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NANOPREPARATIONS TO OVERCOME MULTIDRUG RESISTANCE IN CANCER

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Abstract

Multidrug resistance is the most widely exploited phenomenon by which cancer eludes chemotherapy. Broad variety of factors, ranging from the cellular ones, such as over-expression of efflux transporters, defective apoptotic machineries, and altered molecular targets, to the physiological factors such as higher interstitial fluid pressure, low extracellular pH, and formation of irregular tumor vasculature are responsible for multidrug resistance. A combination of various undesirable factors associated with biological surroundings together with poor solubility and instability of many potential therapeutic small & large molecules within the biological systems and systemic toxicity of chemotherapeutic agents has necessitated the need for nano-preparations to optimize drug delivery. The physiology of solid tumors presents numerous challenges for successful therapy. However, it also offers unique opportunities for the use of nanotechnology. Nanoparticles, up to 400 nm in size, have shown great promise for carrying, protecting and delivering potential therapeutic molecules with diverse physiological properties. In this review, various factors responsible for the MDR and the use of nanotechnology to overcome the MDR, the use of spheroid culture as well as the current technique of producing micro tumor tissues *in vitro* are discussed in detail.

Keywords

Nanopreparations; Multidrug resistance; Spheroid culture; Combination therapy

1. Introduction

Multidrug resistance (MDR) is the most frequent phenomenon by which cancer cells elude chemotherapy. Mechanisms responsible for the MDR can be broadly divided into cellular factors and physiological factors. Cellular factors include altered molecular targets, increased drug metabolism, genetic defects such as polymorphism and gene deletion, reduced apoptosis, and over-expression of efflux pumps whereas physiological factors include cell-cell interaction, higher interstitial fluid pressure, low pH environment, hypoxic region in the tumor core, irregular tumor vasculature, and the presence of cancer cells in areas difficult to penetrate. Most of these factors lead to the requirement of higher doses of chemotherapeutic agents, which demonstrate systemic toxicity. Based on the type of disease

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and given treatment, mechanisms responsible for the demonstration of MDR vary. Cellular mechanisms have been studied extensively using monolayer cell culture. However, it is impossible to study physiological factors responsible for the MDR using the monolayer culture. Numerous researches have been carried out using organ culture to better understand physiological factors. However, obtaining tumor tissue from patients or developing them in lab animals is expensive and time consuming. Therefore, use of micro tumor tissues grown using spheroid culture is gaining increasing interest to study physiological factors.

Use of chemotherapy has proven beneficial in improving survival rate for cancer to some extent. However, it is often associated with severe systemic toxicities. Advances in cancer research have also identified large molecules such as proteins and nucleic acids as potential cancer treatment options. Their stability in biological samples though remains poor. In last three decades, therefore, nanopreparations have been evaluated to improve the delivery of potential therapeutic molecule to cancer site and to improve their stability in systemic circulations while minimizing the exposure to normal tissues to reduce unwanted effects. Many of these nanopreparations provided research community with flexibility to fabricate delivery system according to their target site as well as therapeutic molecule. In many cases, the use of such nanopreparations has resulted in significant improvement in treatment response.

In this review, various factors responsible for the MDR, the use of spheroid as a potential model to study physiological factors responsible for the MDR as well as nanopreparations evaluated to overcome the MDR are discussed in detail.

2. Mechanisms responsible for the MDR

2.1 Cellular factors

Heterogeneity and mutations among cancer cells help develop certain mechanisms, responsible for eluding chemotherapy, which improve their survival odds. As was said, these mechanisms include decreased drug influx, increased drug efflux, increased drug metabolism, increased DNA repair, lack of apoptotic machinery and increased anti-apoptotic machineries (Figure 1). Out of many possible mechanisms, those encountered frequently in clinic and having the most impact on the outcome of chemotherapy are discussed in detail.

2.1.1 ATP binding cassette (ABC) transporters—ABC transporter super family consists of several members subdivided into several subfamilies based on their structure (Table 1). Each member might be identified by various names as several groups were involved in characterization and nomenclature. ABC transporters are membrane proteins with several domains such as nucleotide binding domain (NBD) and transmembrane domain (TBD) [1, 2]. The major function for ABC transporters is to efflux molecules out of cells. As the process is against the concentration gradient, it requires energy which is obtained by ATP hydrolysis at NBD [3, 4].

Amongst all the members of ABC superfamily, ABCB1 (P-glycoprotein), ABCC1 (MRP1) and ABCG2 (BCRP) are most frequently over-expressed proteins in cancer cells. Over expression of such drug transporter proteins on cancer cells leads to reduced intracellular accumulation of chemotherapeutic agents resulting in requirement of administration of higher doses [4–7]. Researches carried out by various groups have identified various substrates belonging to one or more groups such as taxols, anthracyclines, mitoxantrone, topotecan, and etoposides for drug efflux proteins belonging to ABC superfamily [7, 8]. As these proteins have broad substrate specificity and can efflux drugs belonging to various different classes, this phenomenon is referred as the multidrug resistance (MDR) [5, 9–11].

From initial discovery of ABC efflux transporters to subsequent importance in maintaining homeostasis, many researchers have identified their significant role in developing multidrug resistance for cancer cell survival upon chemotherapeutic treatment. Doyle *et al*, identified that the breast cancer resistant protein (BCRP), an ATP dependent transporter, plays vital role in demonstrating drug resistant phenomenon against various drugs such as doxorubicin, daunorubicin and mitoxantrone in MCF-7/AdrVP cells [12]. Numerous other researchers have associated reduced accumulation of chemotherapeutic agents in cancer cells with overexpression of MDR1 gene *in vitro* [13–15]. Abolhoda and Wilson *et al.*, demonstrated significantly higher MDR1 gene activation within 50 minutes in patients receiving doxorubicin via isolated lung perfusion circuit [16]. Such data have given insight about rapid adaptability of tumors to survive upon chemotherapeutic treatment. Requirement of higher dose to achieve similar toxicity post over-expression of ABC transporters makes them an ideal target to overcome MDR demonstrated by tumors. Further, various approaches utilizing nanopreparations to overcome MDR such as inhibition of ABC transporter expression using siRNA, use of ABC transporter inhibitors along with chemotherapeutic agents, or to bypass ABC transporters are discussed.

2.1.2 Defective apoptotic machineries—Apoptosis, programmed cell death, is portrayed by cell shrinking, plasma membrane blebs, DNA damage by condensation and fragmentation, organelle integrity maintenance and finally signaling and subsequent removal by phagocytes [17, 18]. There are two pathways defined for the apoptosis, (a) the extrinsic pathway which depends on caspase activation signals generated by attachment of ligands to the surface receptors (example, death receptors) and (b) the intrinsic pathway which is activated by several stimuli at the mitochondria causing the activation of caspase-dependent and caspase-independent pathways resulting in cell death [19]. These two pathways are inter-linked in many ways and may even proceed concurrently. The apoptosis is an important phenomenon in the life of a cell and hence it is highly regulated by pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-X_L) moieties. It regularly involves inputs of the tumor suppressor p53 and p63/73 as well as the inhibitors of apoptosis (IAPs) family (survivin, livin) [20–22]. However, the cancer cells undergo several adaptive changes to evade the apoptosis and exhibit survival. Following are a few ways in which the cancer avoids the apoptosis.

Upregulated anti-apoptotic proteins like Bcl-2, Bcl-X_L and Mcl-1 are one way to avoid the apoptosis [23, 24]. These proteins possess the ability to strongly antagonize the pro-apoptotic proteins to induce multi-drug resistance. It has also been shown that a relation exists between the expression of ABC transporters and the over-expression of Bcl-X_L, suggesting that ABC transporters may have pro-/anti-apoptotic features too [25]. Ajabnoor, GM *et al.,* showed in *in vitro* experiments how the long-term exposure of paclitaxel to MCF-7 cells resulted in an increase in the levels of P-gp expression and down-regulation of caspases as a method to avoid paclitaxel-induced apoptosis [26].

Also, upregulated are the IAPs and the PI3K/Akt pathways. The IAPs act against the caspases and prevent their apoptotic processes. In contrast, the PI3K/Akt activation mediates its actions by supporting cell growth and thus encouraging survival [27]. Akt produces cell survival, particularly, through NFκB activation and Bad inactivation. Thus, targeting IAPs and PI3K/Akt may assist in the reversal of MDR. Apart from these intracellular changes, cell surface receptors responsible for the extrinsic pathway of apoptosis are also modified to avoid apoptotic signals. For example, instead of death receptors on the cell membrane, there is an up-regulation of the decoy receptors and even mutated death receptors which do not let the extrinsic apoptotic signal to proceed [28, 29]. Also upregulated is the c-FLIP which inhibits the pro-caspase 8 [25].

It has been shown that defects in the p53 gene are observed in most tumors. The mutations in the gene confer genomic instability and apoptosis resistance to the p53. Recently, MDR was observed in ovarian cancer cells from a patient treated with paclitaxel and cisplatin due to alterations in the cell growth patterns, over-expression in mutant p53, and P-gp [30]. Finally, the repression of the pro-apoptotic factors also plays a major role in the MDR development in the cells. The pro-apoptotic proteins including Bax, Bim among others of the Bcl-2 family have been shown to be down-regulated or mutated in cancer demonstrating resistance [31, 32].

