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Review article

Nucleic acid combinations: A new frontier for cancer treatment

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ABSTRACT

The emerging molecular understanding of cancer cell behavior is leading to increasing possibilities to control unchecked cell growth and metastasis. On the other hand, development of multifunctional drug carriers at the 'nano'-scale is providing exciting new therapeutic strategies in clinical management of cancer beyond the conventional cytotoxic drugs. A new frontier in this regard is the combinational use of complementary agents based on nucleic acids to overcome the limitations of conventional therapy. The existence of tightly-integrated cross-talk through multiple signaling and effector pathways have been appreciated for some time, and the plasticity of such a network to overcome one-dimensional intervention is stimulating development of combinational therapy. The objective of this review is to underline the cutting edge technologies and opportunities employed in combination cancer therapy using nucleic acids therapeutics for successful clinical translation. Here, we provide a detailed analysis of the multifunctional carriers designed for different types of payloads, surveying the biomaterials used to construct the functional carriers. We then provide effective nucleic acid combinations employed to obtain more comprehensive outcomes, highlighting the critical factors involved in successful therapy. We conclude with an authors' perspective on the future of combinational therapy using nucleic acid therapeutics, articulating the main challenges to advance this promising approach to the clinical realm.

1. Introduction

The conventional view of gene therapy, *i.e.*, hereditary single gene defects corrected with functional copies of the native gene, has been expanded to treatment of both acquired and infectious diseases [1–4]. In the case of cancer, where hereditary and acquired defects, as well as infections agents can cause cellular transformations, nucleic acid-based therapy is presenting an effective alternative to traditional chemotherapy. Therapeutic limitations of the latter approach have been appreciated, and they were attributed to robustness in signaling networks that includes redundancies, extensive crosstalk, compensatory and neutralizing activities in disease-causing cells [5–8]. This realization has shifted the drug development paradigm from conventional broad-spectrum cytotoxic compounds to molecular agents selective for specific targets. Anticancer drugs, molecular inhibitors, and nucleic acids have all shown to be effective mostly in monogenic diseases, but these modalities are challenged in the face of cellular heterogeneity and adaptive resistance in cancer [5,7,9]. In many aggressive heterogeneous cancers with adaptive resistance, strategies to target individual signaling pathways have failed to block abnormal proliferation and metastasis due to cellular plasticity enabling the cells to restore the activities of interfered pathways or deployment of alternative pathways for vital cellular activities [6]. To this end, a new strategy employing combinational therapy, which comprises of co-delivery of multiple types of therapeutic agents *via* nanoparticulate carriers, is emerging. This strategy guides the joint payload through multi-dimensional transport routes in cells [10] and it is intended to trigger synergistic effect(s) *via* complementary pathways, generating a greater effect than the sum of the constituent components [11–15] (Fig. 1). Synergistic combination of agents may further overcome possible toxicities associated with clinical doses of individual drugs by allowing lower doses of

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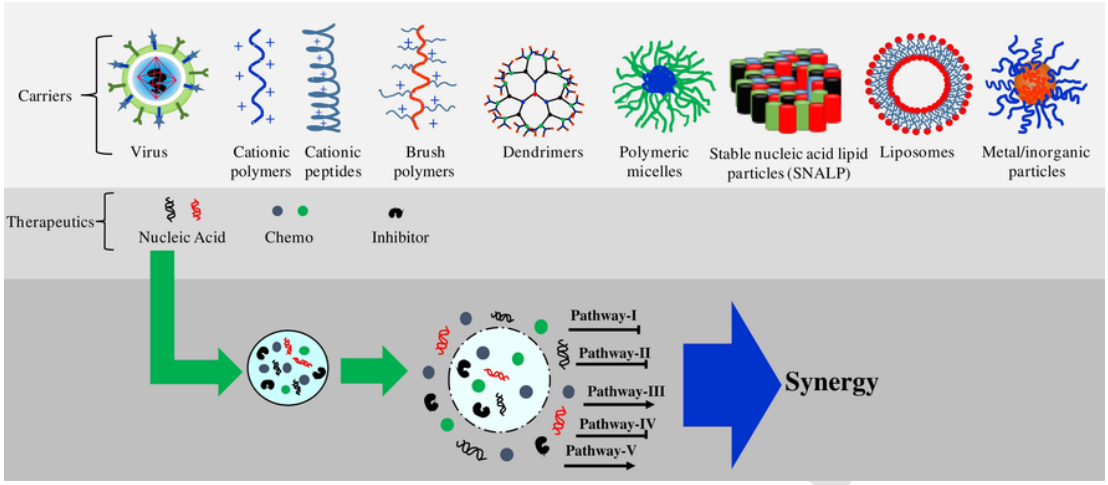

Fig. 1. Combinational nucleic acid delivery. Different types of carriers and therapeutic agents can form nano-sized formulations with therapeutic agents that enter the cell, and release their payload to influence specific pathways that may preferentially result in synergistic anti-tumor activities.

individual components to be employed [16–18]. In an ideal combination therapy, each component could display independent pharmacodynamics, with minimal overlapping of the toxicity spectra, or display joint pharmacokinetics profile if the desired outcomes are optimized in this way.

Multidrug cocktails were the earliest combinations explored and, with the advent of nano-carriers in the 25 to 250 nm range, it is becoming convenient to encapsulate drug cocktails into a single carrier and several of these formulations were approved for clinical applications [19]. Combination of nucleic acids and anti-cancer drugs has recently emerged to specifically tackle the critical issue of multidrug resistance (MDR) [20,21]. While other reviews focused on the latter theme [22,23], we specifically focus on the delivery of nucleic acid combinations (D-NAC) in this manuscript. Nano-carriers are an integral part of this approach, since they can provide physiological protection of the payload (especially critical for highly-sensitive nucleic acids), reduce systemic toxicity (by delivering the cargo to target organ/tissue/cells), and enhance bio-availability after systemic administration. By controlling intracellular trafficking of the payload (Fig. 2), nanocarriers could improve efficacy of the agents by delivering their cargo to the appropriate sub-cellular compartment. Nano-carriers can address one of the fundamental challenges of combination therapy, namely the variable pharmacokinetics and bio-distribution of inherently different constituent agents [23,24]. Therefore, it is crucial to understand nano-carrier design in D-NAC and the emerging molecular opportunities to translate combinational therapy.

2. Rationale for delivery of nucleic acid combinations (D-NAC) in cancer gene therapy

Therapy *via* D-NAC ideally targets multiple pathways associated with signaling networks such as phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor κB (NF-κB), Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT), and various activators of transcription, apoptosis, growth/invasion and angiogenesis [25]. Apoptosis inhibition by over-expression of anti-apoptotic mediators including Mcl-1, Bcl-2, and survivin, and mutation in drug targets, such as MEK, Epidermal Growth Factor Receptor (EGFR) and BCR-Abl, are associated with cellular resistance against conventional therapeutics [13,26–30]. Heterogeneity in the malignant cell populations and a complex web of signaling networks continuously limit the outcome of one dimensional therapies. Three major approaches to combinational therapy include inhibition of specific targets by multiple strategies, abolishing multiple components in a given pathway (to better eradicate a given pathway), and interfering with multiple mechanisms in tumor growth and metastasis [12]. The overall goal is to generate a better efficacy with minimal side effect by delivering multiple types of therapeutic agents [12,22,23]. The combined drug actions could be, preferentially, synergistic, additive, or based on coalition [31], where individual agents are inactive, but show efficacy in combination [32,33].

Effective pairs of agents could be identified by exploring mechanistic insights from the literature or high-throughput screening without introducing a bias in the selection process (Fig. 3) [34–36]. A suitable combination is expected to generate a synergistic effect, but combinations can often exhibit an additive or even antagonistic effect due to variation in protein expression in malignant cells [9,37]. With over-expressed mediators, the higher the available target levels, the greater the effects *via* synergy and usually the lower the synergistic side effects. In many cases, inter-connectivity of signaling pathways does not allow sufficient effect by simply switching to alternate proteins, and/or functional mutations [18,38]. Combination therapy that addresses different mechanisms may overcome this limitation by simultaneously blocking a greater number of pathways. One can envision co-delivery of combination drugs in a single carrier, delivery in a mixture of distinct (separate) carriers, or subsequent delivery of an agent following delivery of another agent in a carrier [23,39]. The pharmacodynamics of the individual agents are likely to play a key role in the choice of the delivery strategy.

3. Vehicles and nucleic acids in D-NAC

Both viral vectors and non-viral carriers are explored in D-NAC [30,40,41], where both approaches displayed significant anti-cancer effects compared to corresponding monotherapies. In combination therapies, viruses are specifically utilized to induce protein expression, specifically in immunotherapy (cancer vaccines), and the combination of suicide gene therapy and cancer vaccines has been shown to be an effective synergistic pair to induce anti-tumor response in both *in vitro* and *in vivo* models [40,42,43]. However, it is difficult to explore viruses in clinical settings due to several limitations such as insertional mutagenesis, toxicity, limited cargo capacity and manufacturing challenges. Safety issues especially have propelled the non-viral delivery to clinical stage, which are more convenient for RNA-based agents as well. Cationic lipids and natural/synthetic polymers are utilized to achieve synergistic effects among therapeutic agents [10,44–50]. The strategies for combination delivery using non-viral carriers include co-delivery (therapeutics are encapsulated into a single carrier), mixed de-

