Teaser Nanotechnologies support combinational treatments of natural products with standard chemotherapies against cancer multidrug resistance, mainly induced by related enzymatic-efflux transporter activities and by a subpopulation of cancer stem-like cells.

Reviews • KEYNOTE REVIEW

Cancer stem cells and nanomedicine: new opportunities to combat multidrug resistance?

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'Multidrug resistance' (MDR) is a difficult challenge for cancer treatment. The combined role of cytochrome P450 enzymes (CYPs) and active efflux transporters (AETs) in cancer cells appears relevant in inducing MDR. Chemotherapeutic drugs can be substrates of both CYPs and AETs and CYP inducers or inhibitors can produce the same effects on AETs. In addition, a small subpopulation of cancer stem-like cells (CSCs) appears to survive conventional chemotherapy, leading to recurrent disease. Natural products appear efficacious against CSCs; their combinational treatments with standard chemotherapy are promising for cancer eradication, in particular when supported by nanotechnologies.

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Introduction

Cancer is one of the main causes of death worldwide [1]. Remarkable progress in the treatment of cancer over the past five decades has been counteracted by the onset of cancer resistance against most therapies [2]. In general, the drug resistance of cancers can be sorted into two categories: intrinsic or acquired. Intrinsic resistance to chemotherapy occurs in patients retaining resistant phenotypes before the start of treatments, whereas acquired resistance can arise during or after treatment of patients who are initially responsive. Often, the acquired resistance against a specific drug can also extend to other drugs [3]. In particular, chemotherapeutic drugs inhibit fast-replicative cells, including cancer cells [4]. Even though chemotherapy constitutes a valid choice for cancer therapy, it is possible that, after repeated treatments, cancer cells not only become resistant to the specific chemotherapeutic agent used, but also cross-resistant to other cytotoxic drugs with different chemical structures or mechanisms of action [5]. This phenomenon, called MDR, is one of the most difficult challenges for cancer treatment [6].

Cancer cells can develop MDR through several mechanisms, including (i) activation of Q7 detoxifying systems; (ii) increased drug efflux; (iii) decreased drug uptake; (iv) activation of DNA repair mechanisms; and (v) evasion of drug-induced apoptosis. The increase in drug efflux is the most important mechanism related to drug resistance [7]; moreover, drug efflux increase is
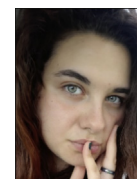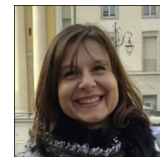
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GLOSSARY

Androgen deprivation therapy (ADT) a type of treatment for PC that blocks the effects of androgens and can slow PC growth.

Cancer stem cells (CSC) subpopulations of cancer cells sharing similar characteristics as normal stem or progenitor cells, such as self-renewal ability and multi-lineage differentiation, to drive tumor growth and heterogeneity. Throughout cancer progression, CSCs can further be induced from differentiated cancer cells via adaptation and crosstalk with the TME as well as in response to therapies, contributing to their heterogeneous phenotypes.

Chemopreventive agents chemicals or substances that impact carcinogenesis by either blocking DNA damage during the initiation stage or by arresting or reversing processes during promotion and progression.

CSC surface markers surface protein used to identify CSCs; also present on human embryonic stem cells and adult stem cells.

Cytochromes P450 (CYPs) superfamily of enzymes containing heme as a cofactor that function as monooxygenases [1–3]. In mammals, these proteins oxidize steroids, fatty acids, and xenobiotics, and are important for the clearance of various compounds, as well as for hormone synthesis and breakdown. P450 is derived from the spectrophotometric peak at the wavelength of the absorption maximum of the enzyme (450 nm)

Hedgehog (Hh) pathway essential for normal embryonic development; has crucial roles in adult tissue maintenance, renewal, and regeneration. Secreted Hh proteins act in a concentration- and time-dependent manner to initiate a series of cellular responses that range from survival and proliferation to cell fate specification and differentiation.

Immunotolerance a state of unresponsiveness of the immune system to substances or tissue that have the capacity to elicit an immune response in a given organism.

Intratumoral androgen synthesis an intratumoral synthesis of testosterone and dihydrotestosterone from weak androgens produced by the adrenal glands and possibly de novo from cholesterol.

Keratinocytes the major cell type of the epidermis, the outermost skin layer; comprise 90% of epidermal cells.

Metastatic castration-resistant prostate cancer (MCRPC) when PC has spread to parts of the body other than the prostate, and is able to grow and spread despite treatment.

Multiple drug resistance (MDR) antimicrobial or anticancer resistance shown by cancer cells to at least one antimicrobial or anticancer drug. The development of MDR to chemotherapy remains a major challenge in the treatment of cancer. Resistance has developed against every effective anticancer drug and can develop by numerous mechanisms, including decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms, and evasion of drug-induced apoptosis.

Notch signaling an evolutionary conserved pathway in multicellular organisms that regulates cell-fate determination during development and maintains adult tissue homeostasis. The Notch pathway mediates juxtacrine cellular signaling (contact-dependent signaling) wherein both the signal-sending and receiving cells are affected by ligand–receptor crosstalk, by which an array of cell fate decisions in neuronal, cardiac, immune, and endocrine development are regulated.

Phase I metabolism step of the metabolism of drug or xenobiotic involving chemical reactions, such as oxidation (most common), reduction, and hydrolysis.

Phase II metabolism involves reactions that chemically change the drug or phase I metabolites into compounds that are soluble enough to be excreted in urine. In these reactions, the molecule (drug or metabolite) is attached to an ionizable grouping. This is called conjugation and the product is called a conjugate. Metabolites formed during Phase II are unlikely to be pharmacologically active.

Procarcinogen any substance that is transformed into a carcinogen by metabolism.

Stochastic model tool for estimating probability distributions of potential outcomes by allowing for random variation in one or more inputs over time.

Taxanes chemotherapeutic agents, including paclitaxel and docetaxel, that produce antitumor activity by causing stabilization of cellular microtubules, thereby inhibiting cell division; originally identified from plants of the genus Taxus (yews).

Vinca alkaloids set of antimitotic and antimicrotubule alkaloid agents originally derived from the periwinkle Catharanthus roseus.

Xenobiotic a chemical substance found within an organism that is not naturally produced or expected to be present within that organism.

often combined with the upregulation of enzymes involved in the metabolism of anticancer agents [8]. Therefore, enzymes and efflux transporters expressed by cancer cells appear to be crucial not only for their proliferation, but also for their resistance to clinical treatments. Furthermore, research also suggests that CSCs, a subgroup of cancer cells characterized by stem-like properties, significantly contribute to chemoresistance and cancer relapse, being able to self-renew and differentiate into heterogeneous cancer cell lineages in response to chemotherapeutic agents [9].

In this review, we describe several CYP enzymes and efflux transporters related to cancer, given that MDR can arise from their combined upregulation. In addition, we describe current strategies proposed to tackle MDR, taking into account the contribution of CSCs and the importance of the nanotechnologies in the design and development of new therapeutic systems.

CYP enzymes in cancer cells

CYP is a superfamily of enzymes that contribute to the metabolism of exogenous and endogenous compounds in our body, particularly during Phase I [10]. The liver and small intestine show the highest concentrations of CYP enzymes [11], although they are also expressed in other healthy tissues [1]. However, some are overexpressed in tumor tissues. For example, the CYP1 family includes CYP1A1, CYP1A2, and CYP1B1 [12], among which CYP1B1 appears abundantly expressed in the prostate, breast, and uterus; moreover, it is frequently overexpressed in tumor tissues [13]. In terms of the CYP2 family, CYP2J2 is mainly expressed in the heart [14], but is also upregulated in multiple cancers [15,16]. CYP17 constitutes a family of enzymes involved in androgen synthesis, the expression of which is increased in prostate carcinoma [17], and implicated in the pathogenesis of prostate

cancer (PC) [18]. In general, CYP enzymes can induce different effects in cancer cells depending on the type of substrate and protein interactions. CYP activities can induce not only carcinogenesis, but also the activation or degradation of anticancer drugs, as well as contributing to MDR.

CYP and carcinogenesis

Specific ligands able to activate the aryl hydrocarbon receptor (AhR) induce the transcription of CYP1 enzymes with carcinogenic effects. For example, the xenobiotic 2,3,7,8-tetrachlorodibenzo-r-dioxin (TCDD) induces AhR to regulate the transcriptional activation of CYP1A1, CYP1A2, and CYP1B1, which have the ability to bioactivate procarcinogenic compounds into carcinogenic derivatives [19].

The expression of the CYP1 family can also be regulated by estradiol. In particular, levels of CYP1B1 are increased by estradiol in breast cancers expressing estrogen receptors (ERs) [20]. When expressed in cells, CYP1B1 contributes to transform estradiol into a potential carcinogen, inducing its conversion to 4-hydroxyestradiol, which can be transformed into estradiol-3,4-quinone by peroxidase [21,22], forming an estrogen-deoxyribonucleoside adduct with the ability to induce DNA damage [23]. CYP1B1 is also able to promote cancer cell proliferation, migration, and invasion via WNT/β-Catenin signaling (see later) [24].