2.1.3 Altered DNA repair pathways—Chemotherapeutic agents like the alkylating agents (busulfan, cyclophosphamide), antimetabolites (mercaptopurine), anthracycline (daunorubicin), platinum compounds (carboplatin, cisplatin), and epipodophyllotoxine (etoposide) act by damaging DNA directly or indirectly. This makes the DNA repair system a good target for the cancer cells to alter in order to induce MDR. The DNA repair pathways most often targeted are: (a) reversion repairs, (b) base excision repair (BER), (c) nucleotide excision repair (NER), (d) mismatch repair (MMR) and (e) double-strand break (DSB) repair [25, 33, 34].

The reversion repair involves the role of MGMT (O^6-methylguanine-DNA methyltransferase). It plays a role in resistance by acting as a DNA methylating agent. The natural role of the MGMT is to maintain the integrity of the DNA and the irony is that the damage caused by the methylating agents is the substrate for the MGMT. It repairs adducts at the O^6 position of guanine residues and then gets degraded. Similar to the MGMT action, the BER pathway repairs adducts at the N^7-methylguanine position.

The NER involves a complex mechanism of more than 20 proteins to repair the drug-induced large damage on the DNA. This mechanism is responsible for resistance to platinum-based compounds and alkylating agents. Of the several proteins involved in drug repair via NER, the protein ERCC1 (excision repair cross-complementing protein 1) deserves a special mention. A study showed how the cisplatin therapy induced the over-expression of ERCC1 via MAPK pathway to induce drug resistance in melanoma [35]. A few other studies have also shown the relation in between ERCC1 and tumor resistance towards platinum-based drugs [36, 37].

MMR system is responsible for the repair reactions on the DNA drug-induced breaks. The proteins involved in this system act by removing the DNA adducts, however, when the number of adducts is larger than the capacity of the system to repair, it is unable to repair the damage. This leads to the induction of cell death. Thus, it is observed that if the MMR system is down-regulated, the resistance of the cell towards the drugs increases [38]. Double-strand lesions in the DNA are the most harmful for a cell. Homologous recombination and non-homologous end joining repair are the main mechanisms involved in the DSBs repair process. A study carried out to study the effect of DBS repair on cisplatin administration determined that the cisplatin induced resistance in the cells was due to decrease in the activity of non-conservative DSB rejoining mechanisms primarily because of non-homologous repair [39].

2.1.4 Other mechanisms—Cancer cells have an extraordinary tendency to adapt to their environment, due to which they survive and thrive. Besides most commonly encountered phenomenon such as over expression of efflux transporter pumps and defective apoptotic machineries, numerous researches have shown other adaptive mechanisms responsible for the MDR. Recently, Lu *et al.* demonstrated the inhibition in the rate of apoptosis in gastric cancer when survivin gene was over-expressed [40]. Similar study was performed to compare survivin expression and clinical outcome of patient with renal cell carcinomas. In

this study, the over-expression of survivin was associated with increased tumor grade and low recurrence-free survival [41]. Increasing evidence indicating over-expression of survivin in cancer cells only rather than in normal cells, as well as its profound impact on inhibition of apoptosis and clinical outcome makes it an interesting target to overcome the MDR phenomenon [42–49].

Cancer cells have also been shown to acquire drug resistance against chemotherpuetic agents aimed at vital regulators such as tyrosin kinases, controlling proliferation, angiogenesis, and DNA repair. Cancer cells achieve this feat either by carrying out mutations or by altering protein expression [50–52]. Other similar examples include mutations of folate transporters leading to resistance towards toxic folate analogues [53].

2.2 Physiological Factors

In recent years, a lot of attention has been drawn to the physiological factors causing resistance to cancer therapy, which involve tumor physiology, tumor microenvironment and 3D structure of the solid tumors. The solid tumor microenvironment differs in multiple characteristics from the normal tissue. The physiological factors primarily responsible for the MDR and under investigation are high interstitial fluid pressure (IFP), hypoxia and low extracellular pH (pHe) [54–57]. These factors are inter-related and considered to affect one another (Figure 2).

2.2.1 Interstitial fluid pressure (IFP)—In normal tissues, the process of angiogenesis is well regulated by pro-angiogenic and anti-angiogenic factors acting in coordination. The blood vessels thus formed are highly organized and they efficiently meet the requirements of nutrients and oxygen for the tissue. Also well defined in the normal tissues is the lymphatic system which drains the fluids and metabolic products from the tissue interstitium. However, in solid tumors, the balance between the angiogenic factors is disturbed and leads to the development of an unorganized network of vasculature. In essence, the tumor vasculature is dilated and tortuous with presence of loops, blunt-ends and arteriolar-venous (AV) shunts [58, 59]. These abnormal characteristics along with poorly formed fenestrations lead to leaky tumor vessels and irregular blood-flow within solid tumors [60, 61]. Because of this leaky vasculature, fluids as well as proteins are released into the interstitial space. The absence of the lymphatic system results in accumulation of these and as a consequence an increase in interstitial fluid pressure (IFP) up to 100 mmHg as compared to normal interstitial pressure (equal to the atmospheric pressure) is observed [55, 62, 63]. Furthermore, the tumor cells also compress the blood vessels, which exacerbates the problem [64]. A feature of the IFP is that it is homogenous in the center of the tumor and near the periphery it becomes fairly equivalent to the normal values [65]. Also, because the blood vessels are poorly formed within a tumor and the network is heterogeneous, the rate of cell proliferation is greatly reduced in the areas of tumor distant from the vessels [66].

Multi-drug resistance (MDR) can be observed in the solid tumors as an effect of the unique tumor physiology as detailed above. A large number of chemotherapeutic drugs are administered through intravenous injections and thus they depend on the systemic circulation to reach the respective targets. The irregular tumor vasculature and the high IFP can obstruct successful drug delivery [67–69]. Because of the decreased blood flow, transcapillary fluid flow and the convective transport, the drugs, especially large molecules like proteins, peptides and antibodies, do not extravasate and distribute in the tumor uniformly. Another important feature of the tumor tissues is deep localization, cells located far from the blood vessels, (up to 200 μm) are posing difficulty for drugs to penetrate and reach the viable cancer cells [61]. Small molecules, which rely on the diffusion for transport, are affected by the concentration gradients, drug characteristics, the ECM, and by

metabolism in the tumor tissue. Studies have shown that sequestration in endosomes and binding with ECM components and/or DNA has direct relation to poor distribution of drugs like doxorubicin and mitoxantrone [70]. Moreover, binding of the targeting ligands like antibodies to the antigen-presenting cells on the tissue periphery prevents further penetration into the tissues [71, 72]. Some anti-cancer drugs act exclusively on rapidly-dividing cells. In case of the tumor tissue, the cells that are farther from the blood vessels are usually non-proliferating and furthermore, these cells may be exposed to only a low dose of drug because of limited penetration and distribution [73]. Such factors also contribute to the drug resistance. Additionally, the rapidly proliferating cells near the blood vessels may be killed by the cytotoxic drugs leading to exposure of the deeply-localized cells to the tumor vasculature which are inherently drug resistant in nature.

2.2.2 Hypoxia and low extracellular pH (pHe)—Another physiological factor contributing to the MDR is hypoxia. It has been estimated that up to 50–60% of locally advanced tumors will have hypoxia. The pathogenesis of tumor hypoxia is well explained by Vaupel *et al* [74, 75]. As compared to the normal tissues where the demand-supply of the O_2 is balanced, the tumors have low supply because of the irregular blood vessels and the lack of sufficient RBCs [76]; the imbalance further affected by the higher consumption rates of the cancer cells. According to Vaupel, there are 3 major mechanisms involved in the development of hypoxia. 'Perfusion-limited O_2 delivery' due to structural and functional abnormalities of the microvessels leads to transient ischemic hypoxia or acute hypoxia. 'Diffusion-limited O_2 delivery' or 'chronic hypoxia' is due to worsening of the diffusion mechanisms, and finally 'anemic hypoxia' is either tumor- or therapy-associated decline in the oxygen carrying capacity of the blood. Chronic hypoxia develops because of the increase in cell distance of around 100–200 μm from the blood vessels, which is beyond the distance O_2 can diffuse through. Often as a result of this, the pO_2 in the hypoxic regions is less than 5 mm Hg [77–79]. Tumors are characteristically heterogeneous in terms of their hypoxic regions but they usually form a hypoxic/necrotic core and an outer shell of rapidly proliferating cells [61]. Low pO_2 in tumors forces them to rely on pathways different to aerobic respiration to obtain the necessary ATP for all cellular processes, the major being the anaerobic glycolysis pathway. In 1956, Warburg observed that tumors produced high amount of lactate via the glycolysis pathway, a phenomenon termed as 'Warburg effect' [80]. Indeed, the hypoxic cells prefer glycolysis rather than oxidative phosphorylation as it is more efficient pathway that produces of CO_2 and carbonic acid [80–82]. The absence of the lymphatic system obstructs the clearance of the above acidic products of glycolysis metabolism. Low extracellular pH (pHe) thus ensues [54, 55, 83]. In contrast, the pH inside the cells is relatively normal due to the action of the ion pumps like V-type H^+ ATPase (proton pump), the Na^+/H^+ exchanger and the Na^+-dependent Cl^-/bicarbonate exchanger. The pHe may drop to 5.8–7.2 while the normal blood/tissue pH is about 7.4 [84].

