EXPERT OPINION

Recent trends in cancer drug resistance reversal strategies using nanoparticles

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Introduction: Resistance to chemotherapy is a major obstacle in the successful amelioration of tumors in many cancer patients. Resistance is either intrinsic or acquired, involving mechanisms such as genetic aberrations, decreased influx and increased efflux of drugs. Strategies for the reversal of resistance involve the alteration of enzymes responsible for drug resistance, the modulation of proteins regulating apoptosis mechanisms and improving the uptake of drugs using nanotechnology. Novel strides in the reversal of drug resistance are emerging, involving the use of nanotechnology, targeting stem cells, etc.

Areas covered: This paper reviews the most recent cancer drug reversal strategies involving nanotechnology for targeting cancer cells and cancer stem cells (CSCs), for enhanced uptake of micro- and macromolecular inhibitors.

Expert opinion: Nanotechnology used in conjunction with existing therapies, such as gene therapy and P-glycoprotein inhibition, has been shown to improve the reversal of drug resistance; the mechanisms involved in this include specific targeting of drugs and nucleotide therapeutics, enhanced cellular uptake of drugs and improved bioavailability of drugs with poor physicochemical characteristics. Important strategies in the reversal of drug resistance include: a multifunctional nanoparticulate system housing a targeting moiety; therapeutics to kill resistant cancer cells and CSCs; cytotoxic drugs and a tumor microenvironment stimuli-responsive element, to release the encapsulated therapeutics.

Keywords: aptamers, cancer stem cells, EPR, L-buthionine-(*S,R*)-sulfoxime, multidrug resistance, nanocarriers, P-glycoprotein, small molecule sensitizers

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1. Introduction

The era of chemotherapy began in 1940 with first uses of nitrogen mustard and antifolates, continues till date with development of targeted therapeutics such as Gleevec (imatinib) and Avastin (bevacizumab) [1]. Though in recent years, we witnessed new strides in the treatment of cancer with advent of targeted therapeutics and nucleotide-based therapeutics, resistance to existing chemotherapeutics agents is also on rise. This fundamental problem of resistance has emerged as the major limiting factor, tremendously compromising treatment success and survival of the patient [2]. Resistance in the realm of cancer chemotherapy can be divided into two elaborate categories: *de novo* also termed as intrinsic, and acquired. In intrinsic resistance, because of inherent genetic predisposition cancer cells are protected from apoptosis induced by chemotherapy, radiotherapy or receptor-mediated cell death [3]. In this type of resistance, tumors fail to respond to first-line chemotherapy and exhibit complex therapy-resistant phenotypes with initial treatments. Acquired resistance culminates on a period of time and usually occurs in tumors that are

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Article highlights.

- Even though chemotherapeutic agents are successful in treating wide variety of tumors, incidence of resistance which can be either intrinsic or acquired is a major limiting factor for the successful use of chemotherapeutic agents.
- Nanoparticles, when carefully designed, have potential to control the drug release profile suitable for optimal cytotoxicity and subsequently circumvent the resistance problem by sensitizing cancer cells to chemotherapeutic agents.
- Various small molecule inhibitors including nucleotides such as microRNA (miRNA), small interfering RNA (siRNA) and aptamers can be utilized for sensitizing tumor cells for chemotherapeutic agents. These agents can be used in conjunction with nanoparticulate strategy to overcome the problem of drug resistance.
- Cancer stem cells (CSCs) have innate drug resistance mediated by drug detoxifying enzymes and multidrug resistance (MDR) transporters and are potential targets for reversal of resistance. Proper delivery of stem cells using nanoparticulate technology would overcome the drawback of undesired differentiation leading to resistance.
- The most important strategy in overcoming problem of resistance is to design a multifunctional nanocarrier which can accommodate a tumor-targeting moiety, therapeutics to sensitize or kill drug-resistant tumor cell and CSCs, chemotherapeutic agent and an element which can be stimulated by tumor microenvironment and release the therapeutics spatially at the tumor site.

This box summarizes key points contained in the article.

highly responsive to initial treatment but manifests a complex therapy-resistant phenotype on tumor recurrence. These phenotypes exhibit sequential genetic changes rendering them resistant to not only previously used drugs but also cross-resistance to other new agents with entirely different structure and mechanism of action [4]. Understanding the mechanisms involved in both *de novo* and acquired resistance is essential to develop effective ways to overcome resistance to chemotherapy.

Studies on cancer resistance mechanisms revealed aberrations in the genetic makeup of the cancer cell by itself or on treatment with chemotherapeutic drugs. Three major mechanisms of drug resistance in cells were elucidated based on the mechanisms of antibiotic resistance in microorganisms. The first mechanism involved reduced uptake of hydrophilic drugs like 5-fluorouracil, methotrexate, cisplatin and cytarabine as these drugs require transporters to enter the cells [5-7]. Second, cellular changes that reduce the capability of cytotoxic drugs to exert their effect, like evasion of apoptosis, altered metabolism of drugs, enhanced repair of DNA damage and changes in the cell cycle [8]. The third mechanism is the enhanced efflux of hydrophobic drugs by energy-dependent transporters called adenosine triphosphate (ATP)-binding cassette (ABC) transporters [9].

The above-mentioned mechanisms are implicated in almost every type of cancer with the mechanism of enhanced efflux being the most predominant both in clinical and in laboratory resistance. For example, in breast cancer the major mechanisms of resistance involve overexpression of β-tubulin, breast cancer resistance protein (BCRP) and multidrug-resistant proteins such as P-glycoprotein (P-gp), altered levels of enzymes such as topoisomerases, aldehyde dehydrogenase and glutathione *S*-transferases (GSTs), mutations of *p53* gene, alterations in DNA repair processes due to mutations in breast cancer susceptibility gene 1 (BRCA1) and inhibition of cell death responses by overexpression of genes such as *survivin, Bcl-2*, etc. [10]. Although, a detailed discussion of mechanisms of resistance pertaining to all cancers is out of the scope of this review, above-mentioned mechanisms are commonly seen in other clinically relevant cancers.