Cyprodinil is a fungicide widely used in agriculture, particularly for grape and apple production. The presence of these fruits in our diet can induce cyprodinil exposure in children. Even if this fungicide is rapidly excreted as a metabolite in urine, its accumulation in liver induces activation of the CYP1 family, with consequent carcinogenic effects [1]. In particular, cyprodinil facilitates AhR translocation into the nucleus of hepatocytes, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and can then bind xenobiotic response elements (XREs), inducing the transcriptional activity of CYP1A1 [25]. Thus, the expression levels of CYP1A1 induce oxidative stress and activation of environmental carcinogens [1].

In chemical warfare, diphenylarsinic acid (DPAA) is used as respiratory irritant. Its accumulation in liver and kidney induces carcinogenesis in rats by promoting the mRNA expression of CYP1B1. This enzyme is involved in DPPA metabolism, inducing hepatocarcinogenesis [26].

Activation of anticancer prodrugs able to inhibit CYP1B activity is a promising approach against cancer, given that this is the main CYP1 enzyme detected in a range of malignant tumors and shows high levels of expression of both mRNA and protein in metastatic diseases. CYP1B mRNA is also expressed in normal tissues, even if the protein is generally not detected [27]. Thus, anticancer prodrugs selectively activated by CYP1B could result in anticancer effects against cancer cells.

Further research supports the role of CYPs in tumor formation; therefore, their inhibition could be a promising strategy for cancer treatment. For example, PC is influenced by androgen activity. Therefore, androgen deprivation therapy (ADT), also known as castration therapy, is proposed as a treatment against PC [28]. CYP17, a family of enzymes involved in androgen synthesis, is implicated in the pathogenesis of PC [18]. In particular, ADT frequently induces a state called 'metastatic castration-resistant prostate cancer' (mCRPC), where the cells provide themselves with intratumoral androgen synthesis via CYP17, the expression of which is increased in prostate carcinoma [17]. In this case, the extragonadal inhibition of androgen synthesis appears promising as therapeutic approach. Abiraterone acetate is an inhibitor of CYP17 enzymes approved for patients with mCRPC [29]. However, similarly to orteronel (another CYP17 inhibitor), it induces adverse effects during treatment, such as hypertension, hypokalemia, cardiac disorders, and liver function test abnormalities, requiring their strict monitoring in patients with PC [30]. Therefore, specific targeting of this type of drug in prostate tumors is required to limit such adverse effects.

Involvement of CYP enzymes in activation or degradation of anticancer drugs and MDR

Enzymes of the CYP2 and CYP3 families are involved in the activation or degradation of anticancer drugs. For example, tamoxifen is an antiestrogenic drug used for therapy against hormone-positive breast cancer. CYP2D6, involved in the metabolism of ~25% of currently used drugs, metabolizes tamoxifen into its metabolite 4-hydroxy-tamoxifen (~10%), which shows an affinity for the ER of two orders of magnitude higher than the parent compound. By contrast, ~90% of tamoxifen is degraded to the inactive metabolite N-demethyltamoxifen by CYP3A4/5, which are involved in the metabolism of >50% of clinically used drugs [11]. These enzymes also activate cyclophosphamide, an alkylating agent used in chemotherapy against breast cancer. In particular, ~90% of cyclophosphamide is converted to its active metabolite 4-hydroxy-cyclophosphamide. Analogously, the antitumor prodrug ifosfamide is activated to 4-hydroxy-phosphamide [11]. Tegafur is the prodrug of 5-fluorouracil and its chemotherapeutic activation is induced by CYP2A6, known to metabolize important therapeutic drugs, toxins, and procarcinogen compounds [11].

However, the presence of CYP enzymes in cancer cells can have negative effects on chemotherapy drugs by promoting their degradation. For example, docetaxel, which belongs to the taxane family, promotes tubulin stabilization, allowing the inhibition of the division of rapidly growing cells. Thus, docetaxel has a wide spectrum of anticancer activity, although its therapeutic effects are limited by its oxidation to the inactive metabolite t-butylhydroxy-docetaxel via CYP3A4 and CYP3A5 [11,27]. Similarly, the antimitotic paclitaxel is another taxane used as first-line therapy for small cell lung, breast, and ovarian cancers; its therapeutic activity can be reduced by its degradation to a hydroxylated metabolite 30-fold less toxic than the parent compound. The enzymatic degradation of paclitaxel is performed by CYP2C8, which is known to participate in the metabolism of important drugs [11,27].

CYP3A and CYP2C8 have an important role in the development of resistance to chemotherapy drugs. Taxanes induce their own degradation in cancer cells by promoting the expression of CYP3A and CYP2C8 [31]. Indeed, taxanes are ligands of the steroid xenobiotic receptor (SXR), which regulates the expression of CYP3A4 and CYP2C8 through transcriptional activation [32]. This phenomenon, often accompanied by the overexpression of efflux transporters (see later), is one of the most important steps in the development of MDR. SXR has been frequently studied for its involvement in the control of CYP3A4 expression [33]. Ligand binding to the receptor causes the dimerization of SXR with the 9-

cis-retinoid X receptor (RXRα). The resulting heterodimer induces transcriptional activation of CYP enzymes by interacting with its response element on CYP genes [34]. Paclitaxel activates SXR and induces the expression not only of CYP3A4, but also of CYP2C8 [32]. By contrast, docetaxel induces very weak transcriptional activation of SXR and, thus, appears unable to enhance CYP3A activity [35].

As a consequence of this type of mechanism, repeated chemotherapy treatment often induces cancer cells to acquire MDRproperties, showing resistance to the specific chemotherapeutic agent used and to other cytotoxic drugs [5]. This phenomenon occurs mainly as a result of the combined upregulation and induction of both CYP enzymes and of efflux transporters interacting with the specific chemotherapeutic agent [8]. MDR shows a strict dependence on the overexpression of efflux transporters of the ATP binding cassette (ABC) superfamily. These transporters are energy dependent and act by coupling ATP hydrolysis to active efflux of their substrates against a concentration gradient [36]. They are classified in seven subfamilies ranging from ABCA to ABCG [8]. The ABC transporters mainly involved in the development of MDR are subtypes ABCB1 [Multidrug resistance gene 1 (MDR1) or P-glycoprotein (P-gp)], ABCC1 [Multidrug resistance protein 1 (MRP1)], ABCC2 (MRP2), and ABCG2 [Breast cancer resistance protein (BCRP)].

Currently, >300 compounds are known P-gp substrates, including vinca alkaloids (vinblastine, vincristine, vindesine, and vinorelbine), anthracyclines (doxorubicin and daunorubicin), taxanes (paclitaxel and docetaxel), methotrexate, mitoxantrone, imatinib mesylate, and saquinavir [36].

The expression of MRP1 in normal tissues reduces the accumulation of xenobiotics and their metabolites. Its overexpression in cancer cells induces the efficient efflux of a variety of anticancer drugs, such as vinca alkaloids, anthracyclines, methotrexate, and mitoxantrone, as well as organic ion conjugates [8,36].

In the same family of MRP transporters, MRP2 is involved in the transport of organic ions from the liver into the bile in health bodies, whereas its overexpression in cancer cells causes cisplatin efflux, inducing resistance to this drug [37].

BCRP was detected in some in vitro cancer models, following mitoxantrone exposure. This transporter is known to be specific for several drugs, such as mitoxantrone, topotecan, irinotecan, methotrexate, saquinavir, flavopiridol, and imatinib [8,37].

Significant correlations occur between efflux transporters and CYP enzymes related to Phase I metabolism and involved in the detoxification of anticancer drugs. For example, imati-

nib, paclitaxel, docetaxel, doxorubicin, vincristine, etoposide, teniposide, and vinblastine are substrates of both CYP3A4 and P-gp [38]. Moreover, CYP3A inducers or inhibitors can produce the same effects on P-gp [8]. Taxanes trigger their own degradation via the transcriptional activation of CYP3A [32]. This phenomenon results from the activation, by the anticancer drug, of nuclear receptors involved in the expression of CYP enzymes. The transcriptional activation of CYP enzymes operated by this type of anticancer drug is related to their ability to promote the transcriptional activation of efflux transporters, with the consequent severe induction of MDR. CYP3A4 and P-gp are known to share this type of combined upregulation [39,40].

In terms of the correlation between Phase II metabolism and efflux transporters, the glutathione S-transferases (GSTs) in normal tissues work to conjugate GSH with substrates requiring higher polarity for their elimination from the body. These conjugates are effluxed from cells by MRP1 and MRP2 transporters [41]. In cancer cells, GSTs are upregulated in the case of anticancer drug resistance because of the concomitant overexpression of these enzymes and MRP transporters [8].