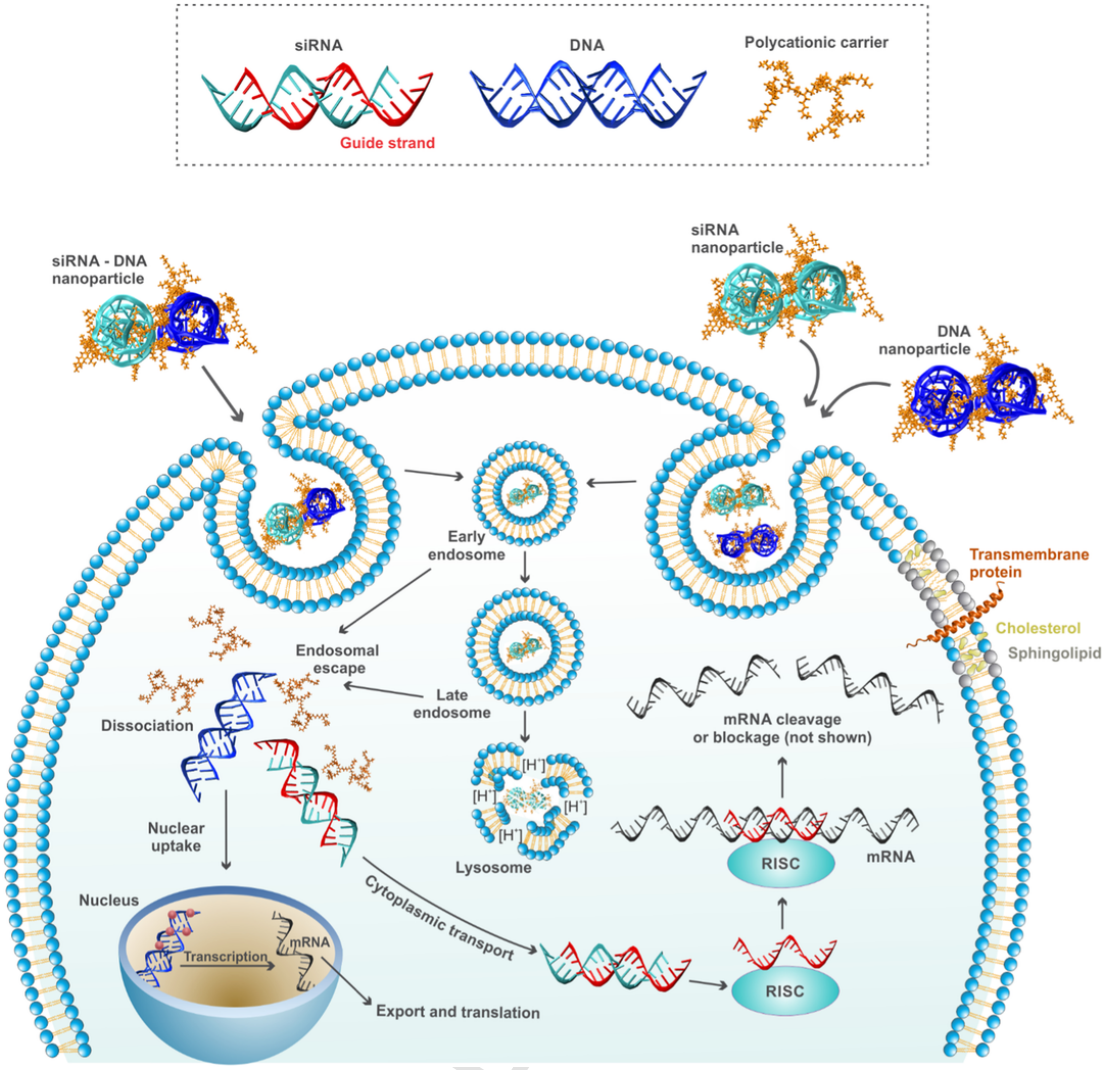

Fig. 2. Intracellular trafficking of nucleic acid therapeutics. The nucleic acids could be loaded and delivered with the same carrier to couple the pharmacokinetics of multiple agents (top left) or delivered *via* different carriers to optimize delivery efficiency or timing for each agent (top right). Depending on the functionality of the nucleic acid, cytoplasmic or nuclear delivery might be needed.

livery (individual therapeutics are encapsulated in different carriers) and subsequent delivery (one therapeutic is encapsulated whereas the other one is free) [23,39]. While the co-delivery is usually performed when nucleic acids payloads display similar characteristics (*e.g.*, a combination of different plasmids, or a cocktail of siRNAs targeting different proteins), mixed and subsequent delivery could be used to deliver different types of nucleic acids and even a combination of nucleic acids and small molecule drugs. Facile and versatile chemistry is the promising aspect of non-viral carriers for combinational cancer therapy (see Fig. 4 for representative carriers). Due to the lipid bilayer envelope and an aqueous phase at the core, liposomes could potentially encapsulate different nucleic acids, as well as small molecule drugs simultaneously. A liposome-like multicomponent system, stable nucleic acid-lipid particles (SNALPs, with a solid lipid core), has relied on the concept of 'combinational' delivery system; SNALP supramolecules incorporate different building blocks based on the needs of delivery. While the primary structure of the SNALP is based on lipid bilayer, novel cationic lipids with increased fusogenic properties have been introduced that are capable of binding to nucleic acids *via* interionic interactions [51]. In 2005, Heyes et al. reported a series of new cationic lipids with different saturation degrees, among which 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA) with two double bonds per alkyl chain showed significant efficiency in cellular uptake after incorporation into SNALP structure [52]. The same cationic lipid was later improved to create DLin-KC2-DMA, which showed *in vivo* activity at siRNA doses as low as 0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates [53]. The other components of SNALPs could include PEG (to create stealth properties and enhanced permeation and retention effect), cell penetrating peptides (to enhance cellular internalization), and monoclonal antibodies (for active targeting). Cationic lipids, liposomes, and peptides have been additionally used for nucleic acid delivery; however, SNALP is proposed to combine their capabilities for a more efficient and reliable delivery.

3.1. Cationic lipids and lipoplexes in combinational delivery

Lipid-based nano-carriers liposomes were the initial approach employed to introduce exogenous nucleic acids to host cells. Commercial lipofection reagents (*e.g.*, Lipofectamine® 2000, Oligofectamine®, DharmaFECT®, *etc.*) and DOTAP:cholesterol based cationic liposomes are most effective among lipid-based delivery agents [41,54–56]. These lipofection reagents were explored to deliver a wide array of nucleic acid combinations based on both RNA and DNA (Fig. 4). With non-ionic formulations, the aqueous core makes it possible to entrap multi-

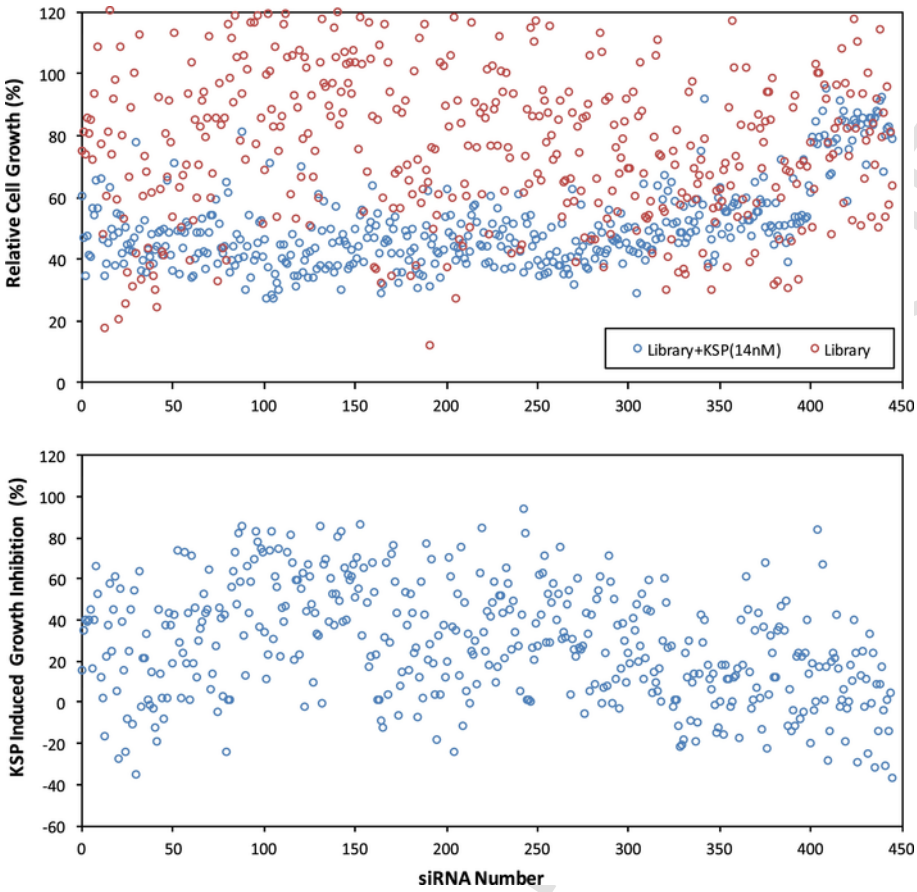

Fig. 3. Screening of an siRNA library to identify synergistic pairs of effective siRNAs. A library of 446 siRNAs against apoptosis-related proteins (Dharmacon siGENOME Human Apoptosis siRNA library) was screened with MDA-435 cells (a poorly differentiated breast tumor line expressing both epithelial and melanocytic markers) in the absence and presence of kinesin spindle protein (KSP) siRNA (**top graph**). A low concentration (14 nM) of KSP siRNA was chosen to minimize its effects on its own. The presence of an apoptotis-related siRNA could (i) abolish (KSP induced growth inhibition < − 20%; *i.e.*, antagonistic effects), (ii) not influence (KSP induced growth inhibition between − 20% and 20%), or (iii) enhance (KSP induced growth inhibition > 40%; additive or synergistic effect) the effect of the KSP siRNA (**bottom graph**). The latter class of siRNA combinations is desirable for complementary enhancement of therapeutic efficacy (unpublished).

ple nucleic acids at desired ratios; however, unlike small molecule drugs, nucleic acids cannot penetrate the lipid membrane and the liposome needs to be destabilized in order to release its payload. DOTAP/cholesterol liposomes were employed for successful co-expression of functionally synergistic tumor suppressor genes, FUS1/p53 in human non-small cell lung carcinoma (NSCLC) cells and its xenograft model [57]. The commercial reagent Lipofectamine® 2000 (not suitable for animal models) has been most commonly used in combinational delivery, though other carriers (Table 1) have also shown effective performance in *in vitro* and *in vivo* models. Cationic charge density, hydrophobic tail conformation and the spacer length could be optimized for co-entrapment and co-delivery, but chemically-modified lipids especially with cationic functionalities (*e.g.*, polyethyleneimine (PEI), polyamidoamine (PAMAM) and polylysine (PLL) derivatives) are particularly attractive to tailor the liposomes for nucleic acid payloads [44,58]. Being localized at the inner and outer aqueous interfaces of liposomes, the cationic moieties could act as binding sites for anionic nucleic acids through electrostatic interactions [44,45]. The cationic groups can also provide a spacer for anchoring specific motifs and binding ligands to generate cell specificity for active targeting. With liposomes that bear nucleic acids on the outside surface, displacement of nucleic acids with other anionic species such as heparin sulfate [59] is always a concern, which results in premature release of the payload, as well as its rapid digestion of nucleic acids in serum. While maintaining proper balance of active agents after loading multiple nucleic acids in carriers is always a concern in combinational delivery, having a secondary nucleic acid may lead to improved pharmaceutical effects; DNA supplementation in short interfering RNA (siRNA) formulations of liposomes were noted to enhance the silencing activity of siRNA, not due to gross morphological changes in liposomes but possibly due to altered dissociation/release of the nucleic acids from the lipoplexes [60]. A similar observation was also made with polyplexes [61], where the presence of a 'helper polyanion', a plasmid DNA in this case, was able to significantly improve the silencing activity of polyplex formulations. It must be pointed out that the structural features of the polyplexes were vastly different after the addition of helper polyanion DNA in this study (unlike the lipoplexes investigated above), and that a relatively ineffective carrier (PLL) was used as the nucleic acid carrier.