MDR is a phenomenon often attributed to the hypoxia in the tumors [85]. Radiation therapy requires O_2 so as to generate reactive oxidative species (ROS) within the cells and lead to the cell death via DNA damage. The absence of the O_2 renders the radiation therapy ineffective against the hypoxic cancer [86] with well-oxygenated cells requiring just one-third of the dose to achieve similar levels of cell death [55]. Similar resistance is observed against drugs that are dependent on the pO_2 such as the alkylating agents (melphalan), the antibiotics (bleomycin) and the podophyllotoxin derivative (etoposide) [87, 88].

The interactions between the tumor cells and its microenvironment as described above induce several proteomic/genomic changes in the cells with poor prognosis [74, 89, 90]. Hypoxia-inducible factors (HIFs), esp. HIF-1, are activated in hypoxia accounting for transcription of more than 70 genes responsible for tumor survival and proliferation [79, 91]. Along with low pHe, HIF-1 induces the expression of vascular endothelial growth factor

(VEGF), nitric oxide synthase (NOS), carbonic anhydrase, transforming growth factor (TGF-β), erythropoietin, interlukin-8, matrix metalloproteinases (MMP) among others [89, 92–94]. Song *et al.,* [95] showed that HIF pathway caused hypoxia-related resistance to cisplatin and doxorubicin in non-small cell lung cancer. Under the harsh environment of anoxia, the cell cycle may get arrested in either G1/G2 or S phase and cause resistance to the cycle-selective cytotoxic drugs (5-FU, paclitaxel) and PARP inhibitor (Veliparib). Hypoxia also induces an increase in the DNA repair enzymes and confers resistance to the DNA-damaging agents like alkylating agents or platinum compounds (cisplatin). Down-regulation of BAX and BID (apoptotic agents) and genetic mutations in the p53 gene confer resistance to the apoptotic machinery under hypoxic conditions [87, 88].

One of the most important effects, albeit indirect, of hypoxia is the expression of the ATP-binding cassette (ABC) transporters, which efflux the drugs out of the cells. Various ABC transporters like P-gp, MRP-1 and BRCP confer resistance to the tumors against drugs like doxorubicin by active efflux mechanisms as shown in sections above [96–98]. It has also been shown that hypoxia-induced resistance of pancreatic cancer cells to gemcitabine is primarily through the activation of the PI3K/Akt/NF-κB pathways and also through the MAPK (Erk) signaling pathway [99]. It is also suspected to up-regulate the carbonic anhydrases (CA-IX) [100], and the subsequent study conducted by Koukourakis *et al.,* [101] showed that this produced resistance to chemoradiotherapy of squamous cell head and neck cancer. The CA-IX was located around the areas of necrosis proving that hypoxia induced the CA-IX.

Low pHe and normal pHi will result in the presence of a pH gradient across the cellular membranes. It has been shown that low pHe contributes to drug resistance *in vivo* and *in vitro* [102]. This pH gradient leads to the ‘ion trapping’ phenomenon [102, 103]. Ion trapping is a cause of extensive permeability difference between ionized and non-ionized forms of a drug. In the case of a weakly basic drug, the extracellular acid environment would make it ionized. The uncharged species are usually able to cross the plasma membranes, but the ionized form of the weakly basic drug would be trapped outside. This form of resistance is typically seen with cytotoxic drugs like anthracyclines (doxorubicin) and vinca alkaloids (vincristine) [102, 104]. This ion trapping is thus dependent on the pHe as well as the pKa of the drugs. For the weak bases as above, the pKa is around 7.5–9.5 and thus at low pHe (5.8–7.2), there are more ionized species formed unable to pass the plasma membranes. In contrast to this, the weakly acidic drugs like chlorambucil (pKa: 5.78) and melphalan (pKa: 1.83 & 9.13) easily cross the tumor plasma membrane [102, 105]. Vukovic has also shown that even though paclitaxel (taxane) and topetacan (topoisomerase inhibitor) are not structurally subjects of ‘ion trapping’, low extracellular pHe impaired their cellular uptake [106]. Moreover, the low pHe provides a harsh environment for the cells thus inducing changes in gene expression, autophagy, apoptotic potential and drug resistance. For example, it has been shown that low pHe leads to loss of p53 functions and clonal selection and expansion of the tumor populations [107]. In addition, studies carried out by LeBoeuf [108] showed that when Syrian hamster embryo (SHE) cells were cultured in low pHe medium, the rate of morphological transformation brought on by carcinogens increased significantly.

2.2.3 Other mechanisms—Studies have indicated that cancer cells grown in the form of solid tumors generate more mutations than the cells, which are grown in the form of monolayers giving an indication of the effect of tumor microenvironment on the genetic instability in tumors [109, 110]. One can also consider the interactions in the tumor stroma. The stroma comprises of the cells like fibroblasts, myofibroblasts, and immune cells. These cells also secrete cytokines and growth factors similar to the cancer cells, hence affecting the tumor microenvironment and the tumor’s growth [111–113]. Thus, the solid tumor

comprising of the cancer and non-cancer portions play a dynamic role on how the drugs will act [114].

A study carried out on human lung cancer line (INER-51) grown as spheroids showed increased levels of P-gp and decreased retention of doxorubicin by 3-folds exhibiting multicellular resistance [115]. As such, it has been shown that cell culture as spheroids exhibits resistance more relevant to the solid tumors *in vivo* as compared to the cells grown in monolayer form *in vitro* [116, 117]. Moreover, with solid tumors (10^8–10^9 cells/ml), the relative cell death by the same quantity of the drug may be less than when cells are grown in dilute tissue cultures (10^4–10^5 cells/ml) [54, 118]. Cell adhesion-mediated drug resistance, caused due to tumor cell – microenvironment interactions, is observed in several cancers and leads to decreased cellular proliferation, decreased apoptosis, alterations in drug targets, integrin signaling cascades & cytoskeletal rearrangements [119]. It has been shown that ECM renders small cell lung cancer (SCLC) resistant to etoposide-induced DNA damage and apoptosis by in a β1 integrin dependent manner. Apart from that, high levels of collagen IV, tenascin, and fibronectin were observed in the SCLC samples from 23 patients [120, 121].

It is not just the presence of these factors in solid tumors that makes it difficult to treat, but the variations in these factors result in unpredictable response to the treatment. Most research aimed at understanding and overcoming MDR phenomenon have focused on cellular factors giving little importance to the impact of physiological factors on the outcome making even very promising approaches ineffective. This is quite evident as survival rates for patients with advanced cancer remains low even after significant advances in utilization early detection techniques, understanding of many altered molecular pathways, discovery of highly potent chemotherapeutic agents, and use of promising nanomedicines. Therefore, it appears very important to overcome few if not all physiological factors along with molecular mechanisms to overcome not just multidrug resistance but to improve response to the treatment as well. Few ways by which physiological factors can be studied involve the development of tumor xenografts in animals, organ culture, and spheroid culture. Tumor xenografts represent an ideal setting to test physiological factors; however, it is far from high throughput. Organ culture also provides platform to study many physiological factors. Hurdles still remain in many areas, such as collection of enough tissues, and its shipments. On the other hand, spheroid culture, using which micro tumor tissues can be produced in bulk, is gaining increasing interest. In this review, use of spheroids to evaluate physiological factors, such as limited penetration due to cell-cell interaction and higher interstitial fluid pressure, hypoxia, and the presence of cells in diverse cell cycle stages is also discussed.

3. Nanopreparations in cancer research

Nanotechnology offers great advantages to the delivery of the drugs, genes and gene products to the solid tumors. The nanoparticles are usually composed of non-toxic, biodegradable constituents and possess varying loading capacities depending on the type of systems. They have a small particle size, exhibit prolonged circulation and can be targeted to required sites of action in the body. The main advantages of the nano-preparations are that they help deliver drugs with adverse solubility and stability characteristics, reduce non-specific accumulation and toxicity of the drugs, improve the pharmacokinetics and bioavailability and increase local drug concentrations at the target tissues [122]. Several types of nanocarriers are used in tumor-targeted delivery including micelles, liposomes, dendrimers, carbon nanotubes, nanocrystals, polymeric nanoparticles and others [96]. Early nanocarriers were optimized for stability and to achieve higher circulation time. This resulted in various nanoparticles with significantly improved antitumor activity. In last decade, in attempts to make nanopreparations even more effective, scientists have exploited

characteristics such as over-expression of various proteins and tumor micro-environment. These attempts have resulted in antibody-modified nanopreparations, receptor specific ligand-modified nanopreparations as well as pH-responsive multifunctional nanopreparations. In this review, the successful use of these nanoparticle-based drug delivery systems specifically targeting to overcome MDR is discussed.

