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Multidrug Resistance: Physiological Principles and Nanomedical Solutions

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Abstract

Multidrug (MDR) resistance is a pathophysiological phenomenon employed by cancer cells which limits the prolonged and effective use of chemotherapeutic agents. MDR is primarily based on the over-expression of drug efflux pumps in the cellular membrane. Prominent examples of such efflux pumps, which belong to the ATP-binding cassette (ABC) superfamily of proteins, are Pgp (P-glycoprotein) and MRP (multidrug resistance-associated protein), nowadays officially known as ABCB1 and ABCC1. Over the years, several strategies have been evaluated to overcome MDR, based not only on the use of low-molecular-weight MDR modulators, but also on the implementation of 1-100(0) nm-sized drug delivery systems. In the present manuscript, after introducing the most important physiological principles of MDR, we summarize prototypic nanomedical strategies to overcome multidrug resistance, including the use of carrier materials with intrinsic anti-MDR properties, the use of nanomedicines to modify the mode of cellular uptake, and the co-formulation of chemotherapeutic drugs together with low- and high-molecular-weight MDR inhibitors within a single drug delivery system. While certain challenges still need to be overcome before such constructs and concepts can be widely applied in the clinic, the insights obtained and the progress made strongly suggest that nanomedicine formulations hold significant potential for improving the treatment of multidrug-resistant malignancies.

Keywords

Nanomedicine; Drug targeting; Multidrug resistance; MDR; Drug efflux pumps; ABC transporters; Pgp; MRP; Pluronics; siRNA

1. INTRODUCTION

In spite of significant advances in understanding the etiology and progression of cancer, and in developing novel diagnostics and therapeutics, both the incidence and the mortality rates of malignancy remain to be extremely high. One of the main reasons for this is

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chemoresistant cancer recurrence. Chemoresistance may either be innate, i.e. existing since the beginning of therapy, or acquired, i.e. developed during the course of treatment. Its significance can be illustrated by the fact that almost all non-small cell lung cancer patients treated with chemotherapy eventually develop resistance against the anticancer agents used [1]. The biological background of chemoresistance is complex and generally includes one or more of the following mechanisms: inhibition of apoptosis, induction of DNA repair mechanisms, alterations of drug target structure, modifications in cell membrane composition (leading to reduced drug uptake), and last but not least, elevated expression levels of drug efflux pumps. Regarding the latter, a major problem is cross-resistance, which relates to an increased expression of broad-spectrum drug transporters present within the cancer cell membrane, which are not only active against a single drug or chemically-related drugs, but against a whole range of chemotherapeutic agents, even to agents which have not yet been administered to the patient. This phenomenon is referred to as multidrug resistance (MDR), and the proteins involved in this process are called MDR proteins.

2. PHYSIOLOGICAL PRINCIPLES OF MDR

The history of MDR proteins started in 1974, when Victor Ling and Larry Thompson described a stable colchicine-resistant cell clone derived from a CHO cell line by a single-step selection, and discovered that the resistant cells did not allow colchicine to enter the cytoplasm [2]. The selected cells were also found to be resistant to demecolcine, actinomycin D and vinblastine. It was furthermore observed that although colchicine uptake by sensitive cells was passive, resistance was an active process, as it could be inhibited by cyanides, azides and dinitrophenol [3]. It was further proven that the main difference between naive and resistant cells was the expression of a 170 kDa plasma membrane glycoprotein called P-glycoprotein (Pgp; with the first P referring to permeability) [4]. It rapidly became apparent that there are other active membrane transporters, distinct from Pgp, which are involved in multidrug resistance. In 1990, for instance, a 95 kDa membrane protein responsible for anthracycline resistance in MCF-7/AdrVp(100) cells was described [5], which later became known as BCRP (Breast Cancer Resistance Protein), and in 1992, Cole and coworkers identified and cloned another phosphoglycoprotein which was highly overexpressed in doxorubicin-resistant H69AR cells and named it MRP (Multidrug Resistance-associated Protein) [6]. It was soon clear that all of these proteins share some sequence- and functional homology, and belong to ATP-binding cassette (ABC) superfamily of proteins.

2.1: ABC OF MDR TRANSPORTERS

ABC proteins are P-type membrane ATPases, distinguished by highly conserved amino acid sequences located in their nucleotide-binding domain (so called Walker A and Walker B motifs), separated by the 'ABC signature' motif LSGGQQ/R/KQR [7]. They constitute one of the largest protein families identified to date, are present in almost all cells of all taxonomic groups of organisms, and are engaged in various membrane transport processes, such as substrate uptake, product excretion and osmoregulation (including transmembrane ion movement). In prokaryotes, ABC proteins form oligomeric complexes, while eukaryotic ABC proteins are usually composed of a single polypeptide [8]. The inventory of human ABC genes contains 48 elements, and to fulfill standards of human genetic nomenclature, they were subdivided into seven families, A to G, each labeled as ABC followed by a family letter and a number [8]. Using this system, Pgp is now generally referred to as ABCB1, while BCRP and MRP are known as ABCG2 and ABCC1, respectively. It should be mentioned in this regard that there are several more ABC proteins, especially from the ABCC subfamily, which are involved in multidrug resistance, but we here primarily focus only ABCB1, ABCC1 and ABCG2, as their clinical significance is broadly accepted and extensively documented.

One of the key characteristic features of ABC transporters is their molecular architecture. The basic unit of the protein is a set of 6 hydrophobic membrane-spanning helical fragments forming a so called transmembrane domain (TMD), followed by a hydrophilic cytoplasmic nucleotide-binding domain (NBD) harboring amino acid sequences distinctive for ABC proteins. Such a structure is doubled in most eukaryotic transporters, forming a TMD1-NBD1-TMD2-NBD2 single polypeptide assembly. ABCB1 is a good example of a canonical eukaryotic transporter [9] (Figure 1B), but the molecular structure of other MDR proteins can vary quite a bit. ABCC1 protein contains an additional N-terminal transmembrane domain (TMD₀), consisting of five helical fragments linked to the core of the molecule by a L₀ loop (Fig 1A) [10]. This fragment of the protein is important for its stable expression and function [11], as well as for proper membrane trafficking [12]. ABCG2 is a representative example of the so-called 'half-transporters', consisting of a single TMD and a single NBD domain, but in reverse order (i.e. NBD is the N-terminal domain; see Figure 1C) [13]. Unlike ABCB1 or ABCC1, which function as monomers, ABCG2 requires homo-oligomerization, most likely octamerization, to form an active transport unit [14].