Many of resistance reversal strategies involve suppression of proteins inhibiting apoptosis, use of demethylating agents, alteration of enzyme levels responsible for drug resistance, targeting *p53* and most importantly overcoming drug resistance by ABC transporters [11]. The most important strategy applied to suppress proteins inhibiting apoptosis is the use of antisense oligonucleotides to target anti-apoptotic genes like *Bcl-2* and *Bcl-X_L* [12,13]. Another approach to enhance the apoptosis involves the use of small peptides resembling the *N*-terminus of second mitochondria-derived activator of caspases (SMAC) protein, which binds and inhibits a family of inhibitors of apoptosis (IAPs) [14]. MLH1 gene deficiency due to hypermethylation of promoter region results in mismatch repair and was observed in many sporadic tumors, and a correlation between MLH1 gene deficiency and resistance to tumors was also reported in clinical studies [15]. Thus, replacement of MLH1 gene with either using demethylating agents such as 5-azacytidine or 2′-deoxy-5-azacytidine or by gene transfer were proved to enhance the sensitivity to cisplatin, temozolomide, doxorubicin and epirubicin [16-18]. Alteration of enzymes like γ-glutamyltransferase, which is involved in deactivation of chemotherapeutic agents and thus contributing to resistance were explored as successful strategies to overcome drug resistance. L-Buthionine-(*S,R*)-sulfoxime (BSO), a potent and specific inhibitor of γ-glutamyltransferase was shown to increase sensitivity to platinum-containing agents, alkylating agents, arsenic trioxide and anthracyclines [19-21]. A tumor suppressor gene called *p53* is a potential therapeutic target implicated in drug resistance and restoring the wild-type *p53* activity can revert the malignant phenotype and enhance the drug sensitivity [11]. When p53 gene was transfected into non-small cell lung cancer (NSCLC) cells with different endogenous expression, the cells showed enhanced sensitivity to paclitaxel and cisplatin [22]. The most widely investigated strategy to overcome drug resistance is definitely overcoming the drug efflux by ABC transporters. The strategies include use of small molecule inhibitors, hammerhead ribozymes, antisense oligonucleotides, short interfering RNA, peptides and antibodies to inhibit various ABC transporters leading to multidrug resistance (MDR) [8]. Salinomycin, a monocarboxylic polyether used earlier as

agricultural antibiotic is shown to kill cancer stem cells (CSCs) and was also able to induce massive apoptosis in various drug-resistant human cancer cells [23]. 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), a heat shock protein 90 (HSP90) inhibitor is potentially indicated for the treatment of refractory multiple myeloma and is in Phase III clinical studies [24]. Schematic illustrations of various above-mentioned strategies are given in **Figure 1** and **Figure 2**.

Myriad of strategies which emerged over the past 30 years though helped us understand mechanism of resistance in a great deal, there is still a need for an effective clinical tool to be proved to overcome resistance. To circumvent the tumor resistance to chemotherapy, an ancillary strategy involving use of nanomaterials/nanotechnology to append the already existing strategies is of great value. Nanotechnology can overcome drug resistance by various mechanisms. For example, allowing specific targeting of drugs and nucleotide therapeutics to tumor, enhancing cellular accumulation of drugs in tumors refractive to chemotherapy, enhancing immunity to protect normal tissues from tumor invasion and improving bioavailability of drugs with poor physicochemical characteristics [25]. Especially, with the emergence of therapeutic strategies involving microRNA (miRNA), small interfering RNA (siRNA) and gene therapy, effective delivery of these molecules to the site of intended action has gained wide importance.

This review is an attempt to critically analyze most recent trends in nanotechnology implied for reversal of drug resistance. The major emphasis is given to acknowledge the role of novel delivery strategies in overcoming resistance using miRNA, siRNA, small molecule inhibitors, peptides, monoclonal antibodies and gene therapy. Several cardinal aspects of resistance reversal like reversibility and sustainability of therapeutic effect after treatment with preclinical and clinical evidences are also discussed.

2. Strategies for reversal of drug resistance

2.1 Nanocarriers in reversal of resistance

Nanocarriers are excellent platforms to enhance the intracellular accumulation of chemotherapeutic agents in tumors, which eventually results in reduced systemic toxicity and circumvents the problem of drug resistance. Nanocarriers achieve these objectives by both active and passive targeting. Nanocarriers extravasate through the leaky and highly permeable tumor vasculature and accumulate in tumor interstitium by a phenomenon termed as enhanced permeation and retention (EPR) effect [26]. Various properties of nanoparticles such as size, shape, surface charge and its dynamic and continuous interactions with components of vasculature, govern the ability of nanocarriers to exhibit EPR effect. The cross talks that take place between nanoparticles and tumor microenvironment are highly dependent on physicochemical properties of nanoparticles and largely influence their *in vivo* behavior [27]. Drug-resistant tumors exhibit exclusive tumor microenvironment often characterized by features such as low extracellular pH, hypoxia, lack of adequate lymphatic drainage and changes in expression/regulation of oncogenes, tumor suppressors and apoptosis mediators [28]. Nanocarriers utilize the above-mentioned features and overcome the drug resistance by eradicating the minimal residual disease (MRD) population of cells, which are responsible for recurrence of cancer [3]. Passive targeting strategy using nanocarriers involves EPR effect and the active targeting strategy is pursued using tumor cell-specific ligands.

The most prevalent and clinically relevant form of tumor resistance is the MDR. MDR is mediated by several ABC transporters like P-gp, MDR-associated protein 1 (MRP1), ABCG2 (also known as mitoxantrone resistance protein (MXR) and BCRP [8]. Several MDR inhibitors were developed to overcome drug resistance, however, nanoparticles were shown to be better in terms of higher efficacy and lower toxicity. The nanocarriers proposed included polymer–drug conjugates, surfactant micelles, lipid-based nanocapsules and nanoparticles, liposomes and polymeric nanoparticles [29]. A list of selected examples of recently investigated nanocarriers for reversal of resistance is provided in **Table 1**.

Tumor cells from blood vessels that are more than 100 – 150 μm were shown to be oxygen deprived because of inadequate oxygen diffusion and thus lead to chronic hypoxia and necrosis [30]. Hypoxic cells have intrinsic properties that reduce chemotherapeutic efficacy and have internal mechanisms which induce MDR [31]. It was also reported in various studies that hypoxia also increased P-gp expression. The primary mechanism by which hypoxia induces MDR was attributed to hypoxia inducible factor (HIF-1α) [32]. Interestingly, HIF-1α was reported to be associated with growth factor and growth factor receptors like EGFR, HER2, etc. in a positive feedback loop [33]. EGFR-targeted poly(D,L-lactide-co-glycolide)/poly (ethylene glycol) (PLGA/PEG/EGFR) nanocarriers were developed for delivery of paclitaxel/lonidamine to treat multidrug-resistant human breast and ovarian cancer cells. EGFR-targeted nanoparticles were shown to actively target tumor cells which are overexpressing EGFR on induction of hypoxia [34].

The rapid turnover of recycling endosomes and lysosomes and their more acidic environment as compared with cytoplasm and the nucleus favors sequestration of chemotherapeutics eventually leading to drug resistance [35]. Low pH in various subcellular organelles is an attractive signal to target MDR cancer cells. In an attempt to exploit this property, polymeric micelles with pH-induced ligand were developed. The micelle consisted of a block copolymer made of polyHis-*b*-PEG and PLLA-*b*-PEG-*b*-polyHis-biotin. When the micelle encounters an environment with slightly acidic pH (pH < 7.2, tumor pH), the biotin which was buried inside micelle will be exposed to surface and gets internalized by virtue of biotin receptor-mediated endocytosis. After internalization these micelles will be subjected to endosomal or lysosomal pH (pH < 6.5) leading to the destabilization of micelles and enhances the cytosolic drug release [36,37]. Similar approach was applied to target MDR cells using TAT

