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Metabolic and non-metabolic pathways that control cancer resistance to anthracyclines

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ABSTRACT

Anthracyclines Doxorubicin, Epirubicin, Daunorubicin and Idarubicin are used to treat a variety of tumor types in the clinics, either alone or, most often, in combination therapies. While their cardiotoxicity is well known, the emergence of chemoresistance is also a major issue accounting for treatment discontinuation. Resistance to anthracyclines is associated to the acquisition of multidrug resistance conferred by overexpression of permeability glycoprotein-1 or other efflux pumps, by altered DNA repair, changes in topoisomerase II activity, cancer stemness and metabolic adaptations. This review further details the metabolic aspects of resistance to anthracyclines, emphasizing the contributions of glycolysis, the pentose phosphate pathway and nucleotide biosynthesis, glutathione, lipid metabolism and autophagy to the chemoresistant phenotype.

1. Introduction

Anthracyclines are a family of antitumor antibiotics used in children and adult patients to treat a wide variety of solid tumors, including breast, lung and stomach cancers, leukemia and lymphomas [1,2]. Despite their efficacy, their use is limited by toxicity to normal tissues and treatment resistance. The major side effect of anthracyclines is cardiotoxicity due to cumulative doses [3]. On the long term, other organs, *e.g.*, the brain, kidneys and the liver, can be affected [4], and secondary cancers can be induced [5]. Tumor resistance to anthracyclines involves mechanisms that can differ from tumor to tumor. Here, we discuss cellular resistance mechanisms with a special focus on those that involve tumor metabolism.

Anthracyclines possess a common structure that consists in a tetracyclic ring with quinone-hydroquinone groups linked to (3*S*, 4*S*, 5*S*)-3-amino-4,5-dihydroxyhexanal (Daunosamine) by a glycosidic bound (Fig. 1) [5,6]. The presence of Daunosamine accounts for the hydrophilic properties of the drugs [6]. Specific anthracyclines are distinguished by minor chemical changes that nevertheless profoundly influence their half-lifes, capacity to bind to DNA, targetable tumor types and toxicities [2,5].

The first anthracyclines were identified by an Italian team who, between 1950 and 1960, was analyzing different natural compounds for their activity against murine cancer cells [7]. The first anthracycline, Daunorubicin (DNR, Fig. 1A), was isolated from a red pigment produced by bacterium *Streptomyces peucetius* species [8]. Experimentally, DNR induced important tumor regression in mice [1,7], and its clinical efficacy was rapidly demonstrated in a clinical trial conducted between 1963 and 1964 on patients with acute leukemias [1]. Unfortunately, its development was halted because of its high cardiac toxicity [3]. In order to avoid, or at least to limit, this toxicity, several anthracyclines were produced by genetic and chemical modifications [1,8], which led to the synthesis of more than 2,000 compounds, among which only few have been approved for clinical use [2,5]. Doxorubicin (DOX) was derived from DNR by genetic modifications in bacteria [9]. The main difference between DNR and DOX is the presence of a hydroxyl group on the carbon 14 of DOX (Fig. 1B) [8]. Due to its broad spectrum of action on liquid and solid tumors, DOX largely replaced DNR for anticancer therapy, but, unfortunately, heart toxicity remained a strong issue [9]. Among efforts to overcome this side effect, liposomal formulation was the one showing better results: when used in ovarian and breast cancers, this drug formulation had better therapeutic and safety profiles than conventional treatment [9].

Epirubicin (EPI) was then designed by introducing a chemical modification to DOX consisting in an inversion of the 4′-hydroxyl group on the amino-sugar moiety [10] (Fig. 1C). The activity of EPI in tumors is equivalent to that of DOX [5]. However, EPI is less toxic and is more easily eliminated in urine because it is more glucuronidated than DOX [2,11].

The last anthracycline used in the clinics is Idarubicin (IDA). It was

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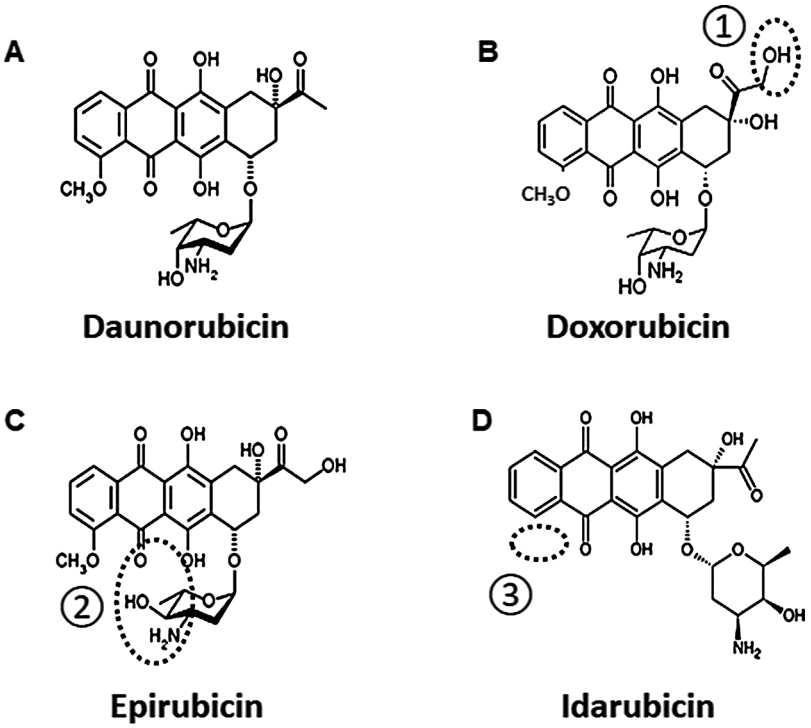

Fig. 1. Chemical structure of anthracyclines. *A*, Daunorubicin (DNR). ***B***, Doxorubicin (DOX). Compared to DNR, DOX is characterized by the presence of a hydroxyl group (OH) ①. ***C***, Epirubicin (EPI). Dotted line ② indicates an inversion of the 4′-hydroxyl group on the daunosamine amino-sugar moiety of EPI compared to DOX. ***D***, Idarubicin (IDA). Dotted line ③ indicates the absence of a methoxy group at carbon 4 on the structure of IDA compared to DNR.

generated by a chemical modification of DNR consisting in removing the methoxy group at carbon 4 (Fig. 1D). Compared to DNR, IDA is characterized by a high lipophilicity and cellular uptake due to its chemical structure that, additionally, allows oral delivery [12].

