Overcoming multidrug resistance with nanomedicines

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Introduction: Cancer remains the leading cause of death worldwide. Numerous therapeutic strategies that include smart biological treatments toward specific cellular pathways are being developed. Yet, inherent and acquired multidrug resistance (MDR) to chemotherapeutic drugs remains the major obstacle in effective cancer treatments.

Areas covered: Herein, we focused on an implementation of nanoscale drug delivery strategies (nanomedicines) to treat tumors that resist MDR. Specifically, we briefly discuss the MDR phenomenon and provide structural and functional characterization of key proteins that account for MDR. We next describe the strategies to target tumors using nanoparticles and provide a mechanistic overview of how changes in the influx:efflux ratio result in overcoming MDR.

Expert opinion: Various strategies have been applied in preclinical and clinical settings to overcome cancer MDR. Among them are the use of chemosensitizers that aim to sensitize the cancer cells to chemotherapeutic treatment and the use of nanomedicines as delivery vehicles that can increase the influx of drugs into cancer cells. These strategies can enhance the therapeutic response in resistant tumors by bypassing efflux pumps or by increasing the nominal amounts of therapeutic payloads into the cancer cells at a given time point.

Keywords: cancer multidrug resistance, drug targeting, multidrug resistance efflux pumps, nanomedicine


1. Introduction

Cancer is a leading cause of death worldwide, causing 8.2 million deaths in 2012 according to the World Health Organization (WHO). The WHO expects that the annual cancer cases will rise from 14 million in 2012 to 22 within the next two decades [1]. Therefore, cancer treatments stand in the forefront of the scientific arena. These treatments involve a selection of one or more interventions, such as surgery, radiotherapy, chemotherapy and novel biotherapy modalities. Such novel biotherapy modalities include the use of mAb or small molecules for specific blockage of cellular pathways.

Chemotherapy often fails since tumors can resist chemotherapeutic agents due to multidrug resistance (MDR), also termed multiresistance [2]. Recognizing the different broad characteristics of MDR, the terms extensively drug-resistant and pan drug-resistant have been coined to account for MDR although no consensus has yet been reached on the precise definitions and use of these terms [3].

MDR can arise in numerous mechanisms, which can be non-cellular or cellular-based. Non-cellular mechanisms involve limited vascular accessibility and rapid clearance, which leads to inadequate intratumor drug concentration, or factors of the tumor cells’ microenvironment, which inhibit damage-induced apoptotic signaling. Cellular mechanisms, which lead to MDR, involve enzymatic degradation,
which leads to drug inactivation, or transport-based mechanism, which involves the efflux of drug from the cell by various energy-dependent membrane transport proteins, thereby limiting the drug from reaching to therapeutic concentrations inside the cell [4]. In order to overcome MDR, oncologists usually change chemotherapeutic regimens or use combination therapy. In many cases, this approach does not prevent treatment failure and cancer recurrence, due to the fact that tumors are composed of diverse population of cells, which are genetically unstable, and may develop resistance to different chemotherapeutic agents during time. Several strategies have long been used in order to overcome the obstacles mentioned above. First, many efforts have been made to find analogs of routinely used chemotherapeutic agents, which would have wider activity and lower toxicity. Yet, very few of these analogs become clinically useful, due to the fact that interrupting the original molecular structure of the drug usually resulted in a reduced anticancer activity and did not reduce the risk of toxicity [5,6].

The second strategy is administration of MDR modulators, also known as chemosensitizers, in combination with anti cancer drugs. The mechanism of action of these MDR modulators is usually blocking efflux pumps of the ATP-binding cassette (ABC) transporter family [7,8]. The main shortcoming of this approach is that these MDR chemosensitizers are in many cases toxic at the concentration inhibiting resistance [9].

Drug delivery strategies hold great promise, especially nanosized particles that are designed to overcome MDR. Notably, in recent years, new and innovative approaches have been developed to reverse drug resistance using nanoparticles (NPs) in order to increase the therapeutic effectiveness and to decrease potential toxicities.

Paul Ehrlich has coined the idea of a ‘magic bullet’. This concept laid the foundation for development of targeted drug delivery: personalized and tailored drugs that precisely target a specific molecular pathway within the cancer cell [10]. Since then, numerous types of nanoscaled drug delivery systems (DDS) have been devised and evaluated for delivery of therapeutic payloads to tumors. Nanoscaled DDS have several outstanding properties: they can carry multiple drugs molecules and/or imaging agents; their high surface area-to-volume ratio enables high ligand density on their surface for active cellular targeting strategies; they could also be utilized to increase the local drug concentration by carrying the drug and release it in a controlled manner on binding to its target [11]. The family of nanoscaled DDS includes polymer conjugates, polymeric NPs, lipid-based carriers such as liposomes and micelles, protein-based carriers, including immune-conjugates and spheres, dendrimers, carbon nanotubes and inorganic NPs such as gold, silver and TiO₂ [11,12].