3.2. Cationic polymers and polyplexes in combinational delivery

Cationic polymers, the leading materials in D-NAC, can be configured into multiple forms, including micelles, hollow polymersomes and homogenous nanoparticle (NP) polyplexes [49,62,63]. They were broadly effective in several type of cancers (Table 1) after being employed in their native form or after derivatization with functional moieties (Fig. 4) [15,30]. Low molecular weight PEI (LMW; < 2 kDa) was derivatized with a broad range of hydrophilic and hydrophobic moieties to transform them into effective carriers [46,64]. Hydrophobic modification of LMW PEIs with aliphatic/aromatic moieties imparts lipophilicity to already existing buffering capacity. Synergism between these features enables improved self-assembly during complexation

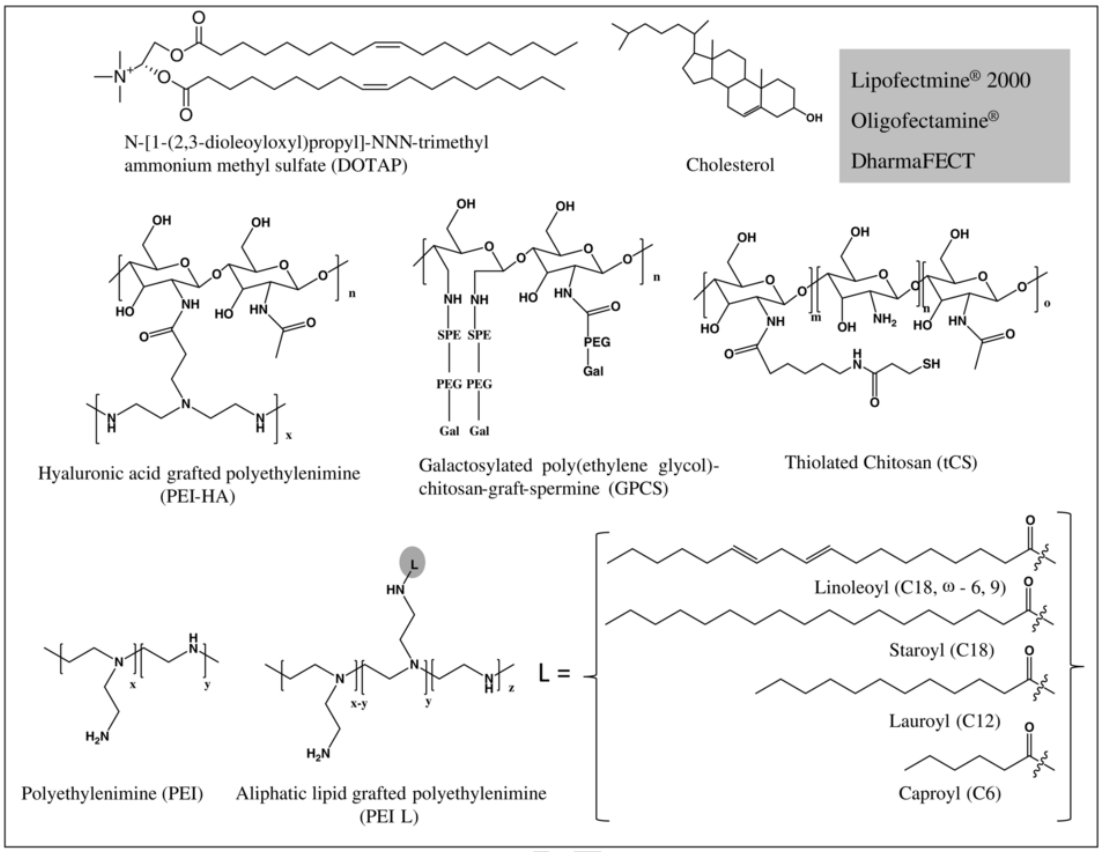

Fig. 4. Chemical structures of the main lipids, polymers and their derivatives utilized in the D-NAC.

with nucleic acids while enhancing binding to hydrophobic domains of plasma membrane that ultimately enhances internalization [65,66]. LMW PEIs grafted with aliphatic lipids (C8 to C18) generate relatively non-toxic PEI derivatives, and even small hydrophobes (C3) appear to be functional for nucleic acid delivery [67]. As with single agents, these polymeric derivatives displayed superior delivery of siRNA cocktails with a single carrier and enabled down-regulation of target genes without interfering with each other [30,68]. Chemical modification of higher MW PEI (10 kDa) with the natural polyanion hyaluronic acid (HA) was proposed to generate more compatible derivative, since it can neutralize excess cationic charge density to decrease cellular cytotoxicity, while still preserving siRNA encapsulation capacity [69]. PEI-HA was utilized to formulate a dual-functional (CD44/EGFR-targeting) nano-carrier for systemic delivery of p53- and miR-125b expressing plasmid in a mouse model of lung cancer [70]. Chitosan (CS) is another natural cationic polymer extensively studied as an alternative of PEI due to its perceived biocompatibility. Chitosan derivatives, galactosylated poly(ethylene glycol)-chitosan-*graft*-spemine (GPCS) copolymer and thiolated chitosan (tCS), are typical CS derivatives employed in D-NAC [14,15]. Here GPCS was utilized to deliver two different tumor suppressor genes to hepatocellular carcinoma and tCS was used to deliver poly(siRNA) to prostate cancer cells. In both approaches, CS derivatives were able to generate significant anti-cancer activity.

Rather than constructing polymeric conjugates for D-NAC, a simpler approach to designing multifunctional particles is to formulate the particles with multiple polycationic species, *i.e.*, blending (Fig. 5). The polycations usually act to neutralize the anionic charges of nucleic acids along with forming polyion bridges among the NA. Polymer-polymer interactions may further stabilize the formed polyplex formulations. Along with anionic nucleic acids, one can incorporate other anionic species (*e.g.*, HA) in polyelectrolyte complexes, or coat the already formed particles with polycationic species. In the latter case, functional ligands (*e.g.*, specific cell-targeting agents or anti-fouling agents) could be incorporated into the surface coating.

3.3. Nucleic acids in combinational delivery

Different strategies could be used to simultaneously deliver multiple nucleic acids based on the design of nucleic acids (Fig. 6). It is challenging to formulate different nucleic acids into a single carrier due to variations in ionic charge density, size, and stiffness of the constituent molecules, and this becomes more significant while employing smaller molecules like siRNA [15,72]. This approach to RNA interference (RNAi) technology has quickly become a dominant player in preclinical and clinical studies based on its synthetic mediator, siRNA. However, small size, low charge density and high chain stiffness limits siRNA's complexation ability with the carriers. To resolve these challenges and to protect the nucleic acid against early degradation in serum, many studies have reported using either chemically modified or encoded (*e.g.*, co-expressing plasmid construct) siRNAs [14,15]. Chemical modification of siRNA has been developed to design multimeric and polymeric siRNAs with higher MW and higher electronegative charges density (Fig. 7). Insertion of labile bonds among multimeric species is desirable to exploit intracellular reductive environment, such as end-capping siRNAs with thiol groups produce multimeric siRNA species held together with labile disulfides [14,72,73]. The multimeric siRNAs may form more stable complexes with cationic carriers, before undergoing reductive cleavage to release siRNAs. The initially released species will presumably be a mixture of individual siRNAs as well as oligomers, which may further breakdown intracellularly.

Formulating a combination with DNA and siRNA is challenging due to differences in physical and electronic properties (*e.g.*, charge distrib-

Table 1
Summary of nucleic acid combinations employed in cancer therapy.

Combination	Cancer type/ model	Vehicle	Targets	Outcome	Ref.
LETM1/CTMP	Hepatocellular carcinoma *In vivo*: H-ras12V mice	GPCS	Co-expression of LETM1 and CTMP, activate AMPK and Akt1 pathways	Induce mitochondria- mediated apoptosis	[15]
LKB1/FUS1	Lung cancer *In vitro*: A549 and H460 Cells *In vivo*: BALB/c mice	DOTAP/Cholesterol NPs	Co-expression of LKB1 and FUS1, upregulates p-p53, p-aMPK and silence p-mtOR	Induce apoptosis and enhance survival of mice	[54]
FUSI/p53	Lung cancer *In vitro*: NSCLC cells *In vivo*: H322 lung cancer mouse	DOTAP: cholesterol NPs	Silence MDM2, and upregulate Apaf-1	Induce apoptosis and suppress tumor growth	[57]
shRNA-EGFR/PTEN	Glioblastoma *In vitro*: U251-MG cells *In vivo*: BALB/c mice	Lipofectamine	Express shRNA-EHFR/PTEN: down regulate EGFR and up-regulation of PTEN	Induce apoptosis and suppress tumor growth	[13]
shRNA-STAT3/ LKB1	Ovarian cancer *In vitro*: SKOV3 cells *In vivo*: BALB/c mice	Lipofectamine	Express shRNA-STAT3 and LKB1: upregulate p21 and p-p53, silence MMP-2 and MMP-9	Induce apoptosis, reduces invasion, migration and tumor growth	[41]
shRNA-SURVIVIN/ GRIM-19	Laryngeal cancer *In vitro*: Hep-2 cells *In vivo*: BALB/c-nu/nu nude mice	Lipofectamine	Express shRNA-SURVIVIN and GRIM-19: silence SURVIVIN and STAT-3	Inhibit the growth and induce the apoptosis of Hep-2 cells	[120]
shRNA-STAT3/ GRIM-19	Thyroid carcinoma *In vitro*: SW579 cells *In vivo*: BALB/c nude mice	Lipofectamine	Express shRNA-STAT3 and GRIM-19: silence STAT3, MMP-2, MMP-9, VEGF expression	Induce apoptosis, reduces invasion, migration and tumor growth	[123,124]
p-gp/Mcl-1/ RPS6KA5 siRNA	Breast cancer *In vitro*: MDA435 *In vivo*: Nude Mice	PEI-Derivatives	Down regular target gene	Induce apoptosis and inhibit tumor growth	[26,30]
VEGEF/Bcl-2 or SURVIVIN-siRNA	Prostate cancer, Breast cancer *In vitro*: PC-3, MDA-MB-231 cells *In vivo*: Mouse model	GC, LPEI25	Silence VEGF, Bcl-2, SURVIVIN expression	Increase apoptosis, and inhibited tumor growth	[14] [73]
TTK/CDC20, CDC20/SURVIVIN	Breast Cancer *In vitro*: MDA-MB-231 Cells	PEI-Derivatives	Silence TTK, DCD20 and SURVIVIN expression	Inhibit cell growth	[68]
VEGEF-siRNA/ HER2-siRNA	Breast cancer *In vitro*: MCF7 Cells	Lipofectamine	Silence VEGF and HER2 expression	Inhibit invasion, proliferation and induce apoptosis	[145]
VEGEF-siRNA/ HER2-siRNA	Gastric Cancer *In vitro*: SGC-7901 cells	Lipofectamine	Silence VEGF and HER2 expression	Inhibit cell proliferation, migration, and induce apoptosis	[146]
psiRNA-VEGEF-C + A	Mammary cancer *In vitro*: BJMC3879 cell *In vivo*: BALB/c mice	Electro-transfer	Silence VEGF-C and VEGF-A expression	Reduce metastasis and enhance survival of mice	[185]
VEGEF-siRNA/KSP-siRNA	Hepatocellular carcinoma *In vitro*: Hep3B cells	Lipofectamine	Silance KSP, Cyclin D1, Bcl-2, SURVIVIN	Inhibit growth, migration, invasion and induce apoptosis of HCC cells	[148]
Bcl-2/Mcl-1	Lung cancer *In vitro*: MSTO-211H, NCI-H28 and NCI-H2052 cells	INTERFERin™	Silence target gene expression	Increase anti-cancer activity	[149]
Bcl-2/SURVIVIN-siRNA	Human bladder cancer *In vitro*: T24 cells	Lipofectamine	Up-regulate caspase-3 activities	Inhibit cell proliferation and induce apoptosis	[150]