2. Modes of action of anthracyclines

Despite their use to treat a variety of tumors, several studies were needed to understand the mode of action of anthracyclines. It has been widely proved that anthracyclines passively diffuse through plasmatic and nuclear cell membranes [13]. However, recent studies demonstrated that the proteasome is a nuclear transporter of DOX: cytosolic DOX forms a complex with the proteasome that is translocated from the cytoplasm into the cell nucleus owing to the fact that the proteasome possesses nuclear translocation signals [2,14]. This process requires ATP. Once inside the nucleus, the high affinity of DOX for DNA dissociates the complex, and DOX, like other anthracyclines, intercalates with DNA bases to form adducts and to stop the activity of DNA and RNA polymerases, consequently blocking DNA and RNA synthesis and triggering apoptosis [6]. Of note, the toxicity of anthracyclines can also be mediated by alterations of the proteasome. Indeed, DOX binding to the proteasome reduces its chymotrypsin-like protease activity, which reduces the processing and degradation of regulatory proteins that control cell growth, thus triggering apoptosis [14].

Anthracyclines were also found to induce cytotoxicity by inhibiting topoisomerase II (TOPO II) [4], the enzyme that decreases DNA supercoiling by cutting DNA double strands during transcription and replication. Anthracyclines indeed block the catalytic activity of TOPO II. Thereby, they stabilize DNA breaks, thus contributing to inhibition of DNA replication and, consequently, initiating cell death [4,6]. Anthracyclines are also capable to generate reactive oxygen species (ROS) through a redox reaction in the presence of cytochrome P450 reductase and NADH dehydrogenase. This reaction converts the quinone group of anthracyclines in a semiquinone-free radical that causes DNA damage through oxidation and membrane damage through lipid peroxidation [2,6,15,16].

Anthracyclines can also induce cell death mediated by ceramide, a sphingolipid resulting from the covalent coupling of sphingosine with fatty acids by ceramide synthase and serine palmitoyltransferase (SPT). A first evidence of the existence of this pathway was provided by the observation that DNR triggers apoptosis by stimulating cycles of sphingomyelin hydrolysis with concomitant ceramide generation [17,18]. Ceramide production was proposed to involve *de novo* synthesis *via* activation of ceramide synthase [17] or, alternatively, neutral sphingomyelinase (SMAse) [18]. Similarly, Denard et al. [19] recently observed that the treatment of cancer cells with DOX induced *de novo* ceramide synthesis. Mechanistically, they reported that, in response to DOX, ceramide accumulation in the endoplasmic reticulum (ER) led to the translocation of transmembrane protein c-AMP-responsive element binding protein 3 like 1 (CREB3L1) from the ER to the Golgi apparatus, where it was cleaved by site-1 and site-2 proteases, thus releasing its cytoplasmic NH_2-terminal domain. Upon release, the free CREB3L1 protein fragment can translocate into the cell nucleus where it acts as a transcription factor that stimulates the transcription of cyclin-dependent kinase inhibitors, which ultimately blocks cell proliferation. The whole pathway was blocked when DOX-treated cells received either ceramide synthase inhibitor fumonisin B1 or SPT inhibitor myoricin [19].

3. Antitumor use of anthracyclines in the clinics

Since their discovery, anthracyclines have been widely used in antitumor therapies, not only as single treatments but also in combination (Table 1). DOX is currently used as a standard therapy in breast cancers, sarcomas, leukemias, Wilm's tumors, Hodgkin's disease and non-Hodgkin's lymphomas [2,20], whereas EPI is indicated to treat breast, stomach, lung, ovarian and prostate cancers, as well as soft tissue sarcomas [2,11]. DNR is mainly used for the treatment of myeloblastic and lymphoblastic leukemias [20], and IDA is indicated for the treatment of acute leukemias, myelomas and breast cancers [2]. To limit cardiotoxicity, the recommended cumulative doses are between 450 and 500 mg/m^2 for DOX, 900 mg/m^2 for EPI [21], 550 mg/m^2 for DNR and 160 mg/m^2 for IDA [2].

Both DOX and EPI can improve disease-free survival and overall survival when used as adjuvant treatments in breast cancer. For example, the association of DOX or EPI with 5-Fluorouracil (5-FU) and

Table 1
Anthracyclines used in anticancer therapy.

Tumor type	Anthracyclines used	References
Breast cancer	Doxorubicin, Epirubicin, Idarubicin	[9,12,180]
Ovarian cancer	Doxorubicin, Epirubicin	[181,182]
Acute Leukemia	Daunorubicin, Doxorubicin, Idarubicin	[9,24]
Wilm's tumor	Doxorubicin	[158]
Non-Hodgkin lymphoma	Doxorubicin	[183]
Gastric carcinoma	Doxorubicin, Epirubicin	[9,184]
Lung cancer	Doxorubicin, Epirubicin	[1,185]
Prostate cancer	Epirubicin	[1,186]
Soft tissue sarcoma	Doxorubicin, Epirubicin	[187,188]
Hodgkin's disease	Doxorubicin	[9]

Cyclophosphamide in young women reduced the rate of annual breast cancer death by 38% [22]. Another clinical trial in breast cancer patients reported that the postsurgical treatment of patients with Docetaxel + DOX + Cyclophosphamide reduced the risk of relapse by 28% and improved overall survival by 30% at 5 years compared to 5-FU + DOX + Cyclophosphamide [2,21,23]. This study also showed an improvement of disease-free survival rates observed after 10 years, which were estimated at 62% for the first combination and 55% for the second [21]. In the case of acute myeloid leukemia (AML) in adults, the actual adopted strategy is 7 days of Cytarabine (100–200 mg/m^2/day), followed by 3 days of DNR or IDA. This regimen contributes to a complete response rate between 60% and 85% in patients younger than 60 years [24].

4. Resistance to anthracyclines

Anthracyclines are among the most widely used chemotherapeutic agents due to their multifaceted cytotoxic effects on cancer cells [25]. Consequently, tumor resistance to anthracyclines is a multifactorial clinical issue that involves diverse mechanisms. They can be distributed in five groups: 1) multidrug resistance (MDR), 2) DNA repair, 3) TOPO II activity, 4) cancer stemness, and 5) metabolism (Fig. 2).