MDR transporters, as all ABC proteins, are vanadate-sensitive ATPases [8]. Both NBDs are involved in ATP-binding and hydrolysis, which is coupled to a conformational change in the protein (with hydrolysis being the rate-limiting step of the catalytic cycle [15]). The ATPase activity of MDR proteins is azide- and ouabain-insensitive, and can be stimulated by drugs to which a given protein confers resistance, as was clearly shown for ABCB1 [16] and later on also for other family members. MDR transporters are located in apical (ABCG2 [17] and ABCB1 [18]) or basolateral (ABCC1 [19]) domains of the plasma membrane of polarized cells. The lipid milieu is an important factor influencing protein activity. It was clearly shown that ABCG2 is located in lipid rafts, as its activity significantly decreases in cholesterol-depleted cells [20]. Furthermore, knockdown of caveolin 1, a lipid raft protein, restores drug-sensitivity of ABCG2-expressing cells [21]. Similar results were observed for ABCB1 in cyclodextrin-treated cells [22], while ABCC1 seems to be a raft-independent protein [23]. The tissue and organ distribution of MDR proteins is remarkable: they are expressed predominantly in epithelial cells, forming an interface between the organism and its external environment, such as for bronchial or gastrointestinal tract cells, as well as between tissues and the vascular compartment, such as for Sertoli cells, syncytiotrophoblasts and blood-brain barrier endothelium (see Table 1).

MDR transporters are also present also in excretory (canalicular hepatocytes, proximal tubule epithelium) and secretory (adrenal gland, lactiferous duct) cells. ABCG2 is furthermore highly expressed in stem cells of different organs, being responsible for a so-called 'side-population phenotype', which is marked by a reduced uptake of Hoechst 33342 [40]. No MDR proteins have been detected in connective tissues, neurons or glial cells, with the exception of astrocytes, where ABCC1 is present in a reasonably high amounts [41]. It is not surprising that tumors evolving from cells and tissues where their basic expression is significant, i.e. in particular epithelial cells, generally develop multidrug resistance. However, MDR proteins have also been detected in tumors derived from cells where they are physiologically absent, as is for instance the case for gliomas, which express relatively high amounts of ABCB1 [41].

2.2: TRANSPORT PROPERTIES OF MDR PROTEINS

The most intriguing feature of MDR proteins is their ability to transport - and thus confer resistance to - a broad spectrum of chemically unrelated compounds. It is also striking that the resistance profiles of these proteins overlap quite significantly (see Figure 2). All discussed proteins, for instance, are involved in anthracycline resistance, and it is worth noting that ABCC1 and ABCG2 were originally discovered in doxorubicin-selected cells

[13] [42] [43]. Both ABCB1 and ABCC1 are responsible for Vinca alkaloid and podophyllotoxin resistance [43] [44] [45], but glutathione is a required co-factor for ABCC1 to act [46]. ABCB1 and ABCG2 are also able to efflux camptothecin derivatives [47,48] and tyrosine kinase inhibitors [49,50]. Antifolate resistance is mediated by both ABCC1 and ABCG2, but the former is only able to efflux unmodified methotrexate [51], whereas the latter can transport both methotrexate itself [52] and its di- and triglutamate derivatives [53]. Mitotic spindle poisons are specifically effluxed by ABCB1, as only this protein is able to transport both taxanes [54] and epothilones [55], and resistance against alkylating agents is solely associated with ABCC1 overexpression [56].

When looking for convenient tools to study MDR protein function, a number of fluorescent dyes have been identified as more or less specific substrates for each of these transporters. ABCB1, for instance, is able to transport fluorescent NBD-lipid analogues [57] and rhodamine-based dyes, such as rhodamine 123 [58] and tetramethylrhodamine [59], and also leuco-esters of fluorescent dyes, such as acetoxymethyl esters of calcein or Fura-2 [60]. ABCC1 effluxes bulky fluorescent anions, like calcein [61], 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [62] and Fluo-3 [63]. This protein also exhibits a floppase activity against NBD-lipids [64,65]. The best-known artificial fluorescent substrate of ABCG2 is Hoechst 33342 [66].

Based on their resistance profiles, the conclusion was drawn that transporters demonstrate some preference to a widely defined group of compounds. Accordingly, ABCB1 is considered to transport mainly bulky cationic lipophilic substances, while ABCC1 is believed to be a transporter of organic anions. ABCG2 functions somewhere being in-between. Several conceptual models have been built to explain the mode of action of MDR proteins, the most important of which are: (I) a pump model, in which the transported substance is moved directly from the internal aqueous phase, i.e. the cytoplasm, to the external aqueous phase, i.e. extracellular fluid (the most probable model of ABCC1 action); (II) a 'molecular vacuum cleaner' model, in which the substrate partitions into a lipid bilayer from where it is recognized by the transporter and exported to the extracellular aqueous phase (the most widely accepted model for ABCB1); and (III) a flippase model, in which the substrate partitions in the inner leaflet of the lipid bilayer and is flipped to the outer leaflet by the transporter, so the whole transport process is limited to the lipid phase (proposed for ABCB1). These models are extensively reviewed in [67]. However, the molecular basis for the observed and unusually broad substrate specificity spectra – although the word 'specificity' may seem a bit odd in this context – of these proteins are still partially elusive, and they are being intensively investigated.

2.3: PHYSIOLOGICAL FUNCTIONS OF MDR PROTEINS

Significant effort has been put in identifying the endogenous substrates of MDR proteins, in order to elucidate their physiological role. No natural ABCB1 substrates have been identified thus far, at least not in vivo, although this protein has been shown to be able to transport certain steroid hormones [68] and cytokines [69]. Some additional light was shed on this by analyses in animal models. Abcb1a knockout mice, which have two orthologues of ABCB1, are phenotypically normal, vivid and fertile, although they are 100-fold more susceptible to ivermectin, an anthelmintic neurotoxin which is usually non-toxic for mammals, as it does not permeate the blood-brain barrier [70]. The toxicity of vinblastine was also found to be increased, by a 3-fold, in these animals. Also Abcb1a/Abcb1b double knockout mice seem to be unaffected by these genetic disruptions when kept in standard conditions, although significant alterations in the biodistribution of certain drugs, e.g. digoxin, were noted in these animals [71]. Taking these data together and keeping in mind the tissue distribution of ABCB1 (see Table 1), it seems that the main physiological role of this protein is to protect vital organs and tissues from the deleterious effects of xenobiotics.