Table 1 (*Continued*)

Combination	Cancer type/ model	Vehicle	Targets	Outcome	Ref.
VEGF-NET-1 siRNA	Hepatocellular carcinoma *In vitro*: HepG2 cells *In vivo*: BALB/c mice	Lipofectamine	Silence VEGF expression	Inhibit growth and angiogenesis of HCC, suppress tumor growth	[151]
IGF-IR-siRNA/ EGFR-siRNA	Colorectal cancer *In vitro*: DLD-1, Caco2 cells	Oligofectamine	Up-regulate caspase-3/7 activities	Inhibit cell proliferation and induce apoptosis	[55]
pU6-EGFR-shRNA/ pU6-IGF1R-shRNA	Nasopharyngeal cancer *In vitro*: CNE2 and TW03 cells	Lipofectamine	Silence EGFR and IGF1R mRNA and protein expression	Induce apoptosis and chemo-sensitivity	[160]
miR-34/let-7	Lung cancer *In vitro*: NSCLC cells *In vivo*: Mice model	DharmaFECT 1	Repress oncogene expression	Inhibit cell proliferation and tumor growth	[56]
plasmid (wt-p53 + miR-125b)	Lung cancer *In vitro*: SK-LU-1 cells *In vivo*: KP mice	HA-PEI/PEG	Increase APAF-1 expression and caspase-3 activity, Silence Bcl-2	Reduce tumor progression and proliferation	[70]
miRNA/siRNA	Lung cancer *In vitro*: KP cells *In vivo*: KP Mice	Polyplexes	Restore P53 activity, reduce KRAS gene expression and MAPK signaling	Increase apoptosis, Inhibited tumor growth	[164]
tK/mGM-CSF/ mIL-2	Colon cancer *In vivo*: BALB/c Mice	Virus	Express tK/mGM/mIL-2	Enhance antitumor immunity	[42]
GC-CSF/IL-2	Squamous cancer *In vitro*: SCCVII *In vivo*: C3H/HeJ mice	DISC viruses	Secret GC-CSF/IL-2	Suppress tumor growth	[43]
HSV-tk/mIL-2	Colon carcinoma *In vivo*: BALB/c Mice	Virus	Express HSV-tK/mIL-2	Reduce metastasis of colon carcinoma into liver	[40]
IL-12, pro-IL-18, and ICE cDNA	*In vivo*: BALB/c mice	Gene gun	Induce INF-γ pathway	Enhance antitumor activity	[161]

Abbreviations: LETM1: Leucine zipper/EF hand-containing transmembrane-1, CTMP: carboxyl-terminal modulator protein, GPCS: galactosylated poly (ethylene glycol)-chitosan-graft-spermine, VEGF: Vascular endothelial growth factor, Bcl2: B-cell lymphoma2, GC: glycol chitosan NPs, KRAS: Kirsten rat sarcoma viral oncogene homolog, LKB1: liver kinase B1, STST-3: signal transducer and activator of transcription 3, FUSI: tumor suppressor candidate 2, DOTAP: N-[1-(2,3-dioleoyloxyl) propyl]-NNN-trimethylammoniummethyl sulfate, MDM2: murine double minute-2, Apaf-1: apoptotic protease-activating factor 1, EGFR: epidermal growth factor receptor, PTEN: Phosphatase and tensin homolog, HER-2: human epidermal growth factor receptor 2, NET-1: Neuroepithelial cell-transforming gene 1 protein, KSP: kinesin spindle protein, PEI25: Polyethylenimine (MW 25,000 Da), IGF-IR: insulin-like growth factor I(IGF-I) receptor, HSV-tk: herpes simplex virus thymidine kinase, mIL-2: mouse interleukin-2, mGM-CSF: mouse granulocyte macrophage colony-stimulating factor, GC-CSF: granulocyte-macrophage colony-stimulating factor, DISC: 2-defective infectious single-cycle (DISC) viruses, IL2: Interleukin-2, NSCLC: non–small cell lung cancer, VEGF-C: Vascular endothelial growth factor-C, VEGF-A: Vascular endothelial growth factor-A.

utions, dipole moments, polarizabilities and proton affinities), but a co-expression system for a therapeutic gene and a short hairpin RNA (shRNA) can be a convenient solution [13,57]. Viral vectors loaded with multiple genetic material have been initially designed especially in tumor vaccination [40,42,43], for example with immune-stimulatory molecules for anti-cancer immunity. In some studies, DNA-intercalating feature of anti-cancer drugs (*e.g.*, doxorubicin) was exploited for encapsulating and delivering complementary agents [74,75]. These complexes are formulated by allowing DOX intercalation into double-stranded 5′-GC-3′ or 5′-CG-3′ base pairs, which are then condensed with cationic polymers and dendrimers [76]. This strategy offers better control on the efficiency of loading and delivery of payload.

4. Specific nucleic acid combinations for cancer therapy

The innate resistance of unresponsive tumors is usually attributed to tumor heterogeneity. In 2015, Sottoriva et al. proposed a 'Big Bang' model of tumor initiation that suggests after initial oncogenic mutation, future generations acquire further mutations, which are present in discrete populations of cells, leading to spatial heterogeneity [77]. A more diverse pattern has been reported in other types of cancers. Amir et al. studied two human acute lymphoblastic leukemia samples with viSNE technology, and reported a large, irregular population of cells that were more different than similar [78]. The sub-population with intrinsic resistance to therapeutic assault would survive and outgrow other cells due to the selection pressure; this will promote relapse, which results in abundance of originally minor cells [79]. This "Darwinian clone selection" has been well-documented in different types of cancer in response to a variety of molecularly-targeted drugs [80]. On the other hand, plasticity of cancer cells that enables adaptation to molecularly targeted drugs could explain the acquired resistance. In addition to point mutations, availability of a variety of pathways leading to enhanced proliferation and survival could be responsible for the intracellular adjustments required. Our understanding of intracellular signaling cascade is evolving, and new "cross-talk" between different pathways and previously seemingly unrelated proteins are reported on a regular basis.

Latest reports have led to elucidation of a key role of JAK/STAT signaling pathway in development, proliferation, differentiation, and survival of cancer cells [81]. JAK is associated with a variety of transmembrane receptor families [82], and propagates signaling to cytoplasmic proteins such as the STAT proteins. Over-activation of JAK family members has been implicated in tumorigenesis [83], and persistent activation of STAT3, and to a lesser extent STAT5, has been shown to increase proliferation, survival, angiogenesis and metastasis in a variety of human cancers [84,85]. Similarly, PI3K/Akt pathway regulates pro-

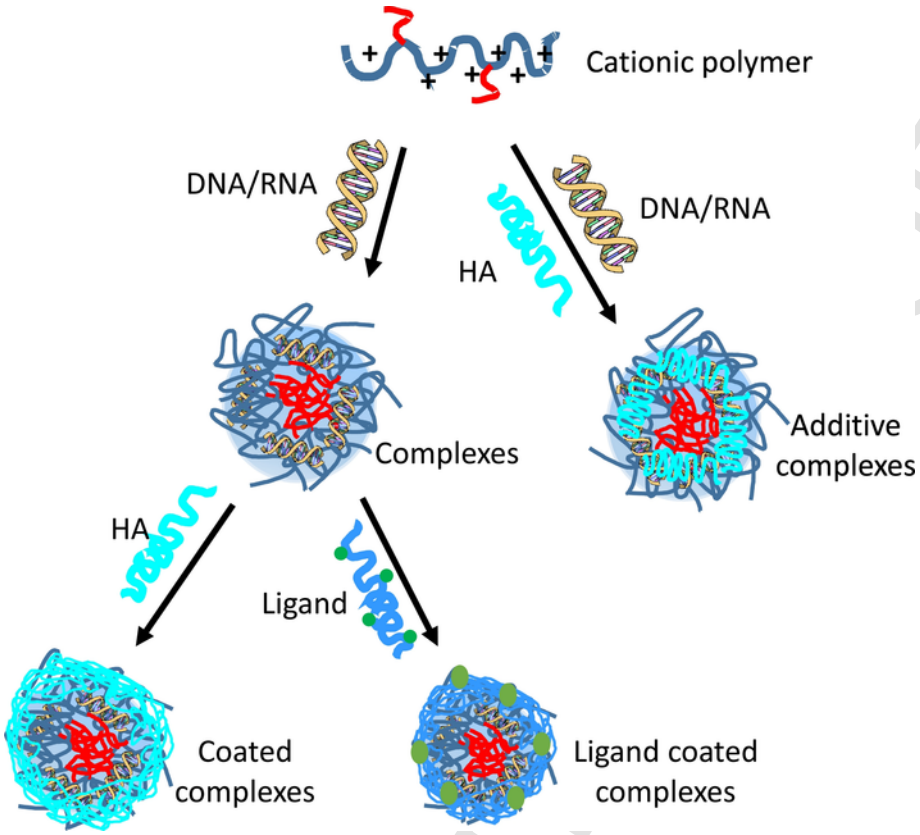

Fig. 5. Schematics of NP formulation through coating and additive processes. Polyplexes formulated using cationic polymer and a mixture of polyanions (*e.g.*, HA and nucleic acid) leads to additive nanocomplexes, while treating of polymer/nucleic acid complexes with an additional polyion leads to coated nanocomplexes. While additive polyplexes are expected to display enhanced dissociation, surface coating optimizes interactions with serum components and membrane/cellular surfaces [65,71]. Surface coating procedure can be repeated several times for Layer-by-Layer (LbL) coated NPs.

liferation, survival, motility and morphology, and has been associated with cancer pathogenesis [86]. Studies indicate that receptor tyrosine kinases (RTKs), including growth factor receptors, play a major role in activation of this signaling pathway [87]. PI3K/AKT pathway is one of the most commonly disrupted pathways in human breast cancer, and the PI3K-dependent activation of the serine/threonine kinase AKT and its downstream effectors are some of key factors in cell survival mechanisms. In cancers that are sensitive to RTK inhibitors, PI3K signaling could be initially lost; however, other routes soon emerge to activate the PI3K signaling [88,89]. Binding of growth factors to cell surface receptors could also activate Ras/Raf/MEK/ERK pathway. Mutations in KRAS, BRAF, MEK1 or MEK2 result in growth factor-independent ERK1 and ERK2 activation, which results in enhanced cell proliferation and survival [90]. Activated ERK also translocates to the nucleus to activate transcription factors, including FOS. The ERK1 and ERK2 MAPKs are activated by mitogens and are up-regulated in several human tumors [91].