Overcoming MDR with drug delivery strategies is a broad topic. Given the massive amount of scientific data and the ample number of papers in the field, we focused on selected subjects that reflect the implementation of drug delivery strategies to resist MDR. Specifically, we briefly survey the MDR phenomenon and provide structural and functional characterization of key proteins that account for MDR. We next describe passive tissue targeting and active cellular targeting of drugs using nanomedicines. We also provide a mechanistic overview of how changes in the influx:efflux ratio result in overcoming MDR. Finally, we present our view of the field and discuss future outlook.

2. MDR: a multi-faceted phenomenon

MDR is a multidimensional phenomenon that is manifested by resistance to many different chemotherapeutic agents and is possibly caused by multiple operating mechanisms. Alternatively, it may also occur by the same mechanism that confers resistance to a wide repertoire of drugs. Drug resistance can be manifested in numerous modes, including a diminished drug influx, activation of the cellular DNA repair mechanisms, metabolic modification and detoxification of the given drug and enhancement of drug efflux, which mostly occurs though ATP-driven extrusion pumps belonging to the ABC superfamily. The latter is the most prominent mechanism since overexpression of ABC transporters is prevalently observed in cancers [13,14].

ABC transporters are transmembrane proteins that are expressed on the plasma membrane and may also be seen on the organelles membranes. They perform crucial physiological functions and are responsible for transporting foreign substances and toxins across the membrane by exploiting energy from ATP hydrolysis [15]. To date, almost 50 human ABC transporters are known, classified into seven families (ABCA to ABCG) by the Human Genome Organization. From which, the most commonly associated with MDR phenotype

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<td>- Cancer multidrug resistance (MDR) is most likely one of the major hurdles for successful treatment, and comprehensive understanding of protein extrusion pumps may pave the way for novel strategies to overcome MDR.</td>
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<td>- Enhancement of drug efflux mostly occurs through ATP-driven extrusion pumps members of the ATP-binding cassette (ABC) superfamily, and their structure-function relationship characterization is crucial for molecular and clinical translations.</td>
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<td>- Increasing the influx of drugs using targeted nanocarriers will shift the balance between influx and efflux occurring via the ABC transporters and may result in an effective therapeutic benefit.</td>
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<td>- The use of MDR inhibitors (also known as chemosensitizers) blocks the ABC transporters and eventually cumulates to cancer cell death.</td>
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<td>- Combinational treatment with MDR inhibitors together with targeted nanomedicines entrapping therapeutic payloads or even MDR inhibitors and chemotherapeutic agents entrapped in the same nanomedicine are expected to result in a potent therapeutic benefit.</td>
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This box summarizes key points contained in the article.
are the ABCB1 (also known as MDR1/P-glycoprotein [P-gp]), ABCC1 (also known as MDR-associated protein 1 [MRP1]) and ABCG2 (also known as breast cancer resistance protein [BCRP]). These protein pumps can significantly decrease the intracellular concentration of a wide plethora of distinct cytotoxic molecules, which are characterized by different structural, biochemical and mechanistic features, and thereby enhance the MDR phenomenon [16,17].

2.1 ABCB1/MDR1/P-gp
Almost 40 years have passed since P-gp was cloned and characterized by its ability to confer MDR phenotype to ovarian cancer cells that had developed resistance to chemotherapeutic drugs [18]. Subsequently, P-gp, encoded by the ABCB1/MDR1 gene, was the first human ABC transporter to be described [19], and historically also named MDR1. It is one of the most studied transmembrane transporters in humans and has a central clinical importance (for comprehensive reviews, [20,21]).

P-gp has an exceptional broad specificity, transporting hundreds of neutral and cationic hydrophobic compounds as small as 330 Da up to 4000 Da with varying functions and structures. P-gp is found in only a limited number of tissues, mainly in the liver, kidney, ovary, testes, small and large intestines and capillary endothelial cells in the brain [22]. Additionally, it is expressed in cancer cells (especially leukemia, esophageal carcinomas, ovarian adenocarcinoma, pancreatic adenocarcinoma and non-small-cell lung cancer), where it functions as an energy-dependent efflux pump accounting for decreased drug accumulation and thereby confers MDR [23]. For example, P-gp is expressed in acute myeloblastic leukemia (AML) in one-third of the patients at the time of first diagnosis, and in more than half of the patients at first relapse. Evidently, its expression level negatively correlates with the likelihood of relapse [24], and its inhibition by cyclosporine A increases the remission chance and patients’ survival [25]. P-gp plays a major role in MDR also in non-cancer-related drug treatments as a result of its wide-ranging effects on the absorption and excretion of a variety of drugs. The ABCB1/MDR1 gene exhibits a considerable single nucleotide polymorphism (SNP), with total of > 50 SNPs and three insertion/deletion polymorphisms, which accounts with differential bioavailability of substrates and extent of drug resistance [26].