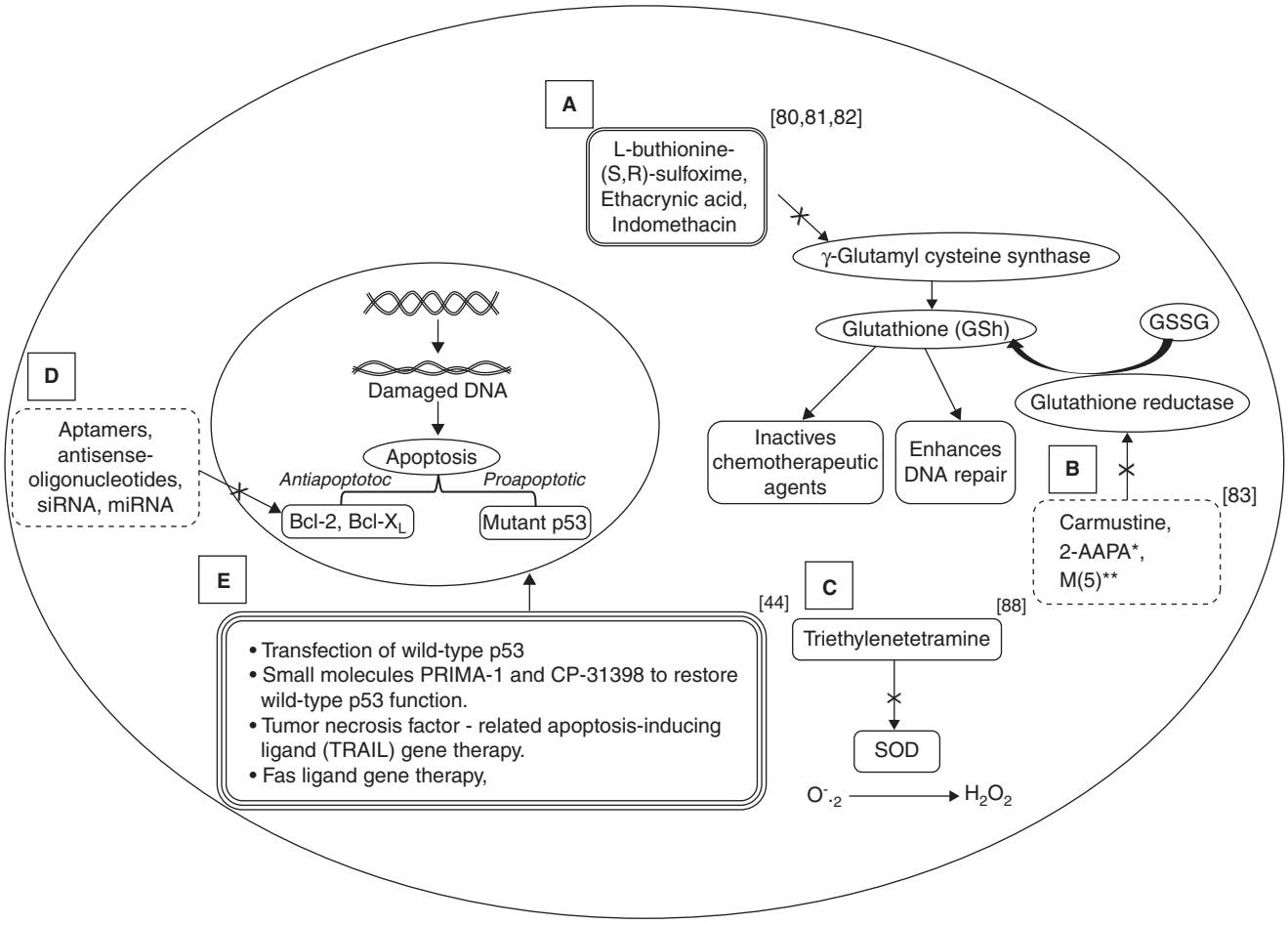

Figure 1. Schematic overview of various mechanisms to overcome drug resistance involving small molecule sensitizers, nucleotides and gene therapy. A. Inhibitors of γ-glutamylcysteine synthetase. **B.** Inhibitors of glutathione reductase (*2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acid, ** 6-[2′-(3′-methyl)-1′,4′-naphthoquinolyl]hexanoic acid). **C.** Inhibitors of superoxide dismutase (SOD). **D.** Nucleotides (aptamers, miRNA, siRNA) in reversal of resistance. **E.** Gene therapy for reversal of resistance.

peptide. TAT, which replaces biotin, is a non-specific cell-penetrating peptide that enhances the cellular uptake of micelles. This TAT-based polymeric micelle was also proved to be effective *in vivo* for various solid tumors including drug-sensitive and drug-resistant phenotypes [38].

Newer strategies to overcome MDR involved a combined approach using both ultrasound and nanoparticles. Daunorubicin-loaded titanium dioxide nanoparticles were investigated for their effectiveness on MDR leukemia K562/A02 cells with concomitant exposure to ultrasound. The results have shown that this strategy resulted in significant reversal of MDR tumor by enhancing the uptake of daunorubicin by MDR cells [39]. Novel polymers such as cyanoacrylates have been investigated in reversal of MDR. Doxorubicin was encapsulated in polyisohexylcyanoacrylate nanospheres and its cytotoxicity and accumulation was studied in a doxorubicin-resistant rat glioblastoma model. The results have indicated that the reversal of doxorubicin resistance was completely achieved and the mechanism involved a bypass of P-gp rather than the inhibition of P-gp [40]. Magnetic Fe_3O_4 nanoparticles loaded with cisplatin showed the reversal of cisplatin resistance. The IC_{50} of cisplatin was reduced by ~ 2.2-fold when cisplatin-resistant ovarian carcinoma cells were treated with magnetic Fe_3O_4 cisplatin nanoparticles [41]. In our lab we have also observed that IC_{50} of cisplatin was reduced by ~ twofold when complexed with surface-modified PAMAM (poly(amido amine)) dendrimers in cisplatin-resistant ovarian cancer cells (unpublished data).

Squalene, a polyterpenoid and precursor in biosynthesis of cholesterol, exhibited the ability to form self-assembled nanocarriers in water when conjugated to chemotherapeutic agents [42]. 'Squalenoylation' of chemotherapeutic agents, gemcitabine and paclitaxel, resulted in nanoassemblies in water with advantages of improved cytotoxicity and stability [43,44]. Squalenoylated gemcitabine formed nanoassemblies when dispersed in water with a mean diameter of