3.1 Nanopreparations to overcome cellular factors responsible for the MDR

3.1.1 Targeting ABC transporters—Over-expression of drug efflux transporters is one the most widely encountered multidrug resistance phenomenon in clinic. These drug efflux transporters are also expressed in normal tissue to maintain homeostasis of various processes. It is, therefore, necessary to inhibit these transporters in tumor tissues while minimizing the exposure to normal tissues. To achieve this and to increase the localization of therapeutic molecules in tumor tissues, nanoparticles are widely used. Majority of our understanding has been drawn from work done on P-gp (MDR1). To overcome drug efflux mechanism, it is necessary either to bypass transporter recognition, inhibit its expression or inhibit its function.

Numerous studies have been carried out to bypass exposure of drugs to efflux transporters. Cuvier *et al.,* demonstrated equivalent doxorubicin nuclear localization in sensitive and resistant cells when delivered using nanospheres compared to free doxorubicin delivery [123]. Others have also demonstrated the reversal of drug resistance in P338/ADR cells when doxorubicinis delivered using polyisobotylcyanuacrylate (PIBCA) and polyisohexylcyanoacrylate (PIHCA) nanoparticles [124]. More recently, Susa *et al*., showed higher cytotoxicity of doxorubicin (Dox) in various cell cancer cell lines *in vitro* when the drug is delivered using a dextran-based polymeric nano-system [125]. In another study, Dox-tethered responsive gold nanoparticles were prepared and tested for the reversal of the MDR on MCF-7/ADR cells. These nanoparticles were designed to release drug cargo in acidic environment of organelle. They demonstrated significant nuclear localization of doxorubicin as well as reversal of the MDR when delivered using Dox-tethered responsive gold nanoparticles [126]. The utilization of the approach where nanoparticles are designed to bypass drug efflux transporters requires therapeutic molecules to remain incorporated in nanoparticle system until internalization. However, most nanoparticle systems are fabricated to accumulate in tumor microenvironment and release their cargo, which should allow maximum penetration and exposure to cells. Also, powerful tools have resulted in identification of many non-toxic potent efflux transporter inhibitors as well as identification of RNAi, using which expression of drug efflux transporters can be inhibited. Therefore, many groups have turned attention to co-deliver therapeutic molecules with either drug transporter inhibitors or their gene silencer.

Advances in molecular modeling and protein characterization techniques have resulted in identification of highly selective and potent drug transporter inhibitors (Table 2). Again, most of our knowledge about the potential of the drug efflux transporter inhibitors has been drawn from P-gp. Our group evaluated the co-delivery of tariquidar with paclitaxel using long-circulating liposomes for the reversal of the MDR. The incorporation of tariquidar in the liposomes showed efficient P-gp inhibition when examined on SKOV-3 and SKOV3TR cells (Figure 4). The treatment with the combination therapy demonstrated an improved substrate accumulation in SKOV-3 resistant variant as well as complete reversal of MDR measured by determining IC_{50} value. Specifically, the IC_{50} of paclitaxel treatment for SKOV-3TR was reduced from 2743 nM to 34 nM when cells were treated with the tariquidar-paclitaxel-liposomes as compared to free paclitaxel (Figure 5) [127]. Many other groups have demonstrated the potential of the co-delivery of anticancer drug with drug efflux transporter inhibitor for the reversal of the MDR [128–133]. However, mixed results

are obtained in early clinical trials [134–136]. Specially, tariquidar, a highliy selective and potent P-gp inhibitor, when combined with doxorubicin or paclitaxel showed limited clinical activity to restore sensitivity in advanced breast cancer patients [136]. It has, therefore, become very important to identify targets, study mechanisms and evaluate approaches to overcome the drug resistance phenomenon.

On the other hand, nucleic acid based approaches are gaining increasing interest as well. Wang *et al.*, demonstrated tumor growth inhibition of KB-A-1 cells by inhibiting MDR-1 gene levels with antisense oligodeoxynucleotides delivered in hydroxypropyl-chitosan nanoparticles [147]. Potential of siRNA to selectively inhibit gene of interest has been recognized as one of the most powerful tool to alter protein expression. However, its stability in biological matrices remains challenging. Numerous groups have utilized nanoparticle systems to protect enzymatic degradation as well as have customized sequence to target protein of interest to reverse the multidrug resistance in cancer [148, 149]. In a recent review, Abbasi *et al.*, have discussed the attempts to inhibit P-gp using RNAi for the reversal of the MDR [150]. In our lab, micelle-like nanoparticles were prepared using DOPE-modified PEI to deliver siRNA against P-gp. This formulation demonstrated significant loading of siRNA, improved transfection efficiency, P-gp down-regulation as well as improvement in therapeutic effects of doxorubicin in resistant cells [151]. Potential tools like siRNAs and effective delivery systems have opened possibilities of targeting numerous cellular proteins.

3.1.2 Targeting apoptotic machineries—The apoptotic machinery depends on the pro-apoptotic and the anti-apoptotic proteins and the imbalance in their expression renders the cells resistant to the drugs, as shown above. Hence, these proteins are naturally a target for the novel anti-cancer therapies. One such target is the anti-apoptotic protein Bcl-2. Bcl-2 protein functions by its inhibitory actions on the release of the cytochrome C from the mitochondria and thus preventing downstream apoptotic pathways. Bcl-2 is expressed highly in the tumors as a method to prevent apoptosis. Bcl-2 down-regulation will be a link to reduce the drug resistance in the tumors. Beh *et al* sensitized the HeLa cells to paclitaxel by downregulating the Bcl-2 mRNA and protein. They used the RNAi therapy by introducing cationic nanoparticles to deliver Bcl-2-targeted siRNA [152]. Similarly, mesoporous silica nanoparticles as well as cationic liposomes were used to deliver a combination of doxorubicin with Bcl-2 siRNA to improve cytotoxicity effects in resistant tumors [153, 154]. Cheng *et al.* developed a combination therapy of Bcl2-siRNA-doxorubicin in polymer-based nanosystems targeting the folate receptors and achieved 60% cell death at highest doxorubicin concentrations in tumor models [155]. Yet a different study used doxorubicin in combination with Bcl2-siRNA and MRP1-siRNA in liposomes to form liposomes-siBcl-2-siMRP1-doxorubicin. The use of two different siRNA with doxorubicin achieved up to 95% SCLC cell death *in vitro* [154].

An interesting molecule that acts as a pro-apoptotic agent is ceramide. It has been shown that administration of ceramide along with a drug restored the apoptotic signaling and prevailed over MDR in the tumors. The same group delivered ceramide and paclitaxel intracellularly by means of biodegradable polymeric nanoparticles, specifically poly(ethylene oxide) – modified poly(epsilon-caprolactone) (PEO-PCL). They reported an increase of up to 100 times in the sensitivity of the MDR SKOV-3 ovarian cancer cells by this approach [156–158].

Perhaps, the most important tumor suppressor gene is p53 which is invariantly downregulated in all tumors. Reinstating the functions of p53 or similar tumor suppressors within the tumor can aid in reducing the drug resistance. Plasmid DNA nanoparticles were used by Deng *et al.,* to express both p53 as well as FUS1 to exhibit the activation of the

apoptotic protease activating factor 1 (Apaf-1) dependent apoptosis pathway in human non-small cell lung cancer cells [159]. Moreover, some groups showed the activation of apoptotic pathway by the transfection of the p53 gene using small cationic lipid nanoparticles and poly($_{D, L}$-lactide-co-glycolide) (PLGA) respectively [160, 161]. It was also shown that such a p53 gene therapy mediated through transferrin-targeted nanoparticles sensitized the head and neck cancer xenografts towards radiation therapy [162]. The activation of apoptotic pathways in such manner can also prove useful in combination of regular chemotherapeutic agents. On these lines, the delivery of an epigenetic drug, 2′-deoxy-5-azacytidine (DAC), simultaneously with doxorubicin to the cancer cells was carried out through the lipid-polymer nanoparticles. While DAC caused the expression of tumor suppressor genes in the tumors, the doxorubicin acted as a traditional chemotherapeutic to hinder the tumor growth and stimulate apoptosis [163]. Likewise, the therapeutic efficacy of cisplatin in a human H322 lung cancer orthotopic xenograft mouse model was improved by a combination treatment of *FUS1*-nanoparticles and cisplatin [164].

c-FLIP obstructs tumor necrosis factor-α (TNF-α), Fas-L, and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis rendering the cells resistant to the drugs [165]. Several approaches have been made to work around this form of resistance using novel nano delivery systems in combination with orthodox chemotherapeutics like doxorubicin [166, 167].