P-gp is a 170 kDa transmembrane protein, comprising an N-terminal half molecule containing six transmembrane helices (called transmembrane domain [TMD]), then a large cytoplasmic domain with an ATP-binding site (named the nucleotide-binding domain [NBD]), followed by a second half with six transmembrane helices and an ATP-binding site that shows over 65% of amino acid similarity with the first half of the polypeptide [16,27]. The structure of the human P-gp has not been solved by X-ray crystallography so far, yet low resolution mammalian structures, at a limit of ~ 20 Å, were determined by electron microscopy [28,29]. The X-ray structure of a murine P-gp, which shares 87% sequence identity with the human P-gp, was reported a few years ago. However, it is incomplete, missing an intermediate linker sequence that is proved to be essential for substrate recognition and ATP hydrolysis. In addition to this structure, further murine P-gp structures were solved in a complex with cyclic peptide inhibitors of the protein, characterizing the unique protein−drug interactions and yielding informative crucial data regarding poly-specificity [30]. Molecular dynamics simulations, which have been performed with the murine P-gp structure, shed important insights on the efflux mechanism [31]. The murine P-gp X-ray structure was solved in an apo inward-facing conformation (Figure 1A) [30] and reveals that the two sections of the protein are positioned in a pseudo twofold molecular symmetry. All these murine structures unquestionably enhance our understanding and provide opportunities for structure-function and dynamic studies of the human P-gp.

2.2 ABCC1/MRP1
The MRP1, encoded in humans by the ABCC1 gene, was first cloned in 1992 and was identified because of its ability to provide MDR to a lung cancer cell line, H69AR [32]. MRP1 is a 190 kDa protein, member of the ABC superfamily of transmembrane proteins that is able to exploit the energy of ATP-binding and hydrolysis to efflux chemotherapeutic drugs. Being expressed in a wide variety of solid and hematological malignancies, MRP1 is also found in most normal tissues throughout the human body, where it physiologically protects the body from endobiotics and xenobiotics, with higher levels in the lung, small intestine, placenta, testes, skin, kidney, colon, peripheral blood mononuclear cells and skeletal muscle [33,34]. It functions as a multispecific anion transporter for many organic substrates, including oxidized glutathione, cysteinyl leukotrienes, activated aflatoxin B1, sulfate conjugates of steroid hormones, bile salts and glucuronide conjugates. MRP1 has a few splice variants as a consequence of alternative splicing of exon deletion, yet all maintain the original open-reading frame [35].

 Elevated levels of MRP1 (both mRNA and protein) have been found consistently in a variety of mostly solid tumors, correlating with poor clinical outcome. Specifically, a role of MRP1 in MDR in prostate cancer, non-small-cell lung cancer, several types of breast cancer and neuroblastoma has been found [36]. At the latter case, it was shown that high expression level of the ABCC1 gene is an independent prognostic indicator of poor prognosis and correlates with the expression of the MYCN oncogene for which ABCC1 is a downstream target [37]. In hematological malignancies, MRP1 plays a role in AML and acute lymphoblastic leukemia (ALL), with respect to non-satisfactory therapeutic response and adverse effects in response to administration of chemotherapeutic drugs [36]. These observations indicate that inhibition or downregulation of MRP1 should have a positive
Figure 1. Structures of MDR-associated proteins P-gp, MRP1 and ABCG2 are shown. A. X-ray structure of P-gp in front (left) and back (right) views is presented. Helices 1 – 12 are labeled. The N- and C-terminal halves of the molecule are colored yellow and blue, respectively. Helices 4 and 5 and helices 10 and 11 crossover to form intertwined interfaces that stabilize the inward-facing conformation. Horizontal bars represent the approximate positioning of the lipid bilayer. B. Illustrations of homology model-based structure of MRP1 in the outward-facing conformation, where individual monomers are shown in blue or cyan (left). The model was constructed using the X-ray structure of the homodimeric exporter Sav1866, following a multiple sequence alignment that took into account closely related ABCC family members. The TMD is presented by an enlarged zoom-in view together with the membrane, and Y324 is highlighted in yellow (right). Atomic coordinates of the protein were downloaded from [43], and the model is visually presented using VMD [105]. C. Illustrations of homology model-based structures of ABCG2 in the open (left) and closed (right) apo inward-facing apo conformations. The models were constructed using the X-ray structures of *E. coli* MsbA and mouse P-gp as the templates, respectively. The TMD’s α-helices of each ABCG2 monomer are labeled with numbers 1 – 6 in different colors. Individual ABCG2 monomer is shown in red or cyan. The putative boundaries of the lipid bilayer are indicated with straight lines. A coupling helix in the intracellular loop connecting helix 4 and helix 5 of one ABCG2 monomer interacts with the NBD of the opposite monomer.

