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MULTI-FUNCTIONAL NANOCARRIERS TO OVERCOME TUMOR DRUG RESISTANCE

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Abstract

The development of resistance to variety of chemotherapeutic agents is one of the major challenges in effective cancer treatment. Tumor cells are able to generate a multi-drug resistance (MDR) phenotype due to microenvironmental selection pressures. This review addresses the use of nanotechnology-based delivery systems to overcome MDR in solid tumors. Our own work along with evidence from the literature illustrates the development of various types of engineered nanocarriers specifically designed to enhance tumor-targeted delivery through passive and active targeting strategies. Additionally, multi-functional nanocarriers are developed to enhance drug delivery and overcome MDR by either simultaneous or sequential delivery of resistance modulators (e.g., with P-glycoprotein substrates), agents that regulate intracellular pH, agents that lower the apoptotic threshold (e.g., with ceramide), or in combination with energy delivery (e.g., sound, heat, and light) to enhance the effectiveness of anticancer agents in refractory tumors. In preclinical studies, the use of multi-functional nanocarriers has shown significant promise in enhancing cancer therapy, especially against MDR tumors.

Keywords

Multi-drug resistance; multi-functional nanocarriers; drug delivery; resistance modulators; energy delivery

1. INTRODUCTION

According to the Global Cancer Report issued by the World Health Organization, there are over 10 million new cases of cancer each year and over 6 million annual deaths from the disease [1]. Although there has been tremendous progress in the prevention, detection, and treatment of cancer over the last fifty years, adequate therapy remains elusive due to late stage diagnosis, inadequate strategies for addressing aggressive metastasis, and a lack of clinical procedures for overcoming multi-drug resistant (MDR) cancer [1-3]. Multi-drug resistance in cancer refers to a state of resilience against structurally and functionally unrelated drugs [4]. MDR can be intrinsic (inherent) or acquired through chemotherapy exposure [4]. The development of MDR contributes to the persistence of the disease in spite of high dose and combination chemotherapy, which is often the last treatment option [5]. However, this often leads to toxic side effects and poor clinical outcomes. The majority of clinically approved chemotherapeutic

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agents target cell growth patterns and are not selective for cancer cells. Chemotherapy regimens are often delayed, reduced, and altered due to low white blood cell counts (neutropenia). Alterations in a chemotherapy regimen undoubtedly contribute to the development of acquired MDR cancer.

1.1 Tumor Microenvironment and MDR

Although the true clinical prevalence of MDR is difficult to assess as only a minority of cancer patients are tested for the MDR phenotype, MDR is implied in most cases of non-responsive recurrence and has been clearly associated with treatment failure in many different types of cancer in patients with diverse demographics [6-17]. MDR has been linked to poor prognosis and reduced survival in gastric cancer, gliomas, sarcomas, breast cancer, ovarian cancer, pancreatic cancer, and hematological malignancies, including childhood acute lymphoblastic leukemia and acute myeloid leukemia [6-17].

To understand the physiological mechanisms of MDR, one must have an appreciation for the microenvironment of tumors and for the selection pressures that contribute to tumor progression (Figure 1). These selection pressures include hypoxia and changes in the regulation/expression of oncogenes, tumor suppressors, and apoptotic factors [18]. Cellular responses to concurrent selection pressures and chemotherapeutic agents determine if the cell will become quiescent (G_0 phase), engage in apoptosis, or develop MDR [19]. Unlike their common diagrammatical depiction, the structure of a tumor is not composed of neatly compartmentalized regions. Tumors are commonly depicted as a core-shell structure with a hypoxic/necrotic core and a proliferating outer shell of cells. Although tumors are highly heterogeneous, the anatomy of a tumor more closely resembles a haphazard pattern than a core-shell structure [20]. The outer regions of tumors tend to be highly vascularized, but these blood vessels are unorganized and discontinuous, resulting in high permeability and “leakiness”; a phenomena that is exploited in passively targeted delivery and referred to as the *enhanced permeability and retention* (EPR) effect [20,21]. Due to the diffusion limit of oxygen, tumor cells that are more than 100–150 μm from blood vessels tend to be oxygen deprived, leading to chronic hypoxia and inevitable necrosis [22,23]. Acute hypoxic regions can also be created when blood vessels are closed, which often occurs in the microenvironment of a tumor due to compression, tumor cell invasion, and discontinuity of the epithelial cells lining the vessels [20,23]. Re-opening of these vessels re-oxygenates these hypoxic regions [23]. As a result of the leaky vasculature and impaired lymphatic drainage in tumors, hypoxic regions are associated with high interstitial fluid pressure relative to the low interstitial fluid pressure of well-vascularized regions [20,24]. Hypoxic cancer cells also have a substantially reduced intracellular pH level relative to normal cells [23,25]. This acidic cellular environment is associated with the activation of a subset of proteases that contribute to metastasis [23]. Additionally, hypoxic cells revert to anaerobic metabolism, obtaining ATP through the conversion of glucose to lactic acid instead of through oxidative metabolism (known as the Warburg effect) [26].

By virtue of their location in the tumor, hypoxic cells are less likely to accumulate therapeutically relevant doses of chemotherapeutics through the blood supply [27]. Drugs that exert their effect through reactive oxygen species production, drugs that are cell cycle dependent, and therapies that target rapidly dividing cells are more likely to be ineffective in hypoxic cells due to decreased oxygen and slowed cell cycle progression [20,25,27,28]. Hypoxia is also associated with resistance to radiation therapy as this relies on the production of free radicals from oxygen to induce cell death [25]. In addition to the inherent properties of hypoxic cells that decrease chemotherapeutic efficacy, hypoxic cells have active mechanisms for inducing MDR and, thus increasing their probability of survival [23,25,28].

Hypoxia in cancer has been associated with increased metastatic potential, increased drug resistance, and poor prognosis [20,22,23,29-32]. A study conducted in the 1990's on 37 patients with cervical cancer monitored intra-tumor oxygen levels, apoptotic index, and metastasis and found that hypoxic apoptotic-resistant tumors resulted in higher degrees of metastasis compared to apoptotic sensitive hypoxic tumors and non-hypoxic tumors [30]. This study defined tumor hypoxia as pO_2 levels below 10 mm Hg [30]. The frequency of recurrence and metastasis in patients with hypoxic, apoptotic-resistance tumors was double the frequency in patients with hypoxic, apoptotic sensitive tumors and non-hypoxic tumors [30]. In various studies hypoxia has also been shown to increase P-glycoprotein (P-gp), one of the primary drug efflux pump involved in MDR. In one such study, a hypoxic exposure time dependent increase in P-gp RNA, mRNA, and protein was demonstrated for OKF6, T84, Caco2, and HMVEC cell lines [27]. Up to a 7-fold increase in P-gp was evident after 18 hours of hypoxia (pO_2 20 torr) [27]. Hypoxia Inducible Factor (HIF) appears to be the primary mechanism by which hypoxia induces MDR and metastasis [28,33]. HIF upregulates target genes by binding to an enhancer region, the hypoxia-response element (HRE), in the target [34]. Antisense oligonucleotide inhibition of HIF-1 α prevented the hypoxia induced upregulation of P-gp suggesting that the mechanism of hypoxia induced P-gp is via a HRE on the P-gp (MDR1) gene [27]. Similarly, the use of RNA interference to silence HIF-1 α RNA in NSCLC cell lines has been shown to reverse hypoxia induced cisplatin and doxorubicin MDR [35]. HIF-1 α is also induction of genes that regulate angiogenesis; such as VEGF (vascular endothelial growth factor), LEP (leptin), and TGF- β 3 (transforming genes involved growth in extracellular matrix metabolism such as MMP2 (matrix metalloproteinase 2), FN1 (fibronectin 1), and collagen type V; and genes involved in glucose metabolism, apoptosis, cell proliferation, and cell survival [36].