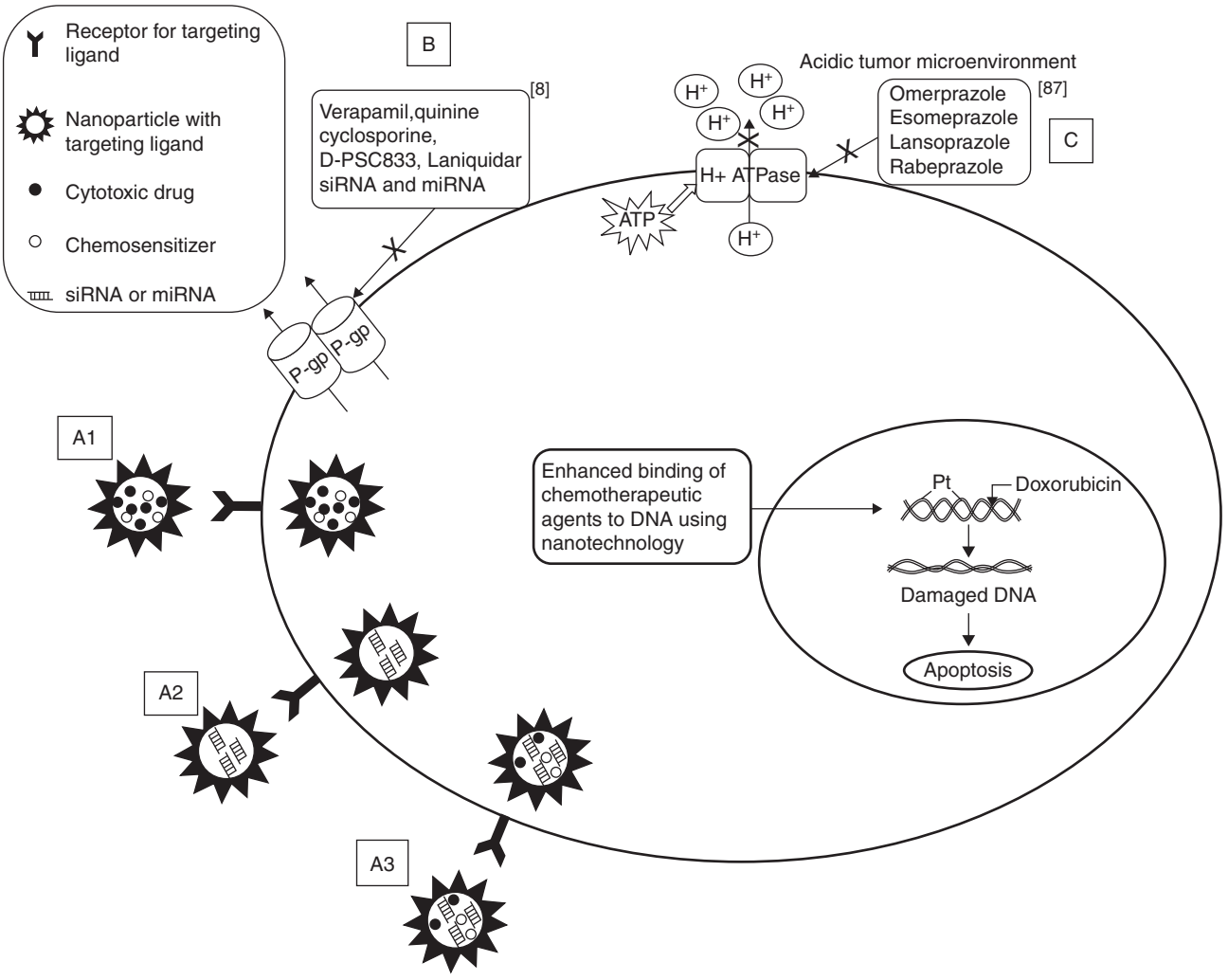

Figure 2. Schematic illustration of drug resistance reversal strategies involving use of nanocarriers, P-glycoprotein (P-gp) inhibitors and proton pump inhibitors. A1. Ligand-conjugated polymer loaded with chemotherapeutic agent or chemosensitizing agent. **A2.** Ligand-conjugated polymer with nucleotide (miRNA or siRNA). **A3.** Multifunctional nanocarrier housing nucleotide, chemosenstizer and chemotherapeutic agent. **B.** P-gp inhibitors of various generations. **C.** Proton pump inhibitors for reversal of resistance.

130 nm and exhibited ~ 3.2-fold higher toxicity in resistant leukemia cells in comparison with gemcitabine. These nanoassemblies caused S-phase arrest followed by apoptosis, and increased survival time *in vivo* [43]. Because of its advantages in cancer therapy, squalenoylation strategy was also used to design multifunctional pharmaceutical theragnostics involving gemcitabine as drug and magnetite nanocrystals as imaging agents. Nanocomposites were injected into L1210 tumor-bearing mice and when magnetically guided displayed considerably greater anticancer activity than free gemcitabine [45].

A multifunctional approach in engineering of nanocarriers based on use of both active and passive strategies, housing various components for reversal of resistance such as a selective targeting moiety, chemosensitizer, tumor microenvironment stimulative moiety and efflux pump inhibitor would result is an efficient nanoparticle to circumvent drug resistance. For example, a 'Quadrugnostic' nanoparticle system was proposed containing four synergistic elements: a specific targeting moiety, a chemotherapeutic drug, drug-resistance inhibitor and a diagnostic-imaging aid [46].

2.2 miRNAs and siRNAs in reversal of resistance

The tumor suppressor protein, p53, apart from playing the central role in cell cycle arrest and cell death also upregulates the genes such as *Fas* (or CD95) and *Bax* to promote apoptosis [47]. It was reported that the gene encoding p53, *TP53*, is mutated in approximately 50% of human cancers and these mutations have been correlated with lack of response to chemotherapeutic agents like doxorubicin in patients with advanced breast

Table 1. Selected examples of nanocarriers used in reversal of resistance.

Nanocarrier	Mode of reversal of resistance	Cytotoxic agent/s	Tumor type	Ref.
PEG-*b*-PLA polymeric micelles	Increase in cellular accumulation	Paclitaxel	Ovarian cancer	[113]
Magnetic Fe_3O_4 nanoparticles	Combination therapy with MDR1 shRNA	Daunorubicin	Leukemia	[114]
PLGA nanoparticles	Combination therapy with chemosentitizer verapamil	Vincristine	Breast cancer	[115]
Long circulating liposomes	Combination therapy with chemosentitizer tariquidar	Paclitaxel	Ovarian cancer	[116]
PNIPAM-co-PS nanofibers	Enhanced intracellular uptake	Daunorubicin	Erythroleukemia	[117]
Poly(*n*-butylcyanoacrylate) nanoparticles	Inhibition of P-gp function	Paclitaxel	Ovarian cancer	[118]

P-gp: P-glycoprotein; PLGA: Poly(D,L-lactide-co-glycolide); PNIPAM-co-PS: Poly(*N*-isopropylacrylamide)-co-polystyrene.

cancer [48]. In a study conducted by our research group, it was found that single or multiple pretreatment of MCF-7 cells (having no p53 mutations) with TP53 gene using sigma ligand conjugated-PAMAM (generation 4) dendriplexes at N/P 10, followed by doxorubicin treatment led to decrease in IC_{50} values of doxorubicin to half (from 29.66 to 10.94 nM). Although, when similar treatments were given to NCI/Res-ADR cells (having mutated p53) only about 10% decrease in the cell viability was observed after multiple treatment with TP53 dendriplexes (at N/P 10), when compared with doxorubicin treatment alone at the concentration tested (unpublished data). Various strategies in sensitizing cancer cells to chemotherapy by *p53* gene therapy are shown in **Table 2**.

miRNAs belong to a group of non-coding, endogenous RNAs with 19 – 25 nucleotides which after generation and processing get incorporated into RNA-induced silencing complex (RISC) and downregulate the post-transcriptional gene expression [49]. A recent study showed that anti-apoptotic factor *Bcl-2* was directly suppressed by miR-15a and miR-16-1 miRNAs, indicating the potential of miRNAs in anticancer therapy [50]. miRNAs, miR-17-92 cluster, which have six miRNAs, are involved in development of lung cancer and B-cell lymphoma [51,52]. These reports suggest the role of miRNAs in regulation of various cancer-related pathways leading to cell survival, migration and invasion, epithelial to mesenchymal transition and sensitivity of tumor cells to chemotherapeutic agents [53].

