IMPROVEMENT OF CONVENTIONAL ANTI-CANCER DRUGS AS NEW TOOLS AGAINST MULTIDRUG RESISTANT TUMORS

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Abstract

Multidrug resistance (MDR) is the dominant cause of the failure of cancer chemotherapy. The design of antitumor drugs that are able to evade MDR is rapidly evolving, showing that this area of biomedical research attracts great interest in the scientific community. The current review explores promising recent approaches that have been developed with the aim of circumventing or overcoming MDR. Encouraging results have been obtained in the investigation of the MDR-modulating properties of various classes of natural compounds and their analogues. Inhibition of P-gp or downregulation of its expression have proven to be the main mechanisms by which MDR can be surmounted. The use of hybrid molecules that are able to simultaneously interact with two or more cancer cell targets is currently being explored as a means to circumvent drug resistance. This strategy is based on the design of hybrid compounds that are obtained either by merging the structural features of separate drugs, or by conjugating two drugs or pharmacophores via cleavable/non-cleavable linkers. The approach is highly promising due to the pharmacokinetic and pharmacodynamic advantages that can be achieved over the independent administration of the two individual components. However, it should be stressed that the task of obtaining successful multivalent drugs is a very challenging one. The conjugation of anticancer agents with nitric oxide (NO) donors has recently been developed, creating a particular class of hybrid that can combat tumor drug resistance. Appropriate NO donors have been shown to reverse drug resistance via nitration of ABC transporters and by interfering with a number of metabolic enzymes and signaling pathways. In fact, hybrid compounds that are produced by covalently attaching NO-donors and antitumor drugs have been shown to elicit a synergistic cytotoxic effect in a variety of drug resistant cancer cell lines. Another strategy to circumvent MDR is based on nanocarrier-mediated transport and the controlled release of chemotherapeutic drugs and

P-gp inhibitors. Their pharmacokinetics are governed by the nanoparticle or polymer carrier and make use of the enhanced permeation and retention (EPR) effect, which can increase selective delivery to cancer cells. These systems are usually internalized by cancer cells via endocytosis and accumulate in endosomes and lysosomes, thus preventing rapid efflux. Other modalities to combat MDR are described in this review, including the pharmaco-modulation of acridine, which is a well-known scaffold in the development of bioactive compounds, the use of natural compounds as means to reverse MDR, and the conjugation of anticancer drugs with carriers that target specific tumor-cell components. Finally, the outstanding potential of *in silico* structure-based methods as a means to evaluate the ability of antitumor drugs to interact with drug transporters is also highlighted in this review. Structure-based design methods, which utilize 3D structural data of proteins and their complexes with ligands, are the most effective of the *in silico* methods available, as they provide a prediction regarding the interaction between transport proteins and their substrates and inhibitors. The recently resolved X-ray structure of human P-gp can help predict the interaction sites of designed compounds, providing insight into their binding mode and directing possible rational modifications to prevent them from becoming P-gp drug substrates. In summary, although major efforts were invested in the search for new tools to combat drug resistant tumors, they all require further implementation and methodological development. Further investigation and progress in the abovementioned strategies will provide significant advances in the rational combat against cancer MDR.

1. Introduction

Resistance to anticancer drugs is the result of a number of distinct mechanisms including impaired drug uptake, drug compartmentalization, drug metabolism, functional bypass and alterantive compensatory pathways, alterations of membrane lipids and target proteins, inhibition of apoptosis, alterations in the tumor microenvironment (TME) including acidification, dysregulation

of gene expression, and increased efflux from cells (Fig. 1) (Gottesman 2002; Pasello *et al.*, 2019; Bar-Zeev *et al.*, 2017; Kopecka *et al.*, 2019; Gacche and Assaraf 2018; Li *et al.*, 2016; Zhitomirsky and Assaraf 2016; Taylor *et al.*, 2015; Niewerth *et al.*, 2015; Gonen and Assaraf 2012).

The development of simultaneous resistance to multiple drugs, of distinct chemical structures, different mechanisms of action and differing targets, is known as multidrug resistance (MDR) (Szakács *et al.*, 2006). MDR is a multifactorial process whereby cancer cells become progressively unresponsive to anticancer agents independently of their structures and mechanisms of action. MDR is a major cause of failure of cancer chemotherapy.

One of the most common mechanisms of resistance is associated with the presence of membrane transporter proteins (ABC transporters), which are overexpressed in cancer cells and extrude multiple cytotoxic drugs out of cancer cells, leading to a marked reduction in their efficacy. The extrusion of these MDR drugs proceeds by using the energy furnished by ATP hydrolysis. ABCB1, known as P-glycoprotein (P-gp), ABCC1-6, known as Multidrug Resistance related Proteins 1-6 (MRP1-6), and ABGC2, known as Breast Cancer Resistant Protein (BCRP), are the principal efflux transporters mediating MDR (Gottesman 2002; Takaara *et al.*, 2006; Baguley 2010; Li *et al.* 2016).

ABC efflux transporters are not the sole cause of MDR. Experimental evidence has recently shown that lysosomes can also contribute to the lysosomal sequestration of weakly basic hydrophobic anticancer drugs followed by their efflux via lysosomal exocytosis (Zhitomirsky and Assaraf, 2016). Weakly basic hydrophobic anticancer drugs are sequestered by these acidic organelles, via passive diffusion, due to their acidic luminal pH (pH $\leq$5) and expelled from the cell via lysosomal exocytosis. Basic anthracycline antibiotics, *Vinca* alkaloids including vincristine, tyrosine kinase inhibitors including sunitinib, gefitinib, nintedanib as well as their analogues are typical examples of such lysosomotropic cytotoxic drugs. Some studies have shown that ABC transporters may also be

localized in the lysosomal membrane and thus can mediate the active sequestration of anticancer drugs in these organelles (Yamagishi *et al.*, 2013; Al-Akra *et al.*, 2018).

Hypoxia is another established cause of drug resistance; solid tumors have a poor blood supply in some deep core regions and consequently low O_2 concentrations (Raz *et al.*, 2014; Jing *et al.*, 2019; Leon *et al.*, 2019; Xu *et al.*, 2019). This is one of the mechanisms underlying the resistance of these tumors to a variety of anticancer agents. The precise mechanisms that underlie hypoxia-induced drug resistance are not well understood. It is generally accepted that an absence of O_2 radicals, cell cycle disruption, DNA overreplication, induction of stress proteins, hypoxia inducible factor-1α (HIF-1α) accumulation, p53 promotion and P-gp overexpression, play important roles. Indeed, HIF-1α up-regulates P-gp (Comerford *et al.,* 2002). On the other hand, the efficacy of chemotherapeutic drugs that act by increasing reactive oxygen species (ROS), such as anthracyclines, gemcitabine or platinum-derivatives, is limited in hypoxic tumors because of the lessened capacity to generate ROS. This phenomenon is strongly drug-dependent, and thus the delivery of oxidants to the tumor, and the use of chemotherapeutic agents that are selectively active against hypoxic cells, have been proposed to address this problem (Teicher 1994).

As most investigated mechanisms of resistance are associated with the overexpression of membrane transport proteins, one of the first approaches to tackling MDR was based on the association of antineoplastic agents with inhibitors of P-gp, which is the most well-known drug efflux transporter. Efflux pump inhibitors can be classified into three groups according to their mechanism of action: substrates, inhibitors, and modulators. Substrates are able to saturate the binding sites of the pump, thus preventing drug efflux; inhibitors block the pump by inhibiting ATP-binding to the pump, and modulators reduce drug binding to the pump via a negative allosteric effect (Colabufo *et al.*, 2010; Li *et al.*, 2016).

Preclinical studies have demonstrated that combination therapy, using P-gp inhibitors and anticancer drugs, decreased tumor volume and prolonged the lifespan of animals (Saneja *et al.*, 2014;

Yang *et al.*, 2015; Wang *et al.*, 2019a). However, the co-administration of P-gp inhibitors and anticancer drugs has some limitations; firstly, the unpredictable pharmacokinetics, biodistribution and membrane-transport properties of the two unrelated drugs may lead to their uptake and accumulation in the target cells having different time scales. Secondly, the lack of selectivity of P-gp inhibitors toward cancer tissues can cause the undesirable accumulation of these drugs in healthy tissues which have central physiological roles (Guo *et al.*, 2017).

Alternative approaches have been proposed to overcome P-gp-mediated MDR. These include the design of molecules whose uptake is greater than their efflux rate, or molecules that are able to evade P-gp, or other drugs that are selectively cytotoxic to MDR cells but are not harmful to drug sensitive parental cells (chemosensitzing agents) (Baguley *et al.et al.*, 2010; Pluchino *et al.et al.*, 2012).

In recent years, improvements in our understanding of the mechanisms underlying the acquisition of drug resistance (Assaraf *et al.*, 2019; Wang *et al.*, 2019b) have led to the development of new strategies aimed at circumventing or counteracting well-defined mechanisms of drug-resistance. These strategies include the use of modifications to currently active antitumor drugs as a means to enhance their ability to target tumor cells, which has always been an important objective in the combat against cancer MDR (Bertrand *et al.*, 2014; Danhier *et al.*, 2010; Kutova *et al.*, 2019; Kydd *et al.*, 2017; Pullan *et al.*, 2019; Rosenblum *et al.*, 2018; Swain *et al.*, 2016). The focus of the current review is to summarize the most recent advances in this field.

Figure 1. Key mechanisms of drug resistance in cancer cells.

2. Natural compounds and related structural modifications to surmount cancer MDR.

More than 70% of all anticancer drugs currently on the market were derived from, or inspired by, natural products (Harvey *et al.*, 2015). Furthermore, natural compounds currently play a crucial

role in drug discovery (Newman and Cragg, 2012; Newman and Cragg, 2016; Li *et al*., 2019a). The reasons for this are the great structural diversity and mechanisms of action shown by natural products, as well as the development of new technologies that facilitate the analysis and screening of more complex natural samples (Harvey *et al.*, 2015; Feher and Schmidt, 2003; Thomford *et al*., 2018). There are several classes of natural compounds that have been studied as means to reverse MDR, and these include flavonoids, curcumins, alkaloids, steroids and terpenoids (Mishra and Tiwari, 2011).

2.1. Flavonoids

Flavonoids are an important class of polyphenols that can be found in different parts of plants (Li *et al.*, 2016). They can be divided into chalcones, flavones, flavanones, flavonols, anthocyanins and isoflavones according to the substituents present on the aromatic ring and their oxidation status (Panche *et al.*, 2016). Flavonoids have antioxidant, anti-inflammatory and anti-mutagenic properties (Ferreyra *et al.*, 2012). They have also been found to inhibit the MDR efflux transporters P-gp, MRP-1, MRP-2 and BCRP (Gupta *et al.*, 2014; Ye *et al.*, 2019). Some flavonoids act on both the expression and activation of P-gp. Quercetin is one of the most commonly studied flavonoids with MDR-modulating properties (Fig. 2), and has been found to interact with the substrate-binding site or the ATP-binding site of P-gp, MRP1 and BCRP (Shih *et al.*, 2000; Li *et al*., 2018b). It has recently been found to increase the accumulation of rhodamine 123 and doxorubicin (DOX), and to increase the chemosensitivity of MDR human hepatocellular carcinoma cells (Chen *et al.*, 2018a). The flavonoids kaempferol and naringenin (Fig. 2) have also been reported to inhibit P-gp; kaempferol has been shown to significantly decrease the level of P-gp in KB-V1 cells (Limtrakul *et al.*, 2005), while naringenin increased the concentration of the anti-hypertensive calcium channel blocker felodipine (Sandeep *et al.*, 2014). Similarly, icaritin and baicalein (Fig. 2) have been found to block P-gp (Miao *et al*., 2016); Icaritin has been shown to decrease the expression of the MRD1 gene (Sun *et al.*, 2013), whereas baicalein increased the oral bioavailability of tamoxifen in the small intestine via inhibition

of P-gp (Li *et al.*, 2011). Other flavonoids that have been studied as MDR modulators include chrysin, rutin, genistein, biochanin A and apigenin (Ye *et al.*, 2019).