ABCC1 has been shown to efflux glutathione S-conjugates [72,73], including conjugates of alkylating agents [56], mono- and diglucuronides [73,74], taurates and sulfates of bile acids [73]. Thus, it seems to be a major conjugate transporter, participating in relatively standard detoxification processes. As it is also able to transport glutathione disulfide [75], it also plays a role in cellular redox homeostasis, although it was shown that ABCC1-overexpression does not protect cells against excessive doses of oxidative agents [76]. The most important physiological ABCC1 substrate arguably is leukotriene C4 (LTC4) [77]. This is confirmed by studies in animal studies, showing that *Abcc1*-knockout mice are characterized by an abnormal inflammatory response due to impaired LTC4 transport [78].

The physiological role of ABCG2 has been linked to the final stages of erythropoiesis, as this protein is a major protoporphyrin IX efflux pump, diminishing the risk of oxidative damage to maturing red blood cells by excessive amounts of this heme precursor [37]. High ABCG2 expression levels are also observed in different kinds of progenitor cells [40], suggesting a protective function. In addition, several glucuronide and sulfate conjugates have been identified as ABCG2 substrates, further supporting this hypothesis [53]. Additional convincing evidence in this regard comes from studies using *Abcg2*-knockout mice. When fed on a diet with increased chlorophyll content, these mice started to develop ear skin lesions. It was found that they absorb large amounts of pheophorbide A, a bacterial catabolite of chlorophyll, which accumulates in the skin, leading to photosensitization reactions [79], while in wild-type mice, pheophorbide A is efficiently effluxed from gut epithelium. Finally, ABCG2 has recently also been shown to be a high capacity uric acid transporter, thus being an important factor in urate excretion and (the prevention of) gout development [80].

Taken together, it can be concluded that the main physiological role of MDR proteins is to act as gatekeepers, preventing the organism from intoxication by xenobiotics and/or endogenous toxic compounds. However, they are classical double-edged swords, as chemotherapy is a planned xenobiotic exposure, and as they prevent chemotherapeutics from accumulating in and killing cancer cells. This explains, to a large extent, why the over-expression of MDR proteins is a very poor prognosis factor for cancer patients, which has been proven numerous times since the 1980's [81,82].

3. NANOMEDICAL SOLUTIONS TO OVERCOME MDR

Over the years, many different strategies have been employed to overcome multidrug resistance. Great expectations have been associated with the use of low-molecular-weight inhibitors, but thus far, most of these attempts have been unsuccessful, due to low selectivity, inherent toxicity and pharmacokinetic interactions with anticancer drugs (for a review, see [82]). Here, we focus on nanomedicines, which have also been extensively evaluated to combat MDR in the last two to three decades. Given their prolonged circulation properties and their ability to accumulate in tumors via Enhanced Permeability and Retention (EPR) [83-86], nanomedicines are able to deliver high concentrations of chemotherapeutic drugs and/or MDR inhibitors to tumors. Upon ensuring efficient delivery to tumors *in vivo*, it is important to understand how these carrier materials are taken up by cells, and how effectively they are retained within cells. Cellular retention depends on the balance between drug uptake and drug efflux. Chemotherapeutics or other small molecules rapidly diffuse across the cellular membrane to enter the cytoplasm. Passive diffusion is a highly efficient process, which in principle results in high intracellular concentrations of chemotherapeutics. In resistant cells, however, most of these rapidly internalized small molecules are rapidly sensed by MDR proteins and effluxed out of the cells [87]. This ATP-dependent efflux mechanism is a prominent feature of multidrug resistant cancer cells. As compared to low-molecular-weight drugs, which generally are <1 nm, nanomedicines are

1-2 orders of magnitude larger. This prevents them from being internalized into cells via passive diffusion. Rather, they are endocytosed, and via endo-lysosomal trafficking, carried relatively deep into the cells. Consequently, since endocytosis enables them to bypass drug efflux pumps, their internalization and retention are not negatively influenced by the overexpression of MDR proteins. In addition to this, certain types of nanocarriers, in particular pluronics, possess intrinsic anti-MDR properties, making them highly interesting materials for treating multidrug-resistant tumors. Furthermore, nanomedicines have also been used in combination with low-molecular-weight MDR inhibitors, either via co-administration to resistant cells and tumors, or via co-formulation (i.e. incorporation of both MDR inhibitors and chemotherapeutic drugs within a single nanomedicine system). And finally, in the last couple of years, these combination concepts have been extended to nucleic acid-based therapeutics, and to carrier materials containing both standard chemotherapeutic drugs and anti-MDR siRNA. Below, we provide representative examples for each of these nanomedical solutions to overcome MDR.

3.1. PLURONIC NANOMEDICINES

Pluronics are block copolymers of hydrophilic ethylene oxide and hydrophobic propylene oxide. They are also known as poloxamers. Pluronics are materials of high interest in drug delivery, not only because of their potent drug encapsulating properties, but also because of their intrinsic ability to modify biological responses. These amphiphilic macromolecules can change their chemical nature based on their copolymer composition and arrangement, as usually reflected by changes in their hydrophilic-lipophilic balance (HLB). With respect to MDR, pluronics have been extensively used and evaluated [88-94]. Their drug-carrying ability (in the form of micelles and polymer conjugates), and their ability to exert biological effects, in particular on ABC transporters, MDR proteins and chemo-resistant cancer cells, have gained them wide notice in the nanomedicine field.

The mechanism behind the biological and anti-MDR effects of pluronic block copolymers has been extensively investigated over the years. As exemplified by Figure 3, multiple phenomena form the basis for the ability of pluronics to overcome MDR [92]. It has for instance been reported that they are incorporated into the cellular membrane, and hence change its micro-viscosity. Their role in reducing the ATP levels in cells are also widely described, resulting in a significant reduction in the activity of drug efflux pumps (which energy- and ATP-dependent). Furthermore, their ability to induce the release of cytochrome C and ROS, and to mitigate anti-apoptotic defensive mechanisms, is relatively well known. Besides the fact that they can inhibit glutathione / glutathione-s-transferase detoxification systems, the propensity of pluronics to de-promote the sequestration of chemotherapeutics within cytoplasmic vesicles (for gradual clearance) is also an affirming reason behind their ability to overcome MDR. Based on these and several additional anti-MDR effects (depicted in Figure 3A), a multitude of studies have been performed in which pluronics have been used to overcome multidrug resistance [88,89,92].

