This review provides an overview of the current state of the passively and actively targeted nanotechnological-based DOX formulations, some approved by regulatory agencies and others still in clinical trials.

Doxorubicin: nanotechnological overviews from bench to bedside

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Doxorubicin (DOX) is considered one of the most effective chemotherapeutic agents, used as a first-line drug in numerous types of cancer. Nevertheless, it exhibits serious adverse effects, such as lethal cardiotoxicity and dose-limiting myelosuppression. In this review, we focus on the description and the clinical benefits of different DOX-loaded nanotechnological platforms, not only those commercially available but also the ones that are currently in clinical phases, such as liposomes, polymeric nanoparticles, polymer–drug conjugates, polymeric micelles and ligand-based DOX-loaded nanoformulations. Although some DOX-based nanoproducts are currently being used in the clinical field, it is clear that further research is necessary to achieve improvements in cancer therapeutics.

Introduction

History

Doxorubicin (DOX) is one of the most potent and commonly used chemotherapeutic agents for the treatment of several types of cancer [1]. This drug belongs to the anthracycline family of antibiotics, together with daunorubicin, epirubicin, idarubicin, among others. It was first isolated in 1969 from *Streptomyces peucetius* var. *caesius* by mutagenic treatment of *S. peucetius* – the daunorubicin-producing microorganism [2].

Previously, in 1940 Waksman and Woodruff discovered the first antibiotic with antitumor activity: actinomycin A, an antibacterial compound produced by *Actinomyces antibioticus* [3]. Soon afterward, in 1952, another substance of microbial origin, actinomycin C, exhibited antitumor activity in animal species, drawing special attention to these kinds of microbial metabolites. Five years later, Farmitalia Laboratories began an investigation in search of new biosynthetic antibiotics with antitumor activity obtained from cultures of newly isolated *Streptomyces* or crude isolates and fractions, testing against Ehrlich carcinoma and sarcoma 180 (S180) in the solid and ascites forms in mice. In 1959, the researchers found that a nonidentified *Streptomyces* species produced a

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http://dx.doi.org/10.1016/j.drudis.2016.11.005

GLOSSARY

Active targeting It involves the conjugation of a special moiety to the surface of the carrier, like antibodies, folate residues, monosaccharides (mannose, glucose, fructose) or ligands. These different moieties are recognized by a specific receptor and delivered to the site to which the carrier was designed.

Enhanced permeability and retention (EPR) effect It is the ability of nanoparticles to accumulate in the tumor tissue, owing to the large fenestrations in the tumor vasculature and impaired lymphatic drainage.

Liposomes These vesicles are formed by a concentric lipid bilayer that entraps an aqueous core. The lipid membrane can be formed with phospholipids, lecithin and/or cholesterol. Hydrophobic drugs can be incorporated in the bilayer, whereas hydrophilic drugs can be loaded in the aqueous core.

Passive targeting It involves the development of a drug delivery system capable of avoiding the mechanisms of elimination from the organism (metabolism, excretion and opsonization followed by phagocytosis), thus increasing the circulation time and selectively accumulating in a target tissue. This can be achieved through the manipulation of certain properties, such as the molecular weight and the size of the carrier, charge on the surface and its hydrophilic or hydrophobic nature.

Polymer–drug conjugates The conjugation of a drug to either a natural or synthetic polymer results in a nanocarrier, the three main components are: the polymeric carrier, the polymer–drug linker and the drug payload. Moreover, imaging residues and targeting moieties can be added to this complex structure.

Polymeric nanoparticles These nanocarriers are colloidal polymeric particles with sizes that vary from 100 to 1000 nm that can be made of either natural or synthetic polymers.

Polymeric micelles These nanocarriers comprise amphiphilic block copolymers that self-assemble into spherical structures, with sizes ranging between 20 and 200 nm. They comprise an inner hydrophobic core, in which poorly aqueous soluble drugs are loaded, and an outer hydrophilic corona that helps to protect and stabilize the encapsulated drug.

rhodomycin-like anthracycline complex with remarkable antitumor properties at very low doses (0.05–0.5 mg/kg), indicating the potential of anthracyclines in cancer treatment. The microorganism was later isolated from a soil sample obtained in Apulia, Italy, and was named *S. peucetius*. After several fractionation studies, the main active compound was isolated: daunorubicin, observing that it was found principally in the mycelium. Almost at the same time, another research group in France at Rhône-Poulenc Laboratories independently isolated the same substance from *Streptomyces coeruleorubidus*, naming it rubidomycin, but the name given by the Italians was chosen because it reflected the dual origin [3]. A few years later, clinical trials began and daunorubicin was proved to be successfully applied in the treatment of acute leukemia and lymphoma, although fatal cardiac toxicity was identified as one of the main adverse effects [4]. Arcamone *et al.* continued studying structurally related compounds to daunorubicin with the objective of discovering new successful antineoplastic agents and found that, after a mutagenic treatment of the parent culture of *S. peucetius* with *N*-nitroso-*N*-methyl urethane, the surviving colonies of *S. peucetius* var. *caesius* accumulated a new daunorubicin-related compound: adriamycin (this name was later changed to DOX), and physicochemical characterization was thereby performed [2].

Nowadays, in efforts to obtain commercially significant amounts of DOX, genetic engineering is applied for strain improvement and this cytostatic drug is mainly isolated from *S. peucetius* ATCC 27952. To guarantee cost-effective production, several studies have been carried out, employing different genetic engineering techniques [5]: expression of a global regulatory gene called *afsR* [6]; overexpression of structural sugar and glycosyltransferase genes [7]; introduction of multicopies of resistant genes; granting additional resistance against daunorubicin and DOX [8]; inactivation of DOX-modifying enzymes [9,10]; and of a global antibiotic downregulatory gene known as *wblA* [11]. By means of all these modifications in the biosynthetic pathways of *S. peucetius* ATCC 27952, the production of DOX has been successfully increased through the years [5].

DOX is currently indicated by the FDA for the following neoplastic conditions: acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms' tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkin's disease, malignant lymphoma and bronchogenic carcinoma in which the small-cell histologic type is the most responsive compared with other cell types. DOX is also indicated as a component of adjuvant therapy in women with evidence of axillary lymph node involvement secondary to resection of primary breast cancer (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/050467s070lbl.pdf).