Inhibition of CYP: potential new anticancer therapies

The inhibition of specific CYP enzymes is a new and promising therapeutic strategy against cancer. CYP2J2, expressed in the heart for the metabolism of polyunsaturated fatty acids to cardioactive metabolites [14], is one focus for research because it is upregulated in multiple cancers [15].

The high expression of CYP2J2 in human carcinomas appears as a general phenomenon rather than type specific; thus, its inhibition can hold significant promise for the treatment of neoplastic diseases. Terfenadine induces apoptosis in melanoma cell lines and its derivatives appear as novel selective CYP2J2 inhibitors. In particular, the compound C26 (1-[4-(vinyl)phenyl]-4-[4-(diphenyl-hydroxymethyl)-piperidinyl]-butanone) hydrochloride promotes apoptosis in cancer cells and induces a marked reduction in tumor proliferation and migration [42].

By contrast, adverse cardiac effects induced by CYP2J2 inhibitors are an important problem associated with their use as anticancer drugs. In this case, the design of CYP2J2 inhibitors for cancer therapies requires investigation of their cardiac safety profiles [43]. Alternatively, the use of nanoparticulate systems can obtain the selective targeting of anticancer drugs to their site of action (see later).

TABLE 1

Q2 CYP enzymes the inhibition of which could constitute a strategy against cancer[a]

Enzyme	Pathology	Inhibitor(s)	Inhibitor mechanism
Aromatase (CYP19)	Breast cancer	Formestane, exemestane, letrozole, anastrozole	Inhibits rate-limiting step in androgen–estrogen conversion
17α-hydroxylase, C17,20-lyase (CYP17)	Prostate cancer	Ketoconazole, abiraterone	Inhibits testosterone biosynthesis
25-Hydroxy-vitamin D3-24-hydroxylase (CYP24)	Non-small cell lung and colon cancer	Genistein, trichostatin A, ketoconazole, liarozole	Inhibits 1,25-D$_3$ deactivation
ATRA hydroxylase (CYP26)	Breast and prostate cancer	Liarozole, azolyl retinoids, benzeneacetic acid derivatives, 2,6-disubstituted napthalenes	Inhibits C-4 hydroxylation and deactivation of ATRA (i. e., retinoic acid metabolism)

[a] Abbreviation: all-trans-retinoic acid (Vitamin A).

In addition to CYP2J2, induction of the inhibition of several other CYP enzymes has been studied as an anticancer approach, as detailed in Table 1 [44].

Natural compound derivatives as potential anticancer agents
Significant anticancer activity via CYP2J2 inhibition has been found with naturally derived compounds. For example, tanshinone IIA is a *Salvia miltiorrhiza* constituent that decreases the viability of cancer cells by inhibiting CYP2J2 activity without inducing toxicity effects on normal cells. A similar response was recorded for decursin, a natural derivative compound from *Angelica gigas* [43].

Phytoestrogens, such as kaempferol or resveratrol, are plant-derived estrogenic compounds that are able to suppress TCDD-induced AhR-mediated transcription and to decrease the levels of CYP1A1 and CYP1B1 mRNA expression in breast epithelium tumor cell lines. Therefore, these compounds appear to induce anticancer effects against estrogen-dependent cancers by suppression of CYP1 family expression [1].

Thus, the anticancer mechanisms described for these natural derivatives could be an alternative approach to chemotherapy, which is based on the inhibition of rapidly dividing cells.

Relation of the chemotherapeutic action of plant-derived compounds to mammalian P450 enzymes
Several chemotherapeutic agents, such as vinca alkaloids, taxanes, and camptothecins, are phytotoxins synthesized by plant P450 cytochromes [27]. These compounds evolved as defensive mechanisms of plants against animal predation [27,45,46]. By contrast, the first stages of the evolution of mammalian P450 s enabled animals to defend themselves against phytotoxins [27,31,32]. Therefore, P450 s were at the forefront of plant–animal coevolution at the chemical level [27].

The enhanced expression of these types of enzyme and efflux transporter in cancer cells leads to MDR [27]. Therefore, the existence of phytotoxins, coupled with the ability of cancer cells to induce defence systems against these compounds evolved during plant–animal coevolution, appear to focus cancers on previous evolutionary stages of animal and plant cells. This suggests that cancers derive from an evolutionary stage regression of mammalian cells, indicating new valuable therapeutic approaches against cancers. Therefore, it is important to consider current models describing the origin and development of cancers [47], as discussed herein.

The stochastic model postulates that every cancer cell has the potential to produce new tumors. In particular, initiation and development of cancer depend on sequential acquisition of mutation and epigenetic changes leading to dedifferentiation and regression of cells into a more primitive phenotype. Therefore, this clonal evolutional model suggests an increased ability of clones of cells to become malignant, with the potential induction of cellular heterogeneity and to become invasive and develop metastases far from the original site. These cells can develop resistance to therapeutic treatment, inducing cancer recurrence [48].

The CSC model postulates that only a small subpopulation of stem-like cells is responsible for the development of cancer. Indeed, the stemness properties of CSCs allow them to induce self-renewal and multi-lineage differentiation, generating a varied progeny of highly proliferative cells that comprise the tumor bulk. Therefore, typical behaviors of malignant cancer cells, such as invasion and induction of metastasis, originate from CSCs. According to this model, cancer resistance to chemotherapy is attributed to the CSC subpopulation surviving treatments, leading to recurrent disease even when remission appears complete [49].

In various tumors, CSCs are recognized as a subpopulation of 'colorless' cells, because their upregulation of the efflux transporter BCRP (ABCG2) allows them to expel the DNA-binding dye Hoechst 33342. CSCs appear to be generally quiescent, highly tumorigenic, and pluripotent [49] and are the only type of cancer cells that can form tumors (xenografts) when transplanted into animals [50]. CSCs also appear to be involved in metastasis processes [51].

The origin of CSCs is currently under debate. It is suggested that CSCs can arise from normal stem cells characterized by genetic instability, resulting in dysregulation of the self-renewal program, which leads to tumorigenicity. By contrast, it is also suggested that CSCs can arise from differentiated tumor cells by acquisition of oncogenic mutations conferring them with stemness properties [52]. Recently, an atavistic origin of tumorigenic development were proposed for CSCs [53], suggesting that cancer derives from normal cells in the body that lose their proliferation capacity prematurely because of local hostile conditions. To escape premature and imminent death, these cells reactivate an atavistic stem and progenitor cell lineage encrypted in the dark genome. This strategy should increase their probability of survival by retrieving properties typical of single-cell eukaryotes. Indeed, unlike body cells, the protection of which is generally guaranteed in homeostatic compartments, single-cell eukaryotes evolved adaptive mechanisms over millions of years to protect themselves from exposure to hostile life conditions, such as increased radiation, oxygen and nutrient variations, and harmful chemicals. Thus, body cells exposed to local hostile conditions can induce the reappearance of unicellular features and assume, therefore, surviving atavistic characteristics that lead to CSC [53]. Culturing keratinocytes in a minimal serum-free medium invariably induces them to become malignant [54], a behavior that can be explained by the atavistic model.

CSCs can be recognized and characterized based on specific properties
CSCs, similar to normal stem cells, are able to renew by maintaining an undifferentiated state. However, this process is dysregulated, leading to CSC overpopulation, which drives tumor growth. In particular, an increase in symmetric cell division (which produces two stem cell daughters) with respect to asymmetric division (which produces one stem and no-stem daughter cells) separates CSCs from normal stem cells [47]. Therefore, cancer cells in solid tumors appear distributed according to a hierarchical organization, even if abnormal, where CSCs constitute the apex of the hierarchy, having tumorigenic ability [55]. In this hierarchical organization, CSCs reside in niches constituted by specific microenvironments.

Tumor microenvironments (TMEs) are often characterized by hypoxic conditions, which result from aberrant angiogenesis, high oxygen consumption of growing cancer cells, and closing or impairment of blood vessels induced by tumor cell invasion

[56]. Phase I reduction reactions, normally catalyzed by CYP450 reductases under anaerobic conditions, allow cell survival under these conditions [11]. Hypoxic conditions induce the inhibition of cancer cell proliferation; however, the effects of therapeutic approaches based on drugs targeting highly proliferating cells commonly reduce as a function of distance from blood vessels [57]. Moreover, the poor blood supply reduces the opportunity to accumulate chemotherapeutic drugs in hypoxic regions of cancers at effective concentrations where, furthermore, the cancer cells develop active mechanisms, governed by transcriptional hypoxia inducible factors (HIF), able to induce MDR [58].

Thus, it is difficult to target anticancer drugs to the niches where CSCs reside. Moreover, the hypoxic conditions of these niches enhance the intrinsic quiescence of CSCs [59] as well as inducing their resistance to conventional anticancer drugs and, therefore, leading to MDR [60,61].