An exemplary study in this regard has been published by Chen and colleagues, evaluating the anti-tumor efficacy of methotrexate-conjugated mixed pluronic micelles, in mice bearing multidrug-resistant epidermoid KBv tumors [95]. Mixed micelles were prepared by thin-film hydration, using pluronic P105 modified with methotrexate (P105-MTX), and stabilized using pluronic F127 (Figure 3B). This resulted in relatively high drug loading (10% w/v) and in micelles with a proper diameter for EPR-mediated passive drug targeting (i.e. <100 nm). Upon i.v. injection, EPR-mediated tumor accumulation and endocytosis-based cellular internalization, the pluronic P105 inhibited ABCB1, and thereby ensured efficient intracellular methotrexate accumulation in KBv cells. As shown in Figure 3C, it also enabled effective in vivo antitumor treatment: both F127/P105-MTX and PF-MTX (pluronics-based mixed micelles in which methotrexate was physically entrapped) were

found to be significantly more effective than treatment with free MTX and with saline. Furthermore, in vivo fluorescence imaging was carried out, at several different time points after the i.v. injection of near-infrared fluorophore (DIR)-labeled F127/P105 and F127/P105-MTX, confirming that pluronic block copolymer-based nanomedicines effectively and selectively accumulate in tumors over time by means of EPR (Figure 3D). Many similar studies are available in the literature, as are experiments in which the cellular and molecular effects of these amphiphilic block copolymers are investigated [88-94], together suggesting that pluronics are highly interesting materials for overcoming multidrug resistance.

3.2. STANDARD NANOMEDICINES

As opposed to pluronics, most standard nanomedicine materials are inert, and therefore lack intrinsic pharmacological properties to modulate MDR. However, since their mode of cellular uptake is very different from that of low-molecular-weight anticancer agents, also 'standard' nanomedicines are generally assumed to hold significant potential for overcoming multidrug resistance. Nanomedicines are taken up by cells via different mechanisms, including e.g. clathrin-, caveoli-, and clathrin-caveoli-mediated endocytosis. Irrespective of the exact mode of cellular entry, endocytosed nanomedicines eventually end up in lysosomes, and thus are carried relatively deep into cells, far beyond the reach of trans-membrane localized drug efflux pumps. In theory, this strategy therefore ensures efficient delivery of chemotherapeutic agents into the cytoplasm of cells, without them being sensed and removed by MDR proteins. This as opposed to free (i.e. non-nanomedicine-associated) chemotherapeutic drugs, which upon passive diffusion across the cellular membrane are rapidly sensed and effluxed by MDR proteins (see Figure 4A).

To verify the validity of the above concept, we recently generated four different multidrug-resistant cancer cell lines, and used them to evaluate the potential of three different doxorubicin-containing nanomedicine formulation [96]. The cells employed were A431 human epidermoid carcinoma, B16-F10 murine melanoma, CT26 murine colon carcinoma and SW620 human colon adenocarcinoma cells, which were made multidrug resistant by continuously exposing them (for several months) to IC90 concentrations of doxorubicin, and by harvesting surviving fractions upon several rounds of selection. Real-time PCR, western blot and flow cytometry analyses showed that the main reason for Dox-resistance in these cell lines could be attributed to the overexpression of ABCB1, as well as to elevated levels of ABCG2. Before starting the actual in vitro efficacy experiments to compare the activity of free doxorubicin to that of the three doxorubicin-containing nanomedicines, the Dox-resistant A431-D, B16-D, CT26-D and SW620-D cells were allowed to grow for two passages without exposure to the drug. MTT analyses were subsequently performed in these and parental cells to assess the in vitro efficacy of the four formulations, i.e. free doxorubicin, polymer-bound Dox, micellar Dox and liposomal Dox. All four formulations were used at doses up until 30 μ M, as higher concentrations are unrealistic to be achieved in vivo. As shown in Figure 4B, it was found that in parental cell lines, which are sensitive to doxorubicin, the low-molecular-weight drug was significantly more effective in killing the cells than other nanomedicine formulations. This is due to the fact that passive diffusion across the cellular membrane is much more rapid and efficient than endocytosis. Comparing the IC50-values of the four formulations in the four cell lines shows that there was no clear trend with regard to a nanomedicine formulation that was more effective than the other two formulations. In the multidrug-resistant cell lines, this situation was quite different. Here, in line with concept outlined in Figure 4A, the overall difference in efficacy between the free drug and the nanomedicine formulations was less large. In A431-D, free Dox and polymer-bound Dox were found to be equally effective (as compared to a >4-fold difference in parental A431 cells), and also micellar Dox appeared to be more effective in killing cells relative to the situation in parental A431 cells (Figure 4B). In B16-D, a similar trend was

observed, but due to a larger variability between the three experimental runs, the difference was not significant. In CT26-D and SW620-D, the nanomedicines failed to kill more than 50% of cells, therefore no IC₅₀ values could be determined. These findings show that in certain cell types, certain standard nanomedicines might be able to overcome MDR. They also show, however, that this ability should not be over-estimated, and that it cannot be generalized.

In a similar experimental setup, doxorubicin was covalently attached to polyethylenimine, using pH-sensitive hydrazone linkages, and subsequently bound to PEGylated iron oxide nanoparticles [97]. These particles were tested in wild-type C6-glioma cells and in C6-ADR cells (a Dox-resistant variant which overexpresses several ABC transporters). Upon 4 h of in vitro treatment with 1000 ng/ml of free and nanoparticle-associated doxorubicin, similar drug levels were observed for in parental cells. In C6-ADR, on the other hand, only very low levels of free Dox could be detected, while levels were more than a 6-fold higher upon delivering Dox into the cells using nanoparticles (Figure 4C). Twenty hours later, drug levels in both sensitive and resistant C6 cells remained to be high upon incubation with Dox-containing nanoparticles, whereas upon treatment with the free drug, hardly any Dox could be detected anymore. In line with this, when the cells were treated with the different formulations for 24, 48 and 72 h, NP-Dox was always at least equally efficient in killing cells than was free Dox. In C6-ADR cells, it was even found to be significantly more effective, in particular upon incubation with higher drug doses (see lower panels in Figure 4C). To verify these findings, fluorescence microscopy was performed upon 4 h of incubation. As shown in Figure 4D, in normal C6 cells, both free Dox and NP-Dox were efficiently internalized and/or retained. In C6-ADR cells, on the other hand, hardly any free Dox was detectable intracellularly, while NP-Dox could be detected in reasonably high amounts. Together with the results of the above-mentioned study, these insights illustrate that because of their different mode of cellular uptake, i.e. endocytosis vs. passive diffusion, standard nanomedicines seem to be able to overcome MDR, at least to some extent, and at least under certain circumstances.