Properties

DOX is an anthracycline antibiotic with antitumor activity, originally isolated from *S. peucetius* var. *caesius*. It is an amphiphilic molecule comprising a water-insoluble aglycone (adriamycinone: $C_{21}H_{18}O_9$) and a basic, reducing, water-soluble, amino-sugar functional group (daunosamine: $C_6H_{13}NO_3$) [12]. Anthracyclines are polyketides that contain a tetracenequinone ring structure bonded to a sugar by glycosidic linkage (Fig. 1; http://www.drugbank.ca/drugs/DB00997). Owing to the presence of three main ionizable groups, DOX exhibits three different pK_a values: pK_1 = 8.15, associated with the amino group in the sugar moiety; pK_2 = 10.16, related to the phenolic group at C_{11}; and pK_3 = 13.2, as a result of the phenolic group at C_6 [13,14]. The soluble form of DOX is the hydrochloride salt (DOX HCl), which is a hygroscopic, crystalline, thin, needle-like, orange-red powder, whose aqueous solutions are yellow–orange at acidic pH, orange–red at neutral pH and violet–blue at pH > 9.0 (https://cameochemicals.noaa.gov/chemical/19724). This color shift is due to the presence of a dihydroxyanthraquinone chromophore in the molecule. Any variation in the groups of the chromophore can ultimately lead to a change in the absorption spectrum, thus it depends on several factors: pH, binding ions and their concentration, ionic strength, solvent type and drug concentration. Deprotonation of the chromophore directly affects the UV, visible and circular dichroic (CD) spectra [15]. Its absorption maximums in methanol are reported as 233, 252, 288, 479, 496 and 529 nm [16]. DOX HCl presents a melting point of 229–231°C and log *P* = 1.27. It is soluble in water (~2%) and in

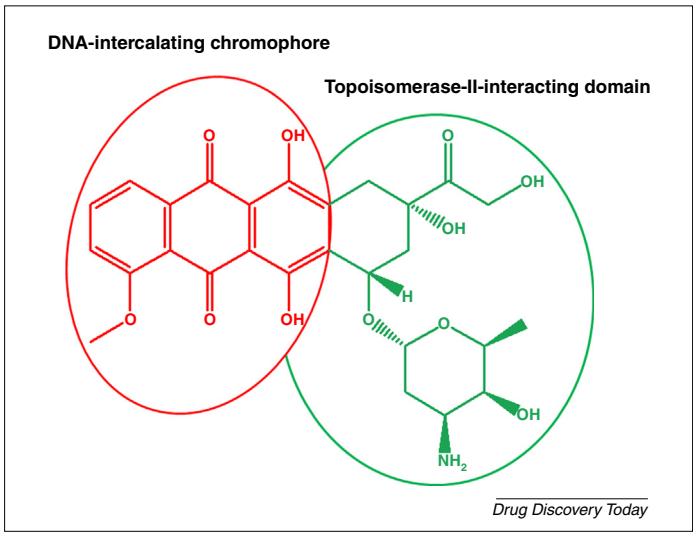

FIGURE 1

Structure–activity relationship of DOX. The cytotoxic effect of DOX is based on DNA intercalation, in which the drug chromophore (red font) is inserted between neighbor base pairs. This moiety could also be involved in free radical formation, because the semiquinone radical can intercalate into DNA, causing DNA damage and lipid peroxidation. However, the main biological event responsible for the cytotoxic effect of DOX is the interference of the catalytic cycle of the enzyme topoisomerase II (TOP II) by the TOP-II-interacting domain (green font). This interference leads to breaks in the DNA strand and to the formation of a DOX–DNA–TOP II ternary complex, in which the enzyme is covalently bonded to the broken DNA strand. This crucial event finally causes apoptosis and cell death.

aqueous alcohols, moderately soluble in anhydrous methanol and insoluble in nonpolar organic solvents (https://pubchem.ncbi.nlm.nih.gov/compound/doxorubicin#section=Top).

Mechanism of action

Among all the different ways in which a chemotherapeutic drug can exert its effect on a malignant cell, it is reported that DOX acts on the nucleic acids of dividing cells by two main mechanisms: (i) intercalation between the base pairs of the DNA strands, thus inhibiting the synthesis of DNA and RNA in rapidly growing cells by blocking the replication and transcription processes [17]; and (ii) generation of iron-mediated free radicals, causing oxidative damage to cellular membranes, proteins and DNA. This occurs because the quinone structure of DOX participates in redox reactions as an electron acceptor, being turned into a semiquinone free radical by several enzymes: mitochondrial NADH dehydrogenases located in the sarcoplasmic reticulum and mitochondria; cytosolic NAD(P)H dehydrogenase; xanthine oxidase; and endothelial nitric oxide synthase [17,18]. This unstable metabolite can provoke injury to the DNA itself or can be converted back to the quinone form, producing reactive oxygen species (ROS), such as superoxide, hydroxyl radicals or peroxide [19]. At the same time, ROS can cause oxidative stress, lipid peroxidation, membrane and DNA damage and trigger apoptosis [20]. What is more, DOX also inhibits the enzyme topoisomerase II (TOP2A), preventing to a greater extent the replication and transcription of DNA and its repair as the relaxing of supercoiled DNA is blocked [21].

Adriamycin®: the first formulation

The first commercially available formulation of DOX was Adriamycin®, approved by the FDA in 1974 (https://dtp.cancer.gov/timeline/flash/FDA.htm). It came as a DOX HCl solution or as DOX HCl lyophilized powder for injection, marketed by Bedford Laboratories™. When used in adjuvant breast cancer therapy, the recommended dose of DOX is 60 mg/m^2, administered as an intravenous bolus on day 1 of each 21-day treatment cycle in combination with cyclophosphamide (CPP) for a total of four cycles [22]. When utilized in a metastatic disease such as leukemia or lymphoma, the recommended dose of DOX as a single agent is 60–75 mg/m^2 intravenously every 21 days, whereas when administered in combination with other chemotherapeutic agents it is 40–75 mg/m^2 intravenously every 21–28 days. In the case of heavily pretreated, elderly or obese patients it is recommended to use the lower dose [23].

Regarding the pharmacokinetics, the distribution half-life is approximately 5 min and the terminal half-life is 24–48 hours (36.6 ± 15.5 hours) [24]; distribution volume in the steady state varies from 391 to 1281 l/m^2 [25] and between 50% and 85% of DOX and its major metabolite, doxorubicinol, are bonded to plasma proteins [26]. Hepatic metabolism and biliary clearance are the main excretion routes, because >40% of the dose appears in the bile in 5 days, whereas only 5–12% of the drug and its metabolites appear in the urine [26].