4.1. Multidrug resistance

4.1.1. Pgp-dependent multidrug resistance

Due to their chemical structure, anthracyclines can create electrostatic and hydrophobic interactions with cell membranes, enabling them to enter inside cells by passive diffusion [26]. Among mechanisms of resistance, multidrug resistance (MDR) characterizes a process independent of the chemical structures of drugs that reduces intracellular drug accumulation [27].

MDR is often due to upregulation and/or amplification of the *MDR1/ABCB1* gene that encodes Pgp, also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1). This 170-kDa glycoprotein is an efflux pump induced by environmental stresses, natural products, drugs, heat shock and unspecific stresses [28,29]. Studies involving DNR and DOX demonstrated that Pgp is responsible for the efflux of these drugs [30–32]. Pgp indeed recognizes and removes them from the lipid bilayer of the cell membrane using energy obtained from ATP hydrolysis. Several approaches have thus been explored to overcome Pgp-dependent MDR. One example includes the formulation of anthracycline nano-delivery systems that have been reported to effectively circumvent MDR both *in vitro* [33] and *in vivo* [34,35]. Doxil, a DOX-carrying PEGylated liposome formulation, was approved by the FDA but, unfortunately, this system does not cover the MDR problem [36]. Other formulations are currently tested in clinical trials, which is the case of the *N*-(2-hydroxypropyl)methacrylamide-DOX (HPMA-DOX) conjugate (NCT00003165) [37,38]. Additional delivery systems are under active research. They include polymeric micelles; peptide/protein conjugates; solid-lipid, magnetic, gold, silica and cyclodextrin nanoparticles; and carbon nanotubes [35]. Derivatives with additional properties compared to the standard anthracyclines have also been reported [39–44]. For example, Chaikomon et al. [45] very recently

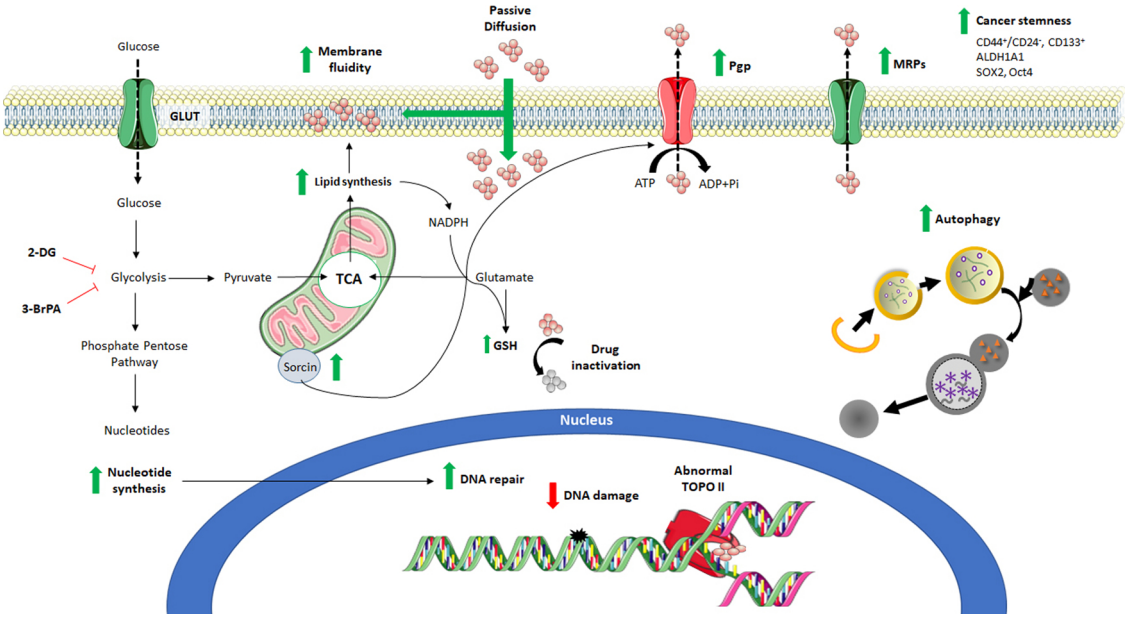

Fig. 2. Major mechanisms of resistance of cancer cells to anthracyclines. After entering inside cells by passive diffusion, anthracyclines can be exported by ATP-binding efflux pumps, such as (sorcin-induced) Pgp/MDR1 and MRPs 1–9. These transmembrane proteins account for reduced intracellular drug accumulation. Significant changes of cancer cell metabolism further contribute to anthracycline resistance: lipid synthesis increases membrane fluidity and anthracycline retention in the cell membrane, more abundant GSH accounts for increases drug detoxification, an increased flux in the pentose phosphate pathway promotes nucleotide biosynthesis for DNA repair, and autophagy, even if controversial results have been observed, plays a relevant role in cancer cell resistance to anthracyclines. Anthracycline-resistant cancer cells can also display an abnormal activity of TOPO II, as well as an increment of DNA repair mechanisms. Finally, stemness can also be increased in anthracycline-resistant cancer cells. Expression of cell surface markers $CD133^+/CD44^+/CD24^-$, overexpression of transcription factors SOX2 and Oct4 and increased activity of ALDH1A1 promote the maintenance, self-renewal and differentiation of resistant cells.

synthetized DexDOX, a DOX conjugation with lipophilic hormone dexamethasone, which successfully escapes Pgp overexpression-induced resistance, thus maintaining the potent cytotoxic effects of DOX in MCF7 human breast cancer cells. Gene-targeted downregulation [46] and pharmacological inhibition [47] of Pgp are other strategies that can decrease cancer cell resistance to anthracyclines. Various agents have been tested over the years. Clinical trials involving first-generation Pgp inhibitors verapamil [48] and cyclosporine A [49] demonstrated increased relapse-free and overall survival in DNR- and DOX-treated myeloma and lymphoma patients [48–50]. Unfortunately, recent Phase III clinical trials involving last generation Pgp inhibitors Tariquidar [51] and Zosuquidar [52] had a limited success, which was mainly due to the toxicity of the inhibitors and to the occurrence of resistances unrelated to Pgp. Overall, the strategy of combining anthracyclines and Pgp inhibitors is promising but will necessitate further drug development.