2.2. Curcumins

Curcumin (Fig. 2) is a major component of the spice turmeric, from the root of *Curcuma longa*; it displays antioxidant, anti-inflammatory and anticancer properties, in addition to MDR modulatory activities (Naberuka *et al.*, 2010; Lopes-Rodrigues *et al.*, 2016). Curcumin has been found to increase the activity of paclitaxel, as well as DOX in adriamycin-resistant MCF-7 cells and taxol-resistant A549 cells (Naberuka *et al.*, 2010). Additionally, curcumin enhanced the sensitivity of tumor cells to vincristine, cisplatin, 5-fluorouracil (5-FU), and 10-hydroxy-camptothecin (CPT) (Yang *et al.*, 2011) and downregulated the expression of P-gp in the vincristine-resistant colon cancer HCT-8/VCR cell line (Zhao *et al.*, 2018; Lu *et al.*, 2013), while also having a promising inhibitory effect on the MDR efflux pumps P-gp, MRP1 and BCRP (Zhao *et al.*, 2013). Despite its general safety, the main limitations of curcumin are its chemical instability, low aqueous solubility and poor pharmacokinetic profile (Zhao *et al.*, 2013). Curcumin analogues with higher chemical stability have thus been prepared, for example, by replacing its β-diketone group with a mono-carbonyl spacer (Liang *et al.*, 2009; Adams *et al.*, 2004; Murakami *et al*., 2017). The β-diketone moiety is a possible target for liver enzymes and can cause curcumin's instability *in vitro* (Zhao *et al.*, 2013). Some of the prepared curcumin analogues have shown improved *in vivo* stability, lower toxicity and similar, or superior, biological activity, including the potential to reverse MDR (Zhao *et al.*, 2013; Liang *et al.*, 2009; Revalde *et al.*, 2015). In addition to chemical modifications, nanodrug systems, liposomes, polymeric micelles and polymer nanoparticles have all been developed to circumvent the poor pharmacokinetic profile of curcumin (Zhao *et al.*, 2018).

Figure 2. Representative flavonoids, curcumin as well as a representative synthetic analogue of curcumin with MDR modulatory activities.

2.3. Alkaloids

There are many classes of alkaloids with MDR modulatory activity, such as piperazine alkaloids, quinoline and isoquinoline alkaloids, as well as indole alkaloids (Joshi et al., 2017). Piperine, a common dietary alkaloid found in black pepper (*Piper nigrum*), is one of the most commonly studied piperidine alkaloids (Fig. 3) as it presents a range of therapeutic activities, including antioxidant, anti-inflammatory, immunomodulatory and anticancer activity (Rather and Bhagat, 2018). Piperine activates apoptotic signaling, inhibits cell cycle progression, influences redox homeostasis in cancer cells, inhibits the self-renewal of cancer stem cells and modulates endoplasmic reticulum stress and autophagy (Rather *et al.*, 2018; Manayi *et al.*, 2018). In addition, piperine is an inhibitor of P-gp, BCRP and MRPs (Qiang *et al.*, 2012) and has an important effect on drug metabolism. These activities imply that piperine can reverse MDR in cancer cells and enhance the activity of many anticancer drugs (Rather and Bhagat, 2018; Manayi *et al.*, 2018).

Lobeline (Fig. 3), a piperidine alkaloid from *Lobelia inflata (known as the Indian tobacco plant)*, has been shown to enhance the activity of DOX in human colon adenocarcinoma cells (Caco-2) by inhibiting P-gp (Ma and Wink, 2008). Tertiary alkaloids stemocurtisine, oxystemokerrine and stemofoline (Fig. 3) have been shown to inhibit P-gp and to reduce the IC_{50} values of some cytotoxic agents (Chanmahasathien *et al.*, 2011).

The anti-malarial drug quinine and its isomer quinidine (Fig. 3) with antiarrhythmic activity, are members of the first generation of P-gp inhibitors (Rijpma *et al.*, 2014). Quinine has been shown to increase the sensitivity of a DOX-resistant human myeloma tumor cell line to DOX (Lehnert *et al.*, 1991). A quinine dimer **1** (Fig. 3) that inhibited the efflux of rhodamine 123 and the transport of radiolabeled paclitaxel in DOX-resistant MCF-7 cells has also been prepared (Pires *et al.*, 2009). Additionally, a set of quinine dimers have been prepared with a triazole heterocycle in the linker, connecting the two quinine moieties. These dimers have been found to inhibit P-gp in DOX-resistant MCF-7 cells (e.g. quinine dimer **2**, Fig. 3) (Kuriakose *et al.*, 2012). The tetrahydroisoquinoline

alkaloid chelidonine (Fig. 3) has been shown to inhibit P-gp and enhance the cytotoxic activity of DOX in Caco-2 cells and the human leukemia cell line CEM/ADR5000 (El-Readi *et al.*, 2013). Similarly, a tetrahydroisoquinoline, glaucine (Fig. 3), has been shown to inhibit P-gp and MRP1 efflux pumps in the drug resistant breast cancer cell line MCF-7/ADR (Lei *et al.*, 2013). Other quinoline and isoquinoline derivatives with MDR modulatory activities include sanguinarine, roemerine, tetrandrine, isotetrandrine, berbamine and hernandezine (Kumar and Jaitak, 2019; Joshi *et al.*, 2017).

Reserpine and yohimbine (Fig. 3), isolated from *Rauwolfia serpentina (Indian snakeroot)*, have been shown to increase the intracellular concentration of DOX, daunorubicin and vincristine in the MDR cell line CEM/VLB100 by inhibiting P-gp efflux (Pearce *et al.*, 1989). In addition, reserpine also inhibited BCRP efflux (Henrich *et al.*, 2006). An ergot alkaloid, bromocriptine (Fig. 3), inhibited P-gp and showed potent MDR reversal activity for DOX, vinblastine, vincristine, vinorelbine and etoposide in several cancer cell lines (Shiraki *et al.*, 2002). Other indole alkaloids with MDR modulatory activities include indole-3-carbinol, indole-3-carboxyldehyde, kopsiflorine, coronaridine and tabernines A-C (Joshi *et al.*, 2017).

Figure 3. Representative piperidine, quinoline, isoquinoline and indole alkaloids with MDR modulatory activities.

2.4. Steroids and terpenoids

Steroids are an important class of natural compounds that primarily act as components of biological membranes or as signaling molecules, although some have also been studied as MDR modulators. β-Sitosterol-O-glucoside (Fig. 4) has been shown to induce a higher accumulation of calcein-AM and rhodamine 123 in CEM/ADR5000 and Caco-2 cancer cell lines, which was thought to be due to its inhibition of P-gp (Eid *et al.*, 2013). Similarly, guggulsterone (Fig. 4) was also shown to increase the intracellular concentrations of daunorubicin and rhodamine 123 in the KB-C2 cancer

cell line, possibly by inhibiting P-gp (Nabekura *et al.*, 2010) and to sensitizeddrug-resistant human hepatocarcinoma cells to DOX (Xu *et al*., 2017). Some cardiotonic steroids (e.g. cardiotonic steroid **3**, Fig. 4) have recently been shown to inhibit P-gp, according to an ATPase assay, and to reverse DOX resistance in the MDR human leukemia cell line CEM/ADR5000 (Zeino *et al.*, 2015). Gracillin, polyphyllin D and 20(S)-protopanaxadiol are other steroids with MDR-modulatory activity (Silva *et al.*, 2016).

Some terpenoids have been reported to possess anticancer (e.g. paclitaxel), anti-malarial (e.g. artemisinin) and MDR reversal activities (Silva *et al.*, 2016). Menthol and aromadendrene (Fig. 4) have been demonstrated to increase the concentration of calcein-AM and rhodamine 123 in the MDR CEM/ADR5000 and Caco-2 cancer cell lines, possibly via the competitive inhibition of P-gp (Eid *et al.*, 2013; Wink *et al.*, 2012), while citronellal and citronellol (Fig. 4) inhibited P-gp and consequently increased the intracellular accumulation of the P-gp cardiac glycoside substrate [^{3}H]digoxin (Yoshida *et al.*, 2005). It has been shown that a series of sesquiterpenes with a dihydro-β-agarofuran structure (Fig. 4) inhibited P-gp-mediated efflux and reversed resistance to daunomycin and vinblastine in MDR cells (Perestelo *et al.*, 2011). Moreover, carnosic acid, carnosol and ursolic acid (Fig. 4) from rosemary leaves, have been found to inhibit P-gp function in KB-C2 tumor cells, thus increasing the intracellular concentrations of daunorubicin and rhodamine 123 (Nabekura *et al.*, 2010). Additionally, carnosic acid sensitized KB-C2 cells to vinblastine, thus reversing MDR (Nabekura 2010). Limonin (Fig. 4), a triterpenoid present in citrus plants, inhibited P-gp and reversed DOX resistance in CEM/ADR5000 and Caco-2 cells (El-Readi *et al.*, 2010). Latilagascenes C and D, cryptotanshinone, balsaminol B, balsaminagenin C and glycyrrhetinic acid are some other terpenoids with reported MDR reversal activities (Kumar *et al.*, 2019).

Figure 4. Representative steroids and terpenoids with MDR modulatory activities

3. Multi-target agents to evade/overcome MDR

The simultaneous interaction of a single molecule with two or more relevant cancer cell targets is a promising approach to circumventing drug resistance. This strategy relies on the design of hybrid compounds obtained either by merging structural features of different drugs in a new molecule, or by conjugating two drugs or pharmacophores via cleavable/non-cleavable linkers. The interest toward multitarget-ligand design is rapidly increasing due to the potential benefits of such bifunctional molecules, including pharmacokinetic and pharmacodynamic advantages over the separate administration of the two individual drug components.

In the following section, we will discuss the potential of dual/multi-target agents in the context of MDR and the challenges that they face. In providing an overview of their anticancer activities, we will underline the key features of two main classes of multi-target compounds: hybrid molecules that contain currently used antitumor drugs and multi-target compounds that inhibit P-gp activity/expression in MDR cancer cells.

3.1. Hybrid compounds containing currently used antitumor drugs

The development of successful multivalent drugs is a very challenging task. The compounds must be chemically and biologically stable, at least during *in vivo* circulation, and should be capable of reaching an intracellular concentration that is sufficient to promote the therapeutic effect of both moieties. In addition, the conjugates should possess enough chemical flexibility to interact with their site of action. Finally, the molecular size and physicochemical properties of the dual-action compounds should be taken into consideration when developing "druggable" molecules.