As with any other chemotherapeutic agent, DOX presents several adverse effects, including cardiotoxicity, reversible alopecia, hyperpigmentation of nailbeds and dermal creases, onycholysis, rash, itching, photosensitivity, nausea, vomiting, mucositis (stomatitis and esophagitis), ulceration and necrosis of the colon, anorexia, abdominal pain, dehydration, diarrhea, hypersensitivity reactions (fever, chills, urticaria and anaphylaxis), myelosuppression, peripheral neurotoxicity and ocular adverse effects (rare) such as conjunctivitis, keratitis and lacrimation. It is worth stressing that dose-limiting toxicities of therapy are myelosuppression and cardiotoxicity [27]. Recent evidence suggests that positively charged DOX preferentially accumulates in the mitochondria of myocytes, apparently because of its high affinity for a negatively charged lipid known as cardiolipin, located predominantly in the mitochondrial membranes abundant in heart tissue [28]. Although the exact mechanism of cardiotoxicity is not clearly elucidated, it is assumed that it is related to the production of free radicals and DOX–iron complexes in mitochondrial membranes, inducing an increase in the permeability of the inner membrane of the heart mitochondria, thus dissipating the membrane potential and releasing pre-accumulated Ca^{2+}, which finally leads to mitochondrial dysfunction, loss of myocytes and cardiac failure [29]. In the past few years, new explanations regarding the mechanisms of DOX-related cardiotoxicity have emerged, including anthracycline-dependent regulation of major signaling pathways controlling DNA damage response, myocyte survival, gene expression modulation, energetic stress and cardiac inflammation [30].

Nanotechnology in cancer

According to the European Commission, a nanomaterial is 'a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size

distribution, one or more external dimensions is in the size range 1–100 nm' [31]. Among scientists, however, the boundaries of this definition are still diffuse, because the conventional physicochemical rules might not be entirely applicable to the nanoscale [32]. First, there is a large number of nanomaterials that tend to form agglomerates with sizes >100 nm, owing to huge surface energies [33]. Moreover, the wide size distribution of these materials challenges any single parameter-based definition, because it might not provide a clear criterion whether a material can or cannot be defined as 'nano' [32]. Therefore, depending on the field in which nanotechnology is applied, the size limit used to determine whether a particle is accepted as a nanoparticle or not can vary. Particularly in the nanomedicine field, materials with dimensions up to 1000 nm are considered to be nanomaterials [34]. With this in mind, the following questions arise: what is the importance of these objects being so minuscule? What advantages do nanomaterials bring to modern medicine?

The first nanosystems were developed to improve diagnosis methods and the efficacy and safety of certain drugs that presented low bioavailability and dose-limiting adverse effects, respectively. These new cancer therapies were designed drawing upon the well-known enhanced permeability and retention (EPR) effect, a unique phenomenon presented by solid tumors, owing to their anatomical and physiopathological features that differentiate them from normal tissues (Fig. 2). These tumors show selective extravasation and retention of the drug-carrying nanomaterials, because the endothelial cells in the malignant blood vessels exhibit large gaps ranging from 100 nm to several hundred nanometers between them, compared with normal blood vessel junctions (5–10 nm). Furthermore, in normal tissues, drug-loaded nanosystems are cleared by the lymphatics, but in solid tumors most of the lymphatic vessels are compressed and collapsed – hence the nanovehicles are selectively retained, resulting in the EPR effect [35,36].

These days, the advances in the fields of biomaterials and physics have enabled the appearance of new applications, such as photothermal therapy with gold nanoparticles or hyperthermia with superparamagnetic nanoparticles [34]. Nevertheless, the main goals of nanomaterials are: active targeting to a specific tissue or cellular type, the development of novel controlled drug delivery systems, evaluating the possibility of co-encapsulating more than one active principle and improvements not only in the pharmacokinetic and pharmacodynamic parameters but also in the safety profile of drugs, as well as relieving some of their side effects. In this sense, taking into account its physiopathological characteristics and the fact that it is one of the most devastating diseases all over the globe, cancer has been one of the most studied fields in which nanotechnology has been applied.

Nanotechnology-based DOX formulations

From the clinical point of view, DOX is considered as one of the most effective chemotherapeutic agents ever developed against a broad range of cancers. As previously described, this first-line drug in cancer therapy, however, presents several adverse effects – its cumulative dose-dependent cardiotoxicity being the most dangerous one – together with other previously mentioned concerning side effects. All these factors make DOX an attractive alternative to work with when thinking of developing a drug-loaded nanotechnological product [37]. Several nanotechnology-based DOX