A. Reprinted with permission from the American Association for the Advancement of Science (AAAS) [30].

C. Reprinted with permission from [50].

MDR: Multidrug resistance; MRP: Multidrug resistance associate protein; NBD: Nucleotide-binding domain; P-gp: P-glycoprotein; TMD: Transmembrane domain; VMD: Visual molecular dynamics.
therapeutic effect, and hence it serves as a major therapeutic target for cancer treatments.

MRP1 (and other ABCC transporters, including MRP2) has a unique 5-unit structure (as opposed to the typical 4-unit of most ABC proteins, including P-gp and MRP4). It is composed of 2 NBDs and 17 transmembrane α-helices forming three TMDs (TMD0, TMD1 and TMD2). The cavity formed in the membrane, through which solutes can be evacuated from the cell, is formed by TMD1 and TMD2, whereas the NBDs bind ATP that is needed for the protein’s function [34,38]. The role of TMD0, at least in TMD2, whereas the NBDs bind ATP that is needed for the diverted effects of specific Y324 mutants on the activity for the diverted effects of specific Y324 mutants on the activity for the diverted effects of specific Y324 mutants on the activity for the diverted effects of specific Y324 mutants on the activity.

A structure of a full-length MRP1 was reported, but the resolution (22 Å) was not high enough to provide atomic resolution details [40]. However, the crystal structure of MRP1’s NBD1 in a complex with ATP and Mg^{2+} was solved, and the NBD1–NBD2 were modeled [41]. These studies paves the way for computational analysis, aiming to predict the three-dimensional structure of MRP1 for the entire protein by homology modeling [42], the common molecular modeling technique for structure prediction. Sav1886 (S. aureus ABC transporter) was used as template to model the TMD1 and TMD2 in conjunction with their corresponding NBDs (Figure 1B) [43]. Two models were constructed: at the first model the sequences of TMD1–NBD1 and TMD2–NBD2 of MRP1 were aligned separately to the Sav1866 sequence by a multiple sequence alignment that included corresponding regions of closely related ABCC family members; in the second alignment that produced the second model, only the sequences of TMD1–NBD1 and TMD2–NBD2 of MRP1 were aligned with Sav1866 and the resulting alignment was optimized manually. The predicted structures were relatively similar and were substantiated by a large body of compelling biochemical and functional experimental evidences that already established the protein’s topology and three-dimensional architecture. The structural model was analyzed by Amram et al. [44], who validated it by performing molecular dynamics simulations of MRP1, allowing the protein to release conformational constrains when embedded in a membrane. The simulations not only legitimized the homology model-based structure but also provided atomic level explanations for the diverted effects of specific Y324 mutants on the activity of the protein.

2.3 ABCG2/BCRP

ABC subfamily G member 2 (ABCG2) belongs to the G-branch of the ABC transporter superfamily that includes five members, ABCG1, ABCG2, ABCG4, ABCG5 and ABCG8. ABCG2 was identified in a breast cancer cell line MCF-7 that displayed an ATP-dependent reduction in the intracellular accumulation of anthracycline anticancer drugs in the absence of overexpression of known MDR transporters such as P-gp or MRP1, and consequently also named BCRP [45]. As P-gp and MRP1, ABCG2 confers to the MDR phenomenon; however, each displays a unique expression pattern. Specifically, ABCG2 is found in the placenta, the epithelium of the small intestine and colon, the liver canalicular membrane and in ducts and lobules of the breast. ABCG2 is able to transport structurally different substrates and can be inhibited by different inhibitors from diverse chemical groups [46,47].

The human ABCG2 gene is located on chromosome 4, consisting 16 exons and 15 introns. The protein’s length is 655 amino acids, and its X-ray structure has not been solved yet. However, it is predicted to be in a reversed configuration to most other ABC transporters, as its NBD is located at the N terminus, and followed by six putative transmembrane helices (together named TMD) at its C terminus [48]. ABCG2 possesses a large extracellular loop between transmembrane helices 5 and 6 and contains three putative glycosylation sites (D418, D557 and D596) [49]. The protein functions as a dimer and is suggested to form higher-order oligomers under physiological conditions since ABCG2 purified from Pichia pastoris was shown to assemble as a tetramer [50]. Based on a series of site-directed mutagenesis analysis, it was shown that helices 1 and 5 of the protein’s TMD are involved in its dimerization process, pointing specific residues accounting for the dimer formation (e.g., T402, G410, G533, C592 and C608) [47]. ABCG2 is expressed in solid tumors (such as esophageal, breast, colon, pancreatic and lung cancers) and is commonly involved in blood cancers (AML and ALL). Thus, it is a reasonable candidate for intense research (e.g., [51,52]), aiming for pharmaceutical targeting interventions toward development of potential inhibitors.