Liu and coworkers (Liu *et al.*, 2019) have successfully attached paclitaxel (PTX) to vorinostat (SAHA), thereby forming multi-target drugs with synergistic anticancer effects. The compounds showed *in vitro* cytotoxicity, in the nanomolar range, in the human colorectal cell line HCT-116 and breast cancer MCF-7 cells. Moreover, the IC_{50} of selected compound **4** (Fig. 5) was lower than that of PTX alone against MCF-7/ADR, demonstrating the effect against MDR cells. PTX-SAHA co-prodrug nanomicelles were also prepared with mPEG2000-PLA1750, which was used as the carrier,

using the thin film method (Liu *et al.*, 2019). The co-prodrug nanomicelles demonstrated significant advantages, including prolonged blood circulation and increased accumulation at the tumor site. Moreover, the *in vitro* drug-release tests showed that nanomicelles had a sustained release effect that could contribute to the reversal efficacy of PTX-resistance *in vitro*. The *in vitro* cytotoxicity was evaluated in HCT-116 cells, MCF-7 cells and MDR MCF-7/ADR cells. The results showed that **4** nanomicelles had better cytotoxicity than PTX, especially against MCF-7/ADR cells (Liu *et al.*, 2019). A study by Xu *et al.,* has examined a series of nitrogen mustard conjugates with the natural cytotoxic compound oridonin (Xu *et al.*, 2014). The hybrids were screened *in vitro* against K562, MCF-7, Bel-7402, and MGC-803 human cancer cell lines. All the conjugates showed anti-proliferative activities that were higher than those of the control alkylating drugs chlorambucil and melphalan. The most potent hybrid **5** (Fig. 5) showed IC_{50} values in the low micromolar range. Significantly, the compound also exhibited potent anti-proliferative activity against MDR carcinoma cell lines (SW620/AD300 and NCI-H460/MX20).

Numerous groups have recently prepared Pt(IV)-based hybrid compounds that can be effectively reduced to Pt(II) equivalents inside (Johnstone *et al.*, 2016). Pt(IV) prodrugs that contain phenstatin, an inhibitor of tubulin polymerization, have been synthesized by Huang and co-workers (Huang *et al.*, 2017a). The Pt(IV) complexes showed better antitumor activities than their Pt(II) counterparts on HepG-2, Bel-7404, NCI-H460 and MGC-803 human cancer cell lines. Interestingly, the hybrid compounds showed significant anticancer activity against cisplatin-resistant cell lines. The most potent conjugate **6** (Fig. 5) exhibited activity against SK-OV-3 and A549 cancer cell lines with a lower resistance index than cisplatin. *In vivo* studies showed that conjugate **6** potently inhibited tumor growth in a NCI-H460 xenograft mouse model (Huang *et al.*, 2017a). Based on the same hypothesis, Novohradsky *et al.,* designed Pt(IV) prodrugs by conjugating oxaliplatin with the histone deacetylase (HDAC) inhibitor valproic acid (Novohradsky *et al.*, 2014). The conjugates displayed activity in both cisplatin-sensitive and -resistant tumor cells, targeting both HDAC and DNA. The same group recently reported the activity of cis,cis,trans-$[Pt(IV)(NH_3)_2Cl_2(OA)_2]$

[Pt(IV)dioctanoate], a Pt(IV) derivative of cisplatin that contains two octanoate units appended to the axial positions of a six-coordinate Pt(IV) center (**7**, Fig. 5) (Novohradsky *et al.*, 2017). This derivative exhibited high cytotoxic activity, with IC_{50} values that were approximately two orders of magnitude lower than those of the cisplatin and Pt(IV) derivatives with biologically inactive axial ligands. Importantly, the conjugate overcame cisplatin resistance and exhibited promising antitumor activity *in vivo*.

Hu *et al.,* reported the covalent conjugation of a platinum(IV) moiety to tamoxifen, an estrogen receptor (ER) modulator, as an approach to selectively enhance platinum concentration in estrogen receptor-positive breast cancers, and to reverse their tamoxifen resistance (**8**, Fig. 5) (Hu *et al.*, 2018). The conjugate not only exhibited potent cytotoxicity against breast cancer cells, but also reversed tamoxifen resistance of TamR-MCF-7 cancer cells. The authors also demonstrated that the ER ligand portion of these conjugates played a targeting role in ERpositive tumors and enhanced the uptake of platinum via an ER-mediated pathway.

Platinum-based drugs have also been conjugated to tyrosine kinase inhibitors (TKIs) with the aim of circumventing TKI resistance, which is predominately mediated by the emergence of secondary mutations in oncogenic kinases (**9**, Fig. 5) (Wei *et al.*, 2016). The hybrids maintained the same specificity towards the kinases as the parent TKI. Notably, these hybrids were remarkably less affected by TKI resistance, and this was presumably due to the unique structure and the observed dual mechanisms of anticancer activity (kinase inhibition and DNA damage). The hybrids were found to escape drug efflux and accumulated in the brain of BALB/c mice more than the original TKI.

Cincinelli *et al.,* have developed hybrid agents by combining a diaminedichloro-platinum (II) complex and camptothecin (CPT) derivatives (Cincinelli *et al.*, 2013). These dual compounds showed growth inhibitory activity against a panel of human cancer cell lines, with potency that was comparable to that of topotecan and, in general, superior to that of cisplatin. Resistance indices were observed to be reduced for hybrid compounds, compared to cisplatin and topotecan, in several human

cancer cell lines. Interestingly, the most active derivative **10** (Fig. 5) was able to overcome cisplatin resistance in the osteosarcoma U2OS/Pt cell line. This hybrid compound exhibited *in vivo* activity, against a human H460 tumor xenograft, that is superior to that of cisplatin. The same group also designed dual-acting molecules that contained a CPT scaffold linked to the active fragment of the HDAC and aminopeptidase A inhibitor psammaplin A (Cincinelli *et al.*, 2018; Shim *et al.*, 2004). Compound **11** (Fig. 5) displayed anti-proliferative activity, in the nanomolar range, on a series of human solid and hematologic cell lines. Notably, the hybrid appeared not to be affected by the presence of P-gp, as the IC_{50} against the A2780 cell line was comparable to the IC_{50} against the resistant A2780-Dox cell line.

Podophyllotoxin-NSAID (non-steroidal anti-inflammatory drug) conjugates have been synthesized by Zhang and coworkers (Zhang *et al.*, 2017a). The most potent conjugate **12** displayed selective cytotoxicity against 5-Fluorouracil-resistant Bel-7402/5-FU cells with an IC_{50} value in the nanomolar range. In addition, all conjugates induced apoptosis, disrupted the microtubule network and showed anti-migratory activity in Bel-7402/5-FU cells. Finally, the compounds modulated MDR-related proteins, and ERK1/2, STAT3 and AKT signaling in Bel- 7402/5-FU cells.

A series of methotrexate (MTX)-diosgenin conjugates has been designed and synthesized to enhance the passive internalization of the antifolate MTX into transport-resistant cells (Cai *et al.*, 2016). The inhibitory effects of these conjugates on dihydrofolate reductase (DHFR) and their anti-proliferation behavior against a transport-resistant breast cancer cell line, MDA-MB-231, were investigated. All of the synthesized conjugates retained the ability to inhibit DHFR after diosgenin substitution. The MTX conjugates were much more potent against MTX-resistant MDA-MB-231 cells than MTX. Conjugate **13**, which contained a disulfide bond, exhibited the most potent anti-proliferative activity and DHFR inhibitory effect (IC_{50} = 4.1 μM and 17.2 nM, respectively).

Figure 5. Representative structures of hybrid compounds.

Zhang *et al.,* have prepared compound **14** (Fig. 6), a microtubule and heat-shock protein 90 (Hsp90) dual inhibitor (Zhang *et al.*, 2013). The authors demonstrated that **14** was not a P-gp substrate. This is noteworthy as many microtubule targeting agents (MTAs) and Hsp90 inhibitors are substrates of P-gp. Interestingly, compound **14** showed similar growth inhibitory activity in P-gp-overexpressing cancer cells and their parental cells. The compound also inhibited tumor growth in a human drug resistant non-small cell lung cancer (NSCLC) xenograft model with the same efficacy as in the parental model, without displaying untoward toxicity in normal tissues (Zhang *et al.*, 2014).

Mistry *et al.,* have described compound **15** as an inhibitor of both topoisomerases I and II (Mistry *et al.*, 2002). This revealed that the compound was more potent in inhibiting the growth of human chronic myeloid leukemia cell line K562 than the references, etoposide and CPT. Moreover, compound **15** exhibited activities against human colon and small cell lung cancer (SCLC) xenografts, MDR cancer cells that overexpress P-gp as well as in tumor cells with lower topoisomerase II expression (Di Nicolantonio *et al.*, 2002).

Topoisomerase inhibitors are frequently used in combination with MTAs. However, the use of MTAs (Skok *et al.*, 2019) or topoisomerase inhibitors frequently leads to the development of drug resistance. Compound **16** (Fig. 6) was developed by Yi *et al.,* to target both microtubules and topoisomerase II (Yi *et al.*, 2015). The compound inhibited microtubule polymerization by binding to the colchicine binding site, thus disrupting spindle assembly and subsequently leading to mitotic arrest. It also inhibited topoisomerase II and caused DNA double-strand breaks. Interestingly, compound **16** showed the same efficiency in drug resistant cancer cells as in their sensitive counterparts.

Another dual microtubule and topoisomerase II inhibitor has been reported by Podolski-Renic and coworkers (**17**, Fig. 6) (Podolski-Renic *et al.*, 2017). The dual compound maintained activity in resistant cancer cells that displayed P-gp overexpression, and induced microtubule depolymerization

and apoptosis. Importantly, compound **17** was able to suppress P-gp activity in MDR cancer cell lines by decreasing the activity of P-gp in a dose-dependent manner, without inducing P-gp expression.

Compound **18** (Fig. 6) was rationally designed to simultaneously inhibit HDAC and phosphatidylinositol 3-kinase (PI3K). It is currently in clinical development in patients with lymphoma or multiple myeloma and advanced solid tumors. To and Fu (To and Fu, 2018) investigated the potentiation effect of compound **18** on Pt drugs in drug resistant cancer cells. ABCC2 (MRP2) stably-transfected HEK293 cells, and two pairs of parental and Pt-resistant cancer cell lines were used to evaluate the reversal of drug resistance. The authors reported a synergistic combination of compound **18** with cisplatin, in cisplatin-resistant cancer cells. In Pt-resistant cancer cells, compound **18** apparently circumvented drug resistance via inhibition of ABCC2 ATPase activity and inhibition of DNA repair. In the presence of **18**, the cellular accumulation of Pt drugs and formation of DNA-Pt adducts were found to be increased, whereas the expression levels of ABCC2 and ERCC1 were inhibited in Pt-resistant cells.

Zhang *et al.,* have synthesized a series of curcumin-BTP (benzo[*b*]thiophene 1, 1-dioxide) hybrids as STAT3 inhibitors with the potential to induce ROS production (Zhang *et al.*, 2017b). The authors hypothesized that a combination of STAT3 inhibition and "oxidation therapy" may overcome MDR. The most potent, compound **19** (Fig. 6), showed potent and selective anticancer activity against MCF-7 and MCF-7/DOX cells and displayed a weak cytotoxic effect on normal MCF-10A breast epithelial cells. Notably, the compound also inhibited STAT3-mediated P-gp expression in MCF-7/DOX cells. *In vivo* experiments showed a significant reduction in the volume of human implanted breast cancer xenografts in mice at a dose of 10 mg/kg, with low toxicity.