CSCs share several properties with normal stem cells, not only in terms of their self-renewal by remaining in an undifferentiated state, but also in terms of the expression of surface markers, or specific enzymes, and the upregulation of particular signaling pathways conferring them high resistance [6,62]. Here, we discuss those pathways that are related to the focus of this review.

Research revealed **CD133** and **CD44** to be the most important surface markers. CD133 is a membrane glycoprotein (prominin-1) located on the surface of actively proliferating stem cells, the function of which is currently unknown [63]; by contrast, CD44 is a glycoprotein of the hyaluronic acid (HA) transmembrane receptor involved in the survival, growth, differentiation, and motility of stem cells. Knockdown of CD44 reduces the proliferation of CSCs and inhibits their tumorigenicity in xenograft models [64].

Aldehyde dehydrogenase 1 (*ALDH-1*) is another marker of CSCs [65] and is often associated with disease progression in several cancers [66]. It is involved not only in detoxification processes, but also in defence against free radicals [66]. Moreover, ALDH-1 is linked to resistance and cancer recurrence following conventional chemotherapy [66,67].

CSC signaling pathways are mediated by membrane receptors that, after activation by specific ligands, induce transcriptional processes as a result of modified gene expression. The **Hedgehog** (Hh) pathway under nondisease conditions is involved in embryonic development, but is inappropriately activated in several human cancers, resulting in the proliferation of CSCs and increase in tumor invasiveness [68].

The **NOTCH** pathway normally contributes to the proliferation, differentiation, and survival of cells, but is also one of the most commonly activated signaling pathways in cancer and is associated with CSCs. The amplification of this pathway induces the progression of cancer and its inhibition can overcome the cancer chemoresistance [69].

WNT/β-catenin signaling is normally involved in embryonic development and cell migration. Nevertheless, alterations in this signaling contribute to sustain CSCs and the proliferation of tumor bulk. Moreover, WNT signaling has been associated with MDR and the immune-escaping ability of CSCs [70].

Finally, other properties that characterize CSCs are an active DNA repair capacity [62], upregulation of efflux systems [71], and relative quiescence [2]. These properties, in addition to upregula-

tion of detoxifying enzymes, such as ALDH [66] and increases in WNT/β-catenin and NOTCH signaling, result in the high resistance of CSCs to conventional therapies (i.e., chemotherapy, surgery, and radiation) [49].

What therapeutic approaches do the models above described suggest?

It is unclear whether the models described earlier are fully representative of reality. Nevertheless, the therapeutic approaches that can be proposed against cancer are dependent on these models in terms of their potential efficacy.

According to the stochastic model, chemotherapy is designed to kill rapidly proliferating cells showing high efficacy. However, this efficacy can be strongly reduced by the onset of MDR. This could be counteracted by using efflux transporter inhibitors, even though this approach does not appear to be feasible from a clinical point of view using conventional formulations [36].

Furthermore, conventional chemotherapy is unable to destroy CSCs, which are relatively slow cycling quiescent cells; nevertheless, their specific elimination is crucial to eradicate the cancer. Thus, combinational treatments involving both a standard cytotoxic therapy and a CSC-targeted therapy suggest promising outcomes for cancer eradication [62].

Diets that are poor in fruits and vegetables, but rich in saturated and trans-fatty acids, sugar, and refined starch are strongly related to an increased risk of cancer [72]. By contrast, diets based on plant products are related to a reduction in cancer risk, indicating the potential of natural products from dietary sources as chemopreventives or new anticancer agents [38]. For example, foods containing phytochemicals characterized by CYP450-inhibitory activity are associated with anticancer and chemopreventive properties [73]. The phytochemicals known to be the most effective in reducing the activity of cancer cells are curcumin, tea polyphenol epigallocatechin-3-gallate (EGCG), resveratrol, lycopene, luteolin, genistein, piperine, β-carotene, and sulforaphane. The anticancer activity of these compounds has been studied for >30 years, before the discovery of CSCs [74], but appears to be strongly related to their ability to destroy CSCs [52,74]. Therefore, characterizing the mechanistic pathways of natural compounds related to their ability to act against CSCs is an important challenge in the fight against cancer. Nevertheless, such an approach could be extended to synthetic compounds (Table 2).

Curcumin is a natural compound derived from turmeric and is able to modulate multiple signaling pathways in a variety of cancers [52]. It interferes with carcinogenic processes in animal models via inhibition of the initiation step or suppression of the promotion and progression stages of cancer development [75,76]. The chemopreventive effects of curcumin also appear synergistic with those of other diet-derived polyphenols [74]. Recently, curcumin was found to selectively target CSCs in human esophageal squamous carcinoma cell lines, by inducing significant loss of ALDHA1+ and CD44+ cell populations [77]. Moreover, in a human hepatocellular carcinoma cell line, curcumin affected the proliferation of cells and induced apoptosis by reducing β-catenin activity and the transcription of target genes of the WNT/β-catenin pathway, typical of CSCs [78]. Therefore, curcumin is considered an attractive candidate for therapies combined with conventional chemotherapeutic agents, given its ability to target the CSC sub-

TABLE 2

Natural and synthetic compounds known to act against CSCs[a]

Compound	Origin	Mechanism	Target CSC	Type of study	Refs
Curcumin	*Curcuma longa* (curcuminoid)	↓ ALDHA1$^+$ cells, ↓ CD44$^+$ cells	Esophageal squamous carcinoma	*In vitro*, human cell line	[77]
		↓ β-catenin activity, ↓ WNT/β-catenin genes transcription, → apoptosis	Hepatocellular carcinoma	*In vitro*, human cell line	[78]
		+ 5-fluorouracil or oxaliplatin (anticancer drugs); ‖ CSC population	MDR colon cancer	*In vitro*, human cell line	[79]
		+ Dasatinib (anti-leukemia) at mRNA level: ↓^{D} ALDH1 markers, CD44 markers, and CD133 markers	MDR colon cancer	*In vitro*, human colon cancer cells	[80]
		+ metformin (anticancer drug): ‖ CD44 markers, ‖ NOTCH1 markers	Oral carcinoma	*In vivo*, mice model	[82]
		+ cisplatin (anticancer drug): ↓ CD133$^+$ cells, ↓ ABCG2	Laryngeal carcinoma	*In vitro*, CSCs	[83]
		+ piperine (*Piper nigrum*, alkaloid): ‖ WNT signaling; ↓ ALDH$^+$ cells	Breast cancer	*In vitro*, mammospheres from breast tissue	[86]
EGCG	*Camellia sinensis* (polyphenol)	+ Quercetin (*Capparis spinosa*, flavonoid) or alone: ‖ HH components	Pancreatic cancer	*In vitro*, pancreatic CSCs	[90]
B2G2	GSE (flavonoid)	‖ NOTCH1, → apoptosis	Prostate cancer	*In vitro*, human cell line	[91]
Silibinin	*Silybum marianum* (flavonoid)	‖ WNT/β-catenin, ‖ CD44 markers expression	Bladder cancer	*In vitro*, human cancer cell line	[92]
		↓^{D} CD44 expression	Prostate and breast cancer	*In vitro*, human cancer cell line	[93,94]
Parthenolide	*Tanacetum parthenium* (sesquiterpene)	‖ CSCs	Breast cancer	*In vitro* model of human cell line (sphere cells and SP)	[97]
Dimethylamino-parthenolide (analog)			Blood and lymph node cancers	Phase I	[99]
RSV	*Vitis vinifera L.* (polyphenol)	+ GSE; # WNT/β-catenin; → apoptosis	Colon cancer	*In vitro*, cell lines; *in vivo*, rodent model	[105]
		‖ viability of cells	Ovarian cancer	*In vitro*, CSCs from ovarian cancer cells	[108]
		+ Etoposide (anticancer drug): ‖ RAD51; (sensitize to drug), → apoptosis	Cervical cancer	*In vitro*, human cell line	[109]
Pterostilbene	*Vaccinium* genus (analog of RSV)	Cytotoxic to CD44$^+$ cells; ↓ CD44 marker expression, ↑ β-catenin degradation	Breast cancer	*In vitro*, human CSCs from cancer cells	[110]
3,5,4′-Trimethoxy-stilbene	Methoxylated analog of RVS	↓^{D} WNT/β-catenin	Breast cancer	*In vitro*, human breast cancer cell line	[111]
Sulforaphane	Cruciferaceae sp.	# WNT/β-catenin	Breast cancer	*In vitro*, breast cancer cells	[112]
		↓ ALDH$^+$ cells	Breast cancer	*In vivo*, re-implantation in secondary mice	[112]
Genistein	*Glycine max* (isoflavone)	↓^{D} HH signaling	Prostate cancer	*In vivo*, prostate tumorspheres	[114]
Honokiol	*Magnolia officinalis* (lignan)	# NOTCH signaling	Colon tumor	*In vitro* and *in vivo* (mouse xenografts)	[115]
WPE	*Juglans regia*	‖ β – catenin expression; ‖ NOTCH1 expression	Colorectal cancer	*In vitro*, human cell lines	[116]
Indirubin	*Indigora suffruticosa* (phytochemical)	‖ WNT signalling	Leukaemia	Leukemia studies	[117]
Casticin (or vitexicarpin)	*Vitex trifolia* (phytochemical)	‖ WNT/β-catenin; ↓ CD133$^+$ cells	Hepatocellular carcinoma	*In vitro*, CSCs from human cancer line	[118]