Since the X-ray structure of ABCG2 has not been determined yet, a few efforts have been made to computationally predict its structural model, both apo and holo. MsbA (an E. coli ABC transporter) and MalK (an E. coli maltose transporter) were used as templates to model the TMD and NBD of ABCG2, respectively. Then, the constructed TMD and NBD were combined, and the model was subjected to molecular dynamics simulation to account for the protein’s native structure [53]. Sav1886 (a S. aureus ABC transporter) and MalK (an E. coli maltose transporter) were used as templates to model the TMD and NBD of ABCG2, respectively, yielding its apo model after geometry optimization. Then, holo models with known ligands (daunomycin, doxorubicin [Dox], mitoxantrone, Hoechst 33342, prazosin, rhodamine and porphyrin) were produced following blind docking methodology [54]. Finally, two homology models of ABCG2 were built, one representing its substrate-free open apo inward-facing conformation based on MsbA (an E. coli ABC transporter) and the other representing its substrate-bound closed apo inward-facing conformation based on the mouse P-gp structure (Figure 1C) [50].

Taken together, comprehensive understanding of the structural features of the key protein extrusion pumps is essential for developing new therapeutic strategies with chemo sensitizers and targeted nanomedicines.
3. Drug targeting strategies

3.1 Passive drug targeting

On systemic administration of a low molecular weight therapeutic, it is rapidly cleared from the circulation and only extremely low doses of the drug reach and accumulate in the tumor vicinity (in the case of a solid tumor) and inside tumor cells (in the case of blood cancers and solid tumors), whereas their localization at healthy organs (liver, spleen, kidneys, lungs and bone marrow) is relatively high, which could enhance adverse effects [11].

Different types of nanoscaled DDS (termed here ‘nanomedicines’) have been designed and evaluated in preclinical models for tumor targeting. Examples of such strategies are

![Figure 2. Schematic representation of nanocarriers targeting cancer. (A) Schematic illustration of various nanomedicines for targeted drug delivery. These DDS are usually composed of a targeting moiety cross-linked to a carrier, which contains an entrapped or conjugated chemotherapeutic agent (B). Adapted with permission from [11]. DDS: Drug delivery system.](image-url)
Presented in Figure 2. These nanomedicines essentially aim to improve the circulation time of the entrapped or conjugated chemotherapeutic agents by relying on the pathophysiological properties of solid tumors, which tend to present a leaky vasculature, in contrast to the vasculature in healthy tissues. This increase in vascular permeability, together with the ineffective lymphatic drainage of solid tumors, allows nanomedicines with sizes up to several hundreds of nanometers to accumulate in solid tumors over time. This mechanism is known as the enhanced permeability and retention (EPR) effect (Figure 3) \cite{11,55,56}. Because of the fact that it essentially only relies on the pathophysiological properties of the target tissue, it is generally referred to as ‘passive drug targeting’. To increase their circulation half-life, nanomedicines are usually coated with hydrophilic polymer such as PEG, which attenuates the uptake of particles by macrophages and monocyte members of the reticuloendothelial system (RES). This further enhances efficient targeting of tumors \cite{57,58}.

The exploitation of the EPR effect is one of the most important strategies for improving the delivery of low molecular weight chemotherapeutic agents to tumors. These primarily include long-circulating liposomes, polymers and micelles. The most remarkable example of such nanomedicines is Doxil™ (Caelyx™ in Europe), FDA-approved PEGylated liposomal Dox, which passively directs the drug into the tumor vicinity by exploiting the EPR effect, and thus increases the local Dox concentration in the vicinity of the tumor and reduces the cardiotoxicity caused by Dox \cite{59}. The addition of PEG moiety endows Doxil with stealth-like properties, which also improves circulation time and stability of the drug carrier. Doxil is widely used in the clinic for several indications, such as breast cancer, ovarian cancer, multiple myeloma and Kaposi’s sarcoma.

Another well-known example of passively targeted formulation approved by the FDA is the albumin-based paclitaxel, Abraxane™. Paclitaxel is a poorly soluble drug and for many
Over the past 15 years, several strategies were developed to improve the delivery of drugs by designing nanomedicines so they can actively bind to specific receptors on malignant cells. These strategies rely on the use of targeting agents, such as proteins (mainly antibodies and their fragments), nucleic acids (aptamers) or other receptor ligands (peptides and carbohydrates), which specifically bind to receptors or antigens uniquely expressed (or overexpressed) at the target site [11]. There are several mechanisms by which active drug targeting can occur. One option is the binding to extracellular matrix surrounding the tumors while the nanocarrier is acting as a drug depot (Figure 3 inset [i]). Another option is to bind with high affinity and specificity to a particular cell surface receptor (Figure 3, inset, [ii]) and release its payload in a controlled manner. The last option is that the nanocarrier will recognize and bind to target cells through ligand–receptor interactions and bound carriers will internalize by various routes, such as receptor-mediated endocytosis (Figure 3, inset, [iii]). Whereas passive targeting aims to improve overall tumor accumulation, active cellular drug targeting is usually designed to improve target cell recognition and uptake and is particularly useful for intracellular delivery of otherwise poorly internalized macromolecular drugs, such as nucleic acid drugs (RNA interference [RNAi], anti-sense and pDNA), which need to be delivered into the cytoplasm of cancer cells or its nucleus to exert antitumor effects [70,71].