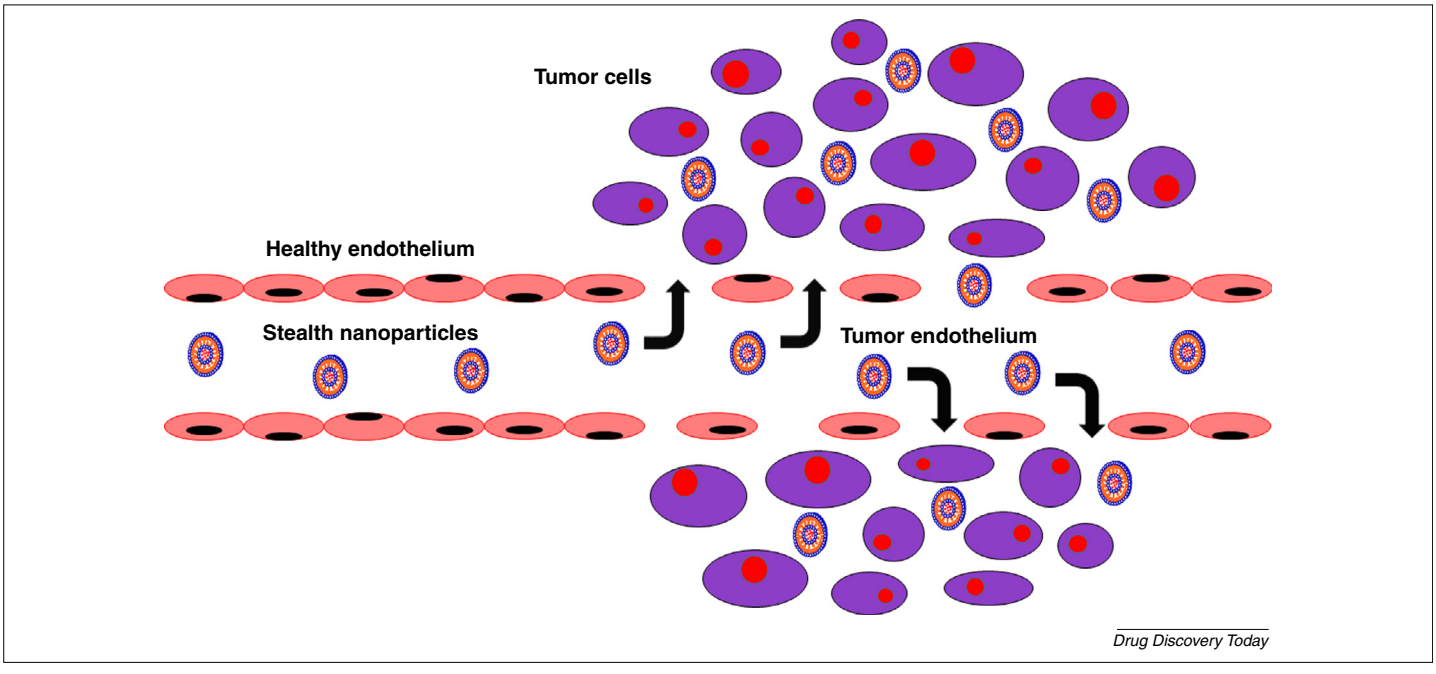

FIGURE 2

Representative scheme of a passively targeted drug delivery system accumulated by the enhanced permeability and retention (EPR) effect. This unique phenomenon exhibited by solid tumors differentiates them from normal tissues, owing to their anatomical and physiopathological features. The endothelial cells in malignant blood vessels present large gaps between them that can vary from 100 nm to several hundred nanometers, when compared with normal blood vessel junctions (5–10 nm). This size difference enables drug-loaded nanocarriers to permeate although tumoral endothelium. Moreover, in normal tissues drug-loaded nanovehicles are cleared by the lymphatics. However, in solid tumors most of the lymphatic vessels are collapsed and compressed, selectively retaining these drug-loaded nanosystems.

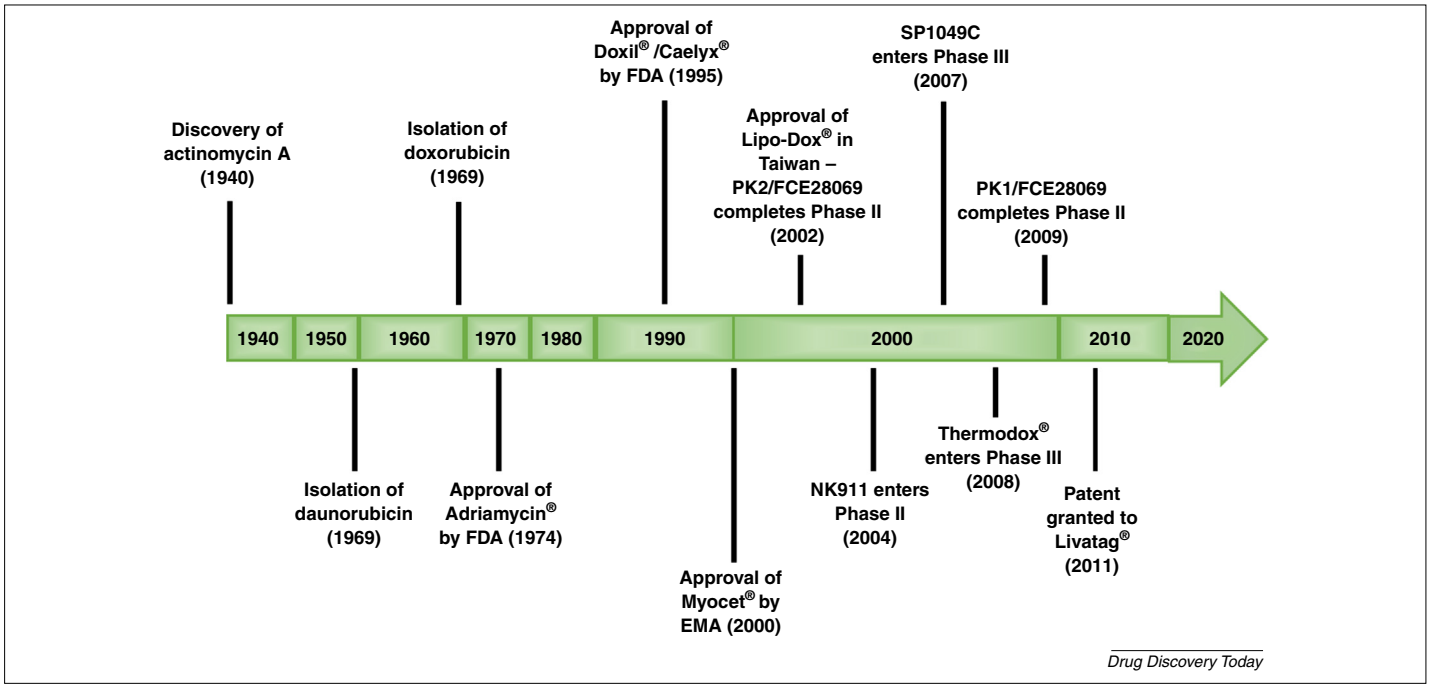

FIGURE 3

Timeline based on the history and pharmaceutical research of DOX. In 1940, the first antibiotic with antitumor activity was discovered by Waksman and Woodruff [3]. Almost 20 years later, the first anthracycline compound with anticancer activity, daunorubicin, was isolated from *Streptomyces peucetius*. After ten years researching structurally related compounds to daunorubicin, Arcamone and co-workers managed to isolate DOX from *S. peucetius* var. *caesius* [2]. In 1974, the first commercially available DOX formulation, Adryamicin®, was approved by the FDA. However, it was not until 1995 that the first DOX-based nanotechnological platform, PEGylated liposomal Doxil®/Caelyx®, reached the market. Since then, several DOX-loaded nanosystems have been clinically studied and, some of them, like Myocet® and Lipo-Dox®, approved by the European Medicines Agency (2000) and by the Department of Health of Taiwan (2002), respectively.

preparations have been designed since the 1990s (Fig. 3), some of them FDA approved, such as PEGylated liposomal Doxil® or liposomal Myocet® and others in clinical trials, like micellar NK-911®, nanoparticles of Livatag® or polymer–drug conjugates PK1 and PK2 (Table 1) [34]. All these formulations will be fully described below.