However, the boundaries between these categorized pathways are fading with each report on a newly identified link among the major proteins in these pathways. For instance, the activation of tyrosine kinase HER2 located on breast cancer cell membrane is shown to trigger phosphorylation of RAF and Ras that regulates Bcl-2 family proteins [92]. MUC1 protein interacts with several cytoplasmic proteins, as well as Ras-MEK-ERK2 signaling pathway [93] and STAT3 (*via* Src signaling pathway) [94]. Activation of mTOR downstream effectors by p38 MAPK pathway [95], the effect of STAT3 activation on Ras and PI3K/Akt pathway [96], and JAK on PI3K and ERK pathways [97,98] are other examples of these inter-pathway cross-talks. Studies even indicate that this crosstalk contributes to a deregulation of PI3K signaling that can lead to tumorigenesis [99]. This could partially explain the preclinical data indicating a failure to induce apoptosis despite effective inhibition of PI3K-Akt components [100,101], which may suggest that a single inhibitor might not create a significant response even in sensitive cells.

The cellular heterogeneity implies that blocking one mechanism of survival would not be effective against the whole population. A subpopulation that does not rely on the targeted mechanism will be selected in this case. Even the cells that do respond could potentially adjust and rely on an alternative signaling pathway. Therefore, simultaneous targeting of multiple carefully selected proteins seems inevitable, and novel drug carriers for co-delivery of multiple agents, with different pharmacokinetics and pharmocodynamics features have to be designed. Proper combination of agents is important to minimize antagonistic effects [9,102,103]. Various combinations of DNA and RNA molecules have been explored to achieve synergistic effect(s) at the molecular level (Table 1). While synergistic agents can be delivered using either a single or mixed carriers, co-delivery through a single carrier is likely to be preferred due to its convenience, and simultaneous delivery of the components of the combinatorial therapy to the site of action. A single carrier has the potential to deliver the payloads at the proper balance to a target site at the same time, if desirable, and generate cumulative activities, if the carrier/payload is stable enough to prevent premature release of any of the payload. Among the nucleic acid combinations, siRNAs are gaining upper hand to complement the action of a DNA-based expression system [104].

4.1. Combinational delivery to up-regulate suppressor genes

Mitochondria, the powerhouse for different cellular activities, is an effective therapeutic target since its activity directly impacts tumorige-

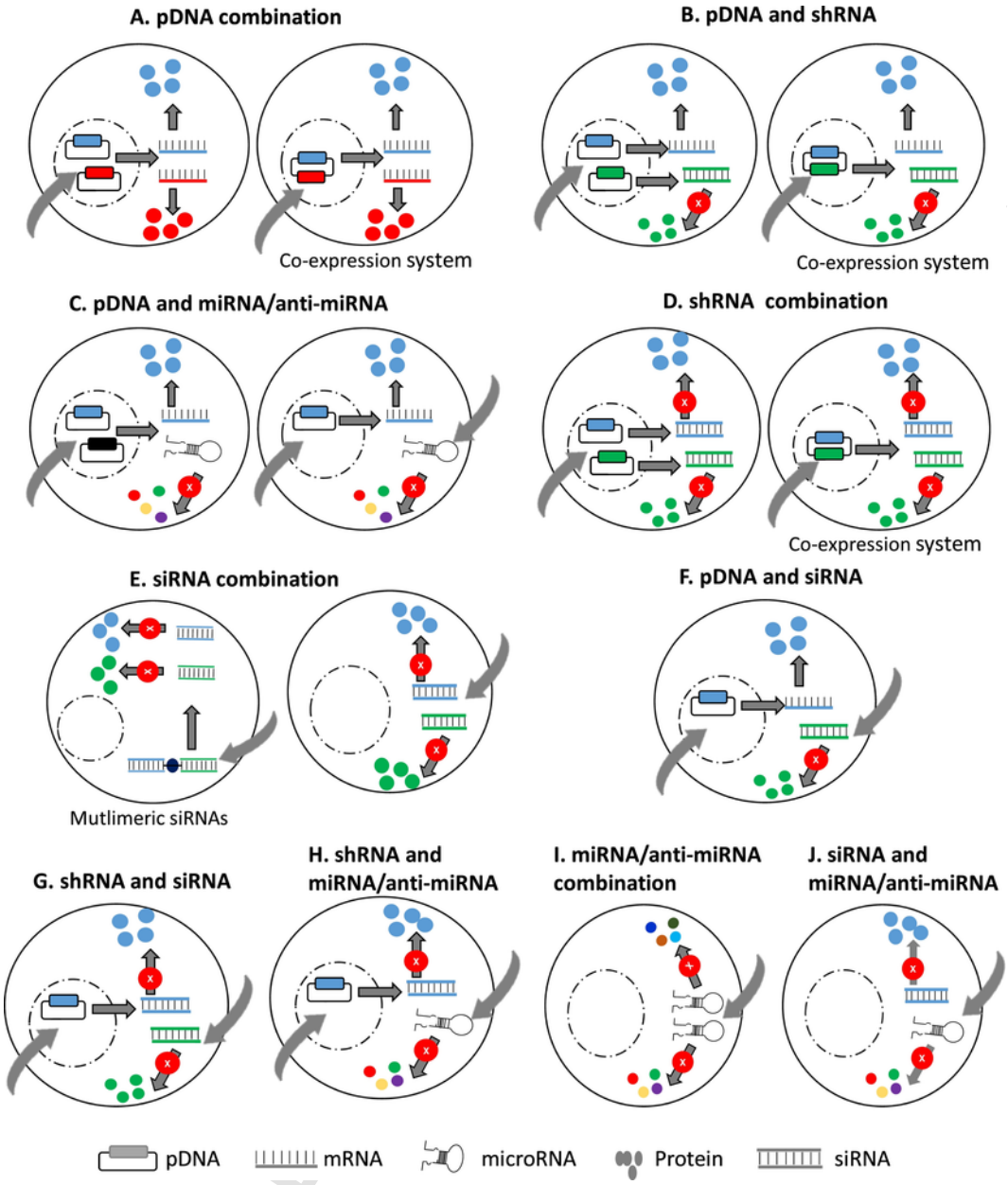

Fig. 6. Schematic representation of possible nucleic acid combinations. (A) Therapeutic protein combination. (B) Therapeutic protein and shRNA combination. (C) Therapeutic protein and miRNA combination. (D) Therapeutic protein and siRNA combination. (E) shRNA combination. (F) shRNA and miRNA combination. (G) shRNA and siRNA combination. (H) siRNA combination composed of individual siRNAs or multimeric siRNAs (I) siRNA and miRNA combination. In the case of pDNA driven expression systems (proteins, shRNA and miRNA), either independent expression systems or co-expression systems could be used. In the case of miRNA delivery, it is possible to deliver miRNA or anti-miRNA reagents to exert a desired therapeutic effect.

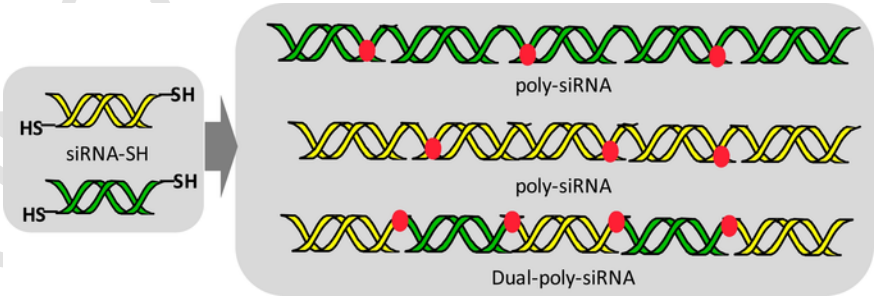

Fig. 7. Schematic for the synthesis of polymerized siRNAs, composed of either unitary siRNA blocks, or dual siRNA blocks. (Adopted from Reference [14]).

nesis [105–108]. Shin et al. delivered an expression system for two mitochondria targeting genes, LETM1 and CTMP, which were linked together by 2A peptide using galactosylated poly(ethylene glycol)-chitosan-*graft*-spermine (GPCS) in hepatocellular carcinoma (HCC) model [15]. LETM1, a mitochondrial inner membrane protein, and CTMP, a binding partner of Akt, are responsible for maintaining the proper morphology of mitochondria, but their over-expression was reported to generate mitochondrial defects that induce cell death [108–111]. The elevated LETM1-2A-CTMP substantially modulated mitochondrial morphology such as swelling and loss of cristae *via* optic atrophy-1 (OPA1) cleavage, which was typically observed in mitochondria due to LETM1 expression [112]. The mitochondria displayed enhanced release of Bax (a pro-apoptotic protein that induces cell death) and cytochrome *C* (that binds to Apaf-1 and activates caspase-3-dependent apoptosis) after LETM1-2A-CTMP delivery. The therapeutic action was driven by the synergistic actions of LETM1 and CTMP overexpression, which was facilitated by self-cleaving 2A peptide sequence [113].