In addition to hypoxia, selection pressures such as mutations in oncogenes and tumor suppressors contribute to the development of MDR. These genetic changes are critical in disrupting cell cycle checkpoints. Disruption of these checkpoints is necessary for cancer cells to evolve into MDR cells by avoiding apoptosis [18,19]. For example, it has been demonstrated that inactivating mutations in the pRb (retinoblastoma) and p53 tumor suppressor protein cascades are sufficient minimal changes to induce MDR in pre-tumorigenic cells that express telomerase [19]. The downstream effects of p53 inactivation have been well-characterized and associated with tumor progression leading to MDR [19,37,38]. These effects include inactivation of pro-apoptotic factors such as BAX and BAK, and activation of anti-apoptotic Bcl-2 proteins [19,37,38].

Cancer cell responses to selection pressures and the development of MDR result in altered expression profiles of many additional genes. For example, many growth factor receptors, such as epidermal growth factor receptor family (EGFR1, HER2, etc), are over-expressed in MDR cancer. EGFR1 over-expression in cancer has been correlated with disease onset, disease progression, and poor survival [39,40]. The incidence of EGFR1 over-expression in ovarian cancer has been reported to be from 30% to as high as 75% [40]. A study of primary human ovarian adenocarcinomas detected EGFR over-expression in 20 out of 35 patients (57%) [41]. Interestingly, HIF-1 α has been associated with growth factors and growth factor receptors in a positive feedback loop [36]. Binding of growth factors to receptors such as EGFR leads to an increase in HIF-1 α ; in turn, HIF-1 α increases the transcription of growth factors [36]. EGFR1 targeting is a common therapeutic approach for types of cancer characterized by EGFR1 overexpression. This is demonstrated by EGFR1 inhibitors such as gefitinib and erlotinib, which are clinically approved for the treatment of advanced NSCLC; erlotinib is also a component of an approved combination therapy for pancreatic cancer [39]. The dynamic and evolving responses of cancer cells to selection pressures lead to changes in the tumor microenvironment that promote MDR.

1.2 Mechanisms of Drug Resistance in Tumors

The mechanisms of drug resistance represent adaptations to cellular stress and toxic insults. The major mechanisms of drug resistance are grouped into five categories as depicted in Figure 2: increased drug efflux, decreased drug influx, DNA repair activation, detoxification, and blockage of apoptosis [5]. Although these categories represent distinct mechanisms, the MDR phenotype is usually the synergistic result of a combination of MDR mechanisms such as blocked apoptosis (decreased ceramide) and increased efflux (upregulated P-gp) [5].

There are over 13 ATP-Binding Cassette (ABC) transporters that are known to contribute to MDR [42]. P-glycoprotein (P-gp, MDR1, ABCB1) expression in resistant cells is the most characterized of the various ABC transporters. P-gp is a 170 kDa protein with 12 transmembrane spanning regions and two cytoplasmic nucleotide binding domains [43]. Membrane-bound P-gp effluxes a broad spectrum of substrates and active efflux requires the hydrolysis of two ATP molecules [5]. In addition to its role in MDR cancer, it is a critical component of the blood brain barrier (BBB), providing neuroprotection [5]. It is also found in the liver, kidneys, placenta, and intestines [5]. A recent study evaluating the cellular onset of MDR identified P-gp over-expression as the primary mechanism of MDR before malignant transformation [19]. P-gp over-expression is associated with poor prognosis in many types of cancer [5,44].

Other ABC transporters that contribute to MDR include multi-drug resistance protein 1 (MRP-1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) [42,44-46]. MRP1 has a vast distribution in normal tissue, including the BBB [5]. MRP1 is a significant ABC transporter in MDR cancer that has been correlated with poor prognosis and broad spectrum efflux [42,44]. Although the clinical significance of BCRP in MDR cancer is less characterized, BCRP has been associated with resistance to classical chemotherapeutic agents such as mitoxantrone and may also be involved in the efflux of tyrosine kinase inhibitors [42,46,47]. In addition to the 13 ABC transporters that are known to contribute to MDR, roles of other ABC transporters in drug efflux and MDR are also being explored. For example, ABCB6 has been associated with MDR in various cell lines. A recent study exploring the expression, cellular localization, structure, and function of ABCB6 identified two different forms of ABCB6 in human cell lines that differed in MW and distribution [48]. Both the high MW form (104 kDa) and the low MW form (79 kDa) localized to the mitochondrial outer membrane with the nucleotide binding domains of the transporters oriented towards the cytoplasm [48]. Conversely, only the high MW form localized to the plasma membrane [48]. Although ABCB6 has been associated with the efflux of chemotherapeutic agents such as cisplatin and paclitaxel, ABCB6 appears to preferentially efflux porphyrins [48]. While the role of ABC transporters such as P-gp, MRP-1, and BCRP in MDR is documented, the clinical success of inhibiting these drug efflux pumps has been inconclusive [49].

Additional mechanisms of MDR include decreased drug influx (which is a spatial characteristic of hypoxic cells); upregulation of DNA repair enzymes, rendering cellular insults ineffective; upregulation of detoxification enzymes such as cytochrome P450 to rapidly metabolize and inactivate internalized drugs; and diversion of apoptosis through various pathways, including the upregulation of apoptotic inhibitors such as survivin and anti-apoptotic Bcl-2 family members [5]. As chemotherapeutic agents aim to induce apoptosis, endogenous ligands that enhance the cell kill efficacy of these agents by decreasing the apoptotic threshold have become attractive experimental targets in recent years. As such, ceramide has emerged as an apoptotic modulator with therapeutic potential. Ceramide (CER) is a second messenger signaling molecule involved in differentiation, proliferation, immune response, and apoptosis [50,51]. Endogenous ceramide is synthesized by sphingomyelin hydrolysis or by de novo synthesis [52]. Ceramide elicits a response to extracellular stimuli such as irradiation, UV-light, *Pseudomonas aeruginosa* infection, and chemotherapeutic agents (including paclitaxel) [51,

53]. Ceramide activates apoptosis inducers such as ceramide activated protein kinase (CAPK) which is also known as the kinase suppressor of RAS (KSR), cRaf (involved in the MAPK/ERK signaling cascade), protein kinase C ζ (PKC ζ), cathepsin D (a lysosomal protease), and the ceramide activated protein phosphatases (CAPPs), which are serine/threonine protein phosphatases (PP1 and PP2A) [54]. PP2A effectors include protein kinase C α , Bcl-2, and Akt/protein kinase B; PP1 effectors include retinoblastoma protein and SR proteins [54].