Figure 6. Structures of dual inhibitors obtained based on the structural features of inhibitors of known biological targets.

3.2. Multitarget compounds modulating MDR efflux pumps.

The group of multitarget compounds that can potentially modulate MDR efflux pumps is mainly comprised of TKIs. Unfortunately, the development of acquired resistance significantly limits the use of TKI in anticancer treatment (Leonetti *et al.*, 2019; Yaghmaie and Yeung 2019; Gotink *et al.*, 2011; Miller 1990). Resistance to TKIs is also caused by the overexpression of ABC transporters, particularly P-gp. Since TKIs interfere with ATP-binding, it can be expected that TKIs may also inhibit the activity of ABC transporters. Indeed, it has been demonstrated that some TKIs act as ABC transporter inhibitors by either suppressing their activity or their expression. A large number of TKIs are currently in different phases of preclinical or clinical trials thanks to their ability to modulate P-gp activity. The list of these TKIs is constantly growing, which serves to highlight their role in overcoming resistance in tumor cells. Here, we will describe some examples of the inhibitory interaction of clinically relevant TKIs with P-gp.

Imatinib (**20**, Fig. 7) is a first-generation inhibitor of the Bcr-Abl tyrosine kinase which also acts as a P-gp efflux inhibitor. This TKI completely or partially reversed MDR to various P-gp substrates (Chen *et al.*, 2010; Dohse *et al.*, 2010; Mukai *et al.*, 2003), downregulated ABCB1 gene and P-gp protein expression and directly interacted with P-gp, producing similar effects to the P-gp inhibitor verapamil (Sims *et al.*, 2013; Chen *et al.*, 2010). Nilotinib, a second-generation inhibitor of Bcr-Abl, is a more potent inhibitor of P-gp activity than imatinib (Dohse *et al.*, 2010; Mlejnek *et al.*, 2017; Villar *et al.*, 2012). A third-generation inhibitor, ponatinib, was able to enhance the cellular accumulation of P-gp substrates in P-gp-overexpressing leukemic cells and thus decrease P-gp expression (Sen *et al.*, 2012).

Gefitinib (**21,** Fig. 7), the first approved epidermal growth factor receptor (EGFR) TKI for cancer treatment, reversed MDR when combined with various P-gp substrates (Leggas *et al.*, 2006; Wang *et al.*, 2017). It directly interacts with P-gp, inhibiting its efflux function, and potentiates the cytotoxicity of various P-gp substrates (Shi *et al.*, 2007; Layney *et al.*, 2012). The second-generation

inhibitor lapatinib is the most effective P-gp inhibitor of all the EGFR inhibitors (Dai *et al.*, 2008; Dunne *et al.*, 2011).

Sorafenib (**22**, Fig. 7), a vascular endothelial growth factor receptor (VEGFR) inhibitor, decreased ABCB1 gene expression as well as P-gp protein expression (Huang *et al.*, 2015a; Hoffmann *et al.*, 2010) and inhibited P-gp efflux activity (Eum *et al.*, 2013).

One of the dual inhibitors with the potential to modulate the MDR efflux pump is a series of conjugates that bear a 1,2,3,4-tetrahydroisoquinoline motif (recurrent in several selective P-gp inhibitors) that is linked to substituted 7-hydroxy-2H-chromen- 2-ones (coumarins). The compounds have been assayed in MDCK cells that stably overexpress P-gp and MRP1 (Rullo *et al.*, 2019). A number of potent and selective P-gp inhibitors were identified, and the most potent compound (**23**, Fig. 7) exhibited nanomolar inhibitory potency (IC_{50} = 70 nM). Molecular docking calculations that were carried out on a human Pgp structural homology model contributed to the gaining of insights into the ligands' binding modes. Some compounds in the series, reversed resistance and thereby restored DOX cytotoxicity when co-administered in MDCK-MDR1 cells with the drug.

Kim and coworkers have designed quercetin conjugates, with a glutamic acid moiety attached at the 7-O position via a non-hydrolysable linker, to reverse cancer MDR via inhibition of P-gp-mediated drug efflux (**24,** Fig. 7). Interestingly, although the compounds displayed considerably higher MDR reversal activity than quercetin, they were not, however, as effective as Pgp-inhibitors as verapamil (Kim *et al.*, 2017a).

Figure 7. Structures of dual-targeting anticancer agents modulating MDR efflux pump.

Nanocarrier-mediated transport and the controlled release of both anticancer drugs and P-gp modulators is a promising and novel strategy that can be used to circumvent MDR, and is currently

being explored (Bar-Zeev *et al.*, 2017; Bar-Zeev *et al.,* 2016; Livney and Assaraf 2013; Shapira *et al.*, 2011).

This approach offers a number of potential benefits. Firstly, the pharmacokinetics will be governed by the nanoparticle or polymer carrier. Another consequence of the co-administration is the fact that NPs allow the drug to exploit the EPR effect, which can increase selective delivery to cancer cells. Finally, there are advantages at the cellular level: NPs are usually internalized by cells via endocytosis and thus present in endosomes and lysosomes. This compartmentalization prevents rapid efflux and also allows the drug to be released in closer proximity to the target and further away from the membrane-bound P-gp efflux transporter. This is particularly attractive for cancer therapeutics as a number of chemotherapeutics act on targets located in the nucleus. Various reports have described the use of polymer- or nanoparticle-based delivery strategies in MDR reversal.

Patil and coworkers (Patil *et al.*, 2009) have investigated the simultaneous and targeted delivery of PTX and a P-gp modulator, tariquidar, using poly(D,L-lactide-*co*-glycolide) NPs to overcome tumor drug resistance. The NPs were surface functionalized with biotin for active tumor targeting. NPs that encapsulated both PTX and tariquidar showed significantly higher *in vitro* cytotoxicity than NPs with PTX alone. *In vivo* studies performed in a mouse model harboring a drug resistant tumor confirmed the *in vitro* results. Treatment with biotin-functionalized NPs that encapsulated both paclitaxel and tariquidar showed considerably higher tumor-growth inhibition at a PTX dose that was ineffective in the absence of tariquidar. A similar approach to the co-delivery of tariquidar and PTX into tumor cells, using long-circulating liposomes, has also been reported (Patel *et al.*, 2011). The simultaneous delivery of this P-gp inhibitor along with PTX by functionalized liposomes, resulted in greater cytotoxicity in SKOV-3TR cells at a PTX dose that was ineffective in the absence of tariquidar.

An analogous approach has recently been followed by Zhang and colleagues (Zhang *et al.*, 2017). In this study, an iRGD-mediated lipid-polymer hybrid nanosystem (LPN) was designed to co-

deliver PTX and the P-gp inhibitor tetrandrine (TET), in a temporal drug release manner, to overcome MDR in ovarian cancer. PTX was first conjugated to a poly (lactic-co-glycolic acid) polymer via disulfide linkages to serve as the core of the LPN. Subsequently, TET was loaded into the LPN via nanoprecipitation and a self-assembly process. Primarily, the incorporation of the iRGD peptide onto the LPN resulted in greater cancer cell targeting and penetration effects. After integrin receptor-mediated endocytosis, the loaded TET was spontaneously and rapidly released to inhibit P-gp. PTX, which was conjugated to the polymeric core, was then redox-sensitively released into the cytoplasm via the reduction of the disulfide bonds (glutathione) and accumulated in the cells. Due to the enhanced cellular uptake and P-gp suppression mediated by TET, a significantly more PTX accumulated in A2780/PTX cells treated with PTX+TET/iRGD LPNs than with either the free drugs or non-iRGD modified LPNs. PTX+TET/iRGD LPNs presented the highest cytotoxicity against A2780/PTX cells and effectively promoted ROS production, enhanced apoptosis and cell-cycle arrest.

Subr *et al.,* investigated the effect of a series of N-(2-hydroxypropyl)methacrylamide copolymers (PHPMA) that bear a P-gp inhibitor, DOX or both, on the viability and the proliferation of the murine monocytic leukemia cell line P388 and its DOX-resistant subline P388/MDR (Subr *et al.*, 2014). Several oxoacid analogues of the ABC-transporter inhibitors, reversin 121, reversin 205 and ritonavir oxoacid esters, were synthesized and conjugated to P(HPMA). Whereas the DOX-PHPMA conjugate failed to show any cytotoxicity against MDR cells, the copolymers that incorporated both the P-gp inhibitor and DOX were found to be effective toward MDR cells. In particular, the cytostatic activity of the conjugate P-Ahx-NH-N=MeOHe-R121(Dox), which contains DOX and the P-gp inhibitor MeOHe-R121, both bound via hydrazone bonds to the carrier, was almost 30-fold higher than that of the P-Ahx-NH-N=Dox conjugate toward the P388/MDR cells *in vitro*. A similar result was observed for P-Ahx-NH-N=MeOHe-RIT(Dox), which exhibited an activity that was almost 10-fold higher than that of P-Ahx-NH-N=Dox.

In a more recent study, Battistella and Klok capitalized on the findings of Subr *et al.,* and have reported dual PHPMA conjugates that carry DOX and the 3rd generation P-gp inhibitor, zosuquidar (Zos) (Battistella and Klock 2017). A maximal P-gp inhibition and enhancement of DOX cytotoxicity in cancer cells was achieved using two orthogonally cleavable linkers. DOX was connected to the polymer backbone via a lysosomally degradable GFLG peptide linker, whereas the P-gp inhibitor was attached via a hydrazone linker designed to be cleaved in endosomes, thereby increasing the cytosolic concentration of the inhibitor in proximity to the P-gp transporter. It was demonstrated that the incorporation of both DOX and Zos in a single polymer carrier enhanced P-gp inhibition compared to a control PHPMA conjugate containing only DOX. At a DOX concentration of 10 μM, treatment of MDR A2780 ADR cells with PHPMA-DOX or PHPMA-DOX-Zos resulted in 8- and 10-fold higher accumulation of DOX, respectively, compared to the free DOX. These results confirmed that attaching the two drugs onto the polymer backbone via orthogonally cleavable linkers enhanced P-gp inhibition, compared to the PHPMA-Dox conjugate, and led to DOX cytotoxicity that is comparable to that observed against drug sensitive A2780 cells. *In vivo* applications have yet to be reported.

4. Combination of anticancer agents with nitric oxide (NO)-donors to circumvent drug resistance

4.1. NO and resistance reversal

Nitric oxide (NO) is a gaseous endogenous messenger that is practically ubiquitous in mammalian tissues and cells. It is a product of the conversion of L-arginine into L-citrulline catalyzed by the enzyme NO-synthase (NOS). Three isoforms of NOS are currently known: eNOS, nNOS and iNOS. The first two are constitutive isoenzymes which, under physiological conditions, produce NO for short periods of time (seconds to minutes), giving rise to low NO concentrations (pM-nM) that regulate protective and physiological functions in the cardiovascular and central/peripheral nervous

systems (Kerwin *et al.*, 1994; Kerwin *et al.*, 1995). The third isoform is an inducible isoform that produces NO for long periods (hours to days) and gives rise to high levels of NO (μM). The NO produced by iNOS is one of the final effectors of the immune response (Kerwin *et al.*, 1994; Kerwin *et al.*, 1995). In this system, it is produced not only by stimulated macrophages, but also by other genuine immune-system cells and cells involved in immune reactions (Bogdan 2001).