The use of old drugs to overcome Pgp-dependent MDR has been recently explored with Metformin, a biguanine mostly used to treat type 2 diabetes but that has also been reported to have antitumor effects [53]. Studies on MCF7 cells revealed that the combination of DOX with Metformin significantly increased DOX cytotoxicity [54–56]. Mechanistically, Metformin-induced sensitization of the cells to DOX was demonstrated to be due to decreased Pgp expression [54], Pgp activity and ATP content, supporting apoptosis [55]. The specific molecular events behind these effects are not clear, but a study by Davies et al. [57] demonstrated that decreased Pgp expression was independent of AMP-activated protein kinase (AMPK) and nuclear factor-κB (NF-κB).

The Pgp system can also be influenced by soluble resistance-related Ca^{2+}-binding protein (sorcin), an oncoprotein involved in Ca^{2+} homeostasis and stress response [58]. The *SRI* gene encoding sorcin is located in the same amplicon than other genes involved in MDR, including ABCB1 and ABCB4 [59]. Usually expressed as an 18 kDa variant, sorcin is present in several subcellular compartments, including the nucleus, cytoplasm, ER and mitochondria [60]. Sorcin localization in mitochondria depends on its interaction with TRAP1, a mitochondrial regulator of the balance between oxidative phosphorylation and aerobic glycolysis. This interaction stabilizes TRAP1, thus correlating sorcin and Ca^{2+} homeostasis to the mitochondrial function. In the cytosol, sorcin is also involved in glucose sensing by binding to and sequestering in the cytosol carbohydrate-responsive element-binding protein (ChREBP), which, in the presence of high cytosolic glucose levels, translocates to the nucleus and promotes the expression of metabolic genes inducing the conversion of excess of glucose into fatty acids. Silencing of sorcin demonstrated its key role in metabolic adaptation to high intracellular glucose levels. In fact, increased Ca^{2+} flux and high level of glucose cannot trigger ChREBP translocation to the nucleus in the absence of sorcin [61]. Sorcin overexpression further activates the cAMP response element-binding protein (CREB) pathway, which increases CREB1 binding to the *Pgp* promoter and, consequently, Pgp expression [62]. Moreover, sorcin is able to bind with high affinity to several chemotherapeutic agents, including anthracyclines, inactivating them, and to change its cellular localization in response to treatment [63]. Although sorcin has been shown to play a key role in MDR *in vitro*, *in vivo* data are lacking and its mechanisms of action are still incompletely understood.

Sorcin has been found to be upregulated in different cancer cell lines, including DOX-resistant K563/A02 leukemia cells [64,65] and DOX-resistant MCF/A02 breast cancer cells [65]. Silencing this protein chemosensitized cancer cells by downregulating Pgp expression (thus reducing drug efflux) [64–66] and by increasing apoptotic cell death through activation of caspases 3 and 12 [67]. However, there is to date no reported pharmacological inhibitor. Considering its role in apoptosis and Ca^{2+} homeostasis, one can fear that interfering directly with sorcin may result in severe adverse effects. For this reason, identifying sorcin targets and unveiling its precise mechanisms of action should be prioritized.

4.1.2. Pgp-independent multidrug resistance

Even if they present a classic MDR phenotype characterized by impaired drug accumulation due to energy-dependent drug efflux, not all MDR cell lines do overexpress Pgp [68,69]. Some cancer cells rather overexpress other members of the ABC transporter superfamily, among which MRP1–9 have been associated with MDR due to their extrusion capacity of chemotherapeutic compounds and their metabolites [70]. MRP1, MRP2, MRP5 and MRP6 are known to be overexpressed in anthracycline-resistant cells [71–74]. However, mRNA and immunohistological analyses of MRP1 and MRP2 in breast cancer samples obtained from patients untreated or treated with neo-adjuvant anthracyclines evidenced no connection between the expression levels of these proteins and clinical drug resistance [75]. In addition, there is no evidence so far of a contribution of MRP5 and MRP6 to clinical MDR, suggesting that other transporter proteins may be involved. Accordingly, some DOX-resistant cancer cells were found to have a high expression of membrane protein P95 in breast cancer [76], lung resistance-related protein (LRP) in AML [77] and lung cancer models [78], and ABC transporter associated with antigen processing (TAP) in lymphoblastoid cell lines [79]. Still, their participation in clinical MDR to anthracyclines has not been firmly demonstrated to date [80,81].

Recent researches focused on microparticles (MPs), *i.e.*, non-exosome small (0.1–1 mm diameter) vesicles derived from the plasma membrane that can bud from a donor cell, circulate and enter into another cell, thus spreading their content to recipient cells [82]. Bebawy et al. [83] demonstrated that MPs are involved in MDR by showing that MPs released by human acute lymphoblastic leukemia (ALL) MDR^+ cells can bind to and deliver functional Pgp to recipient MDR^- cells. Pgp functionality after MP-dependent transfer was validated by treating the cells with DNR. Gong et al. [84] further showed that MPs can sequester DOX and reduce the drug concentration available to target cells. In this study, MPs isolated from DOX-resistant human breast adenocarcinoma cells and from human ALL cells presented a Pgp inside-out orientation, enabling MPs to influx cytoplasmic DOX in order to protect cells from chemotherapeutic cytotoxicity.

4.2. DNA repair

The formation of covalent DNA adducts is a main cytotoxic effect of anthracyclines [85]. Conversely, DNA damage repair is an important contributor to drug resistance. In the context of anthracyclines, Spencer *et al.* [86] took advantage of different cell lines lacking functional proteins involved in each of the five main DNA repair pathways (homologous recombination [HR], mismatch repair [MMR], nucleotide excision repair [NER], DNA strand cross-link repair [ICL] and non-homologous end joining [NHEJ]). They demonstrated that NER and HR are the most important mechanisms for the repair of anthracycline-DNA adducts. These results indicate that cancer cells with an efficient DNA damage repair machinery can overcome the genomic effects of anthracyclines. They further support a strategy that would combine anthracyclines to NER or HR inhibitors, where synthetic lethality could be a possible answer to overcome resistance to anthracyclines.