Recent studies suggest an additional, more dynamic role of ceramide in apoptosis – the creation of protein permeable channels in the mitochondrial outer membrane (MOM) [55-57]. These ceramide channels increase the permeability of the MOM, allow the passage of proteins up to 60 kDa, and are a mechanism for pro-apoptotic protein release (i.e. cytochrome C, AIF, Smac/DIABLO) [55-57]. Sphingosine causes the disassembly of ceramide channels and represents a possible positive feedback loop (as ceramidase hydrolyzes ceramide yielding sphingosine) [55]. In line with ceramide channel formation in the MOM, mitochondria contain the enzymes required for ceramide synthesis and metabolism [55-57]. Pro-apoptotic stimuli have been shown to increase the mitochondrial concentration of ceramide [55-57]. Similarly, ceramide enriched cell membrane domains have been implicated in CD95 (death receptor) clustering [52]. This represents yet another role of ceramide in apoptosis induction. The pro-apoptotic effects of ceramide are diminished when the enzyme glucosylceramide synthase (GCS) metabolizes ceramide to yield a glycosylated derivative, glycosylceramide [58-60]. Interestingly, many MDR cancer cell lines have displayed elevated levels of GCS and glycosylceramide [58-60]. The intracellular decrease of this pro-apoptotic second messenger may contribute to the ability of MDR cancer cells to divert apoptosis. Although many of the mechanisms of MDR have been validated, as discoveries in cellular physiology progress, new factors that contribute to MDR will likely emerge.

2. NANOCARRIERS FOR TUMOR-TARGETED DELIVERY

Nanotechnology offers an unprecedented opportunity in rational delivery of drugs and genes to solid tumors following systemic administration [61,62]. Examples of nanotechnology applied in pharmaceutical product development include polymer-based nanoparticles, lipid-based nanoparticles (liposomes, nanoemulsions, and solid-lipid nanoparticles), self-assembling nanostructures such as micelles, and dendrimers-based nanostructures among others (Figure 3). These engineered nanocarriers offer numerous advantages: small particle size, narrow size distribution, surface features for target specific localization, protective insulation of drug molecules to enhance stability, opportunity to develop nanocarriers that respond to physiological stimuli, feasibility for delivery of multiple therapeutic agents in a single formulation, combination of imaging and drug therapy to monitor effects in real time, and the opportunity to combine drugs with energy (heat, light, and sound) delivery for synergistic therapeutic effects. Regardless of the inherent properties of the drug candidates, the pharmacokinetics and distribution pattern upon systemic administration will be dictated by the properties of the nanocarrier system. For instance, particle size and surface charge of tailor-made nanocarriers regulate the biodistribution and pharmacokinetic properties of the nanosystems in the body.

The advantages of nanocarriers stem from the unique properties that result from their size. Compared to bulk material, nanomaterials have distinguishing characteristics which are largely the result of increased surface area to volume ratio [63-66]. These properties change as the size of the particle changes. The large surface area allows nanoparticles to be held in suspension and increases frictional forces as well as surface adsorption [63-66]. Additional material-dependent changes include the generation of superplasticity, changes in optical properties, changes in solubility, increased catalytic activity, increased heat capacity, superparamagnetism in magnetic nanoparticles, quantum confinement with semiconductor nanoparticles, and the

generation of surface plasmon resonance in metallic nanoparticles [63-66]. Altering parameters such as size, conformation, and charge can have profound effects on how a nanoparticle interacts with and behaves in a biological environment. This versatility of nanoparticles along with appropriate surface chemistry contributes to their usefulness as carrier platforms.

2.1 Passive and Active Targeting Strategies

Classically, drug delivery strategies have been divided into two categories: passive targeting and active targeting. Passive targeting includes the direct/local application of a therapeutic agent (such as injection into the tumor mass) and relies on the microenvironment of tumors to achieve accumulation of a therapeutic agent (such as using the enhanced permeability and retention effect to acquire tumor localization). Active targeting involves the use of targeting residues that are specific for cancer cells such as antigen targeting (antibody), carbohydrate targeting (lectins), and receptor targeting (ligands) [2]. Practically, these two strategies are not divided by a clear line. Active targeting also exploits the mechanisms of uptake involved in passive targeting. For example, the leaky vasculature of a tumor will enhance the uptake of both non-targeted therapies and targeted therapies.

Although specificity is the strategic goal of active targeting, the two are not synonymous. In most cases there will be a degree of residual accumulation, especially in organs of the reticuloendothelial system (RES) [67]. For example, a therapy that uses a vascular endothelial growth factor (VEGF) ligand to target cancer cells over-expressing VEGF will inadvertently target non-cancer cells that express VEGF. Formulations that employ active targeting, especially the use of antibodies, have the potential to be highly toxic to normal cells and may display different toxicity profiles depending on the developmental and immune state of an individual (i.e. different states of health such as infection can change the expression level of receptors in non-cancerous cells).

The preferred clinical approach to treating cancer is to target cells that are rapidly dividing [68]. However, when the microenvironment of a tumor is considered, such as hypoxic regions, it seems that the cells of most concern are the slowly propagating MDR cells. The many different approaches to treating MDR and the dynamic array of formulations indicate that the best targeting strategy (passive or active) is dependent on the design of the formulation and the success of the therapy is more reliant on the synergistic effect of the system components.

2.2 Multi-functional Nanocarriers

The objective of multi-functional nanoparticles is to achieve a compound effect using one system. These systems use variable strategies to attain a combination of targeting specificity, optimized pharmacokinetics, diagnostic/imaging capability, and delivery of multiple therapeutic agents. As evident from a survey of current literature, there are many different multi-functional formulations for cancer therapy and diagnosis. For a therapeutic agent to be effective in treating cancer, a clinically relevant dose must reach the site of the tumor, become internalized by the tumor, and retain in the tumor long enough to have a therapeutic effect. Multi-functional nanoparticulate delivery systems address three parameters: they allow tumor-specific targeting and facilitated uptake through surface modification (such as EGFR1 peptides), they can bypass rapid RES clearance with poly(ethylene glycol) (PEG) modification which allows long-circulation and higher uptake efficiency, and they can be loaded with high concentrations of a combination of therapeutic agents that can divert MDR and elicit a therapeutic effect.

In a recent review by Gottesman *et al.* [49] the strategies for targeting P-gp associated MDR are eloquently categorized as evading P-gp through the use of agents that are not P-gp substrates, engaging P-gp by the use of P-gp inhibitors that block the drug effluxing ability of

P-gp, and exploiting the properties of MDR cells such as receptor over-expression and collateral drug sensitivity [49]. In addition to these categories, there are alternative targeting strategies that focus on non-P-gp associated MDR. Many nanoparticle formulations for the treatment of MDR use a combination of these approaches to optimize the specificity and efficacy of the system (Figure 4).