Many drug-resistant tumor cells have shown to have aberrant expression of miRNAs. Doxorubicin-resistant MCF-7 breast cancer cells showed the role of miRNA-451 in regulation of drug efflux pumps (MDR1) [54]. miR-21 has control over *Bax* to *Bcl-2* ratio in temozolomide-resistant glioblastoma cells [55]. In tamoxifen-resistant breast cancer cells, two miRNAs, miR-221 and miR-222 were involved in negative regulation of estrogen receptor α [56]. In camptothecin-resistant prostate cancer cells, negative regulation of sirutin (SIRT1) was shown to be controlled by miR-34a [57]. Cisplatin-resistant ovarian cancer cells showed that miR-214 is involved in regulation of PTEN/AKT pathway [58]. Aberrant expression of miRNAs in drug-resistant cancer cells was also used as predictive biomarker of tumor response in human cancers treated with chemotherapeutic agents. For example, in patients with metastatic colorectal cancer resistant to 5-fluorouracil and irinotecan, single nucleotide polymorphism in pri-miR-26a-1 and pri-miR-100 were associated with response to treatment and progression time [59]. Hepatocellular carcinoma patients with tumors having low miR-26 expression were shown to respond better to adjuvant IFN therapy [60]. All the above-mentioned mechanisms and the involvement of miRNAs suggest the potential role of miRNAs in reversal of resistance in various types of cancers.

miRNAs are often unstable in circulation, their efficient delivery to drug-resistant cancer cells is of prime importance. In most cases, miRNA was directly delivered by intratumoral injection and it is feasible only for small localized tumors but not for tumors which are metastasized. Trang *et al.* have formulated miRNAs miR-34a and *let-7* in neutral lipid emulsion and were delivered systemically to mouse model of NSCLC. Lipid formulation displayed a 60% reduction in tumor when compared with control miRNA, suggesting that it improves the systemic delivery of miRNA [61]. Other challenges in miRNA applications include non-specific biodistribution, susceptibility of miRNA to serum nucleases and renal clearance [62]. A lysine-containing nanoparticle decorated with lipid chains was investigated to improve the stability of anti-miR-122 after systemic delivery. The chemically stabilized anti-miR-122 was complexed with these interfering nanoparticles (iNOPs) and systemically delivered into mice. Results have shown that 2 mg/kg of anti-miR-122 with iNOPs resulted in 83.2 ± 3.2% silencing of miR-122 and the silencing of miR-122 was long lasting (up to 9 days) and did not provoke any immune responses [63]. Various reported strategies for delivery of anti-miR oligonucleotides are shown in **Table 3**.

siRNA are double-stranded RNA molecules which are ~ 21 nt in length and were shown to cleave the target mRNA by incorporating into RISC [64,65]. Recently, siRNA technology was proved to be very efficient in not only reversing the drug resistance but also improving the sensitivity of chemotherapeutic agents to cancer cells [66-68].

siRNA therapeutics though successful have setbacks such as inefficient systemic delivery, off-target effects, incomplete downregulation of target genes, interactions with endogenous

Table 2. Sensitizing cancer cells for chemotherapy with *p53* gene therapy.

Cancer type	Therapeutic agent/s	Delivery of *p53*	Result	Ref.
Osteosarcoma	Doxorubicin	Baculoviral vector	Combination therapy enhanced cytotoxicity by twofold in comparison with treating alone with either doxorubicin or *p53*	[119]
Human malignant glioma	Doxorubicin, BCNU, cytarabine, teniposide, cisplatin and vincristine	Electroporation using *p53* hygro vector	*p53* gene transfer did not result in consistent pattern of chemosensitization in three glioma cell lines	[120]
Gastric cancer	Epirubicin hydrochloride	Human adenoviral vector	Synergistic tumor inhibition in gastric cancer cells was seen	[121]
Glioblastoma	Cisplatin	Human adenoviral vector	*p53* gene transfer sensitized cancer cells to cisplatin treatment	[122]
Colon cancer	Chlorin e6	Wild-type expression vector	Introduction of wild-type *p53* in colon cancer cells resulted in increased sensitivity to photodynamic therapy	[123]
Prostate cancer	Taxotere	Folate conjugated liposome	*p53* therapy resulted in increase in sensitivity to Taxotere	[124]
Glioma	Acyclovir with HSV-TK gene	Human adenoviral vector	*P53* gene therapy was able to enhance sensitivity of glioma cells to acyclovir-HSV-TK therapy	[125]

HSV-TK: Herpes simplex virus tyrosine kinase.

miRNAs and longevity of silencing capability [69]. Many drawbacks of siRNA therapy can be overcome by efficient delivery of siRNA to target gene. One such strategy which improved the efficacy of siRNA in ovarian cancer is the use of neutral nanoliposome 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) to deliver siRNA which targets a critical platinum resistance gene, ATP7B. *In vivo* studies performed in orthotopic nude mice model of ovarian cancer showed that nanoliposomal siRNA successfully improved the stability of siRNA and shielded siRNA from serum nucleases thus improving its antitumor activity [70]. One of the major obstacles in siRNA delivery is the inability of siRNA to cross lipid bilayer of cellular plasma membrane. To overcome this, siRNA targeted toward P-gp was complexed with lipid modified cationic polymers such as poly-L-lysine modified with stearic acid and polyethyleneimine substituted with oleic acid. These nanoparticles enhanced the transport of siRNA through cellular membrane and resulted in inhibition of P-gp [71].