The role of NO is not limited to maintaining physiological homeostasis, as it has important functions in an extensive number of different diseases, including cancer (Gross and Wolin,1995). It has been shown that low NO concentrations induce cancer cell proliferation, survival, resistance, metastasis, and enhancement of angiogenesis, while high concentrations (μM) reduce cancer progression via several mechanisms, including the induction of apoptosis, resistance reversal, inhibition of metastasis formation, and repression of angiogenesis. Other factors, besides concentration, can influence the effects of NO on tumor growth including the duration of NO exposure and cellular sensitivity (Ridnour *et al.*, 2006; Fukumura *et al.*, 2006).

The toxic effects of NO can be direct or indirect. Direct effects are related to the capacity of NO to react with the metals present in some biomolecules that are essential for cellular life (e.g. iron-sulfur centers of proteins and enzymes containing iron) blocking their functions. Indirect effects are quite complex and are related to the capacity of NO to react with O_2 or $O_2^{-\cdot}$, producing reactive nitrogen species (RNS) that can oxidize, nitrate and nitrosate a variety of biological targets, altering their functions (Wink and Mitchell, 1998). Peroxynitrite ($ONOO^-$) is a highly toxic RNS. It is formed from a reaction between NO and $O_2^{-\cdot}$, and is a potent oxidant and nitrating agent. In the physiological environment, it can generate $OH^{\cdot}$ and $NO_2^{\cdot}$ radicals, which are two strongly oxidant and hydroxylating /nitrating agents (Ferrer-Sueta *et al.*, 2018).

The properties of NO have been exploited in anticancer therapy, in particular, as there is evidence that it plays a role in overcoming resistance. As mentioned above, several resistance mechanisms have been described, and increased drug efflux mediated by ABC transporters, is one of

the most commonly studied. In 2005, Riganti *et al.,* first reported that NO reverses drug resistance in colon cancer cells via the nitration of ABC efflux transporters, which results in the inhibition of their activity and an increased accumulation of the anticancer drug in MDR cells (Riganti *et al.*, 2005). The inhibition of ATPase activity by NO has been confirmed in other tumor cell lines, such as, recently, in ovarian cancer cells (Sinha *et al.*, 2018).

Another mechanism of resistance is drug inactivation. For example, cisplatin can be conjugated to glutathione (GSH) and the drug-GSH conjugate is then extruded from cells by dedicated efflux transporters (Amable 2016). It has been reported that NO can deplete GSH, thus reducing cisplatin inactivation (Bratasz *et al.*, 2006).

Cancer resistance to DNA-targeting drugs can emerge as a result of DNA-repair proteins; NO can induce chemosensitization because it induces the nitrosation and denaturation of several proteins that are involved in DNA repair, thus increasing drug cytotoxicity (Kim *et al.*, 2017b). Furthermore, NO also regulates the chemosensitivity of cancer cells by nitrosylating, and therefore inhibiting, the NF-kB pathway (Huerta-Yepez *et al.*, 2013).

4.2 NO-donors

The difficulties of handling NO, due to its gaseous nature and reactivity, have led to the development of a number of pro-drugs, namely products that are able to release NO under physiological conditions. These products are collectively called NO-donors (NODs). This subject has gained extensive attention with a number of publications (Wang *et al.*, 2002; Grank and Grigor'ev, 2002; Huerta *et al.*, 2008; Wang *et al*., 2005). Classical NODs are generally classified according to their structure. NO-formation from these products can be enzymatic or non-enzymatic. In the latter case, NO release can be spontaneous or mediated by co-factors, for example thiols, heat or light. The use of NODs as NO substitutes in biological applications presents some limitations, the principle of which being the difficult spatial/temporal control of NO release. The perturbation of the physiological values of parameters, such as pH and ionic strength, the formation of toxic side products following

the NO-release reaction, half-life and possible enzymatic mechanisms are all examples of aspects that must be taken into account when selecting the appropriate NOD. Organic nitrates, nitrosothiols, metal nitrosyl complexes, *N*-diazeniumdiolates and furoxan derivatives are the most commonly used NO-donors in MDR studies (Fig. 8).

Organic nitrates ($RONO_2$) are esters of alcohols, the most important of which is glyceryl trinitrate (GTN), commonly known as nitroglycerin (Fig. 8). These products can release NO under the action of sulfhydryl groups or enzymatic activation, giving rise to the related alcohols. Glutathione-*S*-transferase, cytochrome P-450, the membrane-bound enzymes of vascular smooth muscle cells, xanthine oxidoreductase and mitochondrial aldehyde dehydrogenase (mtALDH) are enzymes that are involved in this release. In particular, mtALDH seems to be the key enzyme.

Nitrosothiols (RSNO) are generally unstable products that release NO spontaneously and form the corresponding thiyl radicals, which dimerize to give the related disulfide. Heat, UV light and some catalysts that include metal ions, copper in particular, accelerate this decomposition. Two relatively stable products, *S*-nitroso- *N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (GSNO), are the most frequently used in biochemical studies (Fig. 8).

Metal nitrosyl complexes (M-NO) derive from the interaction of NO with metal centers, in particular iron, which is the principal target for NO bioregulatory functions. These products are thermodynamically stable and kinetically labile species. Sodium nitroprusside (SNP) (Fig. 9), an iron-based nitrosyl compound, is the most important member of this class, is used in clinical practice in cases of acute hypertension and largely employed as an NO donor in biological studies. SNP releases NO under the action of heat, light, thiols and also enzymatically *in vivo*. The reduction and subsequent decomposition of SNP give rise to cyanide ion formation (a maximum of 5 equivalents of CN^- per mole) and this is the reason for the high cellular toxicity of the product.

N-Diazeniumdiolates (NONOates) (Fig. 8) are the most popular sources of NO to be used in biochemical studies. They are obtained in a reaction of one mole of secondary amines or polyamines

with two moles of NO in basic media. Their salts are stable solids and, in neutral or acid buffers, regenerate the parent products. NONOates display half-lives that range from seconds to hours, depending on their structures, and therefore, their use enables good temporal control of NO release. Examples of important members of this class are reported in Fig. 8. NONOates react with nucleophiles giving stable covalent O^2-derivatives, which behave as pro-drugs as they can be enzymatically or metabolically transformed to the parent compounds. JS-K (Fig. 9), the prototype of this class, reacts with GSH and other nucleophiles to produce the related NONOate.

Furoxans (Fig. 8) are a class of heterocyclic compounds that can either release NO spontaneously or under the action of thiol cofactors, depending on their structure. Along with the production of NO, nitrite, nitrate and *S*-nitrosothiols have been also observed. Generally speaking, the presence of electron-withdrawing groups on the ring, in particular at the 3-position, increases the rate and amount of NO production. The exact mechanism of NO release is unknown and only speculative hypotheses have been proposed. In the reaction with thiols in the pH range of 5-9, the extent of NO formation increased with pH and the rates correlate with the pKa of the thiol used. This suggests a mechanism in which the nucleophilic attack of the thiolate anion at the furoxan ring is followed by ring opening and NO release.

3,3-Dinitroazetidine, which bears a 1-bromoacetyl group on the azetidine nitrogen (RRx-001) (Fig. 8), is a recently discovered, potent anti-tumor agent that is able to release NO. It is capable of reacting via bromoacetyl and induce the depletion of GSH and oxidative stress in other thiols, and this could be one of the reasons for its anti-tumor properties. It rapidly penetrates red blood cells where it selectively binds to the β-Cys93 residue of hemoglobin. This binding increases the ability of hemoglobin to produce, under hypoxic conditions, NO from nitrite, which is one of RRx-001's metabolites (Cabrales *et al.*, 2016; Scicinsky *et al.*, 2015).

Interest in photo NO-donors (NOPDs), products that are able to release NO under the action of light, is currently growing. Light can be considered a powerful and minimally invasive microsyringe

for the injection of NO into biological systems. It provides excellent spatial/temporal control as it induces NO-release only in the illuminated region, while the timing and dosage of NO release can be precisely controlled simply by tuning the duration and intensity of the irradiation. NOPDs must satisfy some requirements, including excitation with visible light and the formation of non-toxic side photoproducts, if they are to be sustainable for bio-applications. The principal restriction is the limited tissue penetration of light with wavelengths <600 nm. This limitation can be overcome by the use of the "two-photon excitation" technique (Balzani *et al.,* 2014). In addition, simple derivatives of nitrobenzene that bear appropriate substituents at the *o-, m -,* and *p*-positions are also an important class of NOPDs (Suzuki *et al.*, 2005; Conoci *et al.*, 2006) (Fig. 8).

Figure 8. Structures of NO-donors and the mechanism of NO release from RRx-001

4.3. NODs in combination with anticancer agents to overcome drug resistance

A huge number of studies carried out on a variety of cancer cell lines that are resistant to common anticancer drugs show that high NO levels can overcome MDR. NO levels obtained from 500-1000 mM DETA/NO have been found to reverse cisplatinum resistance in tumor cells as well as epithelial-to-mesenchymal transition following the downregulation of NF-κB/Snail/YY1/ RKIP circuitry via numerous mechanisms (Bonavida *et al.*, 2008; Bonavida and Baritaki, 2011).

The endogenous NO produced at micromolar concentrations by iNOS may also reverse the MDR phenotype. Indeed, small Rho GTPases (RhoA, Rac, and Cdc42) play key roles in the regulation of tumor growth, migration and response to therapy. It was found that RhoA silencing increased the activation of the NF-κB pathway, inducing transcription and iNOS activity, leading to tyrosine nitration in the MRP3 efflux pump and a decrease in the ATPase activity of P-gp. This mechanism induced the accumulation of DOX in both HT29 and HT29-dx colon cancer cells and resulted in the overcoming of drug resistance (Doublier *et al.*, 2008; De Boo *et al.*, 2009).

Glutathione transferases (GSTs) are enzymes that promote the conjugation of GSH to electrophilic xenobiotics. The overexpression of GSTs in tumor cells can induce MDR via two mechanisms: the formation of GSH-anticancer drug conjugates and their active efflux via ABC transporters, and the inhibition of the mitogen-activated protein kinases pathway (MAPKs) (Townsend and Tew, 2003). O^2-(2,4-dinitrophenyl)NONOates are stable products that produce the related NONOates via reaction with GSH (Shami *et al.*, 2006). JS-K (Fig. 8) is a potent anticancer agent that displays high affinity for the GST-π isoform, which is specifically overexpressed in cancer cells. This product and its diethyl carbamoyl analogue (CB-3-100, Fig. 8) have been found to reverse arsenic and cisplatin resistance in a rat liver cell line (CasE) that shows acquired tolerance to arsenic and cisplatin and an overexpression of GSTs (Liu *et al.*, 2004). Other analogues of JS-K have shown a capacity to reverse MDR in DOX-resistant MCF-7/ADR cells (Li *et al.*, 2018a) and in temozolomide-resistant human U87 glioma cells (Kogias *et al.*, 2012). The anticancer effects of JS-K have also been confirmed in a human multiple myeloma xenograft mouse model (Kiziltepe *et al.*, 2007), and in a melanoma mouse model (Huang *et al.* 2018); in both cases, JS-K was found to slow tumor growth and prolong animal survival.