4.3. Altered topoisomerase II activity

DNR and DOX are potent TOPO II inhibitors that block the catalytic activity of the enzyme and stabilize a reactive intermediate where a DNA strand that was cut is covalently bound to the enzyme [4]. Therefore, mutation and/or abnormal expression of the TOPO IIα subunit, suppression of TOPO IIα-mediated apoptotic signaling and a cytoplasmic rather than nuclear localization of TOPO IIα can all account for clinical resistance to anthracyclines [87]. Resistant cancer cells with modified TOPO II expression are called ‘atypical MDR (at-MDR) cells’. They usually present low levels of TOPO II, like initially observed in nuclear extracts of anthracycline-resistant murine and human (Ehrlich ascites) cancer cells [88,89]. Interestingly, a decreased

expression of TOPO IIα and TOPO IIβ has often been associated with mutations that result in deficient drug-DNA-protein interactions [74,90,91]. However, even if TOPO II expression is unchanged, post-translational modifications, including phosphorylation, sumoylation and ubiquitylation, can affect the normal activity of both TOPO IIα and TOPO IIβ and, consequently, anthracycline efficacy [88,92]. Still in the same context, a recent study showed that increased levels of intracellular Ca^{2+} and ROS in breast cancer cells can reduce the efficacy of DOX by hyperactivating intracellular Ca^{2+}-dependent cysteine proteases m-calpains that cleave TOPO IIα, leading to an accumulation of the truncated enzyme in the cytoplasm [93].

In the clinics, several independent trials have shown that high TOPO IIα expression is a good predictor of sensitivity to adjuvant anthracycline therapy [94–100]. In breast cancer in particular, high TOPO IIα expression is regarded as a key determinant of tumor sensitivity to anthracyclines, and a high expression of human epidermal growth factor receptor 2 (HER2) determines the sensitivity to Trastuzumab. Because anthracyclines and Trastuzumab are two main treatments of breast cancer, the expression of the two proteins is often sought simultaneously. However, in some cases, patients with high-expression of TOPO II respond poorly to anthracyclines. This may be due to a compensation of TOPO II inhibition by activation of alternative DNA double-strand break repair systems, such as the Ku pathway [101]. Transcription factor DLX4, which not only stimulates the Ku pathway but also increases TOPO II expression, has been suggested as a marker to identify high TOPO II poorly responding patients [101], but more clinical data are necessary to confirm this hypothesis. These observations collectively suggest that TOPO II is one of the main targets of anthracyclines, but, clearly, the full picture is more complex as other factors seem to be involved. This complicates the identification of reliable makers of sensitivity/resistance to anthracyclines in clinical settings.

In cases of TOPO II mutations, a strategy to overcome at-MDR would be to develop new anthracycline derivatives capable of targeting the mutated enzyme. Accordingly, the analysis of structure activity relationships indicated that chemical modifications of anthracyclines could result in different outcomes for at-MDR patients [102]. These evidences led to the development of a new subclass of anthracyclines characterized by a carboxymethyl group at C-10 instead of two hydroxyl groups at C-10 and C-11, which included Aclarubicin. This anthracycline variant, originally isolated as a secondary metabolite of *Streptomyces galilaeus*, is currently used for cancer treatment in Japan and in China [103].

4.4. Cancer stemness

In general, cancer stem cells (CSCs) and tumor initiating cells (TICs) play a crucial role in tumor resistance, recurrence and metastasis [104]. CSCs and TICs represent a minority of cancer cells in tumors that are characterized by their capacity of self-renewal, tumor initiation, maintenance and differentiation [105]. They promote cellular heterogeneity. In solid tumors, CSCs are often characterized by the expression of cell surface markers $CD133^+/CD44^+/CD24^-$, increased aldehyde dehydrogenase activity ($ALDH^+$) and Hoechst efflux [106,107]. Interestingly, cancer cell populations enriched in CSCs are not only chemoresistant [108,109], but chemotherapy can also increase the abundance of cancer cell subpopulations with CSC-like features [110].

Several independent studies evidenced a direct link between resistance to anthracyclines and cancer stemness. For example, Levina et al. [111] found that DOX-resistant non-small cell lung cancer (NSCLC) cells express stem-related markers CD133 and Oct4, along with high ALDH and canonical Wnt activity. Compared to DOX-sensitive cells, spheroid formation, clonogenicity and tumorigenicity were increased. In another study, primary cell cultures generated from NSCLC biopsies after the treatment of patients with DOX + Cyclophosphamide + Etoposide + radiation therapy and relapse showed an increase in the expression of stem cell markers CD133, ALDH1A1 and members of the Wnt and Notch pathways, compared with matched therapy-naïve primary cell cultures [112]. In the same line, two different studies reported that DOX-resistant breast cancer cells were enriched in CSCs, presented a $CD44^+/CD24^-$ phenotype and had increased ABCB1 expression [113,114]. Their mammosphere-forming efficiency, invasiveness and tumorigenicity were increased, but their treatment with natural phytochemical cardamonin selectively killed the subpopulation of DOX-resistant CSCs [114]. More recently in triple-negative breast cancer cells, Cheng et al. [115] reported that JAK2 inhibitor WP1066 efficiently reverted the stemness-mediated DOX resistance regulated by the Stat3-Oct4-cMyc pathway. In neuroblastoma and osteosarcoma cells, DOX resistance was associated to an increase in clonogenicity, invasiveness, tumorosphere formation and to higher levels of stem cell markers CD133, ALDH1A1, ABCG2 and SOX2 [116,117]. Regarding osteosarcoma, transcription factor Kruppel-like factor 4 (KLF4) was pinpointed as the contributor to stemness-dependent DOX resistance, as DOX-induced stemness was reversed upon *KLF4* silencing [117]. That a CSC phenotype causes cancer resistance to anthracyclines was further established by Santos et al. [118] who reported an association between cancer stem cells, epithelial-mesenchymal transition (EMT), miR-155 upregulation and resistance to DOX in breast cancer cells. Impressively, miR-155-containing exosomes isolated from cancer stem-like resistant cells were able to transfer this resistance to DOX to recipient sensitive cells. These findings illustrate that CSCs play an important role in exosome-mediated DOX resistance in breast cancer cells. It is also worth to mention that a significant increase in the proportion of $CD44^+/ALDH1^+$ cancer cells was reported in tumor biopsies of breast cancer patients after primary DOX-inclusive systemic therapy [119]. This change was positively associated with tumor aggressiveness and progression.

These observations support the role of stemness in cancer resistance to anthracyclines and give a strong incentive to use anthracycline chemotherapy in combination with anti-CSC therapy. Clinical trials involving CSC-targeted therapies and standard therapy are already ongoing [120].