Liposomes

The first nanodrug delivery systems ever described in history were liposomes (see Glossary; Fig. 4a), originally defined as 'phospholipid spherules' by Bangham in 1965 [38]. These vesicles are formed by a concentric lipid bilayer that entraps an aqueous core. The lipid membrane can be formed with phospholipids, lecithin and/or cholesterol and hydrophobic drugs can be incorporated in this bilayer, whereas hydrophilic drugs can be loaded in the aqueous core [39]. Liposomes are usually characterized in terms of size, morphology and surface charge and can be classified as follows: multilamellar large vesicles (MLV; 1–2 μm), large unilamellar vesicles (LUV; 100–200 nm) and small unilamellar vesicles (SUV; 25–50 nm) [40–42]. Drug loading into liposomes can be achieved either passively or actively. The former includes three different methods where the drug is loaded during the formation of the nanocarrier: (i) mechanical dispersion method; (ii) solvent dispersion method; and (iii) detergent removal method [43,44]. Hydrophobic drugs, such as amphotericin B or paclitaxel, can be directly loaded into these vesicles during their formation, attaining high entrapping effectiveness (~100%), depending on the drug solubility in the liposome membrane. However, the

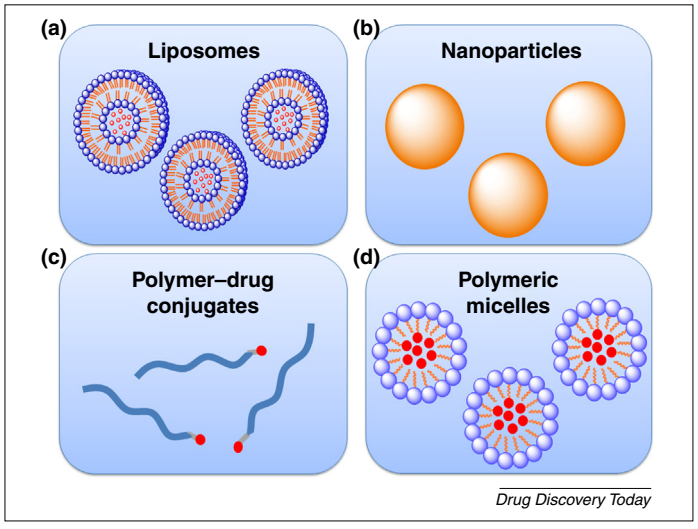

FIGURE 4

Developed nanodrug delivery systems for encapsulation of DOX. Among all the available nanotechnological platforms, only **(a)** liposomes, **(b)** nanoparticles, **(c)** polymer–drug conjugates and **(d)** polymeric micelles have successfully been employed to load DOX and reach clinical stages. Particularly, liposomal formulations, such as Doxil®/Caelyx®, Myocet® and Lipo-Dox®, are the only nanotechnology-based strategy that has reached the market. By contrast, liposomal Thermodox®, Livatag® nanoparticles and micellar SP1049C are currently in Phase III clinical trials, whereas polymer–drug conjugates PK1, PK2 and micellar NK911 are still in Phase II clinical studies.

employment of passive loading for water-soluble drugs is often limited by the trapped volume delimited in the liposome and by the drug solubility, exhibiting lower trapping effectiveness (<30%) [45]. For this reason, water-soluble drugs with ionizable groups, such as DOX, are usually loaded employing active loading techniques, like pH gradients, where the drug is entrapped after the formation of the nanocarrier, obtaining high trapping effectiveness (~100%) [46,47].

Doxil®/Caelyx®

In November 1995, the Oncologic Drugs Advisory Committee (ODAC) recommended FDA approval of Doxil® and, 1 year later, it was commercialized in the USA as Doxil® and in the European Union (EU) as Caelyx®. To date, this product is marketed by Johnson & Johnson and indicated for the treatment of AIDS-related Kaposi's sarcoma (1995), recurrent ovarian cancer (1998), metastatic breast cancer (MBC) (2003) and as monotherapy in patients with elevated cardiac risk and multiple myeloma (2007) [37,48]. It consists of a DOX-loaded PEGylated liposomal bilayer with a size of 80–90 nm, comprising hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL) and methyl-distearoyl phospho-ethanolamine PEG 2000 (DSPE–PEG) sodium salt in a weight ratio of 3:1:1 (molar ratio of 56.51:38.18:5.31) [49]. The researchers who designed Doxil® had previously failed with a non-PEGylated liposomal formulation (OLV-DOX) in a clinical trial performed in 1987, because the pharmacokinetics of the drug showed that it was quickly released from the liposomes in plasma, resulting in undesired cardiotoxicity and, moreover, these liposomes were rapidly cleared by the reticuloendothelial system (RES) of the liver and spleen and, to a lesser extent, by the bone marrow. To overcome these inconveniences, they thought of a PEGylated lipid nanosystem to avoid the liposomes to be captured by the RES, thus extending the circulation time in human plasma. However, as a result of the long-lasting circulation of the PEGylated liposomes to the skin capillaries, a grade 2/3 of desquamating dermatitis known as palmar plantar erythrodystesia (PPE) or foot-and-hand syndrome appears as a dose-dependant adverse effect, inherent to this formulation. It is characterized as a redness, tenderness and peeling of the skin that is more likely to occur in 3-week intervals than in 4-week schemes [50]. In particular, the components of Doxil® have exhibited an adverse effect known as complement-activation-related pseudo-allergy (CARPA) which involves flushing and shortness of breath. Furthermore, it has been suggested that DOX can indirectly induce CARPA [51]. This infusion reaction can be diminished by premedication and by slowing the infusion rate [52]. The researchers also proposed a remote (active) loading approach, based on a transmembrane gradient of ammonium sulfate [with a higher concentration of $(NH_4)_2SO_4$ inside the liposome than in the external medium] that works as the primary force for an efficient and stable remote loading of amphipathic weak bases into already formed liposomes, granting a more stable loading and sufficient levels of loaded liposomes to reach the tumor at therapeutic doses of drug [37].