3.3 MDR MODULATORS AND NANOMEDICINES

Another strategy that has been extensively evaluated is the use of nanomedicines in combination with MDR - and in particular with ABCB1 - modulators. This strategy can either encompass the use of free or nanomedicine-incorporated MDR inhibitors in combination with a nano-encapsulated chemotherapeutic drug, or a nanomedicine formulation containing both MDR modulators and chemotherapeutic drugs within a single drug delivery system. As an example for the former, Wang et al combined liposomes containing verapamil (a classical first-generation ABCB1 inhibitor) with liposomes containing doxorubicin [98]. The formulations were tested in multidrug resistant Dunning Mat-LyLu-B2 rat prostate carcinoma and MESSA/Dx5 uterus sarcoma, at doses more than a 10-fold lower than the IC₅₀ of the mixture of liposomal doxorubicin and free verapamil. It was found that free verapamil plus liposomal doxorubicin was not able to reverse/overcome MDR in these two resistant tumor models. Combining verapamil-liposomes with doxorubicin-liposomes, on the other hand, drastically improved cytotoxicity and therapeutic efficacy. Many similar examples of combinations of MDR modulators with nanomedicine formulations are available in the literature (see Table 2).

Another interesting study in this regard was reported by Patel et al, who used a third-generation ABCB1 inhibitor, tariquidar, in combination with paclitaxel, which is a classic hydrophobic substrate for ABCB1 (see Figure 5A). Both resistant and non-resistant variants of ovarian carcinoma (SKOV-3TR and SKOV-3, respectively) were used in this study, and their ABCB1 expression levels were evaluated using fluorescence-activated cell sorting

[99]. As shown in Figure 5B, more than ten-fold higher ABCB1 expression levels were observed in resistant vs. sensitive SKOV-3 cells. Both cell lines were then treated with free tariquidar, tariquidar-loaded liposomes, paclitaxel-loaded liposomes, and tariquidar-paclitaxel-co-loaded liposomes, for 48 h. The initial IC₅₀ values for paclitaxel were 27 and 2743 nM in SKOV-3 and SKOV-3TR cells, illustrating that an approximately 100-fold higher dose of paclitaxel was required to induce cytotoxicity in SKOV-3TR cells. Upon treatment with tariquidar-paclitaxel-co-encapsulated liposomes, the IC₅₀ shifted to 18 nM in SKOV-3 cells and to 34 nM in SKOV-3TR cells (Figure 5C), clearly showing the benefit of combining ABCB1 modulators with nanomedicine formulations. In summary, this study demonstrates that the co-delivery of the third-generation ABCB1 inhibitor tariquidar and paclitaxel within long-circulating liposomes is possible, and potently reduces MDR in ovarian carcinoma cells.

In another combinatorial anti-MDR approach, Wong and colleagues developed polymer-lipid hybrid nanoparticles (PLN) capable of co-delivering doxorubicin (D) and a BCRP inhibitor, GG918 (Elacridar) (G) [100]. Upon administering single agent PLN, the constructs enter cells via endocytosis, although free DOX, which was partially prematurely released, can enter viadiffusion. While diffusing through the cellular membrane, free Dox is flipped out by drug efflux pumps. However, when administered together with the BCRP inhibitor, efflux pump activity is inhibited, resulting in the retention of higher amounts of free and PLN-associated Dox within the tumor cells (Figure 5D). When administering dual agent-loaded PLN (DG), the local concentration and distribution of both Dox and GG918 matches quite well (since they are both released from the same PLN), and ABCB1 was inhibited even more strongly (Figure 5D). This strategy was further evaluated by determining the clonogenic survival of the multidrug resistant breast cancer cell line MDA435/LCC6/MDR1. Treatment regimens were as follows: PLN loaded with doxorubicin (Dn) or with GG918 solution (Gn), or with both drugs together (DG)n, and they were compared to free doxorubicin (D) and to free GG918 (G). As exemplified by Figure 5E, the results indicated that (DG)n, i.e. nanoparticles loading both doxorubicin and BCRP inhibitor, exhibited the highest degree of anticancer activity, with an IC₅₀ value of 0.34 mg/ml, as compared to 1.54 µg/ml for D, 0.94 µg/ml for Dn+Gn, 0.90 µg/m for Dn, 0.61 mg/ml for Dn+G, 0.44 mg/ml for D+Gn and 0.38 mg/ml for D+G. Fluorescence microscopy images of wild-type and multidrug resistant cells were in line with these findings (Figure 5F). Together, these findings demonstrate that when using a nanoformulation containing both an efflux pump inhibitor and a chemotherapeutic agent, MDR can be overcome relatively efficiently.

3.4 SiRNA-BASED NANOMEDICINES

Increasing numbers of recent anti-MDR studies involve the use of siRNA. Examples e.g. include the co-encapsulation of siRNA and chemotherapeutics within (mostly cationic) polymer- and lipid-based drug delivery systems. An interesting example in this regard has been reported by Xiao-Bing and colleagues, who used micelles based on degradable poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) block copolymers carrying doxorubicin and anti-ABCB1 siRNA (see Figure 6A). The micelles were further functionalized with TAT (as a cell penetrating peptide) and with RGD-4C (as a targeting ligand) [101]. The efficacy of this multifunctional nanomedicine formulation was tested in mice bearing Pgp-overexpressing MDA-MB-435 tumors. The construct was also tagged with a near-infrared dye (Dy677), in order to trace its fate upon i.v. injection (Figure 5A). Confocal microscopy studies showed that standard Dox-containing micelles showed hardly any Dox accumulation within cytoplasm of resistant cells, in particular if scrambled rather than ABCB1-specific siRNA was used. As expected, multi-functionalization proved to be efficient, and the RGD/TAT-double-targeted micelles carrying both Dox and ABCB1-

specific siRNA exhibited the highest levels of cellular internalization (Figure 6B). In good agreement with this, co-incorporating TAT and RGD into the micelles resulted in potent *in vitro* cytotoxicity, inhibiting cell growth by more than 70% (Figure 6C). The conjugates were subsequently also tested *in vivo*, in order to their biodistribution upon *i.v.* injection into athymic nude mice using optical imaging. As shown in Figure 6D, a significant fluorescence of RGD-micelle-Dy677-siRNA and RGD-micelle-Dox-Cy5.5 was observed in tumors at 24 h post injection. In case of non-targeted control micelles, localization to liver and kidney was most prominent, and tumor accumulation was barely detectable. Using multifunctional nanoparticles and a multimodal approach, these findings therefore convincingly confirm that nanomedicines co-containing chemotherapeutics, siRNA and targeting ligands hold significant potential for overcoming multidrug resistance.