2.3 Small molecule sensitizers in reversal of drug resistance

Small molecule sensitizing agents either by inhibiting or modifying the mechanism of resistance in many tumors sensitize resistant tumor cells to chemotherapeutic agents. P-gp inhibition using small molecule inhibitors was the most sought after approach to overcome resistance to chemotherapeutic agents in a wide variety of cancers. Phase I and II trials of P-gp inhibitors were started within 10 years after discovery of P-gp-mediated MDR indicating the significant clinical relevance [8]. The first-generation P-gp inhibitors included drugs such as verapamil, quinine and cyclosporine showed promise initially for P-gp inhibition though at very high doses. In a randomized Phase III clinical trial, addition of cyclosporine improved the reversal of resistance of patients to cytarabine and daunorubicin in patients with poor-risk acute myeloid leukemia [72]. However, the trials conducted later were not fruitful in consolidating the effect of P-gp inhibition by these agents and their treatment also resulted in many toxic side effects [73]. Though, the initial clinical trials did not result in any successful P-gp inhibiting agents, they suggested that P-gp modulation is feasible and encouraged further investigation in this area. The second-generation P-gp inhibition agents were developed with reduced toxic effects and improved efficacy to inhibit P-gp. These included *R*-enantiomer of verapamil, analog of cyclosporine D-PSC833 (valspodar) and these agents have shown specificity toward P-gp without interacting with calcium channels and did not exhibit any immunosuppressive effects [74]. However, these agents have shown marked pharmacokinetic interactions that reduced the clearance and metabolism of chemotherapeutic agents elevating their plasma levels and thus resulting in higher toxicities [75]. To overcome the drawbacks associated with second-generation inhibitors, such as low pharmacokinetic interaction and high transporter affinity, third-generation inhibitors were developed. These inhibitors were specifically designed to evade cytochrome P450 inhibition which was the major cause of pharmacokinetic interaction with previous generation of inhibitors. These included laniquidar, oc144-093 (ONT-093), zosuquidar

Table 3. Strategies for delivery of miRNA and anti-miR oligonucleotides.

Name	Target gene	Delivery system	Therapeutic effect	Ref.
Anti-miR-296	Hepatocyte growth factor regulated tyrosine kinase substrate	Pegylated liposome-polycation-hyaluronic acid nanoparticle with cyclic RGD peptide	Targeted nanoparticles exhibited effective anti-angiogenic activity *in vitro* and *in vivo*	[62]
Anti-miR-122	Cholesterol biosynthesis genes	Hydroxyprolinol linked cholesterol	Decrease in cholesterol levels	[126]
Anti-miR-122	Cholesterol biosynthesis genes	Lipidoids	Enhanced miR-122 expression	[127]
miRNA 34a	*Survivin*	Liposome-polycation-hyaluronic acid modified with scFv	Induction of apoptosis in cancer cells	[128]
Let 7b	*RAS*	Neutral lipid emulsion	Reduction in lung cancer tumor proliferation	[61]

miRNA: MicroRNA; RGD: Arginine–glycine–aspartic acid; scFv: Single chain antibody fragment.

(LY335979), elacridar (GF-120918) and tariquidar (XR9576) [8,76]. The later generations of inhibitors were aimed at inhibiting multiple transporters of the ABC family. Birocidar (VX-710) and GF-120918 were able to interact with P-gp, MRP1 and ABCG2 transporters, respectively [77]. Recently, it was reported that excipients such as Brij 78 (polyoxyethylene 20-stearyl ether) and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), which are components for preparing nanocarriers exhibited a dual role in stabilizing nanocarriers and inhibiting P-gp [78,79]. Brij 78-based solid lipid nanoparticles enhanced the cytotoxicity of paclitaxel and doxorubicin in P-gp-mediated resistant cells, and mechanism involved P-gp inhibition and ATP depletion [78]. Paclitaxel nanocrystals developed using TPGS as the sole excipient has exhibited significant antitumor effect in paclitaxel-resistant cells *in vivo*. The reason for improved antitumor effect was attributed to inhibition of P-gp mediated by TPGS [79].

Glutathione (GSH), a tripeptide is a predominant cellular thiol and its synthesis involves enzyme γ-glutamylcysteine synthetase (γ-GCS)-mediated addition of amino acids glutamic acid, cysteine and glycine to form GSH. GSH was implicated in the development of resistance to various chemotherapeutic drugs including platinum compounds, alkylating agents, anthracyclines [11]. GSH along with increased levels of γ-GCS was shown to contribute to resistance by either inactivating the chemotherapeutic agent, enhancing the DNA repair by providing suitable reducing environment or acting as cofactor in MRP1-mediated drug efflux [80,81]. Thus, depletion of intracellular GSH levels was considered to be an attractive strategy to overcome the drug resistance. The first attempt to achieve this involved use of BSO which is a potent and specific inhibitor of γ-GCS [82]. BSO was shown to reduce intracellular levels of GSH both *in vitro* and *in vivo* and thus augmenting the sensitivity of cancer cells to cisplatin, alkylating agents and doxorubicin [20,83,84]. Human clinical trials with BSO have shown that intracellular GSH levels in circulating white blood cells were reduced by 60 – 80% [85]. Glutathione reductase (GR) is an enzyme which catalyzes reduction of GSSG (oxidized form of GSH) to GSH to maintain a high GSH:GSSG ratio. Inhibition of this enzyme will result in inhibition of thiols redox state which is involved in pathogenesis of various cancers. Thus, GR inhibitors like carmustine, 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylthiocarbonylamino)phenylthiocarbamoylsulfanyl]propionic acids (2-AAPA) were developed as anticancer agents [86].

pH gradient that exists between tumor extracellular environment and the cell cytoplasm is also an attractive target for reversal of resistance in cancer cells. In solid tumors the extracellular microenvironment is significantly more acidic than normal tissues, this acidic environment leads to reduced uptake of many weakly basic chemotherapeutic drugs (low pKa values) as they tend to ionize resulting in poor permeability in tumors. Reduced uptake thus leads to resistance to those chemotherapeutic agents [87]. Vacuolar H^+-ATPases are proton pumps that exist across plasma membrane and membranes across various intracellular components and are responsible for acidic tumor microenvironment. Increased vacuolar H^+-ATPase activity was observed in many human multidrug resistance tumors [88-90]. Thus, to reduce the acidic tumor microenvironment, proton pump inhibitors (PPIs) were introduced. PPIs including omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole, by decreasing tumor acidity were shown to improve the cytotoxicity of chemotherapeutic agents in cancers like melanoma, colon carcinoma, adenocarcinoma and breast cancer. When tumor cells were pretreated with omeprazole both *in vitro* and *in vivo* before administration of chemotherapeutic agents like cisplatin, 5-fluorouracil and vinblastine, sensitivity of tumor cells to these drugs increased significantly [90].

Superoxide dismutase (SOD) is an enzyme which functions to prevent unwanted oxidative damage to cell by converting highly reactive superoxide to less reactive hydrogen peroxide. SOD was reported to be overexpressed in cisplatin-resistant ovarian cancer cells and thus become an attractive target for reversal of drug resistance. A small molecule inhibitor triethylenetetramine (TETA) which can inhibit SOD activity was investigated for reversal of resistance. The results indicated

that a non-toxic concentration of 10 mM of TETA significantly increased the sensitivity of cisplatin-resistant ovarian cancer cell lines to cisplatin [91]. Another interesting class of small molecule chemosensitizing agents is the proteasome inhibitors. Proteasome is a well-elucidated target and plays key role in cancer cell proliferation, drug resistance development and apoptosis due to chemotherapeutic agents. Lactacystin is a naturally occurring proteasome inhibitor which was reported first [92]. But due to lack of potency and specificity several novel synthetic proteasome inhibitors were developed. Bortezomib, dipeptide boronic acid analog, is the first synthetic proteasome inhibitor to receive approval from US Food and Drug Administration for treatment of patients with multiple myeloma [93]. Bortezomib was shown to sensitize various types of cancers like prostate, ovarian, lung, colorectal, breast and non-Hodgkin's lymphoma. In order to preclude occurrence of toxicity due to synthetic proteasome inhibitors, various naturally occurring proteasome inhibitors, like genistein, curcumin, resveratrol and green tea polyphenols, were also reported recently. These naturally occurring agents have shown to sensitize many drug-resistant human cancers [94].