3. NANOCARRIERS FOR COMBINATION THERAPEUTIC STRATEGIES

As the literature is replete with different multi-functional nanoparticle formulations for the treatment of MDR, the following systems are representative examples selected to illustrate the variability in the design and therapeutic approach of these systems (Figure 5). As evident, slight variations in a nanoparticle formulation can lead to profound differences in the therapeutic effect.

3.1 Combination Delivery and Drug Efflux Modulation

A common strategy for overcoming MDR in cancer is co-administration of a chemotherapeutic agent with an MDR modulator. Many formulations that use this approach actively target MDR cells. One such formulation is dual doxorubicin- and verapamil-loaded liposomes, with surface conjugated transferrin [69]. The rationale behind this formulation is that verapamil, a calcium channel antagonist, is also a P-gp inhibitor, albeit at very high doses; it incorporates verapamil in liposomes, reducing the associated cardiotoxicity [69]. Combining verapamil with doxorubicin would increase the amount of doxorubicin retained in MDR cells, thereby possibly increasing the effectiveness of the chemotherapeutic agent [69]. This system used transferrin to achieve targeting specificity as many MDR tumors over-express the transferrin receptor [69]. Comparison of this system with free doxorubicin administration ($IC_{50} = 23.4 \mu M$), non-targeted doxorubicin/verapamil loaded liposomes ($IC_{50} = 21.7 \mu M$), and targeted doxorubicin liposomes ($IC_{50} = 11.5 \mu M$) the transferrin-conjugated doxorubicin/verapamil loaded liposomes ($IC_{50} = 4.18 \mu M$) increased cytotoxicity in a MDR leukemia cell line [69]. To increase the efficacy of doxorubicin, this system exploits the properties of MDR cells (targeting with transferrin) while engaging the MDR pump (verapamil).

A novel polymer-lipid hybrid nanoparticle (PLN) formulation has been created to target MDR breast cancer cell lines with a combination of doxorubicin and the P-gp inhibitor GG918 [70]. This system combined lipids (tristearin and steric acid) with polymers (Pluronic-F68® and a hydrolyzed polymer of epoxidized soybean oil) to create a nanoparticle formulation with improved encapsulation efficiency of lipophilic cationic and non-ionic agents [70]. The study compared free doxorubicin solution, doxorubicin and GG918 solution, doxorubicin solution with GG918 PLN, doxorubicin PLN, doxorubicin PLN with GG918 solution, doxorubicin PLN with GG918 PLN, and a dual loaded doxorubicin/GG918 PLN formulation [70]. Although there was no significant difference in doxorubicin uptake with any of these formulations in comparison to free doxorubicin solution in wild-type human breast carcinoma cells, the most effective formulation in MDR breast cancer cells was the dual loaded doxorubicin/GG918 PLN formulation [70]. The researchers attest that the superiority of this dual loaded formulation compared to the co-administration of doxorubicin PLN with GG918 PLN is due to the spatial distribution of doxorubicin and GG918 [70] (Figure 6). The P-gp inhibitor must act in the same region of the cell where the chemotherapeutic agent is administered for the inhibitor to be maximally effective [70]. The significance of spatial localization is important to consider when designing combination nanoparticle therapies with P-gp inhibitors.

Another approach to obstructing drug efflux is the use of MDR1 targeted antisense oligonucleotides (ASO). One such system with an eloquent multi-functional design that addresses the drug efflux mechanism of MDR and the MDR mechanism of apoptosis inhibition employs PEG-modified liposomes loaded with doxorubicin, MDR1 targeted ASO, and Bcl-2

targeted ASO [71]. This system was evaluated *in vitro* in a MDR human ovarian cancer cell line and *in vivo* in *nu/nu* mice with xenografts of the MDR carcinoma [71]. After treatment with this formulation, immunohistochemistry demonstrated decreased expression of P-gp and Bcl-2 and increased expression of caspase 9 and caspase 3 (markers of apoptosis) [71]. This corresponded with increased cytotoxicity of doxorubicin as demonstrated by decreased cell viability *in vitro* studies and decreased tumor size *in vivo* studies [71]. This system was compared to treatment with saline (or media), empty liposomes, free doxorubicin in solution, PEG-modified liposomes loaded with doxorubicin, PEG-modified liposomes loaded with doxorubicin and Bcl-2 targeted ASO, and with PEG-modified liposomes loaded with doxorubicin and MDR1 targeted ASO [71]. The triple combination therapy was more effective than the other formulations both *in vivo* and *in vitro* studies. The experimental success of this formulation is very promising to the development of other multi-functional therapies that combine targeted ASO with chemotherapeutic agents to address multiple mechanisms of MDR.

3.2 Combination Drug Delivery and Modulation of Apoptotic Threshold

Inhibition of pro-apoptotic factors and/or increased expression of anti-apoptotic factors are characteristics of many MDR cancers. The result is a higher threshold for the induction of apoptosis and increased survival when exposed to cytotoxic insults. Many therapies attempt to exploit this characteristic of MDR cancer through the co-administration of pro-apoptotic modulators or inhibitors of anti-apoptotic factors with a chemotherapeutic agent. The rationale of this approach is that lowering the apoptotic threshold of MDR cells will render chemotherapeutic agents more effective in treating MDR cancer. One such formulation used a polymeric nanoparticle formulation of poly(ethylene oxide)-poly(epsilon-caprolactone) (PEO-PCL) to co-administer ceramide (a pro-apoptotic modulator) with paclitaxel in a MDR human ovarian cancer cell line [72]. Co-administration of paclitaxel and ceramide (10 $\mu\text{mol/L}$) in solution tripled the cytotoxicity of paclitaxel in the MDR cells when administered near the IC_{50} value for these cells (1 $\mu\text{mol/L}$) but was less effective at lower concentrations of paclitaxel [72]. Conversely, the co-administration of paclitaxel loaded PEO-PCL nanoparticles with ceramide loaded PEO-PCL nanoparticles (10 $\mu\text{mol/L}$) increased the cytotoxicity of paclitaxel at lower doses of paclitaxel (0.01 $\mu\text{mol/L}$) [72]. The nanoparticle delivery system appears to increase the cellular retention of therapeutics and is central to the success of this co-therapy in treating MDR cancer [72]. In a similar approach (elevating intracellular ceramide levels) a GCS (glucosylceramide synthase) antisense cDNA therapy was demonstrated to reverse MDR in human breast cancer cells [73]. The pivotal role of the intracellular GCS/ceramide balance was obviated by a study that used siRNA to silence GCS [74]. The silencing reversed MDR in human breast cancer cells by an apparent decrease in MDR1 (P-gp) expression [74].