DOX-resistant MCF-7/Dx human breast cancer cell lines have been treated with nitrosoglutathione, leading to a greater increase in protein glutathionylation, which included proteins of the histone family, compared to MCF-7 cells, while a reversal in drug resistance was also observed (De Luca *et al.*, 2011). By contrast, the same NO-donors have been observed to increase the chemoresistance of C6 glioma cells against BCNU. This confirms that the role of NO in chemoresistance is dual, depending on the cell line type and NO-source (Yang *et al.*, 2004).

Tumor multicellular aggregates are more capable of developing resistance to chemotherapeutics than simple monolayer cultures. GTN and DETA/NO have also been observed to reverse resistance to DOX in a spheroid culture of MDA-MB-231 breast carcinoma cells, and did so via a cGMP-dependent mechanism (Muir *et al.*, 2006).

DOX resistance in human colon cancer cell line HT29-dx, which overexpresses ABC transporters, was reversed in a cGMP-independent manner after incubation with NOS inducers, such as a mixture of cytokines, atorvastatin and classical NO-donors, including GSNO, SNAP and SNP. This behavior was ascribed to the nitration of the tyrosine residue in the MRP3 efflux transporter and the resultant inhibition of this pump protein (Riganti *et al.*, 2005). A series of NO-donor furoxan derivatives have been studied for their capacity to inhibit the activity of P-gp and MRP1 in MDCK cells that overexpress these efflux transporters. When the compounds were co-administrated with DOX, they restored a high degree of antibiotic activity (Fruttero *et al.*, 2010). Two scaffolds that contained 3-phenylsulfonylfuroxan (**25**, Fig. 9) and dinitrooxy NO-donor (**26**, Fig. 9) moieties have been found to increase the cellular accumulation of DOX when co-incubated with this anthracycline in MDR HT29-dx colon cancer cells (Chegaev *et al.*, 2011). Furthermore, the dinitrooxy NO-donor has also been shown to reverse DOX resistance *in vivo* in a breast cancer mouse model (Gazzano *et al.*, 2018).

NO that is derived from propylamineNONOate (PAPA/NO) and diethylene triamine nanoate (DETA/NO) inhibited the ATPase activity of P-gp in human NCI/Adr cells, hence reversing DOX resistance and modifying their resistance to taxol (Sinha *et al.*, 2018).

A hollow microsphere system that contains the anticancer agent irinotecan (CPT-11) and DETA/NO generated NO bubbles that trigger localized drug release and reversed P-gp-mediated MDR when injected into an acidic tumor tissue (Chung *et al.*, 2015).

4.4. Combination of NODs with selected scaffold to overcome MDR

As part of studies investigating the role of NO in cancer resistance, NO-donors have been combined with specific scaffolds of interest as anti-tumor agents. A few examples are listed below.

A number of compounds have been obtained by combining, via appropriate spacers, the 3-phenylsulfonylfuroxan moiety with coumarin derivatives, and these have been tested for their anti-

proliferative activity in sensitive cancer cell lines and three human drug resistant tumor cell lines (A2780/CDDP, MDA-MB-231/Gem, and SKOV3/CDDP). Compound **27** emerged as the best product. Its IC_{50} values in drug resistant lines lie in the 62-140 nM range (Liu *et al.*, 2014). The 3-(*p*-F-benzyl) analogue **28** induced high cytotoxicity (0.5 to 143 nM) in four human drug resistant cancer cell lines (A2780/CDDP, MDA-MB-231/Gem, MCF-7/ ADR, KV-V). Similar behavior, but lower potency, has been displayed by compounds bearing a different substitution pattern at the benzyl moiety and the related seco-B-ring derivatives (Guo, Y. *et al.*, 2018). Interestingly, compound **28** displayed markedly improved anti-proliferative activity in the P-gp overexpressing cancer cell lines MCF-7/ADR and KB-V, compared to their drug sensitive counterparts.

A group of substituted tetrahydroisoquinolines (THIQs, **29**, Fig. 9), linked to 3-phenylsulfonylfuroxan by appropriate chains, were synthesized and studied for their cytotoxicity and effects in reversing MDR in human erythroleukemia K562/A02 cells. While the products triggered moderate cytotoxic effects, some of them elicited more potent reversal activity in this DOX-resistant cell line than that of verapamil (Zou *et al.*, 2011).

Acridonecarboxamide analogues (**30**, Fig. 9), which are potent Pg-p/ABCG2 pump inhibitors, displayed an improved capacity to induce accumulation of DOX in MCF-7/dx cell lines that overexpress P-gp when they were substituted with suitable NO-donor nitrooxyalkyl substituents at the N^{10}-position (Rajendra Prasad *et al.*, 2016).

The NO-donor 3-phenylsulfonylfuroxan moiety gave rise to products that reversed DOX resistance in MCF-7/Adr and K562/A02 cell lines when conjugated to bifendate (DDB) through appropriate spacers. In particular, compound **31** (Fig. 9) was able to increase the concentration of DOX in these cells by inhibiting Pg-p overexpression and blocking its efflux activity (Tang *et al.*, 2012; Ren *et al.* 2016). Subsequently, it has been found that similar products (compound **32**, Fig. 9) inhibited the proliferation of the leukemic K562/A02 cells by targeting several pathways that underlie drug resistance and cell proliferation (Gu *et al.*, 2017).

Hybrid products that are combinations of anti-tumor derivatives of oleanoic acid (OA), a natural triterpenoid, and 3-phenylsulfonylfuroxan elicit potent antitumor cytotoxicity and reversed drug resistance in HCT-8/5-FU colon cancer cells. Compound **33** (Fig. 9), one of the most active in the series, induced the nitration of P-gp, MRP1 and BCRP, as well as inhibiting HIF-1α, Stat3, AKT and ERK signaling (Ai *et al.*, 2015). Nitrooxyalkyl esters of podophyllotoxin have shown a marked potentiation of its anti-proliferative activity against MDR leukemic cells K562/VCR and K562/ADR, compared to the parent compound. An in-depth study of compound **34** (Fig. 9) indicated that, in these cells, the product blocked the G_2 phase, inhibited CDK1 and CDK2 expression and mTOR/STAT3 signaling, induced apoptosis and suppressed P-gp expression (Zhang *et al.*, 2018). Cucurbitacin-inspired estrone analogues (**35** and **36**, Fig. 9) that bear a NO-releasing 4-phenyl-3-methylenoxyfuroxan moiety at the 3-position, exhibited highly potent activity against the erlotinib-resistant HepG2 cells(HepG2-R) (Abou-Salim *et al.*, 2019; Li *et al.*, 2019b).

In view of the great potential of NO in cancer therapy, some important NODs have been covalently attached to currently used anti-tumor drugs in order to develop new, more potent, anticancer agents. DOX, platinum complexes and 5-FU have been the most frequently used drugs, but occasionally other anti-tumor chemotherapeutic agents have also been considered (Huang *et al.*, 2017b). A positive synergistic effect between NO and the considered drugs has been found in a variety of cancer cell lines. Only a few of these studies were specifically aimed at overcoming MDR. In the majority of these studies, DOX was used as the reference anticancer drug. A series of products have been developed in which this anthracycline was combined, via an ester bridge that is susceptible to metabolic cleavage, with NO-donor nitrooxy, furoxan and NONOate substructures (Chegaev *et al.*, 2011; Gazzano *et al.*, 2016). Some of the prepared compounds were more cytotoxic than DOX in colon cancer cells, HT29-dx. One compound (**26**, Fig. 9) emerged as the most promising product. It exhibited a faster uptake and interesting extranuclear distribution, being preferentially localized in mitochondria. In these loci, compound **26** nitrated and inhibited the mitochondria-associated ABC

transporters, decreased the flux through the tricarboxylic acid cycle and the activity of complex 1, lowered ATP synthesis, induced oxidative and nitrosative stress, and elicited the release of cytochrome C and the activation of caspases 9 and 3 in DOX-resistant cells (Riganti *et al.*, 2013). Liposomal formulations of the product were developed and appeared to be effective tools, *in vitro* and *in vivo*, against DOX-resistant breast and ovarian cells/tumors (Pedrini *et al.*, 2014; Gazzano *et al.*, 2018). In a preclinical mouse model of resistant breast tumor, the liposomal formulation significantly reduced tumor growth (Gazzano *et al.*, 2018).

Interestingly, the light-induced release of NO from compound **37** (Fig. 9), in which DOX is attached via a spacer with a nitroaniline photodonor, has been shown to increase toxicity towards the human melanoma M14 cellular line following nitration of critical tyrosine residues in ABC transporters that were overexpressed by these cells (Chegaev *et al.*, 2017). Finally, a light-responsive NO donor has been tested *in vivo* in HeLa tumor-bearing nude mice; passive targeting to the tumor site and significant tumor reduction were observed (Wang *et al.*, 2018a).

Hypoxia-induced drug resistance appears to result, at least in part, from the suppression of endogenous NO production (Matthews *et al.*, 2001; Yasuda, 2008). It has therefore been suggested that NODs may help to overcome this modality of chemoresistance. Studies carried out on human breast carcinoma (MDA-MB-231) and mouse melanoma (B16F10) cells that were incubated with the NOS inhibitor L-NMMA after exposure to hypoxia (1% O_2), showed that the cells increased their resistance to DOX and 5-FU. This effect was reversed by GTN and DETA/NO (Matthews *et al.*, 2001). The hypoxia-induced resistance to DOX that was observed in human PC-3 and mouse TRAMP-C2 prostatic adenocarcinoma cells after incubation under 0.5% O_2 was inhibited by low concentrations of GTN (Frederiksen *et al.*, 2003). Low concentrations of GTN were also effective *in vivo*; it was shown that GTN increased DOX chemosensitivity in human prostate cancer xenografts (Frederiksen *et al.*, 2003). Research carried out on MDA-MB-231 cells (Postovit *et al.*, 2002; Postovit *et al.*, 2004) has shown that hypoxia increases tumor cell invasiveness and metastasis enhancement

by inhibiting cGMP-dependent NO-signaling. A series of hybrids, a 3-phenylsulfonylfuroxan moiety together with 16,17-pyrazo-annulated steroids, has been synthesized and underwent anti-proliferation evaluation in cancer cell lines. Compound **38** (Fig. 9) was found to be active at a concentration of 1 μM on a tamoxifen-resistant breast cancer cell line (HCC1806) (Huang *et al.*, 2015b). Interestingly, a study has shown that SNP significantly reduced cellular injury, triggered in C6 glioma cells by chemical hypoxia, via the activation of Na^{+}-Ca^{2+} exchange (Amoroso *et al.*, 2000).

Figure 9. Representative structures of NO-donor hybrid compounds.