Another major factor shown to have control over resistance is the transcription factor nuclear factor kappa B (NF-κB) and is shown to play a role in tumor cell survival and activation of apoptotic gene products. Thus, inhibition of NF-κB by small molecule inhibitors may result in reduction of anti-apoptotic activity of resistant tumor cells which can lead to either direct cytotoxic activity or sensitization of cells to chemotherapy. A novel NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) was shown to selectively inhibit the translocation of NF-κB into nucleus and prevent its various transcriptional functions. DHMEQ was shown to exert direct toxic effects with significant chemosensitizing activities in resistant tumor cells and was also shown to be non-toxic *in vivo* [95].

Small molecule inhibitors because of their size are often rapidly cleared from blood circulation by reticuloendothelial system (RES) uptake. It is very important to enhance the circulation of these inhibitors as prolonged residence of these inhibitors would ensure maximum sensitization of resistant cancers to chemotherapy. For example, sensitization can be improved by encapsulating BSO, a specific inhibitor of γ-GCS, in porous hydroxyapatite. BSO complexed with doxorubicin and hydroxyapatite reduced tumors in mice with sarcoma 180 because of improved circulation [96]. Similarly, P-gp inhibitor verapamil has shown serious cardiotoxicity, neurotoxicity and drug–drug interaction when administered systemically. Verapamil encapsulated PLGA nanoparticles along with vincristine and these nanoparticles not only reduced drug toxicity but also caused fewer drug–drug interactions [97].

2.4 Aptamers in combination therapy

Aptamers are single-stranded short RNA, DNA or protein ligands which bind with high affinity and specificity to their target molecules. Aptamers are generated *in vitro* by an iterative evolution procedure named SELEX (systematic evolution of ligands by exponential enrichment). Because of advantages like ease of production, lack of immunogenicity, non-toxicity, high specificity and *in vivo* stability, aptamers are widely investigated for target validation, delivery agents to cancer, for tumor imaging and targeted delivery of drugs [98,99].

One of the major causes of resistance to chemotherapy in breast cancer is the overexpression of ErbB2 receptor tyrosine kinase. Peptide aptamers which can interfere with intracellular ErbB2 functions and inhibit the activation of AKT were isolated and investigated for their ability to sensitize chemoresistant breast cancer cells. Results have shown that peptide aptamers strongly inhibited the induction AKT kinase in MCF7 breast cancer cells and this activity sensitized the breast cancer cells toward Taxol [100]. Incidence of cisplatin resistance in prostate cancer was mainly due to poor targeting of cisplatin. To overcome the resistance prostate-specific membrane antigen (PSMA), specific aptamers were functionalized onto PLGA nanoparticles and were loaded with Pt(IV) prodrug. Results have shown an increased uptake of nanoparticles by $PSMA^+$ LNCaP cells by endocytosis when compared with non-targeted nanoparticles, indicating a potential in the reversal of chemoresistance by effective delivery of platinum to prostate cancer cells [101].

NF-κB is a prime target for reversal of resistance because of its key role in mediating resistance to chemotherapy. NF-κB inactivation by a RNA aptamer (A-p50) resulted in overcoming of doxorubicin resistance in NSCLC cells both *in vitro* and *in vivo* [102]. When A549 (NSCLC) cells were infected with adenovirus-mediated A-p50 and co-treated with doxorubicin, cell viability was decreased by 80%. *In vivo* experiments were performed on nude mice xenografted with A549 cells and then treated with doxorubicin to induce *in vivo* chemoresistance. Intratumoral injection with Ad-A-p50 and further treatment with doxorubicin reduced the tumor growth indicating that A-p50 reverses doxorubicin resistance *in vivo* leading to improved cancer cell death [103]. The above studies suggest that combination therapy of resistant tumors with aptamers and chemotherapeutic agents is very attractive strategy with clinical potential to overcome drug resistance.

2.5 Cancer stem cell therapy

CSC theory proposed in 1968 hypothesizes that the cancer-initiating cell is a 'stem cell unable to differentiate'. These CSCs can self-renew, produce tumorigenic daughter cells and also give rise to different cancer cell phenotypes which are non-tumorigenic. CSCs were implicated in occurrence of resistance in various cancers in clinical settings. The recurring tumors were reported to be evolving from the expansion of surviving CSC clones. The CSC has innate drug resistance to environmental toxins including chemotherapeutic agents and is mediated by MDR transporters and detoxifying enzymes [104].

The MDR activity was constitutively expressed in CSC and is independent of drug exposure and downregulated in differentiated tumor progeny [105]. The quiescent tumor

stem cell with constitutive MDR activity is the major barrier for the effective therapy. For example, in melanoma CSC subpopulation, some cells were shown to be ABCB5 and ABCG2 positive thus indicating that alteration of these factors would result in reversal of drug resistance leading to effective chemotherapy [106]. Several oncogenic cascades activated in cancer progenitor cells are involved in crucial functions in regulating self-renewal, survival and invasion of CSCs. These cascades which include telomerase, survivin, Notch, Hedgehog, Wnt/β-catenin are also very important targets for pharmacological intervention to overcome resistance [106].

Targeting the local microenvironment of cancer progenitor cells is an attractive strategy in stem cell therapy. In this aspect, agents that are able to interfere with VEGF transduction system gained importance. Bevacizumab, an anti-VEGF monoclonal antibody was shown to reduce microvasculature density and tumor growth when administered to mice bearing orthotopic U87 glioma cell xenografts. Moreover, it was also observed that this effect is accompanied with a decrease of $CD133^{+}$/NESTIN+ tumor cells [107].

Thus, for a successful cancer therapy, it is very important to differentiate major biological and immunological differences between tumor and normal stem cells. The most clinically relevant target for therapy to overcome drug resistance is the resting cell which is intrinsically resistant and this resistance is independent of chemotherapy-induced gene duplication or gene translocation [108].

Stem cell therapy in order to be effective has to overcome obstacles that hinder their regeneration and has to be delivered to target site to overcome undesired differentiation. Neural stem cells (NSCs) often suffer from drawbacks such as low viability and undesired differentiation. To address this issue a polymeric complex comprising hyaluronic acid hydrogel and PLGA microsphere system was developed to sustain NSCs, which will provide an acquiescent microenvironment for neural regeneration and angiogenesis. This biomaterial scaffold was successful in sustaining NSCs thus showing a good potential for supporting NSCs for brain repair and implantation [109]. Lack of a suitable delivery method of stem cells is a limitation specifically in vascular healing. An optimal stem cell delivery system ensures efficacy in moving cells and arrest them at vascular wall under high shear stress conditions existing in arteries. A classic example of such a delivery system can be described as mesenchymal stem cells (MSCs) coated with gas-filled lipid microbubbles and displaced to specific arterial segment using ultrasound radiation force [110].