Another interesting study demonstrating the potential of using siRNA-based nanomedicines to assist in overcoming MDR has reported by Saad and colleagues [102], who prepared liposomes carrying doxorubicin as a chemotherapeutic drug, and two different siRNA's, targeted against proteins responsible for drug efflux and for anti-apoptotic responses, and thereby attacking multi-drug resistance at two different levels (i.e. both pump and non-pump-related resistance mechanisms). In particular, they incorporated siRNA against ABCC1 (pump-resistance) and BCL2 (non-pump resistance). The latter is a potent anti-apoptotic protein, and is over-expressed in many human tumors. The liposomes they prepared were 100-140 nm in diameter, and labeled with the green dye NBD, to enable microscopic assessment of their cellular uptake. Doxorubicin uptake was monitored on the basis of its auto-fluorescence, and siRNA delivery was visualized by means of siGLO red transfection indicator (i.e. an RNA duplex which is labeled with DY-547). Upon the incubation of multidrug resistant H69AR lung cancer cells, it was observed that the (DOTAP-based) liposomes efficiently entered the cells, and that siRNA and doxorubicin were efficiently delivered to the cytoplasm and the nucleus (Figure 6A). The *in vitro* potential of these constructs was tested using a TUNEL-based apoptosis assay, showing that liposomes containing only ABCC1-siRNA, BCL2-siRNA and/or doxorubicin were much less efficient in inducing programmed cell death than were liposomes containing all three drugs within a single formulation (Figures 6B-C). In line with this, the simultaneous suppression of pump- and non-pump-based resistance resulted in much more efficient cell killing (Figure 6D). Future *in vivo* studies, in which such combinatorial siRNA-based approaches are used to overcome different aspects of multidrug resistance, therefore seem to be strongly warranted.

4. CHALLENGES AND PERSPECTIVES

Multidrug resistance is a serious problem in the treatment of malignancies. Several tumors, such as lung carcinomas and glioblastomas, exhibit a high degree of (cross-) resistance to chemotherapeutic agents, making them notoriously difficult to treat. As described above, and as exemplified by the many studies summarized in this theme issue of Advanced Drug Delivery Reviews, the use of nanomedicines to overcome MDR is relatively promising. We have here outlined several prototypic strategies for employing nanomedicines to tackle MDR, including the use of carrier materials with intrinsic anti-resistance properties, the use of materials modifying the mode of cellular uptake of chemotherapeutic drugs, and the (co-) formulation of chemotherapeutic drugs together with low- and high-molecular-weight MDR inhibitors within a single drug delivery system.

One of the key challenges for these strategies, and arguably for the nanomedicine field as a whole, will be to demonstrate efficacy at the *in vivo* level, in realistic and clinically relevant animal models. For obvious reasons, the vast majority of the work performed to date has been obtained *in vitro*. This has the advantage of enabling relatively large-scale screening

experiments, and of addressing the mechanisms of resistance at the molecular level in relatively great detail. In order to make these efforts meaningful, however, and to foster the clinical translation of anti-MDR nanomedicines, it is imperative to increase both the quantity and the quality of in vivo studies. This is challenging for several reasons, in particular because many of the models routinely used for such purposes are known to poorly predict how well drugs and drug delivery systems will eventually perform in patients. Consequently, establishing tumor models better resembling the human situation seems crucial. Such models would e.g. include the development of standardized animal models for studying multidrug resistance, to enable head-to-head comparisons on the efficacy of different anti-MDR nanomedicines, or the generation of transplant models of resistant human tumor material into nude mice, to assess the in vivo potential of formulations in 'real' patient tumors.

Standardization and head-to-head comparisons are also important for the in vitro situation, as large variations in in vitro efficacy are frequently observed, in particular when comparing the results obtained in different labs. In certain cases, using certain cell lines and certain nanomedicines, highly effective (e.g. up to a hundred-fold) inhibition of MDR is reported, whereas in other cases, improved efficiency is barely detectable. One should keep in mind in this regard that negative results are generally not published, and that there might therefore be much more evidence out there showing that overcoming MDR only works under certain circumstances, and not under others. Consequently, establishing standardized and well-characterized in vitro models for (cross-comparing) anti-MDR studies is highly important.

Another challenge, in particular at the clinical level, will be to predict which tumors become resistant at what stage, and upon which treatments. This should ideally be done using biomarkers, which can e.g. be derived from ex vivo genetic and proteomic profiling of tumors biopsies. In vivo biomarkers reporting on multidrug resistance would even be more ideal. From a clinical point of view, identifying biomarkers in blood (if available), or using imaging probes and protocols to assess and/or predict MDR, would really be highly valuable. Thus far, however, efforts in this regard have been very limited.

A final important issue relates to the general concept of using nanomedicines to overcome multidrug resistance. Given the fact that MDR not only results from the action of drug efflux pumps, but e.g. also from alterations in the apoptotic machinery, the cellular membrane and DNA damage repair, it would be interesting to also look at the potential of nanomedicines to tackle such physiological aspects of MDR. Furthermore, virtually all studies in this area of research are based on cells not responding to standard chemotherapeutics, but it is not known whether cells – and in particular cancer cells, which generally adapt very efficiently to all sorts of conditions – can also develop resistance against nanomedicines.

Therefore, multidisciplinary research, involving scientists working on the genetic and pharmacological aspects of MDR should team up with scientists working on the design and evaluation of nanomedicines, and together with experts on the development of standardized and realistic animal models, they should perform in-depth in vitro and in vivo analyses on the ability of nanomedicines to provide long-term relief of MDR. In our opinion, only such standardized studies, performed by interdisciplinary research teams in clinically relevant animal models, will one day enable us to use nanomedicine formulations in the clinic to overcome multidrug resistance.