3.1.3 Targeting DNA repair mechanism—*NPRL2* is the tumor suppressor gene, expression of which shows anti-tumor activity via the DNA mismatch repair (MMR), cell cycle checkpoint signaling, and regulation of the apoptotic pathways. When *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate : cholesterol nanoparticle was utilized to transfect *NPRL2* gene along with cisplatin *in vitro* and *in vivo*, the cancer resistance to cisplatin was reduced and greater tumor growth inhibition was demonstrated [168]. A combination of doxorubicin and mitomycin C encapsulated in tandem within novel solid polymer-lipid nanoparticles exhibited synergism in cell toxicity in the MDR MDA435/LCC6 human breast cancer cells. Importantly, it was observed that this synergism correlated with higher generation of DNA double strand breaks and subsequent apoptosis [169].

3.1.4 Other cellular targets—As mentioned above, the IAPs play an important role in cellular regulation of drug-induced apoptosis. Perhaps, the most studied IAP is the survivin mRNA and survivin protein, which confers resistance to the cells. Many studies have focused on the use of siRNA directed towards the survivin gene to reduce its expression. It has thus been shown that the co-delivery of the survivin siRNA along with the chemotherapeutic drugs via nanosystems has yielded improved cytotoxicity while reducing the drug-induced resistance in the tumors [170–172]. XIAP, or X-linked inhibitor of apoptosis protein, is responsible for the MDR and cell survival by avoiding apoptosis. Recently, biomimetic polymer-based nano-complexes with cell-penetrating peptides were developed by Jang *et al.,* simultaneously delivering paclitaxel and condensed XIAP shRNA, showing an enhanced efficacy of tumor inhibition in *in vitro* and *in vivo* models [173].

A tyrosine kinase receptor, EphA2, which can also function as an oncoprotein, was targeted by Landen *et al.* using siRNA in combination with paclitaxel to effectively reduce tumor growth in orthotopic ovarian cancer models in mice. To deliver this combination treatment, neutral liposomes using dioleyl-glycerophosphatidylcholine (DOPC) were formulated, achieving 86–91% tumor reduction as a combination therapy [174]. Approaches targeting the PI3K/Akt pathway have also resulted in decrease of the MDR in tumors. One such study involved the combination therapy of PTX and Akt1 shRNA using CLA-coupled poloxamer thermosensitive hydrogel system in breast cancer models MCF-7 and MDA-MB-231 *in vitro*

and *in vivo*. Clearly, the combination therapy synergistically induced apoptosis more effectively than individual treatments [175].

Recently, it has been found that Plk1 (Polo-like Kinase 1) is a potential target for therapy in chronic myeloid leukemia as it is responsible for imatinib resistance [176]. Micelleplexes delivering a combination of paclitaxel and Plk1 siRNA were synthesized using biodegradable tri-block copolymers of poly(ethyleneglycol)-b-poly(ε-caprolactone)-b-poly(2-aminoethylethylenephosphate) (mPEG-b-PCL-b-PPEE). The combination treatment efficacy studies carried out on MDA-MB-435 tumors showed an enhanced growth reduction and indicated that the co-delivery of drug and siRNA is a promising approach [177].

3.2 Nanopreparations to overcome physiological factors responsible for the MDR

As mentioned previously, solid tumors do not have well-defined tumor vasculature as a result of which the blood vessels have a leaky characteristic. The fenestrations and the pores in the endothelial cell linings are 200–600 nm in diameter. As a result of this, the high-molecular weight macromolecules as well as long-circulating nanoparticles tend to accumulate in such sites via the 'Enhanced permeability and retention' (EPR) effect (Figure 3) [122, 178]. Long-circulating nanocarriers refer to the delivery systems which avoid degradation through reticulo-endothelial system (RES) and prolong their half-life in the blood. An illustration would be the FDA-approved Doxil, which is doxorubicin in (PEG)ylated liposomes. (Polyethylene) glycol (PEG) coating on the liposome prevents the RES uptake by conferring steric hindrance.

3.2.1 Lowering IFP and targeting tumor vasculature—As discussed previously, high IFP leads to resistance in tumors. Hence, lowering the IFP seems to be a good rationale to improve blood flow and drug availability to the solid tumor. There are two ways to carry out the objective: (i) to normalize the tumor vasculature and (ii) to reduce the ECM contractility [55].

One target to normalize the tumor vasculature is the VEGF. It is produced in larger quantities in tumors as compared to normal cells and is responsible for the formation of the blood vessels. Moreover, VEGF binds to the endothelial cells of the blood vessels which are genetically stable compared to the cancer cells, thus making it a good target for therapy [179]. Bevacizumab (anti-VEGF antibody) can lead to decreased IFP and improved survival rates in combination with other chemotherapeutics, which shows how significant VEGF targeting is [180]. Using such anti-VEGF antibodies attached to the nanoparticles, the IFP can be lowered and chemotherapeutic drugs can be administered concurrently. Targeting VEGFR (VEGF receptor) can also aid in reducing resistance. Ambasta et al., proposed the development of 'twin nanoparticle' of iron coated with gold and targeting the VEGF-positive cell near to the cancer stem cells. The twin nanoparticle consists of a particle which recognizes cancer stem cell and another conjugated particle that recognizes the VEGF-positive cells. Such a strategy may inhibit the angiogenesis near the cancer stem cell and prevent new tumor formation and metastasis [179]. Inhibiting VEGF expression via use of small interfering RNA (siRNA) has been shown to be anti-angiogenic, reducing resistance of the tumors, inducing apoptosis, and inhibit proliferation [181, 182]. Nanoparticles have thus been utilized to deliver VEGF-siRNA and carry out RNA interference (RNAi). Polyelectrolyte complex (PEC) micelles were used to conjugate the VEGF-siRNA with PEG via a disulfide linkage (siRNA-PEG), and cationic polyethyleneimine (PEI) was used as a core-forming agent. It was shown to exhibit up to 96.5% gene silencing in optimized formulation conditions as well as in the presence of serum *in vitro* [183, 184]. These nanoparticles were also examined *in vivo* in prostate cancer model in mice with a visible inhibition of the VEGF expression and suppression of tumor growth with no inflammatory

response [185]. Another study used a delivery system of long-term sustained release poly ($_{DL}$-lactic/glycolic acid) (PLGA) microspheres encapsulating anti-VEGF siRNA with a carrier (arginine or branched polyethylenimine). The release of siRNA in phosphate buffer (pH 7.4) was sustained for over one month, and the *in vivo* studies in mice carrying S-180 tumors showed suppressed tumor growth [186]. Another study showed that the combination of VEGF-siRNA and interleukin-4 reduced angiogensis and tumor growth in SCID mice bearing U87 human glioblastoma cells [187].

However, one study reported no significant improvements in the delivery of Doxil® after lowering the IFP with a potent VEGFR/PDGFR inhibitor, pazopanib [188]. While the IFP was reduced and antiangiogenic effects were observed in human NSCLC xenografts, the Doxil® penetration was in fact reduced as compared to the control. This observation was in contrast to the proposed theory of improving blood flow and drug availability via lowering IFP. One proposed reason for this was that liposomes and similar nanoparticles rely on the hyperpermeability (EPR) to passively target the tumor, and that normalizing tumor vasculature would in fact reduce the chance for extravasation into the tumor interstitium.

Apart from the VEGF targeting, there have been multiple studies on delivery of chemotherapeutic drugs to the tumor vasculature exploiting presence of several targets. One such study relates to the use of tumor vasculature-targeting peptides PIVO-8 (SNPFSKPYGLTV) and PIVO-24 (YPHYSLPGSSTL) conjugated to liposomal doxorubicin for *in vivo* studies on many types of human cancer xenografts in SCID mice. Through this delivery system, an enhanced efficacy of doxorubicin was observed [189]. Another study incorporated a metal chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) into liposomes and enabled the engraftment of different peptides targeting VEGFR-1(p39-Flt-1) and neurophilin-1 (p24-NRP-1). The study observed that NTA_3-DTDA liposomes encapsulating doxorubicin and engrafted with targeting peptide enhanced the therapeutic efficacy compared to the non-targeted liposomal drug [190]. Yet another peptide sequence (RGD) was successfully used by Nasongkla *et al.,* to prepare polymeric micelles with surface-expressing peptides and deliver doxorubicin to Kaposi's sarcoma cells. The RGD peptide targets $\alpha_v\beta_3$ integrin, present at high levels in tumors and integral to the process of angiogenesis [191]. Nucleolin is a receptor expressed on tumor cancer cells as well as tumor endothelial cells. It can be targeted via the F3 peptide which is specifically internalized by nucleolin via the receptor-mediated endocytosis. The combination of F3 peptide with liposomes delivering eGFP-siRNA was also used, and the eGFP was successfully inhibited in breast cancer cells [192].