Similarly, LKB1 (liver kinase B1), FUS1 and p53, well-established tumor suppressors, were incorporated into a co-expression plasmid and delivered *via* different carriers [54,57]. Each gene induces apoptosis through a different mechanism; FUS1 expression is associated with silencing of protein tyrosine kinases, which induces mitogenic signaling through mTOR pathway [114]. LKB1 is a tumor-suppressor gene associated with p21/p53 gene expression, stability and activity [115,116]. A synergistic effect based on induction of apoptosis and cell cycle arrest was observed in NSCLC model, leading to prolonged survival of tumor-bearing mice after delivery of LKB1/FUS1 co-expression plasmid with cationic liposomes (DOTAP/cholesterol) [54]. Compared to individual delivery, the delivery of co-expression plasmid substantially up-regulated the expression of p-p53, p-aMPK and silenced the expression of p-mTOR in a synergistic manner. FUS1 was also employed to construct a co-expression plasmid with p53 gene [57]. p53 is frequently mutated or deleted in human cancers and exogenous overexpression of wild-type p53 gene has proved effective in tumor growth inhibition [117]. Successful delivery of FUS1/p53 co-expression plasmid to NSCLC model using DOTAP/cholesterol NPs generated synergistic effect in suppressing cell growth *via* inducing apoptosis in *in vitro* and *in vivo* models [57]. The synergism between FUS1 and p53 was reported to be due to FUS1-mediated silencing of murine double minute-2 (MDM2), which is one of the prominent negative regulator of p53 protein [118]. The silencing of MDM2 enhanced p53 accumulation, up-regulated the expression of pro-apoptotic gene Apaf-1, protease-activating factor-1 [119] and ultimately induced apoptosis.

4.2. Combinational delivery to simultaneously up- or down-regulate target genes

Co-delivery of DNA and siRNA pairs is a sophisticated approach since it can simultaneously intervene in two separate pathways protein [13,41,70,120]. However, it is challenging to co-deliver agents of different electro-physical properties. Pan et al. reported an additive effect of co-expressing a STAT3-specific shRNA and LKB1 [41] on inhibition of ovarian cancer growth *in vitro* and *in vivo*, which were revealed in a multitude of assays including proliferation, colony formation, cell cycle distribution, apoptosis, migration, and tumor growth inhibition. LKB1 expression was reported to inhibit STAT3 activation, which is associated with different cellular activities [121,122], so that it acted in concert with direct inhibition of STAT3 with shRNA in this case. Other effects of co-expressing STAT3 shRNA and LKB1 plasmid was up-regulation of p21 and p-p53 expression, and down-regulation of cyclic D1, survivin and Bcl-2. Furthermore, STAT3/LKB1 intervention synergistically suppressed SKOV3 cell migration and invasion, which was due to reduced MMP-2 and MMP-9 expression associated with extracellular matrix turnover [41]. Synergistic effect of STAT3 shRNA with Gene Associated with Retinoid-IFN-induced Mortality 19 (GRIM-19) was also reported to inhibit thyroid and prostate tumor growth in both *in vitro* and *in vivo* models [123,124]. GRIM-19 is a potential tumor suppressor, whose over-expression inhibits cell proliferation and induces apoptosis in human prostate, breast and gastric cancer, and renal cell carcinoma [125–127]. GRIM-19 could also inhibit STAT3 expression by exclusive interaction with transcriptional activating domain of STAT3 and inhibiting auto-regulatory STAT3 driven transcriptional activation [128]. The dual expression plasmid of STAT3 shRNA and GRIM-19 showed significant suppression of cell proliferation, migration and invasion in *in vitro* and *in vivo* models of thyroid carcinoma [123]. In a similar study, co-expression of STAT3 shRNA and GRIM-19 synergistically suppressed prostate tumor growth and metastases when compared with individual treatment with either agent [124]. The combination therapy completely extinguished the activity of MMP-2 and vascular endothelial growth factor (VEGF), two key players in metastasis. A shRNA against survivin, a member of inhibitor of apoptosis protein (IAP) family and key regulator of apoptosis, was also delivered with GRIM-19 [89], with a synergistic effect on proliferation of laryngeal cancer (Hep-2) cells [120]. The effect was promising in both *in vitro* and *in vivo* models compared to corresponding mono-therapies, which indicates an association between survivin and GRIM-19 in cellular signaling. The expression of GRIM-19 could have possibly inhibited the downstream STAT-3 gene as well.

Combination therapy has been also explored in malignant glioblastoma, the most common tumor in central nervous system. Han et al. reported construction of a co-expression plasmid of EGFR shRNA and PTEN, and investigated their synergistic in suppressing cell proliferation, cell cycle and invasion in U251 glioma cells *in vitro* and inhibition of subcutaneous U251 glioma tumors in nude mice [13]. Delivery of the plasmid substantially down-regulated the EGFR expression and up-regulated PTEN expression, while the expression levels of p-AKT, PCNA, Bcl-2, CyclinD1, MMP-2 and MMP-9 were dramatically decreased, leading to diminished AKT activity. In glioblastoma cells, EGFR expression activates specific downstream intracellular signaling pathways that lead to PI3K phosphorylation and activation of AKT, while PI3K is negatively regulated by PTEN [129,130]. These findings suggest that the retarding glioblastoma growth was the combined effect of reduced cell proliferation and apoptosis induction. Along the same lines, shRNA targeting Akt1 (shAkt1) and cDNA of Programmed Cell Death Protein 4 (Pdcd4) were inserted into a dual expression vector, which was delivered into lungs with a sorbitol diacrylate-PEI carrier after aerosolization; a synergistic antitumor effect was evident on the lung tumors with this combination of agents [131].

Combination of siRNA and DNA delivery using a single carrier is beginning to be explored. Non-viral carriers that are typically used for siRNA delivery are usually capable of forming complexes with plasmid DNA as well. While the site of action for siRNA is cytoplasm, getting the DNA to nucleus is one of the major challenges. "Naked" DNA does not easily diffuse into the nuclear envelope; however, interaction with cytoplasmic proteins could trigger importing signaling for nuclear localization. Incorporating peptides or specific ligands are among approaches to enhance nuclear delivery of DNA [132]. One of the approaches reported for co-delivery of DNA and siRNA is formation of micelle-like NPs based on interionic interaction with the nucleic acids. This type of delivery system generally follows the typical micellar core/shell structure, with hydrophobically modified polyamines forming the core after interaction of the polyamine and siRNA (which neutralizes the ionic charge and increases the hydrophobicity), and a hydrophilic moiety [133]. NPs formed by gold/PAMAM nucleic acid complexes showed efficient simultaneous delivery of siRNA and DNA to cytoplasm and nucleus of brain cancer cells, respectively (confirmed by transmission electron microscopy), and superior silencing efficiency compared to Lipofectamine® 2000 [134]. Similarly, a pDNA and

siRNA were condensed with branched PEI and deposited onto poly(lactide-*co*-glycolic acid) (PLGA) NPs for delivery to human mesenchymal stem cells (not cancer therapy) [135]. Lu et al. recently reported a derivative of branched PEI (grafted with arginine-rich oligopeptide) for co-delivery of siRNA and DNA that increased the transfection efficiency 20-folds, and silencing efficiency 2-folds compared to individual delivery of DNA and siRNA, respectively [136]. The exact mechanism of the increased efficiency was unclear. In 2016, Kim et al. reported simultaneous delivery of siRNA against Src Homology Region 2 Domain-containing Tyrosine Phosphatase-1 (SHP-1) and plasmid DNA expressing VEGF using a deoxycholic acid-modified PEI to cardiomyocytes, as a strategy to reduce apoptosis and enhance vascularization [137], which showed synergistic effect over individual deliveries. A recent manuscript has also reported incorporating anionic poly-γ-glutamic acid (γ-PGA) in PEI/nucleic acid complexes, with high transfection and silencing efficiencies in a human hepatoma cell line [138].

4.3. Combinational delivery to silence the over-expressed genes

Delivery of siRNA combinations has been actively explored in combinational therapy to simultaneously down-regulate over-expressed genes associated with tumor growth and metastasis. Several distinct steps involved in tumor formation and growth, such as angiogenesis, abnormal apoptotic process, drug resistance and immune-modulation, have been modulated by this approach.

4.3.1. Inhibition of angiogenesis

Angiogenesis is a fundamental requirement as tumors transform from benign to metastasizing state, characterized by extensive blood vessel formation, and it involves migration, proliferation and differentiation of cells responsible for vascular assembly. VEGF is a potent angiogenic factor targeted in cancer therapy since it stimulates rapid formation of new, leaky vasculature, tumor cell migration, while decreasing response to hormonal/chemotherapy [139,140]. Humanized monoclonal antibody "bevacizumab" against VEGF has been successfully used in many clinical cases individually, and as part of combination therapies [141,142]. Its delivery has been explored for synergistic activity with antibodies against other targets, such as epidermal growth factor receptor 2 (HER-2; a humanized HER-2 antibody "trastuzumab" is approved for treatment of HER-2 positive breast cancers [143,144]). This combination shows substantial effect in suppressing growth and invasiveness in different cancers [145,146]. A synergistic effect of blocking HER-2 and VEGF expression with siRNA combinations was reported in a breast cancer model [145]. A multitude of effects on tumor cells was noted, including changes in cell morphology, suppression of migration and cell adhesion to ECM, and some of these effect were due to alterations in other intracellular regulators such as Pak1, PI3K and HIF1α. These findings are consistent with the therapeutic outcome of VEGF/HER-2 antibody combination in clinical studies [144,147], so that siRNA-based combinations appear to mimic the respective antibody therapy. An HER-2/VEGF siRNA combination was also employed in human gastric carcinoma therapy with similar synergistic results [146]. A substantial knockdown of VEGF and HER-2 mRNAs was observed, leading to inhibiting cell proliferation, migration and inducing apoptosis.

4.3.2. Inhibition of cell proliferation

The RNAi technology that targets the molecular mediators responsible for abnormally regulated cell cycle is another fruitful approach in combinational therapy. KSP, a member of kinesin superfamily of microtubule-based motors, is involved in centrosome separation, bipolar assembly and maintenance during mitosis. A siRNA targeting KSP was explored to synergize with a VEGF siRNA in treatment of different cancers [148]. The delivery of KSP/VEGF siRNA combination to Hep3B cells showed significant down-regulation of the intended target KSP, as well as other important targets regulating cell cycle regulators (*e.g.*, Cyclin D1) and anti-apoptotic proteins (*e.g.*, Bcl-2 and survivin), suggested a highly integrated response to VEGF silencing. In a recent study, we also reported successful delivery of siRNA combinations against cell-cycle regulators TTK protein kinase and cell dividing cycle protein 20 (CDC20) and anti-apoptotic survivin in a triple negative breast cancer model (MDA-MB-231 cells) using cationic lipopolymers [68]. These siRNA combinations primarily silenced the target gene at the mRNA level and resulted in an anti-proliferation activity that was synergistic in nature.