In more than ten Phase I/II clinical trials that included patients with AIDS-related Kaposi's sarcoma, higher response rates with significant lower toxicities were observed in the group of Doxil® when compared with conventional therapy (DOX, bleomycin and vincristine) and a relatively subtoxic dose of Doxil® (20 mg/m^2) was found to be safe and effective [53]. Moreover, in two randomized Phase III clinical trials with more than 100 patients each, the liposomal formulation exhibited the best response rates, in comparison to conventional therapy, because only 23% and 25% of the patients responded partially to the conventional treatment, whereas 53% and 45% exhibited a partial response (PR) when administering the liposomal formulation [54,55]. What is more, in one of these Phase III trials, six patients completely responded to the treatment with Doxil®, whereas only one achieved complete response (CR) with conventional therapy [54].

As for patients with MBC, the results of a multicenter Phase III clinical trial with schemes of Doxil® 50 mg/m^2 every 4 weeks on one arm and free DOX 60 mg/m^2 every 3 weeks on the other arm showed a better toxicity profile with lower risk of cardiac events and congestive heart failure with Doxil® than with free DOX ($P = 0.0006$) and fewer cases of myelosuppression, alopecia and nausea were detected with the liposomal formulation. The efficacy of Doxil® and free DOX were comparable, because the response rates (complete plus partial response rates; 33% vs. 38%), median duration of response (7.3 vs. 7.1 months) and median overall survival (21 vs. 22 months) were similar [56].

In a Phase III randomized multicenter clinical study performed by Gordon *et al.*, long-term survival of patients with recurrent and refractory epithelial ovarian cancer treated either with Doxil® 50 mg/m^2 every 28 days ($n = 239$) or topotecan 1.5 mg/m^2 per day for 5 days every 21 days ($n = 235$) were compared. They found an 18% reduction in median survival of the patients treated with Doxil®, in comparison with those treated with topotecan (62.7 vs. 59.7 weeks, respectively; $P = 0.05$). When analyzing platinum-sensitive patients, they observed that the survival benefit was pronounced (63.6 vs. 57.0 weeks; $P = 0.038$) [57]. On the basis of these results, the PEGylated liposomal formulation was established as the new first-line therapy for recurrent ovarian cancer [58].

With regard to multiple myeloma, a Phase III multicenter randomized clinical trial with 192 newly diagnosed patients was performed by Rifkin and colleagues. They were treated either with Doxil® 40 mg/m^2 and vincristine 1.4 mg/m^2 as intravenous infusion on day 1 together with a reduced dose of dexamethasone (40 mg) administered orally on days 1–4 or vincristine 0.4 mg per day, conventional DOX 9 mg/m^2 per day as a continuous intravenous infusion on days 1–4 and a reduced dose of dexamethasone for, at least, four cycles. This scheme was repeated every 4 weeks, until disease progression, occurrence of unacceptable toxicity, maximal response or transplantation. The results showed that treatment with the liposomal preparation exhibited significantly reduced grade 3/4 neutropenia (10% vs. 24%; $P = 0.01$), reduced need for antibiotics, central venous access ($P < 0.0001$) and growth factor ($P = 0.03$), lower incidence of sepsis and fewer cases of alopecia (20% vs. 44%; $P < 0.001$) but more PPE (25% vs. 1%; $P < 0.001$) when compared with the treatment with conventional DOX. Both schemes, however, exhibited comparable efficacy, because objective response rates (44% vs. 41%), progression-free survival and overall survival were similar [59].

Recently, since February 2013, there has been a shortage of Doxil®, a fact that led to the FDA approval of a generic of this formulation, named Lipodox®, manufactured by Sun Pharma (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm337872.htm). Regulatory approval of

TABLE 1

Most-relevant nanotechnology-based DOX formulations on the market or in clinical stages

Nanosystem	Name	Composition	Size (nm)	Indication	Status	Refs
Liposomes	Doxil®/Caelyx®[a]	Hydrogenated soy phosphatidylcholine/cholesterol/ methyl-distearoyl phospho-ethanolamine-polyethylene glycol 2000	80–90	AIDS-related Kaposi's sarcoma – ovarian cancer – metastatic breast cancer – multiple myeloma	Approved	[37,43,44]
	Myocet®	Phosphatidylcholine/cholesterol	190	Metastatic breast cancer	Approved	[42,54,55]
	Lipo-Dox®	1,2-Distearoyl-sn-glycero-3-phosphocholine/polyethylene glycol	ND	AIDS-related Kaposi's sarcoma – ovarian cancer – metastatic breast cancer	Approved[b]	[48,59,60]
	ThermoDox®	1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine/1-stearoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxypoly-ethyleneglycol 2000	100	Primary liver cancer	Phase III	[75,76]
Nanoparticles	Livatag®	Polyisohexylcyanoacrylate	100–300	Primary liver cancer	Phase III	[49,68]
Polymer–drug conjugates	FCE28068/PK1	*N*-(2-Hydroxypropyl)methacrylamide-doxorubicin	ND	Breast cancer – non-small-cell lung cancer – colorectal cancer	Phase II	[27,70]
	FCE28069/PK2	Galactosamine-*N*-(2-hydroxypropyl)methacrylamide-doxorubicin	ND	Primary or metastatic liver cancer	Phase II	[27,85]
Polymeric micelles	SP1049C	Pluronics® L61/F127	22–27	Adenocarcinoma of the esophagus and gastroesophageal junction	Phase III	[79,80]
	NK911	Doxorubicin-conjugated poly-aspartic acid/polyethylene glycol	40	Metastatic pancreatic cancer	Phase II	[82,83]

[a] This formulation is commercialized in the USA as Doxil® and in the EU as Caelyx®.
[b] Approved in Taiwan.*Abbreviation*: ND, no data.

generic liposomal formulations remains challenging. To date, the only existing document that clearly states how to prove bioequivalence among PEGylated liposomal DOX (PLD) injectable formulations was published by the FDA in 2010 (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf). In these cases, the product-specific guidance defines detailed standards for the evaluation of generic PLD, as the same drug-loading process, composition and equivalent liposome characteristics. According to this, *in vitro* dissolution assays and pharmacokinetic studies in humans should be employed to demonstrate bioequivalence [60]. By contrast, the European Medicines Agency (EMA) published a guideline for marketing authorization of generic intravenous liposomal products developed according to an innovator product (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140351.pdf). However, this general document does not define a specific analytical, clinical or non-clinical strategy, because it provides only general principles for evaluating liposomal products, case by case (http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004011.pdf). Therefore, clearer guidelines are needed for characterization and quality control of nanosimilars (i.e. generic derivatives of nanomedicine products).