The use of charged liposome to target endothelial cells may prove beneficial as demonstrated by the superior accumulation of oxaliplatin in lung tumors when encapsulated in cationic liposomes instead of neutral liposomes, because the tumor endothelial cells are negatively charged and can internalize the cationic liposomes more efficiently [193]. Several studies have utilized cationic liposomes containing drugs like paclitaxel, etoposide, cisplatin, doxorubicin, camptothecin, and oxaliplatin to target tumor vasculature and achieve tumor growth inhibition [193–201]. Another potential target, pericytes, within the vasculature was exploited in a study. Aminopeptidase A (APA) is a marker for the pericytes and when it was used as a targeting ligand for doxorubicin-containing liposomes against neuroblastoma tumors, better efficacy was achieved as compared to the untargeted liposomes [202]. Combining doxorubicin-loaded liposomes with a tumor lymphatics-binding peptide (LyP-1) resulted in increased tumor accumulation and better therapeutic efficacy [203]. Few other illustrations of the use of nanotechnology to target the tumor vasculature and reduce the high IFP as well as reduce angiogenesis are discussed in this review [204].

3.2.2 Targeting hypoxia—Hypoxia and hypoxia-induced factor (HIF) esp. HIF-1α are primarily involved in the MDR of the tumors, and they are exclusively found in hypoxic tumors [205]. The HIF-1α pathway is responsible for many tumor promoter activities and thus targeting HIF-1α is a rationalmethod of drug delivery [206].

Silencing the HIF-1α gene showed the reduced resistance to chemotherapy and it also inhibits the tumor growth [95]. This study showed that the resistance of NSCLC cells to cisplatin and doxorubicin was through HIF pathway proving that it's silencing would revert the resistance. Silencing of the HIF-1α may be achieved by the use of siRNA and RNAi therapy. In recent study, cationic mixed micellar nanoparticles (MNP) to deliver HIF-1α siRNA have been proven to be a suitable nanocarrier in a PC3 prostate cancer xenograft murine model. It would be noteworthy that this system inhibited tumor growth, suppressed proliferation, and was anti-angiogenic in action without any innate immune responses. It was also observed that MNP - HIF-1α siRNA complex reduced the doxorubicin resistance in the PC3 cells both *in vitro* and *in vivo* [207]. A multifunctional carrier was used in another study to target the HIF-1α using siRNA. The carrier, (1-aminoethyl) iminobis [N-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO), showed pH-sensitive amphiphilic cell membrane disruption. It was PEGylated to prevent the non-specific cell uptake and the PEG-modified EHCO/siRNA nanoparticles exhibited good endosomal escape and tumor growth inhibition in human glioma U87 xenografts [208]. A novel drug delivery system was described by Bartholomeusz and his colleagues using the single-walled carbon nanotubes (SWNT) non-covalently coated with HIF-1α siRNA. Intratumoral administration of these nano-complexes in MiaPaCa-2/HRE tumor-bearing mice led to significant down-regulation of the tumor HIF-1α activity [209]. Employing HIF-1α inhibitors can enhance drug efficacy by inhibiting hypoxia-induced drug resistance. A novel HIF-1α inhibitor, JG244, a G-rich oligonucleotide (ODN), was used in combination with T40214 (a p-Stat3 inhibitor) to make ODN/PEI nano-complexes. Mice bearing human prostate tumor (DU145) and murine prostate tumor (TRAMP-C2) were treated with the nano-complexes to demonstrate therapeutic efficacy of the combination treatment and reduction of hypoxia-induced drug resistance [210].

Indirect inhibition of HIF-1signaling is also recommended as a drug therapy for hypoxia [211]. One way of the indirect inhibition is via the mTOR (mammalian Target of Rapamycin) inhibition [212]. The attempt was made to use rapamycin as a mTOR inhibitor, anti-angiogenic against MCF-7 cells *in vitro*. PEGylated and non-PEGylated liposomes were prepared encapsulating the rapamycin. It was observed that the non-PEGylated liposome-drug formulation was more active in terms of anti-proliferative efficacy while the PEGylated liposome-drug was more stable [213]. Iwase *et al.*, prepared liposomal everolimus (rapamycin analog) for *in vivo* targeting and improved therapeutic efficacy towards lung and thyroid cancers [214]. HIF-1 activity can also be influenced by Hsp90 inhibition [206, 211]. Thus, a potent Hsp90 inhibitor, 17-AAG, is being investigated for its synergistic effects with other anti-cancer drugs and it is known to reduce the MDR in multiple tumor lines. Shin *et al.,* prepared multi-drug loaded polymeric micelles for co-delivery of 17-AAG and other poorly soluble drugs against which tumor resistance have been observed [215]. Another Hsp90 inhibitor, geldanamycin, and its fatty acid prodrugs were encapsulated in amphiphilic block co-polymer poly(ethylene glycol)-b-poly(ε-caprolactone) micelles for *in vitro* studies on MCF-7 cells [216].

It has been observed that the EGFR is over-expressed in tumors due to hypoxia, hence making it a good target. Recently, EGFR-targeted nanoparticles were designed by synthesizing a poly (D, L-lactide-co-glycolide)/poly (ethylene glycol)/epidermal growth factor receptor-targeting peptide (PLGA/PEG/EGFR-peptide) construct for incorporation in poly(epsilon-caprolactone) (PCL) nanoparticles. These nanoparticles delivered a

combination of paclitaxel/lidocaine in human breast and ovarian cancer cells with a reduction in the MDR and potentiated the use of the combination therapy [217]. Multiple studies have been carried out to reverse the hypoxia-induced cisplatin resistance in tumors [218]. Thus, multi-hydroxylated metallofullerene nanoparticles were used to load cisplatin, and were targeted to cisplatin-resistant human prostate cancer cells. MDR cell survival decreased and the tumor growth inhibition was observed *in vivo* [219, 220]. Another group proposed a novel macrophage-based nanoparticle system to utilize the inherent hypoxia-targeting capability of macrophages. Nanoparticles (quantum dots and 5-(aminoacetamido) fluoresceinlabeled polyamidoamine dendrimer G4.5) coated with amine-derivatized PEG, through the reduced Schiff base linkage were immobilized to the sodium periodate-treated surface of RAW264.7 macrophages. These nanoparticles can provide drug-loading sites for cytotoxicity [221]. Due to hypoxia, Twist-1, an oncogene, is over-expressed. It also helps escape apoptosis leading to MDR, and thus becoming a valid target for therapy. In these lights, Shen *et al* developed nanoparticles delivering a combination of paclitaxel and Twist shRNA, showing improved cellular uptake and reduced tumor growth and reduced metastasis in *in vivo* breast cancer models [222].

3.2.3 Targeting low extracellular pH (pHe)—pH-sensitive nanoparticles are one of the most widely studied stimuli-sensitive nanoparticles. There have been a number of reviews on these drug delivery systems previously [84, 122, 178, 204, 223, 224].

Most of such systems incorporate a pH-responsive component that gets protonated at the acidic extracellular pHe and eventually destabilizes the nanoparticles and releases the drug load. Lee *et al.,* showed in 2003 how poly(L-histidine) could be used to form block copolymer micelles. The characteristic of the poly(L-Histidine) was that it would protonate at low pH and this results in a change in the CMC of the micelles in the microenvironment destabilizing the micelles to effectively release the encapsulated drugs [225]. In addition, polysulfonamide can also be used to develop such systems targeting tumor pHe [84]. Lee has developed a pH-sensitive micelle system (PHSM) with folate (PHSM/f) incorporating the poly(L-Histidine) core to load doxorubicin and enhanced cytotoxic effects in *in vivo* models [84, 226]. It was highlighted here that the polymer length played an important role in determining the stability of the nanoparticles in the normal pH (7.4) and destabilize at the pHe.

Sawant *et al.* developed low-pH degradable PEG-Hydrazone (Hz)-PE micelles with an aim to improve gene delivery. Analysis of this system carried out with HPLC described intact micelle peaks with retention times of 9.4 min for the micellar samples incubated at pH 7.4. Rather, incubation at pH 5 resulted in the loss of PEG coat on the micelles and this was observed with a cleaved PEG peak at retention time 12.5 min [227].