TABLE 2 (Continued)

Compound	Origin	Mechanism	Target CSC	Type of study	Refs
Salinomycin	*Streptomyces albus* (antibiotic)	+ conventional anticancer agents: # WNT/β-catenin, ↓ ABC transports	Breast cancer	Mice xenografts	[52,119]
		Loss of CSC gene expression	Breast cancer	Breast tissues from patients	[120]
		Targets CD133$^+$ cells	Colorectal cancer	*In vitro*, human cell line	[121]
		# WNT/β-catenin signaling	Osteosarcoma	*In vitro* and *in vivo* studies (mice)	[122]
Metformin	Anticancer drug for patients with diabetes (synthetic)	↓^{D} CD133 markers, CD44 markers, and ALDH1 markers	Pancreatic cancer	*In vivo*, transgenic mice	[81]
DEAB	Synthetic compound	+ conventional anticancer agents: ‖ ALDH1	Breast cancer	*In vitro*, human cell line	[124]
Bedaquiline	Synthetic drug against pulmonary TBC	‖ mitochondrial function	Breast cancer	*In vitro*, human cell line	[126]

^{a} +, combinational treatment; ↑, increase; ↓, decrease; →, induction; ↓^{D}, downregulation; ‖, inhibition; #, interference.

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population within tumors [52]. For example, curcumin combined with either 5-fluorouracil or oxaliplatin induced significant inhibition of a CSC population in MDR colon cancer cell lines. This behavior was confirmed by a decrease in the CSC marker CD44 [79]. Moreover, curcumin in combination with dasatinib, an anticancer agent against leukemia, downregulated the ALDH, CD44, and CD133 markers at the mRNA level in MDR colon cancer [80]. Similarly, metformin, an antidiabetic drug also used for the prevention and treatment of cancer in patients with diabetes, targets and downregulates the CD133, CD44, and ALDH1 markers of pancreatic CSCs [81]. The combination of metformin with curcumin inhibited the CSC-specific markers CD44 and NOTCH1 in a murine model of induced oral carcinogenesis. In particular, the NOTCH pathway was suggested to be involved in the chemoprevention processes mediated by the curcumin–metformin combination [82]. Curcumin also enhanced the anticancer activity of cisplatin against laryngeal carcinoma by reducing the percentage of CD133$^+$ CSCs. In particular, this combined treatment reduced the expression of the ABCG2 transporter in the CSCs [83]. Among the active efflux transporters, ABCG2 is overexpressed in many cancer types and is involved in inducing the tumor resistance of CSCs to anticancer drugs [52,84]. Numerous phytochemicals can induce a significant inhibition of ABCG2 activity [85], which could explain the ability of several natural compounds to eliminate CSCs [52].

In a study of malignant breast stem cells treated using curcumin and piperine, a dietary alkaloid, isolated from black and long peppers [86], both compounds reduced the percentage of ALDH$^+$ cells by inhibiting WTN signaling. However, these compounds, either separately or in combination, showed little or no effect on differentiated cells [74,86,87].

EGCG is the most abundant polyphenol in green tea [74]. Its anticancer properties have been revealed in prostate carcinoma and colorectal cancer, where, in combination with cisplatin and oxaliplatin, it synergistically increased the efficacy of these conventional drugs [88,89]. EGCG acts by modulating CSC HH signaling. For example, in pancreatic CSCs, EGCG inhibited components of the HH pathway and also affected CSC self-renewal, either alone or in combination with the flavonoid quercetin [90].

The procyanidin B2 3,3′-di-O-gallate (B2G2) is a flavonoid and a major bioactive constituent of grape seed extract (GSE); it is able to induce the apoptotic death of human prostate cancer cells. Both GSE and B2G2 were demonstrated to target the self-renewal properties of CSCs in prostate cancer cell lines, via inhibitory effects on the activated NOTCH1 pathway, with B2G2 showing higher potency and efficacy [91].

Silibinin is a polyphenolic flavonoid extracted from the dried fruit of *Silybum marianum*. As well as being well tolerated and free from adverse effects, this phytochemical targets CSCs and induces their inhibition [92]. In particular, silibinin suppressed CSCs in bladder cancer by inhibiting both WNT/β-catenin signaling and CD44 marker expression [92]. Similarly, silibinin downregulated CD44 expression in prostate and breast cancer cells [93,94].

Parthenolide is a sesquiterpene lactone with anti-inflammatory activity and anticancer properties that occurs in the flowers and fruits of *Tanacetum parthenium* (feverfew) [95]. It induces apoptosis in leukemic cells without affecting normal hematopoi-

etic cells [96]. Parthenolide was studied in a model for breast CSC comprising sphere cells and side population (SP) cells from a human breast cancer cell line. Paclitaxel showed better activity against bulk cancer cells compared with sphere cells enriched with breast CSCs. By contrast, parthenolide showed preferential activity against sphere and SP cells from the breast cancer cell line, suggesting that this phytochemical preferentially inhibits breast CSCs [97]. These findings suggest that parthenolide belongs to a novel class of agents for targeting CSCs. Its analog, dimethylamino-parthenolide, designed to improve the solubility and bioavailability of this drug [98], is in Phase I trials for treating blood and lymph node cancers [99].

Resveratrol (RSV) is a phytochemical polyphenolic compound abundantly present in red grapes. It is known for its anticancer properties, which have been demonstrated in several models [100]. For example, the prophylactic use of RSV reduced the size and number of esophageal, intestinal, and colon tumors [101,102]. Moreover, RSV induced apoptosis in prostate cancer cell lines and suppressed prostate cancer progression in transgenic mice [103,104]. Recently, the combination of RSV and GSE was shown to suppress the proliferation of, and induced apoptosis in, colon CSCs by interfering with their WNT/β-catenin signaling pathway [105]. GSE is a dietary supplement associated with anticancer properties [106]. Interestingly, the anticancer effects against colon CSCs obtained with RSV-GSE did not induce the same adverse effects arising after the administration of sulindac, a nonsteroidal anti-inflammatory drug considered promising for colon cancer treatment [105,107]. Therefore, a combination of natural compounds might act synergistically against CSCs without damaging healthy cells. Interestingly, RSV is also able to inhibit the viability of ovarian CSCs at concentrations that are not toxic to normal human fibroblasts [108].

CSCs overexpressing RAD51, a recombinase involved in DNA repair, can appear poorly sensitive to etoposide, an anticancer agent causing DNA fragmentation. RSV inhibits RAD51 expression, decreasing the viability of CSCs derived from cervical cancer cell lines and triggering their apoptosis when treated simultaneously with etoposide. Thus, the ability of RSV to inhibit RAD51 expression sensitizes the CSCs to etoposide [109].

Pterostilbene is an analog of RSV found in blueberries and grapes. It is cytotoxic to CD44+ CSCs isolated from human breast cancer cells, and decreases the expression of CD44 marker to promote the degradation of β-catenin [110]. 3,5,4'-trimethoxy-stilbene, a methoxylated analog of RSV, inhibits the invasiveness of breast cancer cells via downregulation of WNT/β-catenin, a characteristic pathway of CSCs [111].

Studies on breast cancer xenografts revealed the ability of **sulforaphane** (a dietary component of broccoli) to target CSCs (ALDH+) in human breast cancer cells by interfering with their WNT/β-catenin signaling pathway [112]. Moreover, sulforaphane injections in murine xenograft models reduced the number of ALDH+ cells. Interestingly, tumor cells derived from mice treated with this phytochemical were unable to develop secondary tumors after re-implantation into secondary mice, indicating the ability of sulforaphane to eliminate CSCs in vivo [112]. Thus, sulforaphane appears to preferentially target CSCs instead of the tumoral bulk cell population, a preference that is significant for chemoprevention [74].

Isoflavones have been used successfully against prostate cancer by reducing the expression and blocking transcription of genes related to the WNT/β-catenin pathway [113]. The isoflavone **genistein** also suppressed the tumorigenicity of prostate cancer in vivo, possibly by downregulating HH signaling, as evidenced by growth inhibition studies of prostate tumorspheres rich in CSCs [114].

Honokiol is a phytochemical from Magnolia officinalis that reduces the in vitro and in vivo growth of CSCs from colon tumors by interfering with the NOTCH signaling pathway [115]. **Walnut lipid extract** is also able to inhibit the self-renewal capacity of colon CSCs. Recently, walnut phenolic extract (WPE) and its bioactive phenolic compounds were shown to inhibit the expression of CSCs markers, such as β-catenin and NOTCH1, in human colorectal cancer cell lines, although WPE showed higher potency than the bioactive polyphenols [116].