Myocet®

Five years after the approval of Doxil®/Caelyx®, the EMA approved Myocet® (previously named Cephalon®) in the EU and in Canada, a non-PEGylated alternative that, as with Doxil®, showed improvements in its pharmacokinetic and toxicity profile when compared with conventional DOX, and diminished the appearance of the hand–foot symptom produced by the PEGylated preparations [42,61]. It consists of a liposomal membrane of phosphatidylcholine (PC) and cholesterol (CHO), in which the drug is physically entrapped, with a size of 190 nm. It is currently commercialized by Teva Pharma and was approved in the year 2000 as the first-line treatment of MBC [62]. Three crucial Phase III clinical trials evaluated whether this liposomal formulation was superior to conventional DOX or other anthracyclines in terms of cardiotoxicity and efficacy.

In a study performed by Batist *et al.*, the patients ($n = 297$) received either intravenous Myocet® or conventional DOX at a dose of 60 mg/m^2 combined with CPP at a dose of 600 mg/m^2 repeatedly every 3 weeks until progression of the disease or symptoms of unacceptable toxicity were observed. Both groups exhibited similar response (≈43%) and median survival rates (19 vs. 16 months; $P = 0.79$), median time to progression (5.1 vs. 5.5 months; $P = 0.82$) and time to treatment failure (4.6 vs. 4.4 months; $P = 0.30$) but the Myocet® group showed significantly lower cardiotoxicity (6% vs. 21%; $P = 0.0002$) and lesser grade 4 neutropenia (neutrophils <500 mm^3) (61% vs. 75%; $P = 0.017$), as compared to non-liposomal DOX. What is more, the median cumulative dose of DOX at the first occurrence of cardiotoxicity was greater than 2220 mg/m^2 in the Myocet® group, whereas that of conventional DOX was inferior (480 mg/m^2; $P = 0.0001$) [63]. In another Phase III

clinical study ($n = 224$) carried out by Harris *et al.*, patients with MBC were intravenously administered 75 mg/m^2 of Myocet® or conventional DOX every 3 weeks as monotherapy until disease progression or unacceptable toxicities occurred. The results showed that the cardiotoxicity produced by the Myocet® group was significantly lower than that produced by non-liposomal DOX (13% vs. 29%; $P = 0.0001$), whereas the efficacy parameters (overall response rate, time to disease progression and median survival rate) were comparable. Moreover, fewer cases of grade 3 or 4 infections and grade 3 or 4 nausea and vomiting occurred with the liposomal formulation when compared with those produced by conventional DOX. Again, the median cumulative DOX dose at onset of cardiotoxicity was greater with Myocet® than with the non-liposomal preparation (785 mg/m^2 vs. 570 mg/m^2; $P = 0.0001$). There was only one case of grade 2 PPE reported with the liposomal formulation [64]. Two years later, Chan and co-workers designed a Phase III clinical trial, in which they randomized 160 MBC patients to receive either 75 mg/m^2 of Myocet® or 75 mg/m^2 of EPI, both combined with 600 mg/m^2 of CPP. Unlike the previous studies, the liposomal formulation exhibited superior efficacy in comparison with EPI: time to treatment failure (5.7 months vs. 4.4 months; $P = 0.01$) and time to disease progression (7.7 months vs. 5.6 months; $P = 0.02$) were longer, without significant differences in the overall survival rate and both groups presented low cardiotoxicity (11.8% vs. 10.2%). Nevertheless, the Myocet® group showed worse hematological toxicity and a significantly higher level of grade 4 neutropenia (87% vs. 67%; $P = 0.004$) [65].

Lipo-Dox®

In 2002, the Department of Health (DoH) of Taiwan approved the third DOX liposomal formulation that reached the market for the treatment of MBC, ovarian cancer and AIDS-related Kaposi's sarcoma [66]. It is currently manufactured by TTY Biopharm [67] and its lipid composition includes 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and is also coated by PEG. DSPC consists of saturated fatty acids that present a relatively high phase-transition temperature (T_m), making the preparation less prone to drug leakage, enhancing its stability [61].

In a Phase II clinical trial, 32 patients with platinum-sensitive PTX-pretreated recurrent epithelial ovarian cancer were administered Lipo-Dox® 40 mg/m^2 intravenously and, after the DOX infusion, carboplatin (CRP) equivalent to an AUC of 5–6 mg/min/ml for 30 min every day-28 of each cycle to evaluate the effectiveness and toxicity of this formulation. The patients received another cycle only if they did not present any unacceptable toxicity or if they exhibited stable disease or response after cycle 2. The results showed that 62% of the patients [confidence interval (CI) = 95%] achieved an overall objective response rate, a median progression-free survival of 9.1 months (CI = 95%) and an overall survival of 27.9 months (CI = 95%). They observed that the most common grade 3/4 toxicities were anemia ($n = 3$) and nausea/vomiting ($n = 3$), followed by leukopenia ($n = 2$) and thrombocytopenia ($n = 2$) [68].

Furthermore, a recent Phase II clinical study carried out in 45 patients with MBC who failed to respond to taxane-based treatments, treated with Lipo-Dox® 40 mg/m^2, CPP 500 mg/m^2 and 5-florouracil (5-FU) 500 mg/m^2 every 3 weeks, showed that the overall response rate, the median progression-free survival and the median overall survival were 41.9%, 8.2 months and 36.6 months, respectively. The researchers did not observe cases with decrease in the left ventricular eject function but they noted that in 14%, 9% and 1% of the cycles, respectively, grade 3/4 neutropenia, leucopenia and neutropenic fever occurred [69].

It is worth mentioning that all of these clinical studies were noncomparative and that there have been no Phase III clinical trials to date. Except from the higher half-life (~65 hours) and, thus, significantly longer *in vivo* circulation time, there is no other therapeutic improvement achieved by Lipo-Dox® so far, in comparison with Doxil® [61,70].