Several different systems have also been studied and developed. Few of them are highlighted here. A novel strategy was recently developed based on pH-transforming polymer (polymethacrylates, PMA)-grafted poly(amidoamine) (PAMAM) to trigger drug release in response to the acidic tumor microenvironment. Further tumor selectivity was achieved through the folate receptor targeting. As a result, tumor drug accumulation and subsequent tumor growth inhibition was seen in tumor-bearing mice [228]. Poon developed a layer-by-layer (LBL) nanoparticle with a pH-sheddable layer [229]. A trilayer architecture of poly-L-lysine (PLL) modified with iminobiotin, followed by a linker protein, and biotin-end-functionalized PEG made up the LBL. While PEG was responsible for avoiding RES uptake, the iminobiotin-neutravidin bonds were the pH-sensitive portions of the delivery system. On reaching the extracellular environment, the low pH would cause the protonate the iminobiotin decreasing the bonds with neutravidin and in turn exposing the positively charge PLL readily available for cellular uptake. This system was successful in

accumulating in *in vivo* mouse models of MDA-MB-435 and KB subcutaneous tumors [229]. In another study, a novel mitochondrial-targeted zwitterionic oligopeptide liposomal (HHG2C 18 -L) nanocarrier system with multistage pH response to the acidic tumor microenvironment in the exceedingly acidic intracellular compartment was developed [230]. Doxorubicin attached through hydrazone bonds on to SWCNT was used as a competent nanocarrier exploiting the tumor extracellular pHe. The hydrazone bonds are sensitive to the microenvironment and thus on the application to HepG2 tumor cells, the doxorubicin uptake was facilitated and improved cytotoxicity was observed [231]. Similarly, hydrazone-based acid sensitive PEG-PE conjugates were synthesized and described [232].

pH-responsive poly[2-(N, N-diethylamino)ethyl methacrylate] (PDEA) cores in PDEA-block-PEG copolymers were used to load cisplatin for targeting resistant SKOV-3 cell lines *in vitro* and *in vivo*. It was observed that tumor acidic microenvironment-induced rapid release of cisplatin from these nanoparticles was able to overcome the resistance of SKOV-3 cells and resulted in reduced tumor growth [233]. Sawant *et al.,* used cell-penetrating peptides (CPP) and monoclonal antibody 2G4 along with pH-sensitive moieties to make double-targeted micelles. In this case, TATp (Trans-Activator of Transcription peptide) was attached to the surface of PEG-PE-modified liposomes and micelles and further double targeted by attaching the antibody. Hydrazone (Hz) was the pH responsive linker used in this case. In the presence of low pH values the PEG shell was lost on acidic hydrolysis of PEG-Hz-PE and TATp was exposed to the cells thus improving internalization by the cells [234]. A novel multifunctional 3-layered nanoparticle (3NP) was developed with poly(e-caprolactone) (PCL) core, a pH-responsive poly[2-(N, N-diethylamino)ethyl methacrylate] (PDEA) middle layer and a PEG outer layer to specifically release the drug-containing nanoparticle in the tumor interstitium [235].

A unique nanoparticle system was developed by Huang *et al.,* to target the tumor-specific over-expressed marker MMP2 (matrix metalloproteinase - 2) and the low pHe in the tumor microenvironment [236, 237]. This nano-system employed an activatable cell-penetrating peptide (*dt*ACPP) for internalization by cells, a MMP-2 substrate (PGLAG), and a pH-responsive linker. This delivery system (*dt*ACPPD/shVEGF-DOX) was tested for plasmid DNA (sh-VEGF in this study as an anti-VEGF agent) loading, drug (doxorubicin; DOX) loading, cellular uptake, tumor targeting and anti-angiogenesis and cytotoxic activities *in vitro* and *in vivo*. The nanoparticles were observed to target tumors proficiently with little side effects and good anti-tumor activity. The pH-responsive nanoparticles thus facilitate the release of the anti-cancer drugs specifically and rapidly in large amounts in the tumor interstitium to overwhelm the MDR in the tumors.

3.3 Use of spheroid to evaluate physiological factors

Nanotechnology and the field of drug delivery systems have seen significant advances in the development of models to study effects of chemotherapeutic agents. However, spheroid culture is gaining increasing interest to study various physiological factors by growing micro tumor tissues *in vitro* in bulk. Spheroids (micro tumor tissues) grown in vitro, demonstrates various characteristics demonstrated by solid tumors such as glucose gradient, oxygen gradient, hypoxic core and presence of cells in diverse cell cycle stages [238]. Takagi *et al*., also demonstrated superior correlation between gene expression profile of solid tumors with spheroids over monolayer culture [239]. These properties make spheroid culture ideal to utilize in studies focused on not only physiological factors but also cellular factors. However, producing consistent spheroids in bulk for high-throughput screening is challenging, time and labor consuming. Therefore, limited interest has been showed by research community to utilize spheroids to evaluate nanoparticles for multidrug resistance. In our lab, we were interested in evaluating penetration behavior of antibody-targeted doxorubicin-loaded PEG-PE micelles in spheroids. Perche *et al*., demonstrated the formation

of consistent spheroids using non-adhesive liquid overlay technique and significant higher doxorubicin uptake in 2C5 monoclonal antibody-modified PEG-PE micelles. Though observed doxorubicin uptake was higher, the penetration was limited to 50 μm from external border. Essentially it was observed that the limited diffusion of doxorubicin observed clinically was replicated by the non-uniform distribution of the drugs in the spheroids adding a positive attribute in the benefits for using spheroids (Figure 6) [240]. As discussed earlier, cancer cells in tumors can be as far as 200 μm from nearest blood vessels and because penetration can be evaluated for nanoparticles using spheroids, it provides excellent platform to screen for multiple nanoparticle formulations prepared from diverse polymers with diverse targeting moieties.

4. Future perspectives

The cancer MDR is an important reason for the failure of the traditional chemotherapy. As described in this review, it depends on several factors broadly classified into cellular and physiological ones. The MDR is a consequence of the over-expressed drug efflux transporters, defective apoptotic mechanisms, and mutated molecular targets along with the tumor microenvironment complications like poor vasculature, hypoxia and low extracellular pH. Because of such intricacies, the need for higher drug doses and frequent administrations is required resulting in unwanted drug toxicities along with clinical inconvenience. However, because of the unique tumor features, several potential cellular and physiological targets present there, which could be successfully exploited to overcome the MDR.

In last few decades, extensive knowledge has been gathered and significant advances have been made in the field of nanocarrier delivery systems. Many potential therapeutic molecules previously thought to be unviable due to low solubility and/or low stability can now serve as the viable option if nanocarrier delivery platform is used. Nanopreparations have also provided a great platform for the delivery of highly active large molecules, which include nucleic acids and proteins. Unique properties of many nanopreparations can be tuned depending on the type of targets. Advanced knowledge and treatment options are often accompanied with a complicated dosage regimen. In case of MDR, the involvement of numerous mechanisms requires customized nanopreparations with single agent or combination therapy to overcome fundamental mechanism underlying the MDR. In many cases, nanopreparations have demonstrated usefulness in reducing systemic toxicity. Focus has been turned to improve their efficacy while maintaining reduced systemic toxicity. The evolution of nanopreparations has resulted in many successful candidates including surface-modified ones to improve circulation time, as well as make nanopreparations pH-responsive and other stimuli-sensitive. Emerging spheroid culture platform, using which micro tumor tissues can be grown *in vitro*, has also opened doors to extend our knowledge about physiological barriers presented by solid tumors. With the help of spheroid culture, it is now possible to study the effect of surface properties of nanopreparations on their penetration into the tumor mass, which is an important barrier to overcome MDR. Greater efforts are also required to utilize 3D spheroid culture for high-throughput screening for potential therapeutic molecules due to the advantages it offers.

Of course, there are many challenges involved with the nanopreparations in terms of biocompatibility including immune responses, biodegradation (especially of metal-based nanosystems), large scale manufacturing, and batch to batch variability issues. Still, with the ever expanding knowledge in this field, these obstacles can be successfully overcome. Finally, the belief of widely utilizing nanopreparations for cancer therapy is becoming a reality day by day.

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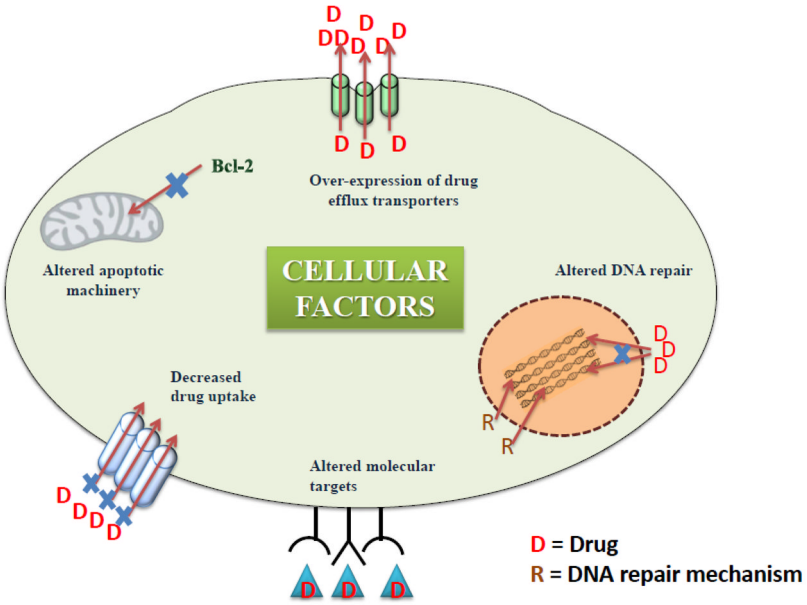

Figure 1.
Cellular factors responsible for the multidrug resistance.