4.3.3. Induction of apoptosis

Over-expression of anti-apoptotic family of proteins (*e.g.*, Bcl-2, Mcl-1, XIAP) in many malignances is reported to contribute to increased cell lifespan [26,149] in addition to drug resistance. siRNAs targeting anti-apoptotic proteins Bcl-2 and survivin show effective synergism with VEGF siRNAs in many cancer [14,150]. Multimeric siRNAs were constructed using labile —S—S— bonding and they displayed better activity with anti-apoptotic Bcl-2 [14] and other targets, especially VEGF [71,72, 14, 72, 73, 150, 151]. Intracellular reductive cleavage of —S—S— bonding of multimeric siRNAs promptly releases constituent siRNA units, which triggers the silencing process of targeted genes, and generates synergistic effect in tumor suppression. The combinational delivery was highly effective at mRNA silencing and significant anti-cancer activities was observed, which was higher than mono-therapy [14,73]. Multimeric VEGF and NET-1 siRNAs were reported for liver cancer treatment with synergistic outcomes in several functional assays [151]. siRNA mediated silencing of NET-1 inhibits cell proliferation and induce cell apoptosis *via* down-regulation of cyclinD1 and Bcl-2 regulators. Delivering a multimeric siRNA bearing siRNAs against solely anti-apoptotic proteins (Bcl-2 and survivin) resulted in higher anti-cancer efficacy than mono-therapies in treatment of bladder cancer T24 cells [150]. In leukemia, several studies also explored combinational delivery of (monomeric) siRNA to enhance apoptosis; siRNAs against WT1/BCR-Abl (using TransMessenger® as carrier) [152], GRI1B/BCR-Abl (using DOTAP as carrier) [153] and c-RAF1/Bcl-2 (using Oligofectamine® as carrier) [154] combinations were explored to inhibit un-controlled cell proliferation and apoptosis induction (Fig. 8). Combinational delivery typically resulted in higher efficacy in both inhibiting proliferation and apoptosis induction, but the effects were not always synergistic or additive. Careful selection of targets was therefore necessary when complementary siRNAs were used in targeting transformed cells. While not all mediators are expected to act synergistically when they are silenced with specific siRNAs, whether a carrier influences the obtained effect, *via* specific effects *via* siRNA delivery and release kinetics or indirect effects on cellular physiology, is a critical issue, and remains to be explored.

4.3.4. Reversal of MDR against drug therapy

The siRNA combinations are often utilized to tackle MDR, which is usually acquired by cancer cells after frequent exposure to less than the lethal doses of a drug, and/or by selective growth of the sub-population already expressing MDR proteins. The fundamental mechanisms behind this type of therapeutic failure are: (1) decreased influx and/or increased efflux of drugs by cell surface transporters, (2) change in drug efficacy due to genotypic and phenotypic changes in cancer cells, (3) blockage of normal apoptosis, and (4) activation of detoxifying systems against the drug. With better recognition of molecular mechanisms involved in MDR, co-delivery of siRNA/drug combinations have demonstrated significant impact to restore cellular sensitivity to drugs [23]. Nucleic acid therapy with siRNAs combinations has been also pursued and, prominently, it has been performed by targeting the efflux protein P-glycorpoetin (P-gp) and anti-apoptotic proteins

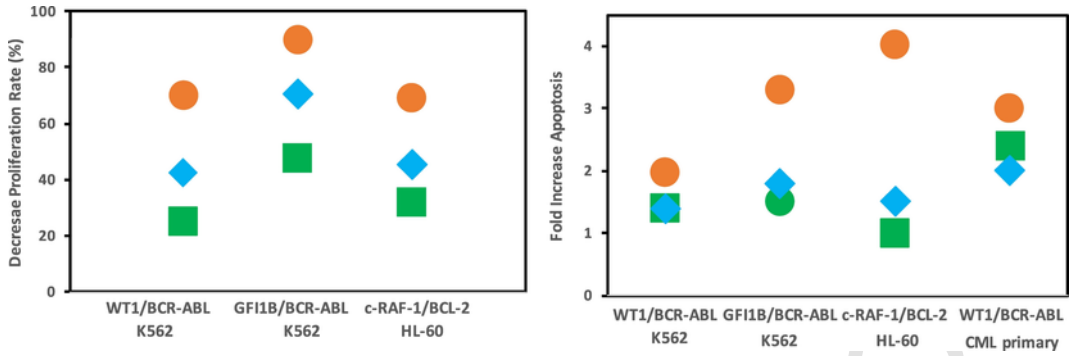

Fig. 8. Effects of combinational siRNA treatment in leukemic cells. The siRNAs indicated on the horizontal axis is added alone (squares representing the first siRNA, diamonds representing the second siRNA) or in combination (circles). The cells used in these studies were K562 (a CML cell line), HL-60 (an AML cell line) or primary CML cells obtained from patients. The decrease in proliferation (**right**; as a percentage of control) and increase in apoptosis (**left**; fold change over non-treated cells) were summarized after our analysis of the reported data. Note the generally beneficial effect of siRNA combinations compared to treatment with individual siRNAs alone.

[26,30,155], since over-expression of anti-apoptotic proteins (*e.g.* Bcl-2, Mcl-1) is a hallmark of increased cell survival and drug resistances [26,149]. Successful delivery of siRNA combinations against P-gp and Mcl-1 or survivin was shown in a breast cancer model using cationic lipopolymers [30]. A higher efficacy of the combination therapy was observed relative to the mono-therapy [30]. A more comprehensive study by our group demonstrated a synergistic effect when siRNA combinations against anti-apoptotic proteins and kinases were employed in doxorubicin-resistant breast cancer model [26]. This study indicated a significant synergistic effect for siRNAs targeting Mcl-1 and Ribosomal Protein S6 Kinase (RPS6KA5). While siRNA targeting RPS6KA5 had no significant impact on the viability of wild-type and resistant versions of MDA-MB-435 cells, combinatorial delivery of siRNAs targeting Mcl-1 and RPS6KA5, halted the tumor growth in a nude mice xenograft model. While tumor growth in the mice treated with Mcl-1 siRNA alone was significantly slower as well, the efficacy of the combination therapy was significantly higher. In contrast to different 'classes' of targets, combinational siRNA delivery solely against anti-apoptotic proteins Bcl-2 and Mcl-1 was also effective in drug-resistant malignant pleural mesothelioma [149].

Solid tumors could develop resistance against EGFR therapy by activating alternative survival mechanisms involving insulin-like growth factor-1 receptor (IGF-1R), which also belongs to receptor tyrosine kinase family and contributes to aggressive tumor growth and poor prognosis [156–158]. As an alternative to antibody combinations targeting IGF-IR and EGFR [159], combinational siRNA therapy was shown to mediate dual silencing of EGFR and IGF-1R at mRNA and protein levels in a colorectal cancer model [55]. This combination inhibited phosphorylation of AKT and ERK1/2, which are known as the major components of downstream signaling pathways of EGFR and IGF-IR. It directly affected cell proliferation and apoptosis, where the therapeutic intervention was substantially higher with combinational delivery as compared to silencing of either target alone. Dual silencing EGFR and IGF-1R using shRNA was also reported as an alternative approach [160]; enhanced apoptosis and increased cell sensitivity were observed to chemotherapy in the case of co-silencing, as compared to silencing single targets in nasopharyngeal cancer cell lines.

4.3.5. Immunotherapy

An exciting therapeutic option in management of cancer is to rely on immune system to eradicate the malignant cells. This will require to sensitize the immune components against malignant cells *via* antigen-dependent vaccination and/or cytokine mediated stimulation. A plasmid for simultaneous expression of IL-2, pro-IL-18 and IL-1b converting enzymes (ICE) were delivered using gene gun to skin tissue surrounding tumor in a murine mammary adenocarcinoma model. This resulted in higher anticancer activity as compared to individual genes as evident by increased level of bioactive IL-18 and INF-γ induced [161]. The immune-modulatory genes granulocyte-macrophage colony-stimulating factor (GC-CSF) and IL-2 were virally delivered in squamous cell cancer model in a separate study [43], but GC-CSF showed suppressive effects in tumor growth with no obvious advantage of combination therapy. The combination therapy has been explored for tumor metastasis by employing suicide (*e.g.*, herpes simplex virus thymidine kinase, HSV-tk) and IL-2 genes as a synergistic pair [40]. This combination has shown a substantial inhibition of metastasis of colon cancer cells to liver and induced a systemic anti-tumoral immunity against parental tumors. A combination of three genes (Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), HSV-tk and IL-2), was subsequently explored in cancer treatment [42]. A substantial induction of long-term antitumor immunity and survival of mice receiving GM-CSF/HSV-tk/mIL-2 treatment had been observed. The underlying synergism observed in this approach was due to amplified CTL activity, probably due to immune effector T-lymphocytes.

4.3.6. Combinational delivery of miRNA

The microRNAs (miRNAs), small non-coding RNA molecules involved in post-transcriptional regulation of gene expression, have been explored for combinational delivery since alterations in miRNA profiles were shown to mediate multiple activities leading to tumor formation, growth and dissemination [162,163]. miRNAs have been delivered in combination with DNA expression vectors and siRNA, as well as miRNA combinations [56,70,164]. Co-expressing plasmid constructs for miRNA expression have been employed in both *in vitro* and *in vivo* models [70]. *In situ* expression of miR-125b and miR-146a were shown to display anti-tumor and pro-inflammatory activity *via* macrophage polarization and activation [165,166]. miR-125b was delivered along with p53 from an expression plasmid to lung cancer cells using CD44/EGFR-targeting NPs formulated using HA, PEI and PEG [70]. The change in tumor microenvironment was evident by an increase in pro-inflammatory mediators. Combinational delivery of a plasmid expressing p53 with miR-125b did not interfere with each other's activities, so that apoptotic effect of p53 reduced tumor progression, which was further enhanced by miR-125b mediated down-regulation of anti-apoptotic proteins such as Bcl-2.

Co-delivery of miRNA with siRNAs could be advantageous due to similarity in composition of the compounds. Successful delivery of a miR-34a mimic (a p53-regulated tumor suppressor miRNA) and a siRNA targeting KRAS was explored in lung adenocarcinoma cells [164]. Adenocarcinoma, the most common Non-Small Cell Lung Cancer (NSCLC) subtype, is associated with frequent mutation in KRAS (20–30%) and TP53 (~ 50%) [167]. Two specific tumor-suppressive miRNAs (*e.g.* let-7 and miR-34) are significantly altered in NSCLC, so that their suppression is particularly relevant to oncogenic phenotype

of these cells [168]. The combination of miR-34a and KRAS siRNA led to tumor regression, more significantly than the individual agents alone; miR-34a targeted gene expression was down-regulated and KRAS expression and MAPK signaling was suppressed. Another study reported co-delivery of the tumor suppressive miR-34 and let-7 for lung cancer therapy [56]. Delivery of exogenous let-7 was reported to prevent and treat KRAS-driven lung tumor, and miR34 can prevent initiation and progression of $Kras^{G12D\ +}/p53R^{172H/+}$ lung tumor and NSCLC xenografts [169,170]. miR-34 and let-7 combination resulted in superior reduction of proliferation and invasion *in vitro*, and systemic delivery of the combination demonstrated suppression of tumor growth *in vivo*, conferring a prolonged survival in the NSCLC mouse xenograft model. The combination delivery was able to silence the over-expressed mRNA levels of Myc, Lin28A and Lin28B.