Effective tumor targeting with mAb has been demonstrated in several FDA-approved products over the past few decades. Remarkable examples of such mAbs are rituximab (Rituxan®) that was approved for treatment of non-Hodgkin’s lymphoma; trastuzumab (Herceptin®), an anti-HER2 mAb that binds to ErbB2 receptors, that was approved for the treatment of breast cancer; and bevacizumab (Avastin®), an anti-VEGF mAb, that inhibits the factor responsible for the growth of new blood vessels, which was the first angiogenesis inhibitor approved for treating colorectal cancer. Later, many antibody-based nanomedicines, such as immune toxins, have been approved for clinical use, namely zevalin, mylotarg, ontak and bexxar [11]. Clinical trials are being executed for a formulation of transferrin receptor-targeted cyclodextrin-based polymeric NP containing anti-RRM2 small interfering RNA (siRNA). For example, the indication of the formulation named CALAA-01, for various solid tumors, completed a Phase I trial [72].

In addition to nanomedicines targeted by antibodies and their fragments, many other targeting moiety is being evaluated for active cellular drug targeting in preclinical and clinical trials. Noteworthy examples are NP clusters coated with hyaluronan (HA), a naturally occurring glycosaminoglycan, which is water-soluble and non-immunogenic. Since HA is the major ligand of CD44, which is expressed on almost all cell types, but is upregulated and undergoes different splice variations in cancerous cells, it can be exploited to actively target tumor cells [73,74]. It has been demonstrated that HA can be covalently attached to lipid-based NPs and can efficiently target epithelial cancer cells and blood cancers expressing HA receptors [73,75-77]. In addition, HA endows the
The use of mAbs specific to tumor neovascular antigens presents an attractive opportunity for the active targeting of therapeutic payloads to tumors for several reasons: blood vessels represent the most accessible route within the tumor for circulating nanomedicines entrapping chemotherapeutics; the formation of new blood vessels (angiogenesis) is a typical feature of many aggressive cancer types; and, unlike antibodies that are specific to the antigens expressed on the surface of tumor cells, vascular tumor-targeting antibodies could be used for many different types of tumors [80]. By targeting these antigens, one may increase drug delivery to tumor endothelium, thereby eliminating tumor blood vessels and revoking tumor cells of oxygen and nutrients. Such antigens have been discovered over the years by various methods: transcriptomic studies, in vivo phage library panning, perfusion-based mass spectrometry-assisted techniques and immunohistochemistry [81,82].

The most promising antibodies reported so far include L19 (specific to the alternatively spliced EDB domain of fibronectin), F8 (specific to the EDA domain of fibronectin) and F16 (specific to domain A1 of tenascin-C) [93,85]. These antibodies have been fused to various cytokines (e.g., IL-2, IL-12, IL-10, TNF-α) and 131Iodine for radioimmunotherapy and are currently evaluated in Phase I and Phase II clinical trials for various indications.

Since tumor vasculature-targeted nanomedicines do not depend on extravasation and penetration across physiological barriers, it is expected that endothelial cell-targeted nanomedicines would demonstrate a significant potential for improving chemotherapy efficacy over cancer cell-targeted nanomedicines. Based on the positive evidence that has been obtained in regard to active targeting to tumor blood vessels [86,88], and on the promising findings that have been reported in Phase I trials for L19-targeted IL-2 [89], it is expected that increasing numbers of endothelial cell-targeted nanomedicines will be evaluated in clinical trials.

4.2 Active drug targeting by stimuli-sensitive nanomedicines

Additional nanomedicine formulations, which are expected to expand in the near future, are stimuli-sensitive systems, which can be triggered to release their payload on exposure to external stimuli, such as heat, light, ultrasound and magnetic fields [90]. These strategies have significant clinical potential in the reversion of MDR, since they are designed to release the conjugated or entrapped chemotherapeutic drug only on exposure to locoregional triggers, thus maximizing drug release and uptake at the desirable pathological site, while decreasing potential damage to healthy tissues.

A prominent example of such nanomedicine is ThermoDox®, temperature-sensitive PEGylated liposomes containing Dox. It is administrated by intravenous infusion and designed to work in combination with hyperthermic treatments. The goal of the ThermoDox approach is to expand the effective treatment zone of these technologies to capture micrometastases, which are most commonly responsible for post-treatment disease recurrence. On local heating to 42°C, the formulation undergoes compositional changes, which allow the release of encapsulated Dox. In addition, when heated, blood vessels in tumors become even more permeable, further increasing the accumulation of liposomes in tumors before releasing the drug payload. This technology is currently at Phase III trial in hepatocellular carcinoma [91].