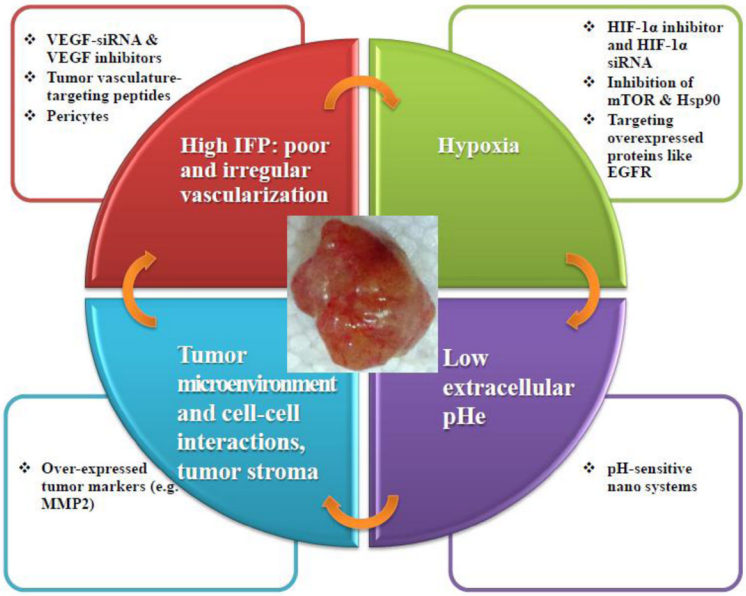

Figure 2.
Physiological factors responsible for the multidrug resistance and their inter-relation.

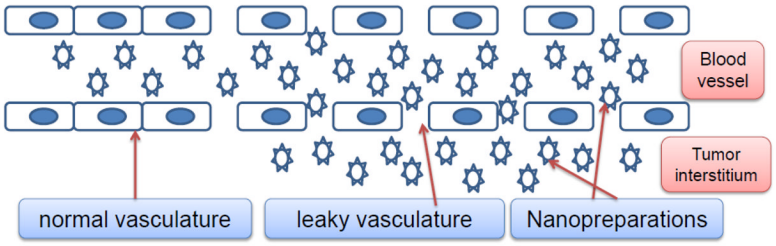

Figure 3.
Enhanced permeability and retention effect. Due to the presence of leaky vasculature in tumor microenvironment and poor lymphatic drainage, EPR effect is observed for nanopreparations with size distribution up to 400 nm.

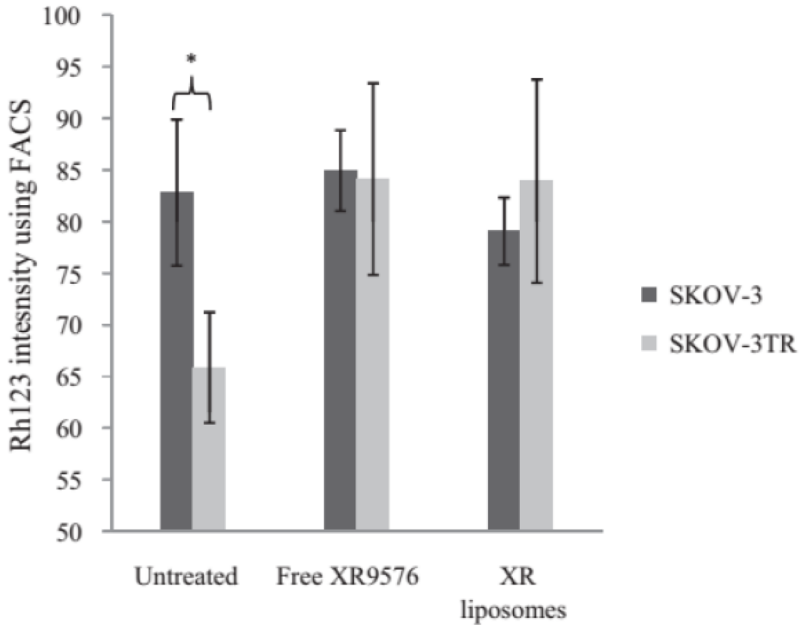

Figure 4.

Rh123 uptake study in SKOV-3 and SKOV-3TR cells. The cells were treated with free tariquidar (XR9576) and tariquidar (XR) liposomes and then incubated with Rh123. FACS analysis showed that the tariquidar retained its activity when in liposomes and effectively inhibited P-gp as shown by enhanced Rh123 intensity. Reproduced with permissions from the authors.

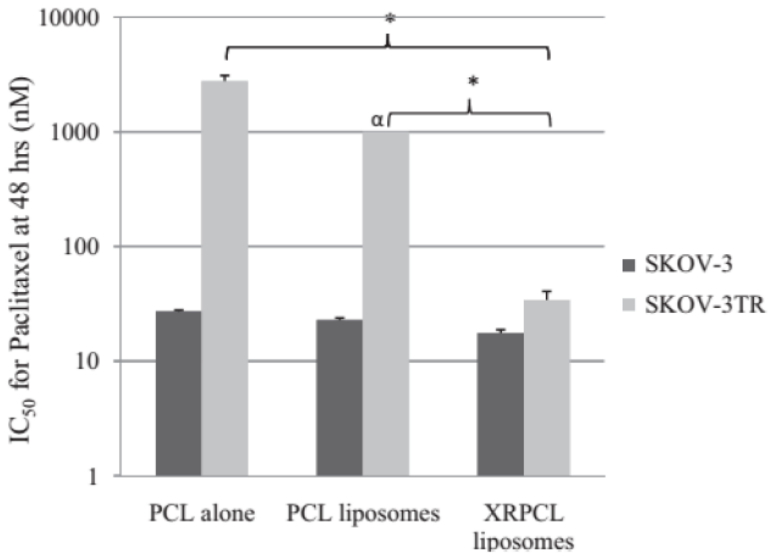

Figure 5.

IC_{50} for paclitaxel in SKOV-3 and SKOV-3TR cells. Cells were treated with paclitaxel (PCL), PCL liposomes and tariquidar (XR)-PCL combination liposomes at various concentrations. Error bars indicate mean ± S.D. *$p < 0.05$. Y-axis is shown in logarithmic scale. IC_{50} for the combination treatment was reduced as compared to PCL alone. Reproduced with permissions from the authors.

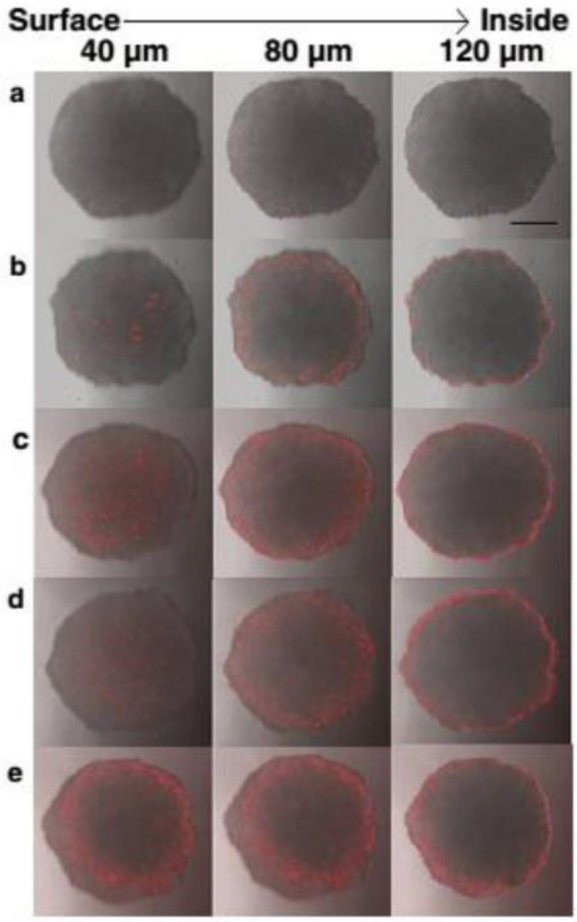

Figure 6.
Z-Stack images of NCI-ADR-RES spheroids by confocal microscopy to study penetration of doxorubicin-loaded micelles. Treatments carried out: (a) HEPES, (b) free doxorubicin, (c) micellar doxorubicin, (d) IgG targeted micellar doxorubicin, (e) 2C5 targeted micellar doxorubicin. Reproduced with permissions from the authors.

Table 1

List of ABC transporters and their substrates [7, 8]

Sub-families	Member	Substrates
ABCB	ABCB1 (MDR1, P-gp)	Doxorubicin, daunorubicin, vinca alkaloids, taxols
	ABCB4	Paclitaxel, Vinblastine
ABCC	ABCC1 (MRP1)	Doxorubicin, etoposide, vincristine
	ABCC2	Doxorubicin, cisplatin, vincristin, etoposide
	ABCC3	Etoposide, methotrexate, doxorubicin
ABCG	ABCG2	Doxorubicin, daunorubicin, mitoxantrone, topotecan

Table 2

List of drug efflux transporter inhibitors

Drug efflux transporter	Inhibitors	Reference
MDR1 (First generation)	Verapamil Cyclosporin A Tamoxifen	[137–139]
MDR1 (Second generation)	Valspodar Biricodar (VX-710)	[140, 141]
MDR1 (Third generation)	Zosuquidar (LY335979) Elacridar (GF120918) Tariquidar (XR9576)	[127, 142–144]
MRP1	Biricodar (VX-710) Imidazothiazole derivatives (N276-12,14, 17) tRA 998006	[141]
BCRP (ABCG2)	Elacridar (GF120918) Imatinib mesylate	[145, 146]