Traditional Chinese Medicine (TCM) might also be a useful source of phytochemicals able to inhibit CSCs. For example, **indirubin** is a compound extracted from Qingdai, a preparation of the TCM derived from the Indigofera suffruticosa and used to treat leukemia. These effects appear related to the ability of indirubin to interfere with WNT signaling [117]. **Casticin** (3',5 dihydroxy 3',4',6,7 tetrahydromethoxyflavone), also known as vitexicarpin, is a component of Viticis Fructus, a TCM prepared from the fruit of Vitex trifolia. This phytochemical, by inhibiting the WNT/β-catenin pathway, was more active against CSCs, isolated as CD133+ cells, than against total cancer cells from a hepatocellular carcinoma cell line. Therefore, casticin could be used as an antitumor drug to selectively reduce CSC self-renewal [118].

Salinomycin is an antibiotic obtained from Streptomyces albus that is also known to be efficacious against CSCs of many types of cancer. The underlying mechanisms of this activity are related to ABC transporters and signaling pathways of CSCs, such as WNT/β-catenin [52]. Salinomycin has been tested in combination with conventional chemotherapeutic drugs, and showed higher efficacy for human cancer eradication in murine xenografts compared with conventional chemotherapeutic drugs alone [119]. Interestingly, salinomycin was identified as a selective inhibitor of breast CSCs. In particular, it reduced the proportion of CSCs in breast cancer cells by more than 100-fold compared with paclitaxel. Moreover, analyses of breast tissues, isolated directly from patients, indicated that salinomycin treatment induced the loss of expression of CSC genes, confirming its specific toxicity for epithelial CSCs [120]. More recently, salinomycin was reported to selectively target CD133+ cell subpopulations of colorectal cancer lines, inhibiting their malignant traits [121]. It was also able to inhibit osteosarcoma by targeting its CSCs via the WNT/β-catenin signaling pathway both in vitro and in vivo, with no severe adverse effects evident in mice [122]. In cancer cell cultures, salinomycin is active in the nanomolar concentration range, indicating its ability to target transformed cells without affecting healthy cells. Preliminary results from salinomycin treatment of patients with metastatic breast, head, neck and metastasized gynecological cancers appear encouraging, although rationally designed clinical trials are needed [123].

In terms of synthetic compounds able to act against CSCs, **diethylaminobenzaldehyde** (DEAB) induced sensitization to conventional anticancer approaches of breast cancer cell lines via

ALDH inhibition [124]. ALDH-1 activity contributes to CSC survival, acting as a detoxifying enzyme [125]. **Bedaquiline,** a drug used against pulmonary TB, inhibits the mitochondrial function of CSCs derived from breast cancer cell lines, blocking their proliferation. Interestingly, this drug does not inhibit mitochondrial respiration in normal human fibroblasts [126].

How can we target CSCs?

Drugs able to destroy CSCs without damaging healthy cells are available and most are naturally derived compounds [52,61,105,127], suggesting that their ability to kill CSCs is related to previous evolutionary stages of the chemical arms race between animal and plant cells [27,52]. The mechanisms of action of these compounds differ from those of conventional chemotherapy, which is designed to kill rapidly proliferating cells. Unfortunately, CSCs are difficult to target, being normally protected in niches inside the tumor bulk, which comprises highly proliferating cancer cells [128]. By contrast, cancer recurrence and MDR appear closely related to CSCs [61]. These aspects highlight the usefulness of combinational treatments involving both conventional therapy and CSC-targeting drugs to definitively eradicate cancers, whereby conventional therapies destroy the tumor bulk, allowing the anti-CSC drugs to reach their therapeutic target. Such a strategy requires the ability to selectively target and concentrate the combined anticancer drugs in the tumor, which could be achieved with nanotechnology. For example, nanomedicines can be obtained by attaching or encapsulating anticancer drugs in liposomes, polymeric micelles, polymeric nanoparticles (NPs), or nanogels [36]. An important property of nanocarriers is ability to accumulate selectively in solid tumors via either passive or active targeting.

Passive targeting

Nanocarriers can passively and selectively accumulate in solid tumors according to the 'enhanced permeability and retention' (EPR) effect [2,36,129], which results from pathophysiological conditions in solid tumors including: (i) hypervascularity, characterized by large spaces between endothelial cells; (ii) excessive secretion of vascular permeability factors, inducing extravasation within cancer tissues; and (iii) uncontrollable rate of cancer cell proliferation, which induces the collapse by compression of intra-tumor lymphatic vessels and consequent impaired lymphatic drainage [2,36,129].

Unfortunately, before reaching their therapeutic targets, nanocarriers can be easily removed from the bloodstream by macrophages of the mononuclear phagocytic system, via opsonin mediation [2,36]. To mitigate this phenomenon, 'stealth' NPs, characterized by a prolonged half-life in the bloodstream, have been formulated by decorating their surface with amphipathic or water-soluble polymers, such as polyethylene glycol (PEG), dextran, or poly(acrylic acid) [2,37,130]. This protective layer repels the absorption of opsonin proteins, blocking uptake by macrophages [36]. NPs have been developed comprising self-assembled cores of a lipophilic prodrug (obtained by conjugation with ursodeoxycholic acid) coated with bile salt (taurocholate or ursodeoxycholate) coronas. These bile salts, used to coat the nanocores, modulate NP uptake in macrophages, suggesting a new strategy to obtain novel pharmaceutical nanoparticulate systems that can

avoid macrophagic uptake [131]. Thus, it might be interesting to verify the behavior of self-assembled NPs of lipophilic prodrugs obtained by the conjugation of conventional chemotherapeutic drugs with agents able to destroy CSCs.

Active targeting

Cancer cells can be characterized by the overexpression of transmembrane receptors able to interact with albumin, folic acid, somatostatin, or transferrin. Similarly, CSCs overexpress the glycoprotein CD44 receptor, which is able to bind HA. The conjugation of this type of compound on the surface of nanocarriers enables their active targeting to specific cancer cells. Alternatively, antibodies against these specific receptors can be incorporated onto the surface of nanocarriers (Table 3). Although active targeting does not allow the delivery of large amounts of anticancer agents in tumoral tissues, it does increase the specificity and drug uptake in the cells compared with passive targeting systems [37,132,133].

Polymeric nanomicelles can be obtained with co-polymers comprising hydrophobic and hydrophilic blocks self-assembled in aqueous environments as hydrophobic cores surrounded by hydrophilic shells. These structures entrap hydrophobic drugs in their core. PEG or immune-tolerant polypeptide can be used as hydrophilic blocks, whereas poly(lactide-co-glycolide) (PLGA), poly(aspartic acid) (PAA), or poly(caprolactone) (PCL) are used as hydrophobic blocks [36,129,134,135]. Nanomicelles can be self-assembled by using bulk hydrophobic drugs conjugated to hydrophilic polymers [129]. Several polymeric nanomicelles loaded with conventional chemotherapeutic drugs have been developed over the past 20 years. PEG-PLGA, PEG-PAA, or PEG-poly(glutamic acid) co-polymeric blocks were used to formulate micelles that were loaded, often by conjugation, with paclitaxel, doxorubicin, or cisplatin. The nanomicelles showed interesting properties, such as prolonged circulation time (due to the PEG hydrophilic shells) and accumulation in tumor tissues (because of the EPR effect) [129,132]. These systems enabled the potentiation of the anticancer effects of drugs, even if they were not able to overcome MDR (Fig. 1a).