5. Acridine derivatives as an example of the pharmaco-modulation of a key scaffold in overcoming drug resistance

Acridines are compounds that have been known ever since the 19th century. The most important agent in this class is amsacrine (m-AMSA, Fig. 10). Amsacrine, and its less active isomer o-AMSA, intercalate into DNA via the acridine ring in a dynamic process (Liu *et al.*, 2007, Jangir *et al.*, 2012). The acridine derivatives that were synthesized after amsacrine, showed a variety of mechanisms of action, including inhibition of both topoisomerases I and II, HDACs, PK, proteasome, Akt1 kinase and PARP-1. One of these derivatives, (N-[2-(dimethylamino)ethyl]acridine-4-carboxamide, DACA, Fig. 10) was found to be highly active against a number of cancer types (Lewis lung murine carcinoma, murine colon carcinoma and human melanoma) (Atwel *et al.*, 1987). DACA is devoid of P-gp-mediated MDR and this may be due to its lipophilic character (Atwel *et al.*, 1987), which allows uptake by tumor. DACA is also devoid of topoisomerase II-mediated resistance, which may be explained by the lack of an anilino side chain and the fact that its interactions with topoisomerase II are different from those of amsacrine (Finlay *et al.*, 1993). The discovery of amsacrine and DACA was the beginning of an intensive research into the use of acridines as anticancer agents in order to

develop new molecules with improved anticancer activity (including those that are active towards MDR cell lines) and less untoward toxicity.

In recent decades, several strategies of modifying amsacrine structure have resulted in compounds with activity towards drug resistant tumor cell lines. These modifications include either relatively minor changes in the amsacrine side chain (e.g. change of type and position of substituents on the anilino ring) or major changes in its structure (e.g. addition of new heterocyclic rings to the acridine moiety and changes in its side chain).

Finlay and co-workers have tested the activity of a group of 9-anilinoacridine derivatives (**38**, Fig. 10), which are structurally similar to amsacrine, towards five MDR human leukemia cell sublines (JL/AMSA, JL/DOX, JB/AMSA, JB/DOX and K/AMSA) (Finlay *et al.*, 1990). After an analysis of the activities towards the JL/AMSA and JB/AMSA cell lines, it was concluded that the resistance factors of compounds **38** were significantly reduced (5-10-fold) compared to amsacrine.

Stefańska *et al.,* have synthesized a group of pyridazinoacridines (**39**, Fig. 10) for use as potential active agents towards resistant tumors (Stefańska *et al.*, 2005). Antitumor activity was tested in leukemia cell lines (sensitive murine L1210 and human K562 and HL-60, as well as resistant sublines K562/DX (MDR-type resistance), HL-60/VINC (MDR-type resistance) and HL-60/DX (MRP-type resistance). Significant improvements in cytotoxicity against resistant tumor cell lines (K562/DX, HL-60/VINC and HL-60/DX) were observed when compared to DOX and mitoxantrone.

Bontemps-Gracz *et al.,* have tested two groups of acridines with fused heterocyclic rings (pyrazoloacridines – **40** and pyrazolopyrimidoacridines – **41**, Fig. 10) in several human leukemia cell lines, including those with P-gp-dependent MDR (K562/DX and HL60/VINC) and MRP-1 dependent resistance (HL60/DX), as well as in the human SCLC-sensitive cell line GLC4/DX with MRP/LRP dependent resistance (multidrug resistance-associated protein dependent resistance/lung resistance related protein dependent resistance) (Bontemps-Gracz *et al.*, 2002). The results confirmed their earlier hypothesis as to the importance of the heterocyclic ring, fused to the acridine ring, in

overcoming MDR. Additionally, the location and type of substituents on this condensed ring significantly influenced cytotoxic activity and the ability to overcome MDR.

A series of bis- (**42**, Fig. 10) and tetra-(**43,** Fig. 10) acridine derivatives have been tested on sensitive (HL-60) and MDR (HL-60/MX2) cancer cell lines (Vispe *et al.*, 2007). The presence of the acridine moiety in their structure led the authors to evaluate the DNA intercalation and topoisomerase II inhibition properties of these compounds. The inhibition of topoisomerase II-mediated DNA decatenation was observed, but the evaluation of cytotoxicity towards cancer cell lines that are either sensitive or resistant to reference topoisomerase inhibitors indicated that topoisomerase was not the only target of these compounds. The authors revealed that some of these molecules also acted as proteasome inhibitors, meaning that they are potent multi-target ligands.

Murahari and coworkers have recently synthesized hybrid molecules that contain the acridone ring and substituted pyrimidine (**44**, **45**, Fig. 10) (Murahari *et al.*, 2017). Their aim was to obtain compounds with the ability to exert anticancer activity by interacting with multiple targets. Absorption titrations with Calf Thymus DNA and gel electrophoresis showed that these molecules displayed anticancer activity that was partly due to DNA intercalation, while the results of a Western blot analysis with Akt kinase showed that they also possessed an ability to inhibit Akt kinase activity and induce apoptosis. Immunoblot analysis of the ABCC1 (MRP1) transporter in MDA-MB-231 cells, which had been treated with the selected compounds, showed reduced ABCC1 (MRP1) expression. Several other groups of acridine-ring containing hybrid molecules, such as those that exert activity against Src and MEK kinases (Cui *et al.*, 2016), topoisomerase II and PARP-1 (Yuan *et al.*, 2017) as well as topoisomerase II and HDACs (Chen *et al.*, 2018b), have recently been synthesized. These dual targeting molecules possess anticancer activity and, as discussed above, have the potential to act towards resistant cancer cell lines. However, this potential has yet to be experimentally confirmed.

Figure 10. Structures of amsacrine and its derivatives/analogs.

6. Other approaches to overcome MDR: glycoconjugation and polymer conjugation

One of the hallmarks of cancer is the so-called Warburg Effect (Warburg, 1956), based on the observation that, among other features, tumors consume larger amounts of glucose than normal cells. This is caused by the overexpression, in human cancers, of several glycolytic enzymes and the insulin-independent glucose transporter (GLUT1) (Altenberg and Greulich, 2004; Medina and Owen, 2002). This unusually elevated flow of carbohydrates towards tumors has garnered much attention from the scientific research community, leading to the design and development of several sugar-based targeted drug delivery systems (Calvaresi and Hergenrother, 2013; Hossain and Andreana, 2019). The final glycosidase-mediated cleavage step of the sugar-containing prodrug is required for active antitumor drug release. The glycoconjugated antitumor agents gained increased selectivity and became less toxic to normal cells than the parent aglycon agents (de Freitas Junior and Morgado-Diaz, 2016; Johansson et al., 2010; Sztandera et al., 2019). More recently, this strategy has also been aimed at counteracting MDR tumors *(*Vogus *et al., 2017;* Wu *et al.*, 2016). Mitragotri and co-workers have developed a delivery vehicle for DOX and gemcitabine (GEM) based on hyaluronic acid (HA) conjugation that optimizes the synergistic effect of drug release and is able to treat triple-negative breast cancer (Vogus *et al.*, 2017). Gao and co-workers have conjugated a (trans-R,R-cyclohexane-1,2-diamine)-2-chloromalonato-platinum(II) complex with galactose (Gal-Pt) and tested it on several tumor cell lines (Wu *et al.*, 2016), including human colon cancer cells (HT29), which are resistant to both oxaliplatin and docetaxel (El Khoury *et al.*, 2016). The Gal-Pt conjugate showed superior cytotoxic potency in HT29 cells compared to a non-cytotoxic dose of oxaliplatin. The important role of galactose was confirmed when a reduction in the cytotoxic potency of Gal-Pt was induced by quercetin, an inhibitor of glucose transporters (Wu et al., 2016).

The covalent conjugation of a low molecular weight drug to a hydrophilic polymeric carrier can lead to an increased therapeutic effect by altering drug pharmacokinetics at the cellular level and

by restricting its endocytosis uptake. Polymer conjugation of conventional chemotherapeutic drugs is a promising approach in cancer treatment offering more effective treatment, improved drug delivery, side effect reduction and decreased drug resistance (Seifu and Nath, 2019). There are many strategies for the development of these polymeric pro-drugs, and they mainly differ in the type of polymer used, which can be simple, such as dextran, cyclodextrin, N-(2-hydroxypropyl) methacrylamide, poly-L-glutamic acid, polyethylene glycol, etc. (Greco and Vicent, 2008), or more complex, such as peptides (Vrettos *et al.*, 2018), dendrimers (Dib *et al.*, 2019; Muniswamy *et al.*, 2019), proteins (Muniswamy *et al.*, 2019; Sasaki *et al.*, 2019), gold NPs (Chen *et al.*, 2016; Eissa *et al*., 2014), quantum dots (Bae and Chung, 2014; Javanbakht and Namazi, 2018) and antibodies (Akkapeddi *et al.*, 2019; Birrer *et al.*, 2019). Several of these strategies have already been successfully used to circumvent MDR (Huang *et al.*, 2016; Kumbhar *et al.*, 2018; Soe *et al.*, 2019). Manjappa and coworkers have devised an integrative approach to improving the *in vitro* cytotoxicity of the antifolate methotrexate (MTX) against the human carcinoma cell lines KB and MDR KBV, significantly reducing its *in vivo* toxicity (Kumbhar *et al.*, 2018). The authors combined gluconic acid conjugation with an encapsulation step into micelles composed of D-α-tocopheryl poly (ethylene glycol) 1000 succinate, as an MDR reversing copolymer, and poloxamer 407 (P-407) to deliver the MTX prodrug to tumor tissues via the EPR effect. The final drug delivery system showed a remarkable IC_{50} value of 5.4 μg/mL, compared to free MTX (85.2 μg/mL), in the MDR KBv cell line. Cao and coworkers have developed a dual nano-drug delivery system, in which DOX is conjugated to a xyloglucan polymer to form stable NPs that were able to encapsulate PTX (Huang *et al.*, 2016). This conjugate system showed synergism between PTX and DOX and a significant effect on the IC_{50} values of the MDR cell line HepG2/DR (0.4 μmol/l for the conjugate *vs* 6.4/15.8 μmol/l for free PTX/DOX). Kim and coworkers have used transferrin conjugated to poloxamer 407 and 123 for the targeted delivery of DOX to the MDA-MB-231(R) cell line (Soe *et al.*, 2019). The authors reported the *in vivo* accumulation of NPs in DOX-resistant tumor cells, and the accelerated and controlled release of DOX from the conjugate, which resulted in higher cytotoxicity via induction of apoptosis.

An alternative strategy to target tumor cells and circumvent some of the drug resistance mechanisms is based on the use of non-covalently bound carriers as encapsulating agents, forming NPs (Wang *et al.*, 2019b). They should be stable, non-toxic, biocompatible, biodegradable and non-immunogenic. They should also entrap lipophilic chemotherapeutic drugs, enhance their membrane permeability and cellular retention, and allow a controlled release within the body. There are several types of carriers in this class that have shown promising cancer-therapy results, including micelles and liposomes. pH-sensitive micelle systems have also been developed (Tian *et al.*, 2018; Wang *et al.*, 2018) and they have shown superior MDR reversal performance against DOX-resistant tumor cells when coupled with polyhistidine or folate polymers (Cao *et al.*, 2019; Li *et al.*, 2015). Liposomes can effectively encapsulate chemotherapeutic drugs and be coupled with innovative strategies to surmount MDR. Ying and coworkers have prepared novel PTX/hydroxypropyl-β-cyclodextrin complex-loaded liposomes that exhibited pH-sensitive PTX release, potent cytotoxicity, and enhanced intracellular accumulation in the PTX-resistant human lung adenocarcinoma (A549/T) cell line (Shen *et al.*, 2019). Talegaonkar and coworkers have prepared hyaluronic acid-coated liposomes that contained imatinib mesylate, and showed that they were 3.5-fold more cytotoxic to the Colo-320 cell line (a CD-44 expressing MDR cancer cell line) than the drug solution *(*Negi *et al.*, 2019). Du and coworkers have devised a rather complex liposome-based drug-delivery system that co-delivered PTX and sorafenib, which is a chemosensitizing agent (Lei *et al.*, 2019). The liposome was based on D-α-tocopheryl polyethylene glycol 1000 succinate and a polylysine-deoxycholic acid copolymer coated with hyaluronic acid. The multifunctional liposome was able to significantly enhance drug accumulation in resistant MCF-7/MDR cells by inhibiting P-gp efflux, and effectively inhibited the growth of tumor cells by 78.5%.