As survivin is an inhibitor of apoptosis that is often over-expressed in MDR cancer cells, many therapies focus on silencing or knocking down survivin expression. One such system used polyamidoamine (PAMAM) dendrimer modified magnetic nanoparticles to deliver antisense survivin oligodeoxynucleotides (asODN) to a variety of cancer cells [75]. The design rationale for this system is that the PAMAM conjugated to the surface of the magnetic (iron oxide) nanoparticles would complex with the asODN and protect the asODN from degradation while the magnetic nanoparticle would function as the foundation for binding, facilitate uptake, and act as an imaging modality [75]. The system was compared to PAMAM magnetic nanoparticles conjugated with nonsense (non-complementary to survivin mRNA) ODN and evaluated in two breast cancer cell lines (MCF-7 and MDA-MB-435) and in liver cancer cells (HepG2) [75]. As demonstrated with RT-PCR and western blot analysis, the asODN PAMAM magnetic nanoparticles silenced survivin mRNA and protein expression in the three cancer cell lines while the nsODN system did not affect the levels of survivin mRNA and protein [75]. Incorporating a chemotherapeutic agent with this survivin silencing system could be a very

powerful approach to treating MDR cancer. Other common therapies include antisense mRNA survivin antagonists and short hairpin RNA targeting survivin [76,77]. Although silencing survivin expression seems to be a successful approach, more studies are necessary to investigate the therapeutic benefit of using survivin silencing in multi-functional formulations.

As demonstrated by the ceramide/paclitaxel PEO-PCL nanoparticle combination therapy, spatial co-localization does not appear to be as critical in combination therapies with chemotherapeutics that use apoptotic modulators as it is when using P-gp inhibitors (such as the doxorubicin/GG918 PLN study). This is most likely due to the membrane-spanning physiology of the P-gp pump compared to the intracellular localization of apoptotic factors. There are many additional targets for multi-functional therapies aiming to modulate the apoptotic threshold, such as p53, and as elucidated in the triple therapy mentioned in the previous section, targeting anti-apoptotic Bcl-2 family members.

3.3 Combination Drug Delivery and Intracellular pH Modulation

Exploiting the distinguishing characteristics of MDR cells is a common theme in the design of nanoparticulate delivery systems. The decreased pH associated with MDR cells is exploited in a variety of ways; some strategies aim to alter the intracellular pH while others rely on the use of pH sensitive constituents to control the release of therapeutic agents. Novel pH responsive polymers, such as poly(beta-amino ester) (PbAE, soluble below pH 6.5), are being incorporated into nanoparticle formulations to localize the release of agents in the acidic cellular environment of tumors and acidic subcellular endosomal/lysosomal compartments [78,79]. A nanoparticle formulation consisting of PEO-PbAE nanoparticles encapsulating paclitaxel has been evaluated in *in vitro* and in *in vivo* models and has been shown to increase intracellular (ovarian cancer cell; SKOV3) and intra-tumor levels of paclitaxel compared to administration of the drug in solution and increase the cytotoxicity of paclitaxel as demonstrated by a higher percentage of cell death *in vitro* and decreased tumor volume *in vivo* relative to paclitaxel solution [78,79]. Due to the inherent spatial requirement for activation (i.e., localized low pH), the PEO-PbAE nanoparticle system and similar pH sensitive nanoparticulate drug carriers have the potential to drastically reduce the toxic side effects associated with chemotherapy.

Drug loaded pH sensitive polymeric micelles have also been developed to target MDR cancer [80]. These micelles were formulated from the two block copolymers poly(L-histidine)- β -PEG-folate and poly(L-lactide)- β -PEG-folate to achieve dissolution below pH 6.8 and folate receptor targeting [80]. The system was used to encapsulate doxorubicin and was studied in *in vitro* and in *in vivo* models [80]. As expected, this system did not demonstrate a higher cell kill efficiency in wild-type breast cancer cells (MCF-7) at pH 7.4 in comparison to free doxorubicin solution, yet there was a dramatic increase in cytotoxicity in MDR breast cancer cells at pH 6.8 (20% cell viability) compared to free doxorubicin solution (85% cell viability) [80]. The *in vivo* studies yielded similar results as assessed by tumor volume measurements [80]. Both the *in vitro* and *in vivo* studies compared this formulation to doxorubicin loaded pH-insensitive micelles and doxorubicin loaded pH-sensitive micelles without folate residues; the pH-sensitive, folate modified system appeared to be superior to the other formulations in both the cellular and animal models [80]. The incorporation of active targeting in the design of pH sensitive carriers may aid the effectiveness of therapies that aim to treat aggressive MDR cancer and could further decrease accumulation in non-cancerous cells and associated cytotoxicity.

4. NANOCARRIERS FOR COMBINATION DRUG AND ENERGY DELIVERY

4.1 Drug Delivery and Ultrasound Therapy

To achieve tumor specific drug release, some researchers have combined the use of ultrasound with nanoparticle carrier systems. In one such study, researchers used high frequency ultrasound to enhance the release of doxorubicin from Pluronic® P 105 micelles in MDR and drug sensitive ovarian cancer cell lines [81]. The study used various concentrations of doxorubicin and compared the use of Pluronic® P 105 micelles, ultrasound (10 minutes of sonication by 69 kHz ultrasound at 3.2 W/cm²), a combination of Pluronic micelles and ultrasound, and free doxorubicin [81]. The highest cell-kill percentage (lowest IC₅₀) was achieved with the combination of doxorubicin loaded Pluronic® P 105 micelles and ultrasound exposure [81]. In a similar study of micellar encapsulated doxorubicin combined with ultrasound, frequency dependent enhancement was demonstrated for both WT and MDR ovarian carcinoma cell lines [82]. In parallel to the development of these systems is the improvement of clinical ultrasound focusing techniques [81]. Combining focused ultrasound with drug loaded nanoparticles may become a promising technique for targeting and eliminating MDR cancer cells. Relying on ultrasound to enhance delivery and to avoid residual toxicity represents an important technique for the design of combination delivery systems.

4.2 Drug Delivery and Hyperthermia

Combining drug delivery with hyperthermia has also been explored to overcome MDR. Combining hyperthermia with folate-receptor targeted liposomes loaded with doxorubicin has been demonstrated to increase cytotoxicity in MDR and drug sensitive cervical carcinoma cell lines relative to free drug administration [83]. The increased cytotoxicity of the combination therapy was more pronounced in MDR cells. Hyperthermia (incubation at 42°C for 1 hour) combined with the folate-targeted doxorubicin loaded liposomes resulted in IC₅₀ values of 0.38 µM for the MDR cells and 0.16 µM for the drug sensitive cells while the IC₅₀ values for free drug were 1.81 µM for MDR cells and 0.543 µM for drug sensitive cells [83].

4.3 Drug and Photodynamic Therapies

Combination therapy using nanoparticle delivery systems has been used to enhance the delivery of therapeutic proteins. The delivery of proteins is inherently challenging due to their high molecular weight, solubility, charge, and often bulky conformation. These parameters additively impair transport across the cell membrane and increase the likelihood of RES clearance. If able to permeate the cell membrane, proteins are highly susceptible to lysosomal degradation. This is the case for the majority of therapeutics including the delivery system itself (i.e., nanocarrier formulation). Therapeutics and targeting systems that enter the cell through the endosomal pathway are most often subjected to lysosomal degradation. To address these issues the technique of photochemical internalization (PCI) has been incorporated with nanoparticle carriers [84]. This technique employs amphiphilic photosensitizers that exhibit preferential membrane localization [84]. Endocytosis of the photosensitizers leads to their incorporation in endosomal membranes [84]. Illumination of the photosensitizers induces reactive oxygen species generation which disrupts membrane integrity and results in the release of the endosomal content into the cytoplasm [84].