Liposomes comprise amphiphilic phospholipids and cholesterol self-associated in spherical vesicles enclosing an aqueous core. These structures allow the encapsulation of both hydrophobic and hydrophilic drugs. Several anticancer formulations based on liposomes are available [36]. Liposomes can enable the co-encapsulation of conventional anticancer drugs and inhibitors of active efflux transporters to overcome the drug resistance of cancer

TABLE 3

Nanocarriers designed for cancer targeting and eradication[a,b]

[a] Abbreviation: MPS, mononuclear phagocytic system.
[b] §, XXX; #, XXX; ##, XXXX; *, XXX; *, XXX; **, XXXX.
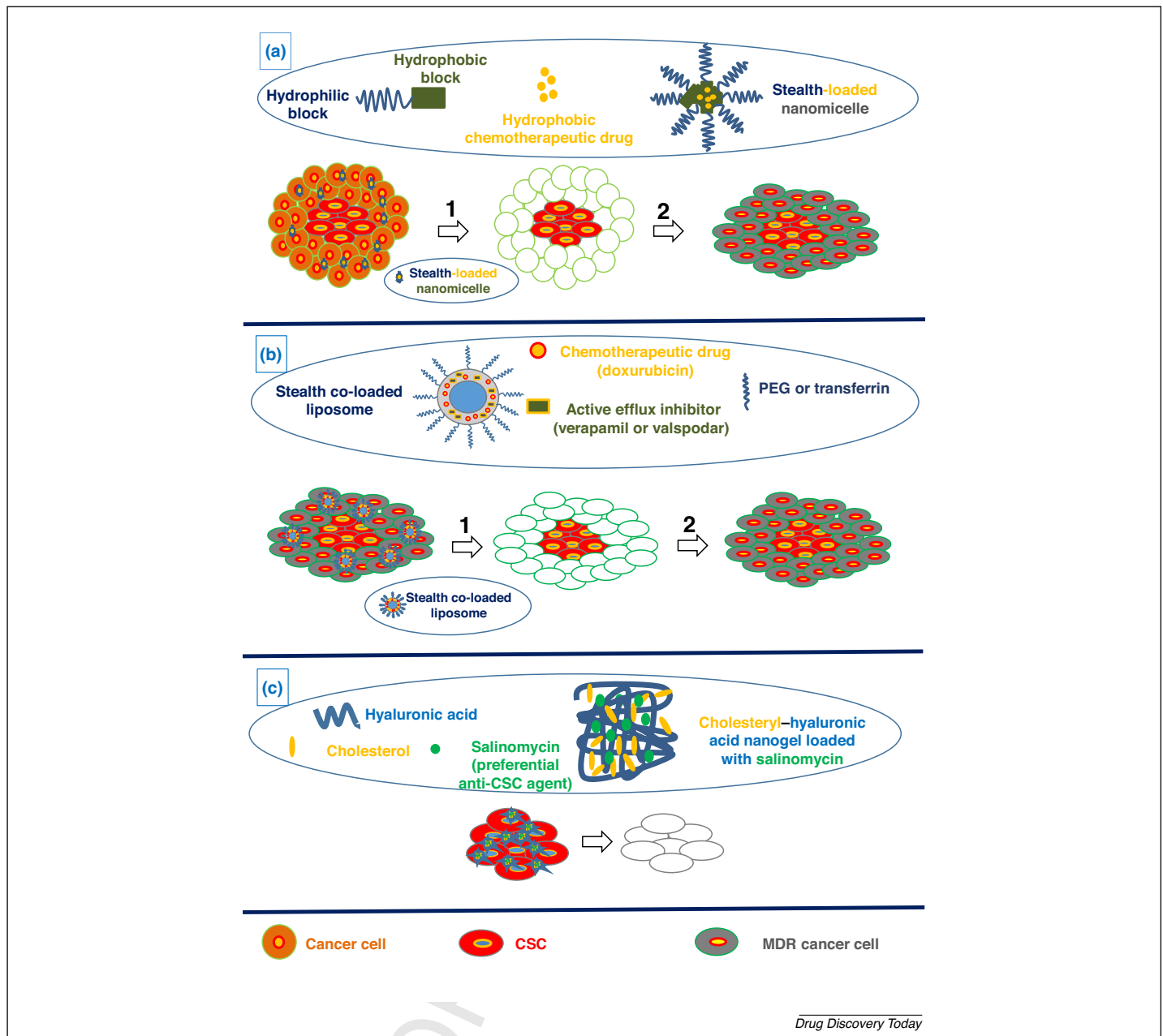
Q3

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FIGURE 1

Q1 Title **(a)** Schematic depiction of stealth nanomicelles obtained with co-polymers comprising hydrophilic (e.g., polyethylene glycol; PEG) and hydrophobic blocks [e.g., poly(lactide-co-glycolide) (PLGA) and poly(aspartic acid) (PAA)] self-assembled in aqueous environments as hydrophobic cores surrounded by hydrophilic shells. The nanomicelles are loaded with conventional chemotherapeutic drugs, such as doxorubicin, paclitaxel, or cisplatin. It is hypothesized that (i) the stealth nanomicelles accumulate the drug in tumor tissue and the cancer cells are killed, whereas cancer stem cells (CSCs) survive; and (ii) the survival of CSCs enables tumor relapse via the generation of multidrug-resistant (MDR) cancer cells. **(b)** Schematic depiction of stealth liposomes co-loaded with a conventional chemotherapeutic drug (doxorubicin) and an inhibitor of active efflux transporters (verapamil or valspodar). It is hypothesized that: (i) the stealth liposomes accumulate the drug in MDR tumor tissue and the active efflux inhibitor allows drug penetration of MDR cancer cells, which that are then killed, whereas CSCs survive; (ii) CSC survival enables tumor relapse via the regeneration of MDR cancer cells. **(c)** Schematic depiction of a nanogel obtained with cholesteryl-hyaluronic acid (HA) and loaded with salinomycin. It is hypothesized that HA induces the active targeting of the nanogel to cancer cells overexpressing CD44 receptor (CSCs); moreover, the cholesterol moieties in the nanogel enable its anchorage in cellular membranes, inducing the efficiency of drug accumulation in cancer cells, with consequent cytotoxic effects.

cells. For example, liposomes co-encapsulating doxorubicin and verapamil (an inhibitor of efflux transporters), decorated on their surface with transferrin (for active targeting) overcame drug resistance in leukemia cells; stealth liposomes (with PEG on their surface) co-encapsulating doxorubicin and valspodar (an inhibitor of efflux transporters) reversed the drug resistance of human breast cancer cells [6,136,137]. An interesting liposomal system, co-encapsulated with doxorubicin and verapamil, was proposed by Tang *et al.*, based on liposomes decorated on their surface with cleavable PEG and octaarginine [138]. PEG was induced to be cleavable in the tumor environment by a reduction-sensitive system based on disulfide linkages; although PEGylation significantly hinders the

carriers from entering cancer cells, it is essential to enable the nanocarrier to escape from surveillance by the mononuclear phagocytic system. Octaarginine is used as cell-penetrating peptide and is normally applied to the surface of carriers [139]. The co-decorated and co-loaded liposomes showed efficient *in vitro* cytotoxic activity against MDR human breast cancer cells. Moreover, this cell line was subcutaneously inoculated in female nude mice and the originated tumors where significantly inhibited in their growth by the liposomal system, which was also able to reduce the adverse effects of doxorubicin and verapamil. These *in vivo* effects were ascribed to the increased accumulation of the anticancer agent in drug-resistant cancer cells and their reduced distribution in healthy tissues [138].

Thus, this type of nanocarrier could be used to both destroy the bulk of drug-resistant cancers and weaken the protection of CSCs in their niches. However, this type of co-encapsulation, lacking specific anti-CSC drugs, does not appear optimized to kill CSCs (Fig. 1b).

Nanomicelles based on PEG conjugates with phosphatidylethanolamine (PE) and vitamin E were used to co-encapsulate paclitaxel and curcumin. In this case, curcumin was used as mediator of chemoresistance, to induce MDR reversal capability and then to sensitize the cancer cells to conventional chemotherapeutic agents. The combination treatment obtained with the micellar co-loaded compounds showed a significant synergistic effect *in vitro* against MDR human ovarian adenocarcinoma (SK-OV-3TR, paclitaxel resistant). This cell line was injected subcutaneously into female nude mice, resulting in tumors the growth of which appeared significantly inhibited by the micellar combination treatment [140]. Given the ability of curcumin to target CSCs, such a system could be useful for destroying CSCs.

The combination of conventional chemotherapy with salinomycin, a specific anti-CSC agent, eradicated cancers in mouse xenografts [119]. However, the poor water solubility of this drug triggered the design of new nanomicelles, comprising PEG-PCL blocks that entrapped salinomycin in their hydrophobic core (SAL-PEG-PCL-nM) to induce its accumulation in tumor bulks via the EPR effect [133]. In addition, to induce active targeting of tumors, PEG-PCL nanomicelles loaded with paclitaxel were formulated by decorating their surface with octreotide (PTX-Oct-PEG-PCL-nM), an octapeptide analog of endogenous somatostatin able to bind somatostatin receptors. Combination therapy performed with both SAL-PEG-PCL-nM and PTX-Oct-PEG-PCL-nM efficaciously suppressed not only breast cancer cells, but also breast CSCs *in vitro*. This combination therapy also showed strong anticancer activity against breast cancer xenografts *in vivo* [133].

Nanogels comprise hydrophilic polymer networks that can be physically or chemically crosslinked, with the latter inducing high stability. Appropriate functional groups can be introduced in the polymers to physically interact with the loaded drugs or to obtain their chemical conjugation [132]. For example, nanogels based on cholesteryl-HA were designed to encapsulate salinomycin by conjugation. Based on HA, this type of nanogel induced active targeting of cancer cells overexpressing CD44. In particular, the nanogel was efficiently internalized in CD44-expressing drug-resistant human breast and pancreatic adenocarcinoma cells via CD44 receptor-mediated endocytosis. The presence of cholesterol moieties in the nanogel allowed its anchorage in cellular membranes, induc-

ing efficient drug accumulation in cancer cells, with consequent strong cytotoxic effects [141]. Therefore, this type of nanocarrier could be promising to obtain the eradication of CSCs in drug-resistant cancers (Fig. 1C).