4.5. Metabolic resistance to anthracyclines

In addition to the above-described strategies deployed by cancer cells to resist to anthracyclines, metabolic changes could also play a significant role. Experimental evidences obtained to date involve 1) glycolysis, 2) the pentose phosphate pathway (PPP), 3) fatty acid metabolism, 4) antioxidant defenses, and 5) autophagy.

4.5.1. Glycolysis

Several studies reported an increased rate of aerobic glycolysis when comparing anthracycline-sensitive to anthracycline-resistant cancer cells. This was the case for CEM/R2 and HL60/R10 ALL cells that were selected for resistance to DNR *in vitro* [121]. In DOX-resistant MCF7/ADR and MBA-MD-231/ADR human breast cancer cells, increased glucose metabolism was correlated to activation of the FGFR4-Frs2-Erk pathway, and inhibition of this pathway significantly reduced both glucose metabolism and chemoresistance. Similarly, DOX-resistant SK-*N*-SH and SK-*N*-Be(2)C neuroblastoma cells were chemosensitized both in normoxia and hypoxia by 3-bromopyruvate (3-BrPA) [122], an agent known to inhibit several glycolytic enzymes [123].

Glycolysis is a major source of ATP in cancer cells and is needed in order to sustain their high proliferation rate. Furthermore, exogenous stress, like chemotherapy, can be faced by cancer cells through overexpression of ABC transporters that actively pump the drug outside of the cells and consume ATP. Therefore, glycolysis inhibition may be an effective strategy to reduce the ATP pool and, thus, impact proliferation as well as drug efflux. 3-BrPA was reported to inactivate ABC transporters in KG-1 leukemia and RPMI8226 myeloma cells, thus sensitizing the cells to DOX [124]. RPMI8226 tumors grew significantly

slower in mice treated with DOX and 3-BrPA compared to DOX alone. Similarly, 2-deoxyglucose (2-DG), another inhibitor of glycolysis, showed a synergistic effect with DOX, sensitizing DOX-resistant MCF-7/MDR breast cancer cells. 2-DG caused a reduction of the ATP/AMP ratio that resulted in increased activation of AMPKα, which, in turn, phosphorylated p53 triggering caspase-3 dependent cell death [125]. Reduced ATP levels also indirectly downregulated MDR-related proteins and reduced the activity of ATP-dependent efflux pumps [126]. In theory, 2-DG would also inhibit the PPP, hence NADPH production and nucleotide synthesis [127], but, to our knowledge, the relative contribution of glycolysis and PPP inhibition to the resensitization of anthracycline-resistant cancer cells has never been addressed.

In highly glycolytic cells, glycolysis is coupled to the conversion of pyruvate, NADH and H^+ in lactate and NAD^+, a reaction catalyzed by lactate dehydrogenase A (LDHA). Interestingly, LDHA was found to be overexpressed in DOX-resistant SW1353 chondrosarcoma cells compared to the parental cell line, while LDH inhibitor oxamate re-sensitized the resistant cells to DOX [128]. A similar observation in K562/ADM leukemia cells further revealed that oxamate inhibited the Akt-mTOR pathway [129], which could, therefore, be as main pathway responsible for the resistance of cancer cells to anthracyclines.

Altogether, the above observations suggest that anthracycline treatments could benefit from a simultaneous inhibition of glycolysis. Because anthracyclines and glycolysis inhibitors target different pathways, synergistic effects could be possible. This strategy could potentially be applied in first intention or after anthracycline-resistant relapses. In Phase II and III clinical trials, glycolysis inhibitor lonidamine, a dechlorinated derivative of indazole-3-carboxylic acid, showed promising results in advanced breast, lung and ovarian cancers [130].

4.5.2. The pentose phosphate pathway and nucleotide biosynthesis

The PPP is branched to glycolysis at the level of glucose-6-phosphate. It is responsible for the production of ribose for nucleotide biosynthesis and of antioxidant NADPH.

Interestingly, both the PPP and *de novo* pyrimidine synthesis have been associated with cancer resistance to anthracyclines. In leukemic cells, Ferretti et al. [131] reported a correlation between MDR and increased expression of the PPP rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) and increased levels of GSH. Along the same line, an independent report showed that the progressive acquisition of DOX resistance by Walker-256 carcinoma cells *in vivo* was accompanied by increased G6PD activity [132]. Gene expression arrays comparing blast persistence (BP) and complete remission (CP) in AML patients after DOX chemotherapy confirmed an increased expression of G6PD in the BP group along with upregulation of nucleotide metabolism enzymes pre-B-cell colony-enhancing factor 1 (PBEF1) and cytidine deaminase (CDA) [133]. In parallel with the PPP, *de novo* pyrimidine synthesis was enhanced in AML cells resistant to DOX, enabling increased DNA damage repair and, consequently, resistance to anthracyclines. That enhanced nucleotide biosynthesis is involved in the resistant phenotype is supported by the observation that DOX-resistant TNBC cells have enhanced activity of carbamoyl-phosphate synthase 2 (CAD), a multifunctional enzyme controlling the first steps of *de novo* pyrimidine biosynthesis [134]. Furthermore, pharmacological inhibition of dihydroorotate dehydrogenase (DHODH), the rate-limiting enzyme of the pathway, sensitized resistant breast cancer cells to DOX-induced DNA damage [134] and effectively suppressed the growth of DOX-resistant AML cells both *in vitro* and *in vivo* [135].

Anthracyclines are also well known to increase mitochondrial ROS (mtROS) production in targeted cells, a mechanism not only accounting for cardiotoxicity but also potentially involved in their cytotoxic effects on cancer cells [6]. In this context, elevated NADPH production by the PPP is directly involved in the recycling of oxidized GSSG to GSH by GSH reductase (GSR) [136]. This mechanism was claimed to account for antioxidant protection against DOX in resistant HT29 colon cancer cells [137]. Accordingly, inhibition of G6PD by dehydroepiandrosterone (DHEA; a non-competitive inhibitor of glucose-6-phosphate for G6PD) or by 6-aminonicotinamide (6-AN; a competitive inhibitor of glucose-6-phosphate for G6PD) reversed the acquired resistance of these cells to DOX. However, to our knowledge, whether direct inhibition of mtROS production could decrease cardiotoxicity and how it would modulate the anticancer efficacy of anthracyclines remains to be addressed experimentally.

