug combinations. Selected examples of combinatorial nanoparticles containing multiple cytotoxic drugs can be found in Table 2.

4.1. Combinatorial nanoparticles co-encapsulating hydrophobic and hydrophilic drugs

Most nanoparticle-based drug combinations are comprised of drug compounds with similar water solubility. Liposomal drug combinations, for instance, typically contain hydrophilic drugs owing to liposome's aqueous core whereas polymeric nanoparticle formulations preferentially carry water-insoluble drugs in their hydrophobic core [68]. The difficulty in co-encapsulating hydrophobic and hydrophilic drugs imposes a major limitation on the possible therapy combinations. To broaden the applicability of combinatorial nanoparticles, Zhang et al. conducted a pioneering work in co-encapsulating hydrophobic and hydrophilic drugs on a polymeric nanoparticle platform [16]. In the study, RNA aptamers were conjugated to the surface of PLGA-PEG polymeric micelles loaded with hydrophobic anticancer drug, docetaxel. The aptamers serve as targeting moieties to prostate cancer cells and at the same time carry the hydrophilic DOX through intercalation. The resulting nanoparticle formulation was able to concurrently deliver DOX and hydrophobic docetaxel to the targeted cancer cells.

Chemical modifications have also been employed to improve drug molecules' compatibility with carrier platforms. Kolishetti et al., for example, conjugated hydrophilic cisplatin to a polylactide (PLA) derivative and co-encapsulated the resulting prodrug with docetaxel in PLGA nanoparticles [15]. The conjugated cisplatin drug detaches from the hydrophobic PLA chain and releases from the polymeric core upon intracellular reduction. The combinatorial nanoparticle formulation of the two mechanistically distinct anticancer drugs showed higher toxicity in prostate cancer than single-drug-loaded nanoparticles. Applying the same principle in modifying drugs' solubility profiles, Aryal et al., chemically modified hydrophilic gemcitabine to enable its co-encapsulation with paclitaxel in a lipid-coated PLGA nanoparticle system [14]. But instead of modifying the hydrophilic compound with a hydrophobic polymer derivative, Aryal et al. covalently joined gemcitabine to the hydrophobic drug with a hydrolysable linker. This approach yielded a water-insoluble drug–drug conjugate that enabled the uniform loading of the two drug species. The conjugate was readily hydrolyzed in mildly acidic condition and the dual-drug-load nanoparticle showed strong toxicity against a human pancreatic cancer cell line (XPA3).

4.2. Combinatorial nanoparticles with precise ratiometric drug loading

Drug-to-drug ratio has been found to govern the efficacy of combination treatments. Multiple studies suggest that the degree of synergism and antagonism of a combination therapy is highly dependent on the relative concentrations between the combined drugs [69,70]. By unifying the pharmacokinetics of their cargoes, combinatorial nanoparticles open the avenue to co-delivering multiple drugs at a predetermined ratio that maximizes the combination efficacy. Currently, dual-drug liposomes with precise molar ratios have been prepared, and their superiority over traditional combination therapy are highlighted by the clinical trials of CPX-351, a 5:1 cytarabine and daunorubicin formulation for acute leukemia treatment [10,71,72], and CPX-1, a 1:1 irinotecan and floxuridine formulation for colorectal cancer treatment [73]. These liposomes demonstrate the ability to maintain the synergistic drug ratios *in vivo* and are more effective than the cocktail administration of the free drugs. In preparing these fixed-ratio, dual-drug liposomes, the empty liposomal carriers are incubated with individual drug solution. Incubation conditions such as liposome-to-drug ratio, solvent, temperature, and incubation time are carefully monitored to encapsulate the desirable concentration of drugs [74].

In polymeric nanoparticles, drug co-encapsulation through non-covalent physical entrapment leads to batch-to-batch variability in drug concentrations. In response to this issue, Aryal et al. devised a strategy to enable consistent and controllable ratiometric drug loading by covalently attaching drugs to polymer chains with uniform length and structure [17]. The polymer conjugation provides a predominant hydrophobicity to overwhelm the drugs' intrinsic solubility. As a proof-of-concept, DOX and camptothecin (CPT) were used to synthesize polymer conjugates with uniform molecular weight. The lipid-coated polymeric nanoparticles prepared from different mixtures of the two drug–polymer conjugates showed consistent size distribution. More importantly, the final loading yield of DOX and CPT follows the same molar ratio as the drug–polymer conjugates used during the nanoparticle preparation. The study demonstrated that the combinatorial nanoparticles were more potent than the cocktail mixture of the single-drug-loaded nanoparticles. The drug–polymer conjugation approach could be applied to other drug types and paves the road to facile assembly of ratiometrically controlled multidrug-loaded nanoparticles.

4.3. Combinatorial nanoparticles with temporally sequenced drug release

The effect of temporally sequenced drug release is most clearly found in a lipid-coated PLGA nanoparticle system developed by Sengupta et al. that combines an antiangiogenic factor, combretastatin, with DOX [18]. In this formulation, the antiangiogenic agent is loaded in the lipid coatings and has much faster release kinetics than the polymer-conjugated DOX. The system takes advantage of the physiology of tumors, whose rapid, uninhibited growth relies on the nutrients supplied by newly formed blood vessels. Once the nanoparticles reach the tumor target, the fast-released combretastatin shuts off the surrounding vessels, enclosing the DOX-encapsulated nanoparticles within the tumor. As compared to a liposomal formulation of combretastatin and DOX, which lacks the sequenced drug release, the lipid-coated PLGA nanoparticle showed superior efficacy and lower systemic toxicity in murine models bearing Lewis lung carcinoma and B16/F10 melanoma. The same treatment philosophy was also applied in a PEG-PLA formulation in which combretastatin was physically entrapped and paclitaxel was covalently attached to the polymeric core. This therapeutic strategy that quarantines and kills tumors from the inside out presents a unique advantage of nanoparticle-based combination therapy [19].

Combinatorial nanoparticles with temporally sequenced drug release also promise better-tailored treatments that optimally deliver the biochemical agents at the appropriate cellular stage. For drug combinations with different action mechanisms, sequencing is particularly important to the drugs' cooperative effect. Many chemotherapeutics are most potent in specific cell cycles, and, therefore, improper sequencing could lead to unintended cell cycle arrest and diminish response to the subsequent drug [75]. Currently several combinatorial nanoparticle formulations have shown differential drug release kinetics among their drug cargoes [15,16,22]. While further investigations are warranted to examine the effect of sequenced drug release on the cellular level, these nanoparticle designs could open the door to more precise multi-modal targeting against molecular pathways of cancer cells.

5. Conclusion and outlook

Multidrug-loaded nanoparticles present a powerful and versatile platform for anticancer drug delivery. The synthesis flexibility

of nanoparticle platforms has enabled unprecedented control in delivering a wide range of therapeutics. Various strategies based on combinatorial nanoparticles opened up many promising options toward addressing cancer drug resistance. Specific chemosensitizing agents, for instance, have been used in combination with chemotherapeutics to suppress MDR with defined mechanisms. In clinical cancer treatment, drugs with different modes of action can be combined in a precisely controlled manner to maximize therapeutic efficacy and to minimize the likelihood of drug resistance development. Enabling uniform and concurrent delivery of drug combinations, maintaining the synergistic drug ratios, and controlling drug exposure sequence are the major advantages of nanoparticle-based combination therapy over the traditional cocktail administration. While batch-to-batch inconsistency and manufacturability are among the key challenges in many combinatorial nanoparticles, we believe that ongoing efforts on the advancement of combinatorial nanoparticles will lead to the ideal combination therapy sought after by physicians and oncologists.

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