5. Nucleic acid combination in clinical use

In the case of local delivery, a carrier might not be needed since a therapeutic can be administered to an exposed tissue such as the eye, lung and skin [171]. However, targeting of therapeutic agents to other tissues often require systemic delivery and a carrier suitable for clinical use is paramount [171]. Clinically relevant RNAi-mediated gene silencing was first reported with a liposomal siRNA formulation in non-human primates in 2006 [172]. Since then, a number of RNAi-based strategies have entered clinical trials [173]. Most carriers are mainly based on SNALPs, with the exception of CALAA-01, a cyclodextrin-based system with transferrin mediated targeting [174,175]. Cationic liposomes such as AtuPLEX and SNALPs represent most advanced carriers since they contain several components [176]. Currently, the only NP system for combination therapy is ALN-VSP (Alnylam), which is a SNALP-formulated siRNAs targeting VEFG and KSP for advanced solid tumor with involvement of liver metastases. Both siRNAs were modified chemically for a reduced immune stimulation. The NPs are 80 to 100 nm in size, have a near neutral charge and distributes primarily to liver and spleen following intravenous administration. RNAi mediated VEGF mRNA cleavage was found in the liver of 2 patients, though this was not conclusive in patients due to the presence of other tissue types in the biopsies. Although no dose response was observed, substantial decrease of tumor blood flow was observed in nearly half of the patients with evaluable liver tumors, which may be due to VEGF down-regulation. It is important to note that the lack of dose response may suggest the possibility of nonspecific effect of SNALP formulation or premedication regime rather than a specific effect of the therapy. Furthermore, patients treated with ALN-VSP showed a decreased spleen volume, which may be indicative of anti-KSP effect [177]. No further reports have been published since 2013, and it is not clear if this formulation is being pursued.

6. Conclusions and future perspectives

It is a widely accepted now that cancer is a heterogeneous disease, which suggests that no matter what mediator we select to target, there is a chance that a sub-population of cells would be unresponsive to the therapy, and be selected to expand into non-responsive cells. On the other hand, the plasticity of the malignant cells highlights the risk of post-treatment adjustments that could render a previously responsive cell population resistant to any treatment. This inherent heterogeneity and plasticity inevitably necessitates combinatorial approaches. While combinational therapy is routine for small molecule drugs, the same cannot be stated for nucleic acids, which is largely due to limited clinical experience with this type of therapeutic agents. The growing interest in combinational therapy arises from the potential of synergism between carefully selected therapeutic agents, which generates higher efficacy compared to the sum of individual constituents with presumably no additional side effects. There is a potentially equal probability of additive as well as antagonistic effect if the therapeutic pairs are improperly selected. Cationic, lipophilic carriers will be vital for the success of such combinations since they can anchor both agents into a single carrier and enhance delivery through the lipophilic plasma membrane. However, due to competitive electronegative charge density, it is a major task to simultaneously load short RNAs and longer DNA-based expression vectors into a single NP. Continuous development of new functional polymers could generate wide varieties of multifunctional NPs. For co-delivery of nucleic acid combinations, release kinetics of individual agents need to be optimized; for example, targeting certain signaling mediators generate more effective actions only after significant silencing of synergistic targets. Subsequent delivery may be more effective than simultaneous delivery in this case [178]. To capitalize on the incredible opportunity for discovering suitable combinational therapies, certain fundamental issues need to be addressed.

6.1. Nano-formulations with proper composition

Nano-formulation of therapeutic agents with carriers generally results in polydisperse NPs, that may display differential activities due to variable stability, cargo content and surface features. Loading efficacy of the carriers may depend on different factors such as cationic charge density, hydrophobicity, and MW, so that exquisite control over starting carrier materials might be needed. Further variations in electronic charge density and solubility among the therapeutic agents create significant variation in loading efficacy. Therefore, formulation stoichiometry may not always be translated into NP composition. It is a challenging issue to design the appropriate vehicle with proper amount of therapeutic pairs to ensure proper efficiency. For an effective systemic outcome, delivery with higher half-life in circulation is more critical than to ensure better accumulation in tumor environment. To minimize opsonization in blood stream and renal-clearance, it is critical for the NPs to be stable and remain homogenous (not segregate into constituents) despite the inherent incompatibilities between the therapeutic combinations [179]. Many delivery experts firmly believe in "one drug, one carrier", implying that there is an optimum carrier for any given drug. This potentially means that any given carrier might be less than optimal for at least one of the components of the combination. This may result in pre-mature release and/or degradation of the component, or insufficient release of the component due to over-stability of the carrier/cargo interaction. Multi-component delivery systems, with the possibility of compartmentalization, could be an effective approach, where lipids, peptides, and/or hydrophilic moieties might provide an optimal milieu for individual components of the combination. Finding the optimal carrier(s) for a combination of nucleic acids will certainly pose a major challenge to overcome before we can effectively utilize this type of combinational therapy.

6.2. Delivery in concert with therapeutic activity

For a better translation of combination therapy to clinical setting, it is important: (1) to completely understand the cell signaling network and especially the crosstalk among pathways, so that the proper combination of therapeutic agents can be chosen, and (2) to match the cellular exposure of individual therapeutics to the order and duration of desired activity [178]. A properly matched combination may lead to strong synergism while an un-match treatment might generate antagonistic effects. So far, most of the efforts in this regard have been arbitrary selections, while more systematic studies are required to identify potential targets for ideal combinations. These targets could be selected to enhance the therapeutic effect by affecting multiple factors involved in the same mechanism, or to affect alternative mechanisms to block other options for cell survival. Due to convenience of administrating

multiple agents at the same time, simultaneous delivery is more commonly pursued for a therapeutic effect, as compared to sequential administration. To realize a proper balance between release kinetics of individual therapeutic agents and their therapeutic activity, NPs derived from layer-by-layer fabrication might be desirable to control the release of different agents at different duration [180].

6.3. siRNA-related off-target effects

Sequence-specific off-target effects related to siRNA (and miRNA) is a critical issue in combinational therapy. siRNA may initiate off-target gene silencing by hybridizing with other mRNA targets and interfacing with the miRNA network [171,181]. Off-target effects induced by binding to proteins, specifically to immune-activating receptors such as toll-like receptors, can lead to inflammation [171,181]. To control specificity of siRNA and minimize off-target potential, careful design of the oligonucleotides is imperative. Dual-targeting may reduce the potential for off-target gene silencing, increase the opportunity to knockdown the desired target gene(s), and provide additive/synergistic effect by both oligonucleotides [171]. Using a breast cancer cell model, we showed that efficiency of double silencing with siRNA is reserved and it is usually similar to the single targeting (Fig. 9). Combinational delivery will be particularly beneficial if the effective dose of the agents could be reduced as a result of synergistic effects. Changes in non-targeted mediators, however, has been often observed after delivering siRNA against other targets (Fig. 9). This will be important if a critical mediator is altered significantly as a by-stander and measures to minimize such an interference might be necessary if the outcome is undesirable. Alternatively, one can attempt to predict the molecular vulnerabilities of malignant cells from known signaling networks [182], and target most effective combinations (*i.e.*, vulnerable) of targets to minimize the therapeutic agents to reduce any possible side-effects.

6.4. Delivery system limitations for nucleic acids

A critical limitation in combinational therapy is the lack of an efficient delivery system for nucleic acids to target specific cells [171]. The most clinically advanced synthetic delivery systems are engineered for well-perfused tissues such as the liver, kidney and some solid tumors, where fenestrated or discontinuous endothelium allow the passage of NPs to target tissues [171,173]. However, delivery to less accessible tissues remains a considerable challenge [181]. Even with systemically present leukemias, there is a need for specific delivery to malignant cells (*vs.* normal hematopoietic cells), whose challenges has been previously articulated in the context of siRNA delivery [173,184]. For selective targeting to tumor or other tissues, careful modulation of physical properties, such as stealth properties of spherical particles, size (< 100 nm in diameter), or affinity (with specific targeting ligands) will be required to control the ideal distribution of therapeutic agents. Target selection is another approach in minimizing unwanted effect in non-targeted tissue. Specific proteins might play a pivotal role in the survival and/or resistance of malignant cells, but they play a significantly less important role in healthy cells. Alternatively, quantitative differences in protein targets between malignant and normal cells may skew the action of nucleic acid therapies where the target level if elevated. Selection of these targets will help limit the effect to malignant cells, since the healthy cells would not respond the same way, even if the targeted proteins are affected.

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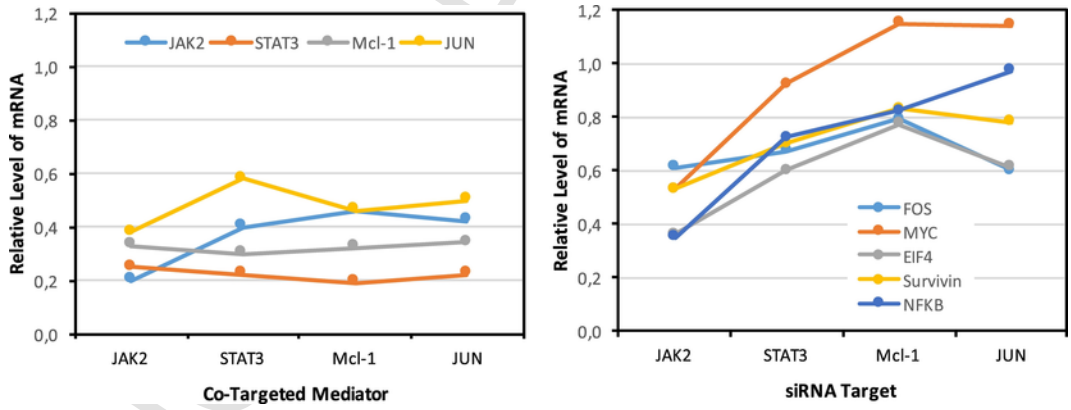

Fig. 9. Dual targeting with siRNA combinations in a breast cancer model.**Left**. Changes in target mRNA levels as a result of single or combinational siRNA treatment. JAK2, STAT3, Mcl-1 and JUN mRNA levels are shown after single or combinational delivery of siRNA (*e.g.*, blue line shows changes in JAK2 mRNA levels after delivering JAK2 siRNA alone or JAK2 combined with STAT3, Mcl-1 and JUN siRNAs). **Right**. Changes in non-targeted mRNA levels as a result of delivering JAK2, STAT3, Mcl-1 and JUN siRNA as a mono therapy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) Note that the indicated mRNA levels were normalized with the specific mRNAs in non-treated cells. (Data adopted from Reference [183]).

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