4.5.3. Glutathione

One of the most explored resistance mechanisms to anthracyclines involves antioxidant defenses in general and GSH in particular [6]. Glutathione directly participates in the reduction of ROS and other oxidized molecules (see section 4.5.2) and contributes to cellular detoxification by a mechanism involving glutathione-*S*-transferases (GSTs).

GSTs belong to a large family of enzymes that catalyze the conjugation of GSH to xenobiotic substrates [138]. The expression of particular classes of GST isozymes has a biological relevance to cancer occurrence, drug resistance and prognosis, and GST polymorphisms account for cancer susceptibility and clinical outcome [139]. The expression of certain GSTs, such as GSTA1-1, has been correlated with the expression of MRP1 and MRP2 and resistance to antineoplastic drugs [70,140,141]. MRPs are cotransporters of GSH, GSSG and GSH-conjugates [142] that are involved in the maintenance of GSH/GSSG homeostasis [70]. Acquisition of resistance to anthracyclines can thus arise from a coordination of drug efflux by MPRs, together with the activity of GSTs [142].

Recently, Drozd et al. [143] reported an upregulation of *GSTM1* and *GSTA1-3* genes in DOX-resistant cervical cancer cells, suggesting an association between the activity of GSTs Mu1, A1, A2 and A3 and resistance. Surprisingly, there was no increase in MRP1 expression in any of the tested cells, indicating that an increased activity of GSTs could be sufficient to cause resistance to anthracyclines. Because high levels of GST have also been detected in anthracycline-resistant tumors [144], several GSH analogues have been developed to inhibit these enzymes. For example, Telcyta (TLK-286) is a GSH analogue used in combination with cytotoxic chemotherapies, including anthracyclines (pegylated liposomal DOX), in a variety of tumors (*e.g.*, ovarian cancers) expressing very high levels of GST Pi-1 [145–147]. Another inhibitor, Telintra (TLK199), has been designed for the prevention of myelosuppression in myelodysplastic syndrome (MDS) [112]. Administered orally to MDS patients, it was efficient and well tolerated in both Phase I and Phase II clinical trials [148,149].

4.5.4. Lipid metabolism

4.5.4.1. Fatty acid synthase. One of the major changes in lipid metabolism associated to anthracycline resistance is overexpression of fatty acid synthase (FASN). This enzyme is responsible for the biosynthesis of palmitate, an important precursor for the *de novo* synthesis of lipid-derived molecules. Conversely, FASN inhibition was shown to restore sensitivity to anthracyclines of resistant osteosarcoma, breast cancer and hepatocellular carcinoma cells [150–153]. FASN mediates drug resistance *via* palmitate production. Indeed, palmitate supplementation mimicked FASN overexpression in causing DOX resistance [150] and counteracted the effects of FASN inhibition [151]. At least two different mechanisms are involved.

First, palmitate limits anthracycline transmembrane uptake by cancer cells. Palmitate indeed modulates the transversal mobility of membrane components by the so called flip-flop mechanism, a major event for DOX entry inside cells [154]. Accordingly, inhibiting acetyl-CoA carboxylase (ACC; the rate-limiting enzyme of palmitate synthesis) with Soraphen altered the lipid composition of the plasma membrane and increased passive flip-flop along with intracellular DOX levels [151]. The increase in DOX uptake was counteracted by exogenous palmitic acid but did not change in the presence of a Pgp inhibitor, thus revealing that it is independent of Pgp activity [151].

Second, FASN overexpression suppresses anthracycline-induced ceramide production by inhibiting the activity of SMase [155]. The exact mechanism by which high FASN expression inhibits SMAse is unknown, but would be mediated by tumor necrosis factor α (TNFα) and the NF-κB pathway. As a consequence of SMAse inhibition, FASN overexpression was shown to block DOX-induced apoptosis mediated by ceramide and caspase 8 in MCF7 breast cancer cells [155].

Small molecule FASN inhibitors of the TVB series are currently gaining interest in the field of cancer resistance. TVB-2640 showed a favorable tolerability profile in a Phase I clinical trial on refractory solid tumors (all tumor types) [156], and is now tested on patients with resectable colon cancer in a Phase I/II trial (Clinicaltrials.gov #NCT02980029). Other FASN inhibitors, TVB-3166 and TVB-3664, are currently in preclinical development. As single treatments, both drugs had anticancer effects *in vitro* on colon, lung, breast, pancreas, prostate, ovary and hematopoietic cancer cell lines and were well tolerated *in vivo* in NSCLC-bearing mice and in mouse models of patient-derived colon cancer xenografts [157,158]. Considering their good tolerability, these compounds could be used as adjuvants in anthracycline therapies.

The importance of fatty acid metabolism in drug response was further confirmed by siRNA-mediate silencing of acetyl-CoA acyltransferase (ACAT1), which restored DOX-induced apoptosis in resistant cells [159]. Not only ACAT1 but also fatty acid metabolism enzymes hydroxyacyl-coA hydrogenase (HADH) and enoyl-CoA hydratase short chain 1 (ECHS1) were found to be overexpressed in proteomic analyses that compared DOX-resistant to DOX-sensitive MES-SA uterine sarcoma cells.

To date, however, no data were published correlating alterations of fatty acid metabolism and tumor resistance to anthracyclines in patients.

4.5.4.2. Membrane fluidity. Cancer cell resistance to anthracyclines has also been associated to alterations of the fluidity of the plasma membrane directly related to its lipid composition. More precisely, increased phosphatidylcholine and decreased phosphatidylethanolamine levels were reported in anthracycline-resistant leukemia cells due to increased phosphatidylethanolamine *N*-methyltransferase (PEMT) activity [160,161]. A different lipid content of the plasma membrane was also reported in DOX-resistant MCF7 breast cancer cells [162]. Other sets of data reported decreased [160], increased [162] or unchanged [161] sphingomyelin levels despite reduced SMase activity in DOX-resistant mouse Friend leukemia cells, human MCF7 breast cancer cells and human K562 leukemia cells, respectively. Plasma membrane cholesterol was reported to be elevated in anthracycline-resistant Hs578 T and MCF7 breast cancer cells, as well as in T-cell ALL [162–164] due to an increase in acetyl-CoA generation [162,165], which resulted in decreased membrane fluidity and mediated cancer cell resistance to DOX [162,163]. One study further reported that scavenging the high level of cholesterol in the plasma membrane of DOX-resistant Hs578 T with cyclodextrins increased plasma membrane fluidity, decreased lipid packing density and inhibited Pgp efflux activity [163]. However, the authors did not demonstrate that cyclodextrins can restore the sensitivity to DOX of the resistant cells. Overall, whether changes in plasma membrane composition are a cause or a consequence of cancer cell resistance to anthracyclines is an open question.