PCI has been used in combination with EGFR1-targeting liposomes to deliver a protein to ovarian cancer cells [84]. To demonstrate proof of concept, the protein saporin was encapsulated in liposomes; saporin is a cytotoxic protein that is captured and degraded in the endosomal/lysosomal pathway [84]. OVCAR-3 cells were incubated with a combination of TPPS_{2a} photosensitizer and the liposome formulation in media for 18 hours, subsequently washed and illuminated [84]. This combination demonstrated higher cell-kill with the combination therapy of PCI and EGFR-targeted liposomes as well as for cationic liposomes

[84]. This system illustrates the importance of multi-functionalization and the synergistic effects that rely on component optimization. Using PCI as a PDT combined with a targeting carrier may be a viable technique for addressing the persistent MDR cells in a tumor population.

5. PROS AND CONS OF NANOTECHNOLOGY APPROACH

The unique properties of nanomaterials can be exploited in designing nanocarrier platforms for the treatment of MDR. The small size of nanocarriers allows them to enter tumor vasculature via the EPR effect. Yet, as with other therapeutics, penetrating the poorly vascularized regions of tumors is an obstacle. An approach to overcome this obstacle is to enhance the residence time of the nanocarrier and therapeutic payload in the tumor mass, relying on diffusion and the by-stander effect to accumulate therapeutic agents in these regions. Another benefit of using nanocarriers is that it is possible to highly conjugate the surface of the particles with bioactive and targeting residues, making multi-functionalization possible. Yet, the enhanced surface absorption of nanoparticles can lead to high RES clearance. Fortunately, PEG conjugation enhances circulation and assists the particles in evading RES clearance. The ability to deliver agents with different properties (i.e., solubility) and the ability to actively target cancer cells are additional benefits of using nanocarriers for the treatment of MDR. One of the major risks of using nanocarriers is that they have not been extensively characterized in a clinical setting (i.e. pharmacokinetics, biodistribution, toxicity). To further complicate this risk, each multifunctional nanocarrier system is unique and must be evaluated as a new formulation. At this stage of development generalizations about pharmacokinetics, biodistribution, and toxicity cannot be inferred for different formulations. Nevertheless, the unique properties of nanocarriers offer promising opportunities for the treatment of MDR tumors.

6. CONCLUSIONS

In addition to the unique properties of nanoparticles in preferentially accumulating at the tumor site *in vivo* by passive and active targeting strategies, the nano-platform also lays a foundation for the development of multi-functional cancer therapeutics, especially in the treatment of refractory disease. With greater understanding of physiological differences between normal and neoplastic tissues and advances in material design, there is an opportunity to develop multi-functional nanosystems that respond to physiological stimuli, enhance delivery of combination therapies as well as the potential to use combination drug therapy with thermal, sound, and light energies. Use of safe and effective multi-functional nano-platforms promises to alleviate many of the challenges in clinical cancer therapy to benefit patients in the future.

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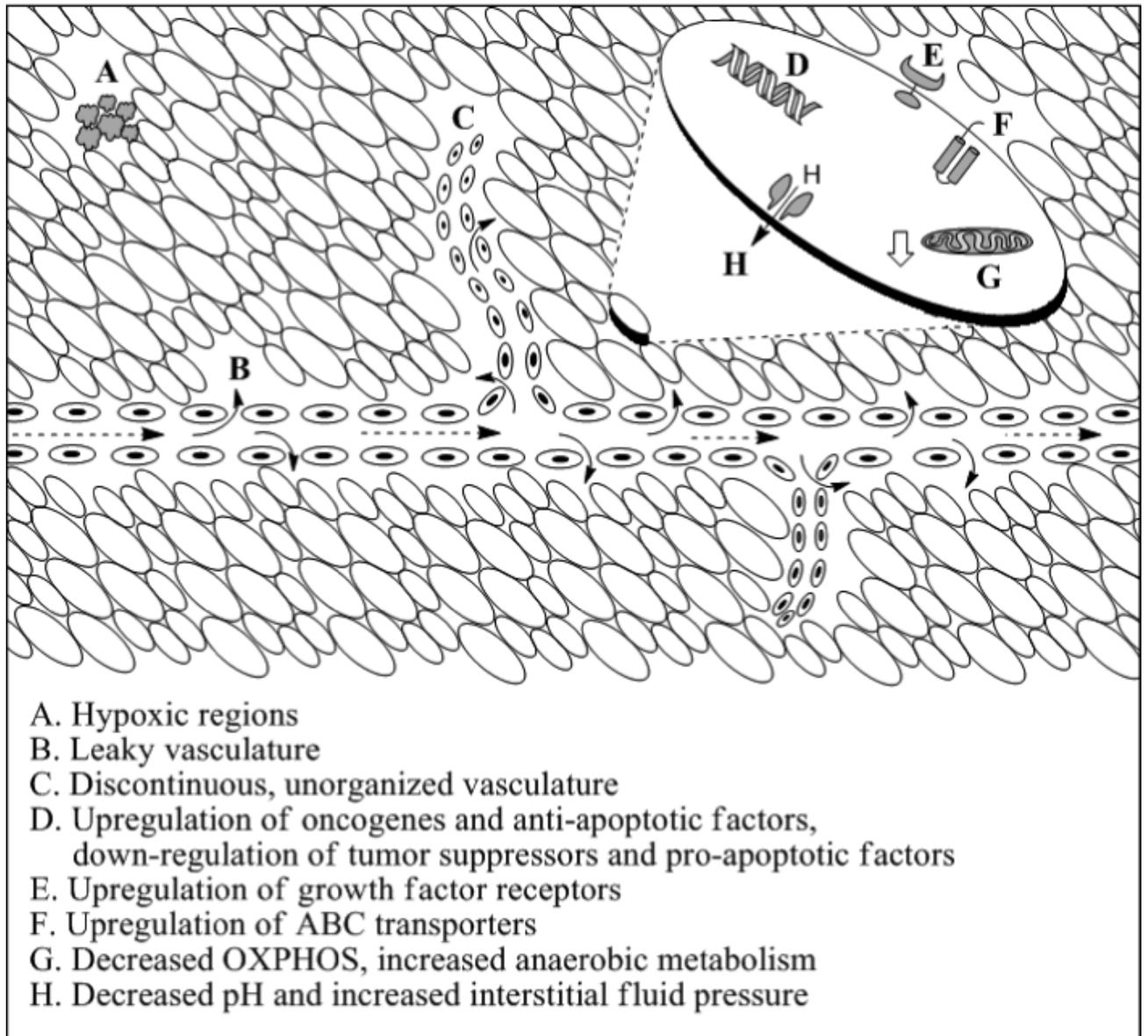


Figure 1.