The TME contributes to the intrinsic chemoresistance of malignant cancers (Assaraf *et al.*, 2019; Taylor *et al.*, 2015; Wang *et al.*, 2019b). A key factor of the TME is its acidosis; the pH values in the extracellular milieu of the TME range from 6.0 to 6.8, with the stronger acidity being present in the more aggressive tumors (Logozzi *et al.*, 2019). The low extracellular pH is a hallmark of the TME

and can be a target for cancer therapeutics that are either aimed at reducing the acidity of the TME or use drug carriers that are specifically activated by acidic pH *(*Cao *et al.,* 2019; Tian *et al.,* 2018; Wang *et al.,* 2018; Zhou *et al.*, 2017). The first approach has been successfully implemented using proton pump inhibitors, such as lansoprazole, to help sensitize cancer cells to conventional anti-tumor drugs and obtain positive synergistic effects that can overcome resistance both *in vitro* and *in vivo* (Azzarito *et al.*, 2015; Taylor *et al.*, 2015).

However, taking advantage of the acidity of the TME and attempts to actively increase antitumor drug concentration in tumor cells, beyond the EPR effect, has been quite challenging (Park *et al.*, 2019). pH(Low) Insertion Peptide (pHLIP) is a family of peptides that insert into cell membranes in a pH-dependent manner (Wyatt *et al.*, 2018). The pHLIP p*Ka* of insertion can be easily fine-tuned (Vila-Vicosa *et al.*, 2018; Weerakkody *et al.*, 2013). It inserts into the membrane in a unidirectional manner, leaving its arginine-containing N-terminus on the extracellular side, and can be conjugated with a variety of agents for both diagnostic and therapeutic applications (Burns *et al.*, 2015; Cheng *et al.*, 2015; Demoin *et al.*, 2016; Moshnikova *et al.*, 2013; Reshetnyak *et al.*, 2011; Wijesinghe *et al.*, 2011; Zhang *et al.*, 2019), including strategies to surmount MDR (Song *et al.*, 2016; Zhang *et al.*, 2017d).

7. Considerations used for *in silico* prediction of drug uptake, bioavailability and ADMET in MDR tumor cells

7.1 In silico modeling of MDR transporters and their ligands.

A great deal of efforts has been invested in the search for potent and specific inhibitors of the efflux activity of the MDR pumps in recent years. Several generations of MDR modulators have been developed, starting with well-known drugs, such as verapamil, cyclosporine A, quinidine, and ending with novel 3rd generation molecules, such as zosuquidar, tariquidar and elacridar (Muller *et al.*, 2008;

Pajeva and Wiese, 2009; Pajeva *et al.*, 2013a). Effective approaches towards the improvement of conventional antitumor drugs and the development of novel drugs should consider a drug's ability to interact with the transporter proteins to possibly avoid efflux. A key element in the search for these improved drugs is an understanding of the way they interact with the transporter proteins. *In silico* methods that, in general, rely on the structure-activity relationships of the drugs and the structure-function relationships of their target proteins can play an essential role in these efforts. Structure-based design methods that utilize 3D structural data on the proteins and their complexes with the ligands are the most effective of these *in silico* methods as they provide a prediction of the interaction between the transport proteins and their substrates and inhibitors.

P-gp is perhaps the most commonly studied ABC transporter and hundreds of biologically active compounds have been reported to act as its substrates and/or inhibitors. The DrugBank database (v.5.1.4) currently contains records of more than 380 drugs that interact with P-gp, including some of the most recent novel drugs approved by FDA (e.g. entrectinib for the treatment of non-small cell lung cancer), alpelisib (breast cancer), erdafitinib (advanced or metastatic bladder cancer) and gilteritinib (acute myeloid leukemia).

The 3D structure of human P-gp has recently been resolved, which opens new perspectives for antitumor drug development. Figure 11 illustrates the 3D structure of P-gp in complex with PTX (Alam *et al.*, 2019). The analysis of the binding pose and the interactions of this antitumor drug with the surrounding amino acid residues provides insight into the binding mode of the compound and could direct possible rational modifications towards preventing its action as a P-gp substrate.

Figure 11. 3D structure of human P-gp in complex with the antitumor drug taxol (PDB ID 6QEX): **A**. General view of the protein. The protein backbone is rendered as a line ribbon; **B**. Close view of the taxol binding site with the 27 amino acids in the protein cavity surrounding the ligand. The binding site of taxol is outlined by its molecular surface, colored in light gray.

The huge protein cavity (Fig. 11A) can accommodate more than one drug, which has been demonstrated using a 3D structural complex of P-gp with two molecules of the inhibitor zosuquidar (PDB ID 6QEE) (Alam *et al.*, 2019). Moreover, this 3D structure shows that the same drug can have more than one binding site and multiple binding modes. Thus, it becomes evident that the availability of reliable information on the potential binding sites of substrates is a key aspect for the successful *in silico* structure-based modeling of the interactions of antitumor drugs with transporter proteins. Utilizing experimental data from various pharmacological tests and assays of P-gp substrates and inhibitors, including rhodamine 123 (R-site), Hoechst33342 (H-site), prazocin (regulatory site), tariquidar and elacridar, a number of binding sites have been proposed for use in 3D homology models of the human P-gp (Pajeva *et al.*, 2013a; Pajeva *et al.*, 2013b). However, except for PTX (mentioned above), there is currently no direct experimental evidence for the possible binding sites of most currently used antitumor agents. Nevertheless, 3D structural data have been used to direct the *in silico* docking of new P-gp inhibitors, as demonstrated in a recently published study on novel Hsp90 inhibitors (Dinić *et al.*, 2019). The analysis of the binding sites of PTX and zosuquidar molecules revealed that their sites partially overlap, suggesting that zosuquidar is involved in a competitive inhibitory mechanism. However, the most recent findings reveal that the transport control of the protein is more complex in nature as it is related to the different roles played by the transport substrates and inhibitors in the structural symmetry of the nucleotide binding sites (Dastvan *et al.*, 2019). The same study proposes a model of P-gp transport and inhibition that includes basal, substrate-coupled and inhibition cycles. The conformational flexibility of the protein raises the question of how relevant the protein conformation used for *in silico* studies is to drug binding. The transport protein undergoes huge conformational changes during the transport cycle as it passes through various states over the translocation pathway between the transmembrane and nucleotide-binding domains. This movement changes the 3D arrangement of the residues surrounding the binding sites in the protein cavity used for *in silico* modeling. The problem has been addressed in a molecular dynamics study of P-gp that demonstrates the need for a critical evaluation of the results

obtained by simulations of large flexible proteins such as P-gp and other MDR transporters (Condic-Jurkik *et al.*, 2018).

Recently, the 3D structure of the human BCRP (ABCG2) has also been resolved in a complex with two antigen-binding fragments of the human-specific, inhibitory antibody 5D3 (Taylor *et al.*, 2017). The X-ray structure of BCRP, similarly to P-gp, has been used for the docking of potential MDR modulators of BCRP efflux (Ji *et al.*, 2019). In a recent study, selonsertib (GS-4997), a selective ASK1 inhibitor with potential antineoplastic activity, has been shown to sensitize ABCB1 overexpressing cells to DOX and PTX, and ABCG2 overexpressing t cells displaying resistance to mitoxantrone and topotecan. Docking studies revealed the possible existence of specific hydrogen-bonding and π-π stacking interactions in the drug binding site of BCRP and outline possible ways by which other ligand structures could be rationally modified.

In summary, *in silico* structure-based methods show great potential as a means to evaluate the ability of antineoplastic drugs to interact with MDR efflux transporters. The recently resolved X-ray structure of human P-gp is especially appropriate for such studies. Despite limitations in the knowledge on the possible binding sites of the most commonly used antitumor drugs, the 3D structure of both P-gp and BCRP could help delineate their drug interaction sites. In addition, the knowledge that has been gained on the PTX site of Pgp could be used to identify possible differences in the interactions of next-generation taxenes that have already been synthesized and tested for their anticancer activity (Ojima *et al.*, 2018). To be successful, such simulation studies should be carefully designed, should be supported with reliable experimental data and subsequently confirmed by pharmacological assays.

7.2 In silico ADMET profiling

Many cytotoxic agents cross biomembranes and enter cells via passive diffusion (Tredan *et al.*, 2007). A deep understanding of this process at the molecular level is therefore essential to efficiently optimize drug uptake, bioavailability and the ADMET of MDR tumor cells (Di *et al.*, 2011; Dickson

et al., 2017; Lipinski *et al.*, 1997). Several approaches have been devised to estimate passive membrane permeation rates, including computational methods, with special emphasis on those based on the solubility-diffusion models (Diamond and Katz, 1974; Dickson *et al.*, 2017; Hummer, 2005; Marrink and Berendsen, 1994). Over the years, this approach has evolved towards correctly describing the inhomogeneous nature of a lipid bilayer, resulting in more complex methods that rely on molecular dynamics simulations, umbrella sampling and the potential of mean force calculations (Dickson *et al.*, 2017; Vila-Vicosa *et al.*, 2017; Yue *et al.*, 2019). The permeability coefficients in many of these methods are derived using the inhomogeneous solubility-diffusion model (ISDM) (Dickson *et al.*, 2017; Hummer, 2005; Vila-Vicosa *et al.*, 2017).

The acidity of the TME can also alter the membrane permeability coefficients of many anticancer drugs. It is common to find antitumor drugs bearing Lewis base groups with pK_a values typically ranging from 7.5 to 9.5 (Gotink *et al.*, 2011; Zhitomirsky and Assaraf, 2017, 2015; Zhitomirsky *et al.*, 2018), that are able to shift their pK_a to lower values when interacting with lipid bilayers (Assaraf *et al.*, 2019; Teixeira *et al.*, 2016). This mechanism allows these compounds to transiently deprotonate, cross the hydrophobic barrier of the membrane and re-protonate to facilitate the final membrane-leaving step. This (de)protonation-concerted mechanism of passive diffusion explains why the acidic TME is, in fact, a drug resistance mechanism and an important barrier to many anticancer agents entering tumor cells *(*Assaraf *et al.*, 2019). Computational methods that aim to correctly describe the pH-dependent membrane permeabilities of antitumor drugs will probably need to couple the ISDM calculations to Constant-pH MD methods (Radak *et al.*, 2017; Teixeira *et al.*, 2016; Yue *et al.*, 2019).

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