4.5.5. Autophagy

In order to circumvent the noxious effects of chemotherapy, cancer cells modulate pathways involved in cell survival and death. In this context, several studies revealed that autophagy plays an important role in chemoresistance to DOX [39,84,166–168]. Autophagy is a well-conserved catabolic degradation process in which damaged or cytosolic organelles in excess are incorporated into double-membrane vesicles that fuse with lysosomes for degradation [169]. Importantly, autophagy has a dual role in cancer: it can cause cell death to protect cells against malignant transformation, but it can also function as a prosurvival pathway by recycling damaged proteins and organelles and providing nutrients in response to metabolic stresses, including exposure to anticancer therapies [170–172]. For what concerns resistance to anthracyclines, the case is not different, and the role of autophagy is still very controversial. On the one hand, some publications show that autophagy promotes DOX resistance and cell survival. For example, Pan et al. [168] observed that DOX-resistant RPMI8226/DOX multiple myeloma cells had increased autophagy and decreased apoptosis compared to matched sensitive cells. Another study in TNBC cells (MDA-MB-231) evidenced that a treatment with DOX induced cytoprotective autophagy, due to an increased expression of the enzyme heme oxygenase (HO1) triggered by activation of the Src/STAT3 pathway, which protected the cells against DOX-induced apoptosis [173]. On the other hand, other studies revealed that inducing autophagy can help to overcome DOX resistance. One of these cases was reported by Lin et al. [167] in human papillary thyroid tumors and *in vitro* models (TPC-1 and 8505-C cells), where treatment with RAD001, a mTOR inhibitor and autophagy activator, increased the expression of LC3-II and autophagosome formation, thereby increasing the cytotoxic response to DOX. The role of autophagy in DOX sensitivity was supported by showing that chemosensitivity was abrogated upon *Atg-5* knockdown: cervical cancer cells and human T cell leukemia cells with defective autophagy due to a deficient expression of Atg5 showed reduced DOX sensitivity [174]. Mechanistic studies are warranted to understand the nature of these apparently opposite observations.

Compared to DOX, the currently available information about the contribution of autophagy to EPI resistance is more consistent. Indeed, increased autophagy was observed not only in EPI-resistant MCF7, MDA-MB-231 and SKBR3 breast cancer cells [175–,176,177,178], but also in EPI-resistant human gastric tumors [179]. In breast cancer cells, increased levels of LC3-II and beclin 1, a protein that mediates autophagosome formation, were positively correlated with EPI resistance. Moreover, either silencing *beclin 1*, *ATG5* or *ATG7* expression with siRNAs [176–,176,177,178] or inhibiting autophagy pharmacologically with hydroxychoroquine [175] resensitized EPI-resistant MDA-MB-231 and MCF7 cells to EPI, enhancing apoptotic cell death [177,178]. These data establish a causal link between increased autophagy and EPI resistance. From a mechanistic standpoint, Sun et al. [176] reported that autophagy and beclin 1 regulator 1 (Ambra1) were induced in EPI-resistant MDA-MB-231 cancer cells. They showed that silencing Ambra1 abolished the growth of EPI-resistant MDA-MB-231 tumor xenografts in EPI-treated mice, but did not report on the effects of Ambra1 silencing in the absence of a concomitant EPI treatment. Altogether, these observations indicate that autophagy is a candidate to target in order to overcome cancer cell resistance to EPI. However, *in vivo* experimental confirmation is needed.

5. Concluding remarks

Cancer cells can develop various mechanisms of resistance to anthracyclines, which also largely reflects the several modes of action of these chemotherapeutic agents (Fig. 2). Three main sites of interest are the cell membrane, the cytosol and the nucleus. At the cell membrane, altered membrane fluidity, ATP-dependent efflux pumps and vesicles restrict drug accumulation inside cells. In the cytosol, GSH inactivates and autophagosomes sequester anthracyclines. In the cell nucleus, altered expression/activity of TOPO II limits DNA damage, while DNA repair is facilitated by nucleotide import. Most of these mechanisms of resistance are under metabolic influence. Indeed, a high glycolytic rate is associated to fast ATP production that optimizes the activity of efflux pumps and DNA repair; a highly active PPP produces reducing equivalents that maintain a high pool of GSH and nucleotide precursors for DNA repair; lipid metabolism modulates the composition of cell and organelle membranes; and sorcin in mitochondria promotes Pgp expression through a yet unknown mechanism, most probably involving Ca^{2+}. Cancer stemness, which provides resistance to anthracyclines, is

also under metabolic control [165].

The majority of the known mechanisms accounting for resistance to anthracyclines have been unveiled years and sometimes dozens of years ago. It is therefore worrying to realize that they are, nowadays, still poorly characterized mechanistically. A main area of uncertainty is how anthracyclines directly or indirectly influence the transcriptional and posttranscriptional pathways conferring resistance. Another one is how to position metabolic changes in the general paradigm: are these changes causes or consequences of the resistant phenotype? Most often, resistance mechanisms have been identified using cancer cells *in vitro* that were chronically exposed to increasing doses of anthracyclines and, most often, cloned. Therefore, whether and how these mechanisms could cooperate and/or compensate each others in clinical tumors is still largely unknown. Identifying potential interplays first depends on the precise molecular characterization of each pathway which, from a metabolic standpoint, is a difficult task. It is, however, an urgent one that should precede the *in vivo* evaluation of treatments combining anthracyclines with antimetabolic drugs, as the final objective is to durably counter chemoresistance in patients.

From a broader standpoint, we believe that, despite many cellular changes associated to chemoresistance have already been identified, our current understanding of the relationship between cancer cell metabolism and resistance to chemotherapy is still very limited. Identifying the precise molecular mechanisms supporting these changes, how they are hierarchized, and how they are coupled is a main task for fundamental research. In our opinion, a global understanding of the interrelationships between cancer metabolism and chemoresistance is needed for the development of successful therapeutic strategies targeting tumor metabolism in order to try to revert cancer chemoresistance in clinical settings.

Conflict of interest

The authors declare that they have no conflict of interest.

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