Schematic illustration of the selection pressures in the tumor microenvironment that leads to development of multidrug resistance. Selection pressures such as hypoxia (A), genetic mutations in regulatory genes and altered regulation of apoptotic factors (D) can lead to cellular adaptation and aggressive MDR characteristics such as increased expression of growth factor receptors (E), increased expression of drug efflux pumps (F), reversion to anaerobic metabolism (G), decreased pH (H), and increased interstitial fluid pressure (H). The abnormal vasculature in the microenvironment of tumors (B and C) contributes to hypoxia (selection pressure) as well as to invasion and metastasis.

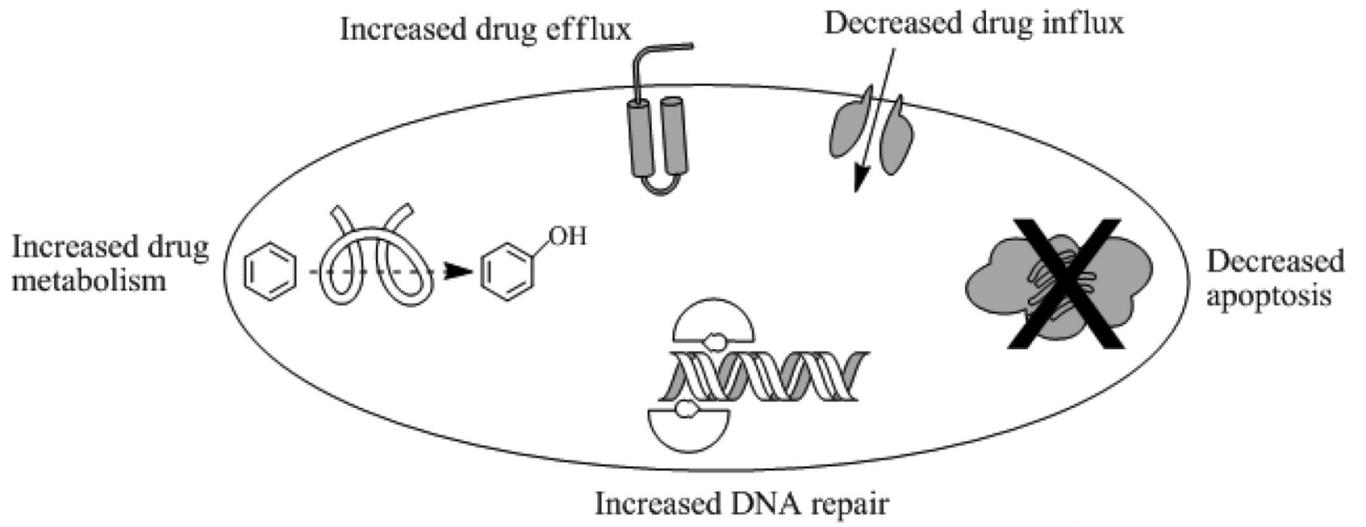


Figure 2. Mechanisms of multidrug resistance development in tumor cells. (Adapted from reference #5).

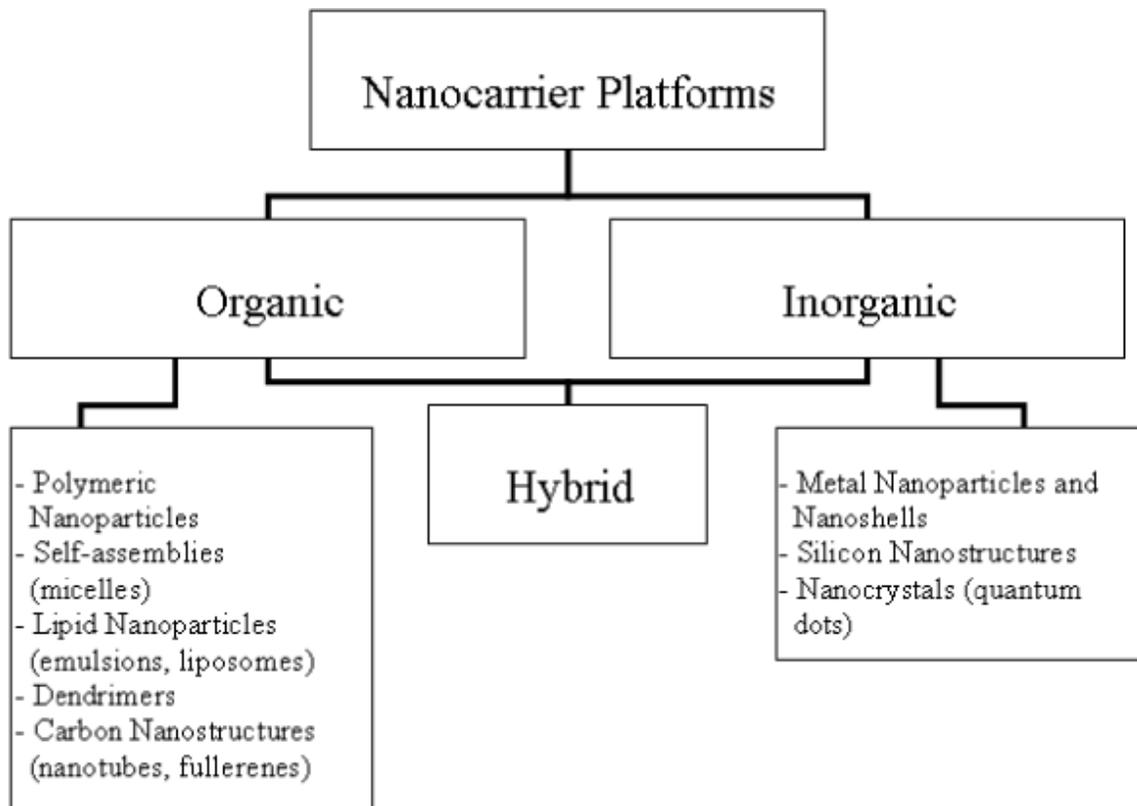


Figure 3. Different types of nanocarrier platforms used in tumor-targeted delivery.

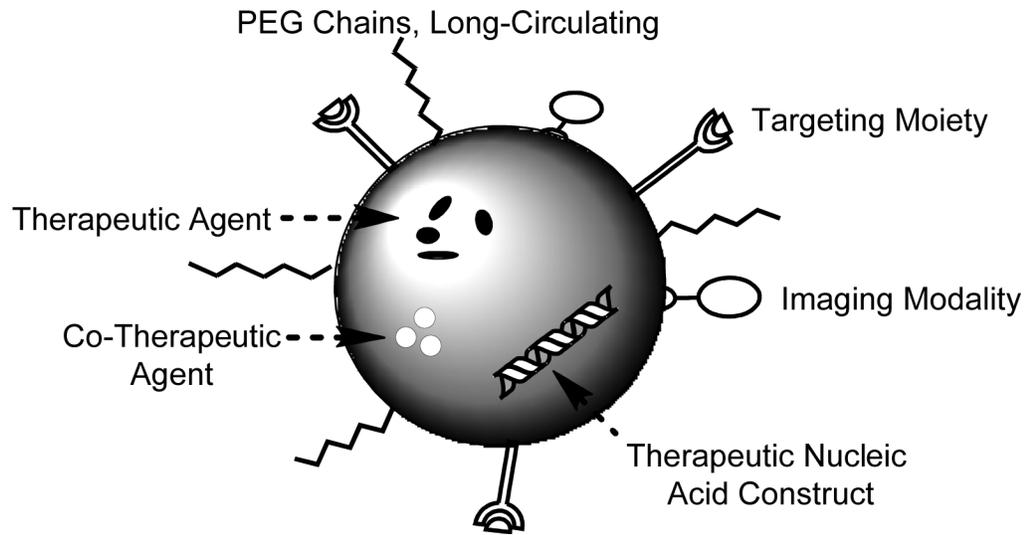


Figure 4.
Schematic illustration of multi-functional nanosystems.