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Figure 1. Schematic molecular architecture of prototypic human ABC transporters.
A. ABCC1, **B.** ABCB1, **C.** ABCG2. TMD – transmembrane domain, NBD – nucleotide-binding domain, L₀ – loop 0.

Figure 2. Resistance profiles overlapping among different MDR proteins.

Anthrcls: anthracyclines, Campto: camptothecin, Epiphtx: epipodophyllotoxins, MetAgs: methylating agents, MTX: methotrexate, PurAns: purine analogues, TKIs: tyrosine kinase inhibitors, VincaAls: Vinca alkaloids.

Figure 3. Overcoming MDR using pluronics.

A. Mechanisms suggested to contribute to the intrinsic anti-MDR properties of pluronic block copolymers: (1) incorporation of pluronics into membranes and decrease of the membrane microviscosity; (2) induction of ATP depletion; (3) inhibition of energy-dependent drug efflux transporters; (4) release of cytochrome C from mitochondria and increase of ROS levels in the cytoplasm; (5) increase of pro-apoptotic and decrease of anti-apoptotic signaling; (6) inhibition of the glutathione / glutathione S-transferase detoxification system; (7) abolishment of drug sequestration within cytoplasmic vesicles. **B.** Design of MTX-conjugated pluronics-based mixed micelles for more efficient treatment of resistant cancers via Pgp efflux inhibition. **C.** Tumor volume and tumor weights were determined for saline, MTX, PF-MTX (in which MTX was physically entrapped) and F127/P105-MTX-treated animals. **D.** In vivo fluorescence reflectance imaging of mice bearing subcutaneous KBv tumor upon i.v. injection of DIR-labeled F127/P105 micelles and DIR-labeled F127/P105-MTX micelles, showing efficient tumor accumulation (arrows). Images are adapted and reproduced with permission from ref [92,95].

Figure 4. Overcoming MDR using standard nanomedicines.

A. Schematic depiction of how standard nanomedicines can be used to overcome MDR. Free drugs are taken up via passive diffusion across the cellular membrane. In MDR cells, they are sensed and externalized by drug efflux pumps. Nanodrugs such as polymers, micelles and liposomes, are taken up by endocytosis, thereby evading drug efflux pumps. **B.** Survival of parental (non-resistant; dashed lines, open symbols) and multidrug resistant (solid lines, closed symbols) cancer cells upon 96 h of incubation with increasing concentrations of free doxorubicin (DOX), polymer-bound doxorubicin (Pol-DOX), micellar doxorubicin (Mic-DOX) and liposomal doxorubicin (Lip-DOX). **C.** Intracellular DOX and NP-DOX accumulation in C6 and C6-ADR cells. Cells were exposed to 1000 ng/ml free and NP-associated DOX for 4 h, and the fluorescence intensity of the cell lysate was determined immediately afterwards (4 h) and 20 h later (24 h). The bottom panels show in vitro efficacy of free vs. NP-DOX in C6 and C6-ADR cells. **D.** Fluorescence images showing the intracellular localization of DOX and NP-DOX in C6 and C6-ADR cells upon incubation with 1000 ng/ml DOX-equivalent for 4 h, followed by 20 h incubation with drug-free medium. Scale bar: 20 μ m. Images are reproduced and adapted with permission from ref [96,97].

Figure 5. Overcoming MDR using nanomedicines and Pgp modulators.

A. Schematic depiction of a Pgp efflux pump located within the cellular membrane effluxing hydrophobic substrates. **B.** Increase in Pgp expression in SKOV-3 and SKOV-3TR cells upon paclitaxel treatment. **C.** IC₅₀ values for paclitaxel in SKOV-3 and SKOV-3TR cells. PCL alone: free paclitaxel. PCL liposomes: Paclitaxel liposomes. XRPCL: tariquidar- and paclitaxel-loaded liposomes. α indicates that the IC-50 for paclitaxel-loaded liposomes in SKOV-3TR cells was not achieved, not even upon treatment with a dose as high as 1000 nM of paclitaxel-equivalent. **D.** Schematic depiction of doxorubicin-containing nanomedicines overcoming MDR when used in combination with the prototypic Pgp inhibitor GG918 (G). The illustration depicts two different scenarios, where the drug and the Pgp modulator are either applied separately (top panel), or in combination (bottom panel). **E.** Cytotoxicity of different formulations containing doxorubicin (D) and/or GG918 (G), in the Pgp-overexpressing human breast cancer cell line MDA435/LCC6/MDR1, measured by clonogenic assay. Results are normalized against cells treated with drug-free medium. **F.** Fluorescence microscopy images of (1) wild-type MDA435/LCC6/WT cells treated with free Dox; and multidrug resistant MDA435/LCC6/MDR1 cells treated with free Dox (2), free Dox plus GG918 (3), and nanomedicines containing both Dox and GG918 (4). Bar: 20 μm. Images are reproduced and adapted with permission from ref [99,100].

Figure 6. Overcoming MDR using siRNA-based nanomedicines.

A. Scheme depicting the design of multifunctional micellar nanomedicines for targeted co-delivery of siRNA and DOX to overcome multidrug resistance. (a) Chemical structure of functionalized copolymers with ligands at the end of the PEO block and conjugated moieties on the PCL block. (b) Assembly of multifunctional micelles with DOX and siRNA in the micellar core and RGD and/or TAT on the micellar shell. (c) Schematic diagram exemplifying the intracellular processing of the targeted micelles in multidrug resistant cancer cells upon endocytosis, leading to cytoplasmic siRNA delivery followed by Pgp down-regulation and endosomal Dox release and nuclear accumulation. **B-C.** Cellular uptake (B) and in vitro efficacy (C) of control micelles (NON), RGD-targeted micelles, TAT-targeted micelles and RGD-TAT-double-targeted micelles bearing Dox and anti-MDR1 siRNA in multidrug resistant MDA-MB-435/LCC6 cells. **D.** In vivo evaluation of the tumor (arrow) accumulation of RGD-targeted vs. control micelles containing either DOX-Cy5.5 (left panels) or Dy677-siRNA (right panels) at 24 h post i.v. injection. Experiments were performed in athymic nude mice bearing MDR1-resistant MDA-MB-435/LCC6 tumors. Images are adapted and reproduced with permission from ref [101]

Figure 7. Overcoming MDR using siRNA-based nanomedicines.