Another example of stimuli-sensitive strategy is pH-sensitive DDS, which responds to physiological differences between the body and the tumor vicinity rather than external stimuli. There are many types of pH-sensitive NPs that incorporate pH-tunable moieties. These moieties can include carboxyl and/or tertiary amino groups, which function as pH sensors because their hydrophobicity is altered by protonation and deprotonation [90]. This property can be very useful when MDR mechanisms prevent the accumulation of the drug within cancer cells. Many studies have shown the ability of pH-sensitive nanomedicines to release their conjugated/encapsulated cargo in a precise pH-responsive behavior, and overcome MDR in highly resistant tumor models.

The downside of stimuli-sensitive formulations is that, in most cases, it is hard to obtain high sensitivity and specificity of stimuli responsiveness. It is often observed that they either already release significant amounts of drug without actually being triggered or they appear so stable that the triggering conditions required to induce drug release might become toxic. Many efforts are being carried out to overcome these obstacles, focusing on improving the stimuli responsiveness of tumor-targeted nanomedicines and developing suitable
instruments to apply more effective and selective stimuli to the target tissue.

5. Mechanistic overview of how changes in the influx > efflux may result in overcoming MDR

Drug resistance mediated by extrusion pumps is a hurdle that holds the key to its solution. In the most direct view, arresting the pump action should restore drug accumulation inside the MDR tumor cell to levels comparable to those of a drug-sensitive tumor cell (Figure 4).

The ongoing search for chemosensitizers that can be applied in the clinic is entering into its third generation. First-generation chemosensitizers were found among drugs already approved for different indications, such as verapamil, cyclosporine A and progesterone. Today, those drugs are used as in vitro benchmarks. They cannot be employed clinically due to dose-related adverse effects, toxicity and, in some cases, solubility limitations [7,92].

The second and third generations of chemosensitizers were drawn from chemical derivatization of the first-generation molecules and from combinatorial chemistry designed predominantly against ABCB1 (P-gp). Examples include many compounds that were under development in pharmaceutical
companies such as VX-710, XR9051, XR9576, MS-209, GF120918, LY335979, ONT-093 and PSC833 (valspodar). Some of the compounds reached clinical trials; although they are less toxic and more potent than the first-generation compounds, they are still prone to adverse effects, poor solubility and unfavorable changes in pharmacokinetics of the anticancer drug. Not all of them are fit to block different pump proteins. Most of them usually function with one or two pumps and thus are not ‘multi-pump’ blockers. PSC833 (valspodar) was one of the prominent candidates that reached clinical trials. Valspodar has been extensively investigated, both preclinically and clinically, and has been found to function as a single-pump blocker (ABCB1). In 2006, it has been reported that valsodar did not improve treatment outcomes and increased the toxicity in a randomized Phase III trial in patients with recurring or refractory treatment. Valspodar was treatment given in combination with the chemotherapeutic drugs vincristine, Dox and the steroid dexamethasone, compared with treatments that exclude the chemosensitizer.

Fluoxetine (Prozac®) is an example of a multi-pump inhibitor (targeting both ABCB1 and ABCC1) that was shown to act in low doses (below its antidepressant therapeutic levels) in several preclinical models of highly resistant tumors (7,8). Fluoxetine and other chemosensitizers operate in a simple kinetic principle. They inhibit the efflux of the drug by either physically blocking MDR extrusion pumps or by binding to some residues that affect the pump to either stop pumping out drugs or jeopardizing its function; this causes the drug that was administered and randomly diffused into the cells to accumulate inside cancer cells (Figure 4A). This will eventually cause cell death since the balance between the efflux and influx is changing toward influx, and in any given time there is more drug accumulated within the cell. This simple biochemical concept (shifting the balance between drug efflux to drug influx) could be further enhanced when nanomedicines are employed acting as ‘local’ drug depot and substantially increasing the influx of the drug into the cells (Figure 4B).

The diversity among pumps proteins, the heterogeneity of cells in a given tumor as well as the variability between patients will probably require treatment with more than one strategy and will promote combination therapy with chemosensitizers and nanomedicines. Over the past decade, tremendous work have been made to overcome MDR pumps with nanomedicines (for recent excellent reviews, [94,95]).