Vinorelbine is a semisynthetic *Vinca* alkaloid that acts against microtubule assembly, whereas parthenolide is a sesquiterpene lactone that has the potential to target CSCs [142]. A combined therapy of vinorelbine and parthenolide encapsulated in stealth liposomes was proposed and tested *in vitro* on both cancer stem-like (SP) and non-SP cells of human breast cancer. Vinorelbine loaded in liposomes showed efficacious effects against non-SP cells, whereas its activity was lower in SP cells. However, a strong inhibitory effect toward these cells was obtained in combination with parthenolide-loaded liposomes (Fig. 2a). Moreover, this combined approach produced a fully inhibitory effect on breast cancer cell xenografts in nude mice [142].

Cabazitaxel is a conventional chemotherapeutic drug that acts as a microtubule stabilizing agent and shows poor water solubility. Silibinin is a well-tolerated polyphenolic flavonoid able to target CSCs and induce their inhibition. Recently, cationic liposomes co-loaded with these compounds were formulated as a combined therapy against prostate cancer cells and CSCs. The liposomes were decorated on their surface with HA to induce their active targeting to CD44 markers, overexpressed by CSCs [127]. These nanocarriers showed efficient anticancer activity in androgen-independent human prostate cancer cell lines, with high cytotoxicity against CD44+ cells, indicating their ability to target CSCs (Fig. 2b). Such a system appears promising as a treatment to eradicate prostate cancer [127].

Polymeric NPs are colloidal biodegradable systems in which the drugs are encapsulated or entrapped in the polymeric matrix. The NPs offer higher stability and ability to control drug release compared with liposomes or nanomicelles. Synthetic polymers, such as PLGA, PAA, PCL, and *N*-(2-hydroxypropyl)-methacrylamide (HPMA) [4], or derivatives of natural polymers, such as chitosan [135], can be used for NP formulations, some of which are under clinical development [4]. Drug loading in NPs can be improved by using a prodrug approach [143], which can also be useful to elude active efflux transporters [144] or to increase the anticancer drug potency [130,145]. Recently, the use of carboxymethyl-hexanoyl chitosan (CHC) was proposed to obtain self-assembled polymeric NPs characterized by both hydrophilic and hydrophobic internal environments. This property allowed the loading of CHC NPs with both the hydrophilic drug cisplatin and the hydrophobic compound demethoxycurcumin, which is a curcuminoid showing higher stability in blood compared with curcumin, and known as an MDR-suppressing herbal extract. The loaded CHC NPs were also decorated on their surface with a CD133 antibody to target and enhance their uptake by lung CSCs. These nanocarriers induced synergistic effects between the drugs, showing high efficiency against MDR lung CSCs (Fig. 2c) [135].

Oil/water (O/W) nanoemulsions based on flaxseed oil were proposed for the co-administration of paclitaxel and curcumin. In particular, the combined therapy showed effectiveness against resistant human ovarian adenocarcinoma cells, allowing the intracellular delivery of both drugs into the cells, the P-gp expression of which was downregulated after incubation with the nanoemulsions [146]. Given the ability of curcumin to selectively target
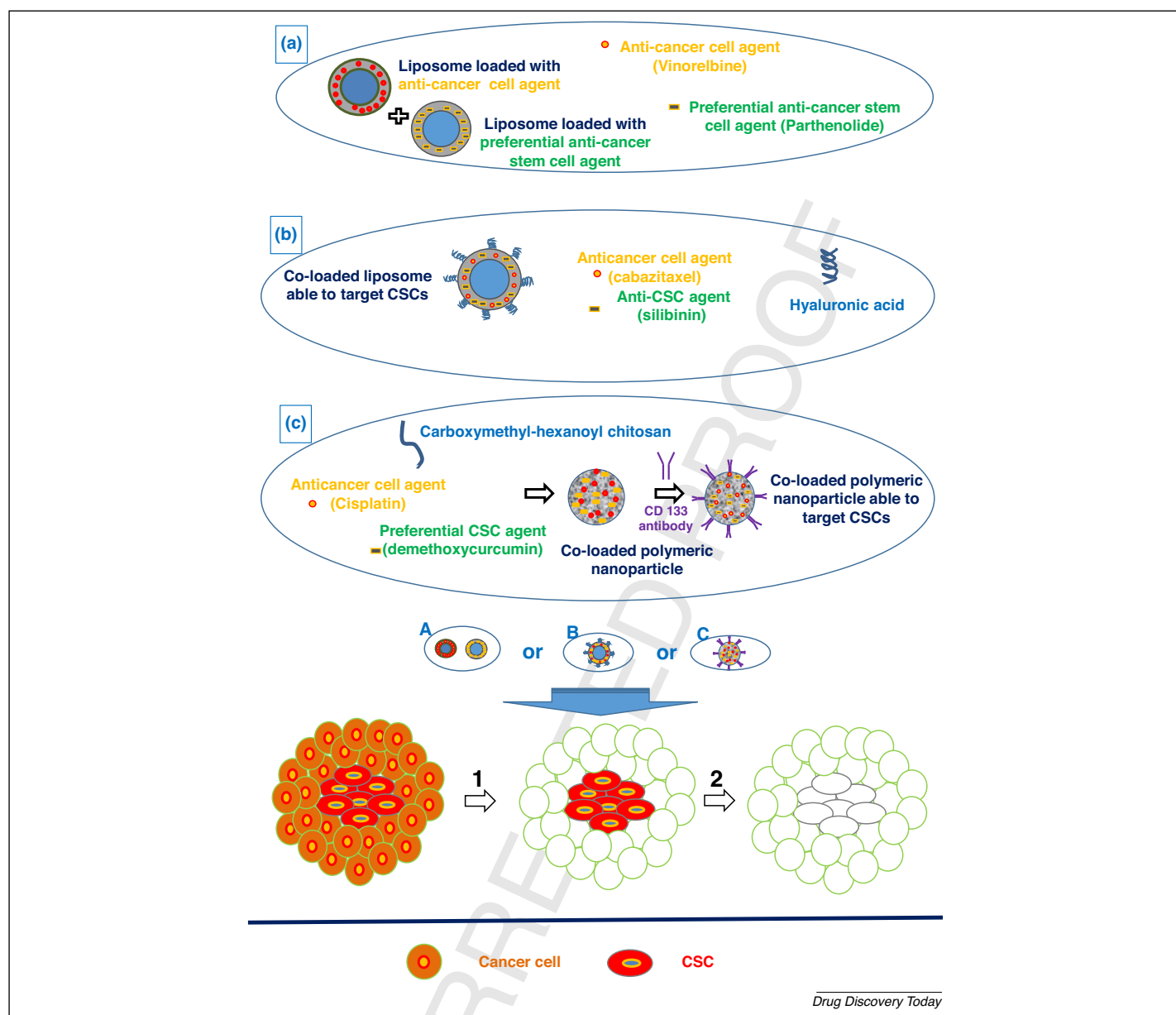
FIGURE 2

Schematic depictions of **(a)** stealth liposomes loaded with agents against cancer cells or preferentially against cancer stem cells (CSCs; vinorelbine or parthenolide, respectively); **(b)** liposomes decorated on their surface with hyaluronic acid (HA) to confer active CSC targeting and co-loaded with both a conventional chemotherapeutic agent and an anticancer stem cell agent, cabazitaxel and silibinin, respectively; **(c)** polymeric nanoparticles (NPs), based on carboxymethyl-hexanoyl chitosan, decorated on their surface with a CD133 antibody to confer active CSC targeting, and co-loaded with both a conventional chemotherapeutic agent and a preferential anti-CSC agent, cisplatin and demethoxycurcumin, respectively. It is hypothesized that these nanosystems: (i) accumulated in tumoral tissue via the enhanced permeability and retention (EPR) effect, where cancer cells are killed, inducing impairment of the bulk tumoral tissue; and (ii) the agents are then able to reach and kill the CSCs. When appropriately surface decorated, nanosystems can selectively target CSCs and induce their internalization, resulting in cancer eradication.

CSCs [77], its role against CSCs will be relevant in this type of combined therapy.

Concluding remarks

Cancer MDR is a phenomenon related both to rapidly proliferating cells, constituting the bulk of tumors, and to CSCs, localized in tumoral niches that are difficult to target. Combined therapies supported by nanotechnologies can weaken the tumoral bulk, killing CSCs, and,

thus, appear promising for cancer eradication. Prodrugs obtained by the conjugation of conventional therapeutic drugs with novel anti-CSC agents could constitute tools to obtain new self-assembled or innovative nanocarriers able to eradicate the target cancer.

Q8

Acknowledgment

Reviews • KEYNOTE REVIEW

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