A. Intracellular localization of doxorubicin- and siRNA-containing cationic liposomes (prepared using NBD-labeled DOTAP) in MDRH69AR human lung cancer cells, showing efficient cellular uptake. **B.** Induction of apoptosis (in green; based on TUNEL staining) upon treatment of cells with the indicated formulations. **C.** Quantification of apoptosis induction. Numbers refer to the formulations depicted in panel B. **D.** Cell viability, assessed using a modified MTT assay, showing the most efficient cell killing upon combining liposomal chemotherapeutics with anti-MRP1-siRNA and anti-Bcl2-siRNA. Images are adapted and reproduced with permission from ref [102].

Table 1
Organ and tissue distribution of the prototypic MDR proteins ABCB1, ABCC1 and ABCG2 in healthy human tissues, as assessed by immunodetection.

+ indicates evidence for expression, – indicates no expression detected. ND: no data available. Numbers between brackets refer to references.

ORGAN / TISSUE / CELLS		ABCB1	ABCC1	ABCG2
Brain	Neurons	– [24]	– [24]	– [17] [25]
	Glial cells	– [24]	– [24]	– [17] [25]
	Blood-brain barrier endothelium	+ [24]	– [24]	+ [25]
Gastro-intestinal tract	Salivary glands	+ [26]	+ [26]	ND
	Esophagus	– [24]	– [24]	– [17]
	Stomach	+ [24]	+ [24]	– [17]
	Small intestine	+ [24] [18] [27]	+ [24]	+ [17] [28]
	Colon	+ [24] [18] [27]	+ [24]	+ [17] [28]
Liver	Hepatocytes	– [24]	– [24]	[17] + [28]
	Bile canaliculi	+ [24] [18] [27]	– [24]	+ [17]
	Biliary ducts	+ [29] – [18] [24]	– [24]	– [17]
Pancreas	Acinar cells	– [24]	– [24]	– [17] + [28]
	Ductal cells	+ [24] [18]	+ [24]	– [17]
Muscles	Striated	– [24]	+ [24]	ND
	Cardiac	– [24]	– [24]	– [17]
	Smooth	– [24]	+ [24]	ND
Lungs	Pneumocytes	– [24]	– [24]	+ [28]
	Bronchioli	+ [24] [27]	+ [24]	ND
Kidneys	Glomeruli	– [24]	– [24]	– [17]
	Proximal tubule	+ [18] [24] [27]	+ [24]	– [17] + [28]
	Distal tubule	– [24]	– [24]	– [17] + [28]
	Collecting duct	– [24]	– [30]	ND
Spleen	Red pulp	– [30]	– [30]	– [17]
	White pulp	– [30]	– [30]	– [17]
Thymus	Lymphoid cells	– [30]	– [30]	ND
	Epithelial cells	– [30]	– [30]	ND
Thyroid gland	Follicular cells	– [30]	+ [30]	ND
	Parafollicular cells	– [30]	– [30]	ND
Adrenal glands	Medulla	[30] + [18]	– [30]	– [17]
	Cortex	+ [18] [30] [27]	+ [30]	– [17] + [28]
Testis	Leydig cells	+ [31]	+ [31]	– [17] [31] + [28]

ORGAN / TISSUE / CELLS		ABCB1	ABCC1	ABCG2
	Sertoli cells	+ [32] – [31]	+ [31]	– [17] [31] [32]
Prostate		– [30]	+ [30]	– [17] + [28]
Ovary	Germinal cells	– [30]	– [30]	– [17]
	Epithelial cells	– [30]	– [30]	– [17]
Placenta	Syncytiotrophoblast	+ [27] [33]	+ [34]	+ [17] [28]
	Fetal endothelium	ND	+ [34]	ND
Breast	Lobules	ND	ND	+/- [17]
	Lactiferous ducts	+ [27]	ND	+/- [17]
Peripheral blood elements	Erythrocytes	– [35]	+ [35] [36]	+ [37] [38] – [17]
	Leukocytes	+ [35]	+ [35]	+ [35]
	Platelets	– [35]	+ [35] [39]	– [17] [35]

Table 2
Overview of literature reports providing evidence on the use of nanomedicine formulations to overcome multidrug resistance.

Table is adapted and reproduced with permission from ref [110].

MDR Modulator	MDR Substrate	Nanocarrier	In vitro	In vivo	Result	Ref
Cyclosporin A	Doxorubicin	Polyalkylcyanoacrylate (PACA) nanoparticles	Dox-resistant leukemia (P388/ADR cells)	-	Improved cytotoxicity	[103]
Verapamil	Doxorubicin	Stealth liposomes	MDR rat prostate adenocarcinoma Mat-LyLu-B2 (MLLB2) cells	PK in Sprague Dawley rats	Improved cytotoxicity Improved PK profile	[104]
Elacridar	Doxorubicin	Polymer-lipid hybrid nanoparticles (PLN)	Human breast carcinoma (MDA435/LCC6/MDR1) cells Clonogenic assay in MDA435/LCC6/MDR1 cells	-	Improved doxorubicin uptake Long-term cancer growth suppression	[100]
Verapamil	Doxorubicin	Transferrin-conjugated liposomes	Chronic myelogenous leukemia (K562/DOX) cells	-	Improved cytotoxicity	[105]
Tamoxifen	Topotecan	Wheat germ agglutinin- conjugated liposomes	Murine glial tumor (C6) cells Transport across BBB (brain microvascular endothelial cells/rat astrocytes) – (BMVECSs/RAs)	C6 tumor-bearing Sprague Dawley rats	Improved cytotoxicity Improved transport and targeting of C6 cells Longer survival of animals	[106]
Tariquidar	Paclitaxel	Poly(D,L-lactide-co-glycolide acid) (PLGA) nanoparticles Biotin-poly(D,L-lactide-co-glycolide acid) (PLGA) nanoparticles	Murine mammary adenocarcinoma (JC) and human ovarian adenocarcinoma (NCI/ADR-RES) cells	JC tumor-bearing female BALB/c mice	Improved cytotoxicity Improved tumor growth inhibition	[107]
Verapamil	Vincristine	Poly(D,L-lactide-co-glycolide acid) (PLGA) nanoparticles	Human breast cancer (MCF-7/ADR) cells Human hepatocellular carcinoma (BEL7402/5-FU) cells	- -	Improved cytotoxicity Improved cytotoxicity	[108] [109]
Tariquidar	Paclitaxel	Stealth liposomes	Paclitaxel-resistant human ovarian adenocarcinoma (SK-OV-3TR) cells	-	Improved cytotoxicity	[99]