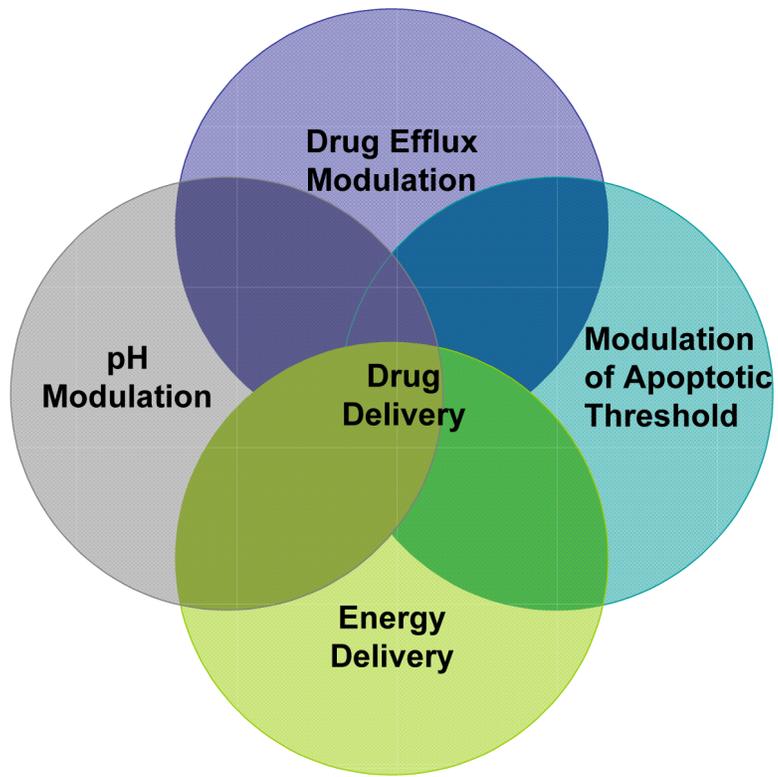


Figure 5.
Combination therapeutic strategies in overcoming tumor multi-drug resistance.

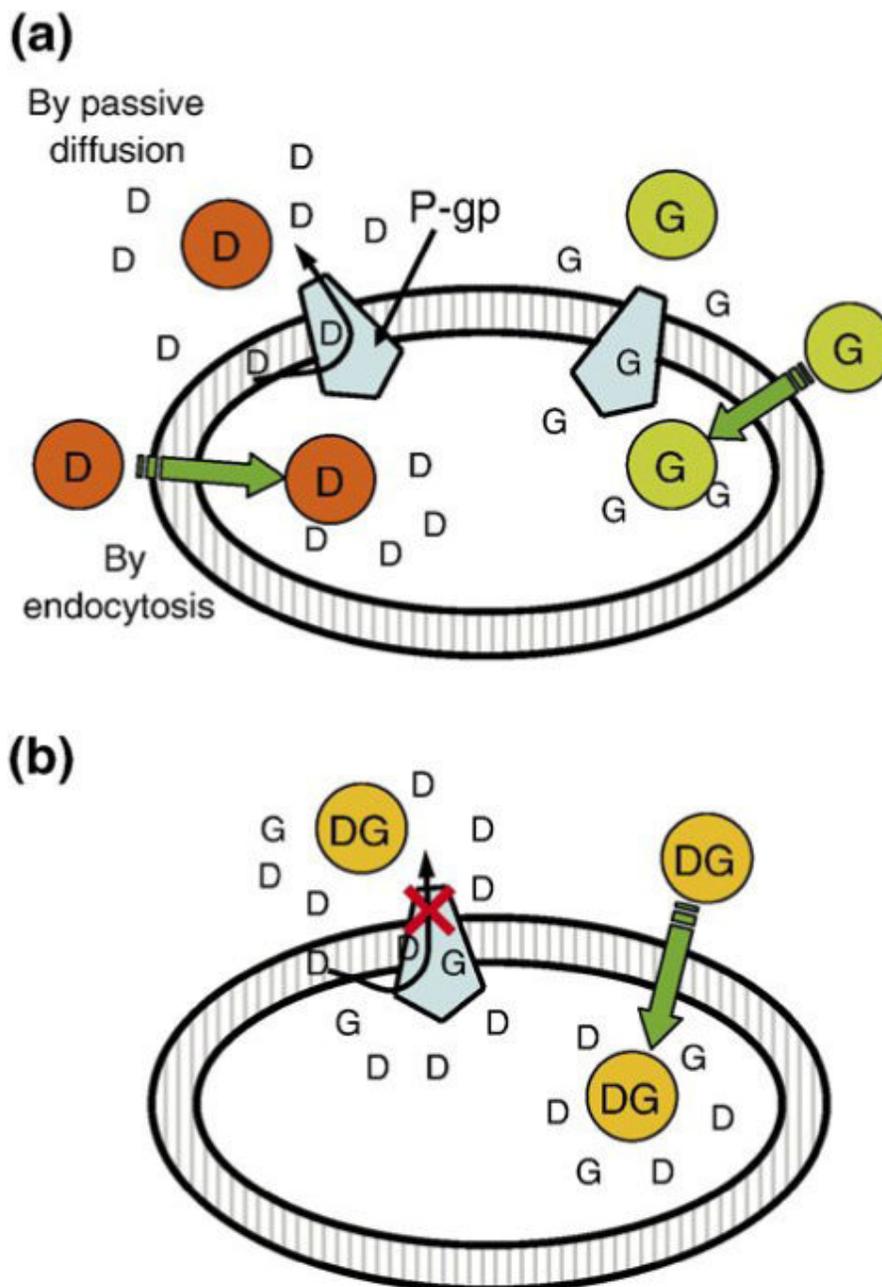


Figure 6. The importance of spatial distribution in combination therapy using a drug efflux inhibitor (G) and a chemotherapeutic drug (D). When separate PLN formulations are used to administer the agents (a) the inhibitor is ineffective in blocking drug efflux. The inhibitor is most effective when both the inhibitor and the drug are loaded into one PLN formulation (b). *Reprinted with permission from the Journal of Controlled Release 2006 Dec 1;116(3):275–84 © Elsevier [70].