3. Expert opinion

While the mechanism(s) of development of drug resistance may vary from drug to drug, application of nanotechnology has shown to overcome drug resistance, in two major pathways, interfering either with the drug transportation across the cell membrane (e.g., drug efflux-related protein such as P-gp and other MDR-associated proteins) or with drug metabolizing enzymes. As a result, the net intracellular drug concentration will be increased several fold and thereby eliciting high cytotoxicity activity. The current trends in the application of nanotechnology for cancer therapy focus on design of nanomedicine to increase the intracellular drug concentration with either single or a combination of chemotherapeutic agents.

Nanoparticles may primarily serve as the drug reservoirs. Leaky vasculature of the tumor tissue allow the escape of these nanoparticles into the tumor tissue and uptake by the tumor cells and lack of lymphatic drainage results in dose dumping at the tumor site. By careful design of the nanoparticles it is possible to control the drug release profile suitable for optimal cytotoxicity. Controlling the drug release from the nanoparticles not only enhances the tumoricidal effect but also alters pharmacokinetic properties of the drug such as bioavailability at the tumor site and its clearance. Moreover, while the hydrophobic drug molecules are generally the substrates for the efflux pumps, colloidal particles are not, resulting in accumulation in the cell. Such an effect is termed as EPR effect [111]. However, there is a significant difference in tumor disposition between small molecules and macromolecular drugs. Because of their size, macromolecular drugs are retained effectively in the tumor site, while small molecules diffuse back into general circulation. In this context, application of nanotechnology assumes significant importance as encapsulation of small and macromolecular drugs into nanoparticles has been demonstrated to increase the drug retention in the tumor tissue [25]. Although, 'passive targeting' approach has shown improvement in tumor therapy, several obstacles have to be overcome. For instance, nanoparticles should have high circulation half-life for adequate localization in the target tissue. This has been achieved by grafting hydrophilic polymers to the nanoparticle surface, minimizing the uptake by RES and increasing the circulation half-life. However, lack of target specificity, presence of leaky endothelium in various organs such as liver, kidney, etc., and drug leakage from the circulating nanocarrier limit the benefit of passive targeting approach [111].

Active targeting, the drug delivery using target-specific ligands, is an efficient approach for target-selective chemotherapy. Several types of ligands such as humanized monoclonal antibodies (antibodies against HER2 receptors for breast cancer, anti-PSMA antibodies for prostate cancer, etc.), small molecule agonists for receptors highly expressed in various cancers (folic acid receptor, transferrin receptor, sigma receptor, biotin receptor, etc.) have been used for imaging and targeted therapy of cancer [112]. Several drug-ligand molecules and ligand-coated drug-loaded nanoparticles are in various stages of clinical trials. The advantage of nanoparticles in targeted drug delivery includes the possibility of attachment of multiple numbers of ligand molecules which increases the target specificity, and drug encapsulation inside the nanoparticles which not only improves the stability of the drug but also carries high drug payload. However, because of receptor heterogeneity and difference in their level of expression in the target tissue, nanoparticles coated with multiple ligands have been developed

for better targeting efficiency. As a further step, multifunctional nanoparticles carrying a combination of different therapeutic moieties and with signal sensitive release properties (such as pH-, temperature-, light- and magnetic field-dependent release) have been developed for better therapeutic outcomes.

Apart from target-specific dose dumping of chemotherapeutics, various molecular interventions such as pro-apoptotic gene therapy, inhibition of anti-apoptotic mechanisms using siRNA, miRNA and using chemosensitizers (both small and macromolecules) have been investigated in the recent past. These interventions are designed either to target the apoptotic pathway or to specific enzyme system in the cancer cells. Impaired balance between pro-apoptotic and anti-apoptotic molecules is common in cancer cells. It is well known that pro-apoptotic gene therapy or inhibition of anti-apoptotic genes enhances the capacity of tumor cells to undergo apoptosis and renders the tumors sensitive to classical anticancer drugs as well as radiotherapy. Alternatively, several small molecule inhibitors such as GSH reductase inhibitors and SOD inhibitors have been developed as a strategy to sensitize the cancer cells to specific chemotherapeutic agents [11]. However, irrespective of the type of sensitization method used, targeted delivery of these sensitizers (both micro- and macromolecules) is the bottleneck in achieving the anticipated therapeutic benefit. It is therefore imminent that in addition to developing novel therapeutics, design of efficient delivery modules should also be developed.

Current strategies to treat drug-resistant tumors use combination therapy such as drugs with different mechanisms, combination of different therapeutic genes or a combination of both, for a possible additive or synergistic effect. It appears that an ideal delivery system may be a complex integrated nanoparticle system that exhibits long circulation half-life based on size, shape, composition; tumor specificity imparted because of multiple ligands, high penetration capacity, optimal drug/gene release characteristics in response to demands of tumor microenvironment, intracellular retention of the therapeutic molecules for a sustained therapeutic effect. Apart from these characteristics, the nanoparticles have to be biocompatible and biodegradable.

In addition to targeting just the tumor cells, several approaches are being developed to target the tumor microenvironment, cross-talk between the tumor cells and their stroma and/or tumor vasculature. In hematologic malignancies, disruption of interaction between stromal cell secreted/derived factor-1 (SDF-1/CXCL12) and its receptor CXCR4 render them sensitive to cytotoxic drugs [3]. Based on the EPR of the tumor vasculature, quite a few nanoparticulate systems encapsulating chemosensitizing, antiproliferative and anti-angiogenic molecules, such as rapamycin, have been investigated. The CSC/TIC (tumor initiating cell) are generally more resistant to chemo-/radiotherapy than non-CSC/TIC. Cell surface markers expressed by CSC are also generally shared by normal somatic stem cells. Although the biomarkers exclusive to the CSC have not been identified yet, telomerase, anti-apoptotic factors, efflux transporters, detoxifying enzymes, oncogenic cascades, including Hedgehog, EGFR, Wnt/β-catenin, Notch and/or polycomb group (PcG) protein chromatin silencing pathways are considered potential drug targets [46,106].

Overall, it is imperative that none of the current therapeutic strategies have the ability to eradicate the tumor cells. Therefore, use of a multifunctional nanoparticle system designed to target various cell populations in the cancer microenvironment and their specific biomarkers would be a promising approach. Ideally, such a system should have i) a targeting molecule, ii) combination of therapeutic molecules to specifically kill CSC or sensitize these cells for chemotherapy and sensitize the drug-resistant cancer cells, and a cytotoxic drug, iii) a module that imparts spatially controlled release of therapeutic molecules in the tumor microenvironment. It is hoped that understanding the subtle differences in signaling pathways between CSC and normal stem cells, and CSC-specific therapeutics combined with developing strategies to design biocompatible, tumor environment-sensitive multifunctional nanoparticles, may be the most promising approach for the future.

Declaration of interest

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