Several direct and indirect strategies have been devised to overcome cancer MDR. For example, using nanomedicines that can deliver vast amounts of cytotoxic payloads and act as a local drug depot in the vicinity of the tumor (if utilizing passive drug targeting mechanisms) or increasing the amount of cytotoxic drugs within cancer cells (if delivered via active cellular targeting). This indirect strategy was demonstrated to bypass MDR efflux pumps. In a recent study, HA-grafted lipid-based nanoclusters entrapping the chemotherapeutic drug Dox was shown to deliver the drug and bypass the efflux pump in a human highly resistant ovarian adenocarcinoma cells (in vitro and in vivo). By knocking down the predominant efflux pump in these cells (P-gp) using siRNAs the authors show that drug accumulation in the cells was independent from the presence of the P-gp pumps, supporting the concept that increasing the influx of a given drug(s) could enhance cell death and bypass the extrusion pump proteins [73].

A direct strategy is to knockdown the pump proteins as a therapeutic approach. This is essentially a sequence-specific chemosensitizer strategy that in combination with chemotherapeutic agent in its free form or entrapped in a nanomedicine will enhance the therapeutic response in resistant tumors [96-98].

The next level was to include siRNAs against pump proteins in combination with a chemotherapeutic agent in the same nanocarrier (Figure 4B). Several combination studies were reported by the group of Nel [99,100]. Multifunctional mesoporous silica NP (MSNP) carriers were used to overcome Dox resistance in a MDR human breast cancer xenograft by co-delivering Dox and siRNA that targets the P-gp extrusion pumps.

These MSNPs had a cationic surface made from polyethyleneimine-PEG-coated particles for the attachment of a series of siRNAs, which was subsequently used in a high-throughput screening assay to find the most optimal siRNA-drug combination for overcoming Dox resistance in MCF-7/MDR cells. This was accomplished by electrostatic attachment of siRNAs to the MSNP surface, which allows siRNA delivery in tissue culture and in blood circulation of tumor-bearing animals following intravenous administration. The phosphonate-coated particle pores allow electrostatic Dox attachment and subsequent release by protons in an acidifying endosomal environment. The resulted delivery strategy showed that co-delivery of P-gp siRNA with Dox enhanced the therapeutic response in the MCF-7/MDR xenograft mouse model [100].

6. Expert opinion

Cancer MDR is most likely one of the major hurdles for successful treatment. Over the past decades, many attempts have been made to better understand protein extrusion pumps related to the cancer MDR and some structural data are already available, whereas some are not. Comprehensive understanding of the structural and functional features of these pumps and improved targeted nanomedicines will enhance the therapeutic benefit in many types of resistant tumors. Taking into consideration that some tumors are intrinsically resistant, whereas others will acquire resistance during the course of treatment, calls for an effective approach to screen and design optimal treatment in a personalized approach [101-104].
Tumor's heterogeneity sets a great challenge for future personalized therapy in cancer MDR. Yet, fast development of high-throughput '-omics' technologies has enabled to decipher the molecular signatures and oncogenic pathways that underlie disease initiation and progression, and to identify molecular profiles that will promote precise tumor classification, prognosis and responsiveness to therapies – all in high '-omics' resolution and with a reasonable cost. Further characterization of MDR biology, and identification of novel molecular biomarkers and therapeutic targets that might be related to MDR energy mechanism and not only the pump proteins themselves could bring the scientific community one step closer to treating some cancer MDRs in the clinic.

Since there are many strategies (most of them not clinically tested, yet), we propose that a candidate molecule acting as a chemosensitizer (be it a small molecule or a sequence-specific RNAi) should meet three in vitro criteria prior to investigating its potential in vivo. The most prominent one is cell demise: treatment of a given MDR tumor cell line, with a combination of the candidate molecule and a chemotherapeutic drug, should significantly enhance cell death, compared with similar treatment with the drug alone. If the a nanomedicine is used, it should release its payload in vitro in a cell-specific manner, that is, only active cellular strategies might benefit from this early screening strategy.

Mechanistic criteria include drug efflux and drug accumulation. Incubating MDR cells with a chemotherapeutic drug in a free form or entrapped in a nanocarrier, with or without the candidate chemosensitizer, should result in higher intracellular drug accumulation in the latter case. Efflux of a free, non-encapsulated chemotherapeutic drug from MDR cells, preferably under unidirectional conditions, should be significantly faster in the absence of the chemosensitizer candidate than when it is present. If a candidate chemosensitizer meets all those criteria, there is merit in expanding the evaluation in several directions: testing, in the same cell line, different chemotherapeutic drugs drawn from those known to be substrates of a given MDR pump entrapped in a targeted nanocarriers; testing different additional MDR cell lines; and testing MDR cell lines with their parent sensitive lines (on availability). Comparative studies of known chemosensitizers used as benchmarks, can stringent the evaluation of the candidate molecule.

Although there are many reported strategies to overcome cancer MDR in preclinical models utilizing nanomedicines, none have been clinically tested, particularly for this indication. However, it is likely that we will witness more strategies that include active cellular targeting approaches in the clinic. Those will first have to show efficacy in mouse xenografts or human tumorgraft models by bypassing the MDR efflux pumps and enhancing specific delivery of therapeutic payloads in a safe manner.

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