ThermoDox®

For the treatment of primary and metastatic liver cancer, thermal ablation techniques, as radiofrequency are one of the most applied therapies. Nevertheless, these kind of techniques exhibit a wide variability in terms of local failure rates [71,72]. In particular, it has been observed that liver tumors larger than 3 cm that had been treated using radiofrequency ablation resulted in one of the most significant risk factors of local recurrence [73]. These relapses were probably caused by untreated areas of microscopic disease at the margins of the treated lesions. It was then proposed that combining chemotherapy with radiofrequency ablation would possibly improve the effectiveness of the treatment in these zones of microscopic disease [74]. In this context, a lyso-thermosensitive liposomal DOX-based product manufactured by Celsion Corporation (ThermoDox®) has emerged as a viable formulation that exhibited encouraging results in Phase I clinical studies and went directly into Phase III clinical trials, but has not yet been approved [75]. These ~100 nm liposomes comprise three synthetic phospholipids that confer sensitivity to temperature, thus rapidly releasing the drug with mild thermal warming (>40°C) in the targeted tumor tissue: DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine), MSPC (1-stearoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine) and DSPE-MPEG 2000 [76].

In 2008, Celsion Corporation started a Phase III, randomized, double-blinded, dummy-controlled clinical trial (NCT00617981) that was last updated in 2014 (https://clinicaltrials.gov/ct2/show/NCT00617981). The pharmaceutical company reported a better clinical outcome in the overall survival in patients with single lesions ($n = 285$) with 50 mg/m^2 ThermoDox® over 30 min plus radiofrequency ablation for ≥45 min versus radiofrequency ablation for ≥45 min plus dummy infusion over 30 min (http://celsion.com/thermodox/). Recently, another Phase III, randomized, double-blinded, dummy-controlled clinical trial known as the OPTIMA study (NCT02112656), the primary and secondary outcome measures of which are overall survival and progression-free survival, respectively, has started to recruit patients with hepatocellular carcinoma to verify these data (https://clinicaltrials.gov/ct2/show/NCT02112656?term=OPTIMA+celsion&rank=1).

Polymeric nanoparticles

Nanoparticles are colloidal polymeric particles with sizes that vary from 100 nm to 1000 nm (Fig. 4b). These nanocarriers can be made of either natural or synthetic polymers, some of them are FDA approved and biocompatible, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), PLGA, poly-ε-caprolactone and

poly(methyl-methacrylate). Their preparation methods can be classified in two main classes: polymerization of the monomers (e.g. emulsion-polymerization method or dispersion-polymerization techniques) and dispersion of preformed polymers (e.g. nanoprecipitation and emulsification-diffusion methods). Drugs can be physically incorporated, chemically bound, ad- or ab-sorbed. When compared with other colloidal systems, nanoparticles present the advantage of being more stable, particularly in body fluids, after their administration [77,78].

Livatag®

On July 2011, Bioalliance Pharma SA (Paris, France) announced that their 100–300 nm DOX-loaded nanoparticles formed with polyisohexylcyanoacrylate (PIHCA), known as Livatag®, had been granted a patent until 2023 [62,79,80]. Currently, this preparation is under development by Onxeo and is in Phase III clinical studies. In a Phase II clinical trial, patients with liver cancer were treated either with Livatag® or with the current first-line therapy. The results showed that this nanoparticle formulation achieved a median survival of 32 months, whereas the first-line treatment reached only 15 months [62]. As expressed before, Livatag® is involved in an international randomized Phase III clinical trial that started in June 2012 and is carried out on over 400 patients with advanced hepatocellular carcinoma all across Europe and the USA (http://www.onxeo.com/site/wp-content/uploads/2016/04/160418EN-AACR-Livatag-Data-Release.pdf). Its main objective is to demonstrate the efficacy of the nanotechnological formulation in patients with hepatocellular carcinoma after intolerance or failure of sorafenib [81].

Polymer–drug conjugates

Among nanocarriers there are two main strategies that are generally applied to deliver the chemotherapeutic agent to the tumor site: physical entrapment of the drug in the hydrophobic core by electrostatic or hydrophobic interactions or conjugation of the antineoplastic drug to a hydrophilic or amphiphilic polymer by covalent linkage. Polymer–drug conjugates (Fig. 4c) usually present an adequate *in vivo* stability and prevent rapid drug release upon blood circulation dilution, in comparison with drug-loaded formulations [82]. In the past decade, several aqueous soluble polymer–drug conjugates have entered Phase I/II clinical trials as chemotherapeutic agents and, particularly, three of them transport DOX: PK1 (Phase II), DOX-OXD (Phase I) and PK2 (Phase II). The latter will be described in the ligand-based DOX-loaded nanoformulations section.

FCE28068/PK1

The first untargeted DOX–polymer conjugate that has reached Phase II clinical trials was developed by Pfizer and is known as PK1 or FCE28068 [34]. This formulation comprises *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer covalently bound to DOX by a peptidyl linker that is cleaved by lysosomal enzymes when taken up by malignant cells via pinocytosis, favoring the release of the active drug at the tumor site. In a Phase I clinical study performed in 36 patients with different types of refractory or resistant cancers, PK1 was evaluated to define the maximum tolerated dose (MTD), the toxicity profile and its pharmacokinetic parameters after 100 cycles (20–320 mg/m^2 DOX equivalent) of intravenous infusion every 3 weeks. The MTD resulted in 320 mg/m^2 and an extended plasma half-life was observed, because a three-order magnitude decrease in the clearance was found, when compared with free DOX. Moreover, no congestive cardiac failure was seen with cumulative doses as high as 1680 mg/m^2. Dose-limiting toxicities were mucositis and febrile neutropenia [83]. In a Phase II clinical trial carried out in 62 patients with breast ($n = 17$), colorectal ($n = 16$) and non-small-cell lung cancer (NSCLC) ($n = 29$); partial response (PR) was observed in ~10% of the cases: three patients with breast cancer and three with NSCLC. By contrast, no patients with colorectal cancer responded [84]. To the best of our knowledge, there has been no other published clinical evolution of this formulation.

Polymeric micelles

Polymeric micelles (PMs) are one of the most studied nanovehicles in diagnosis and therapy of several diseases. These nanocarriers comprise amphiphilic block copolymers that self-assemble into spherical structures (Fig. 4d), with sizes in the range 20–200 nm [85–88]. They are constituted by an inner hydrophobic core, in which poorly aqueous-soluble drugs are loaded and by an outer hydrophilic corona that helps to protect and stabilize the encapsulated drug and that could also be functionalized with different moieties, with the aim of achieving active targeting, pH or temperature response or a combination of them [89–91]. Among DOX micellar preparations, there are two well-known formulations that have reached Phase II (NK911) and Phase III (SP1049C) clinical trials and that are worth describing.

SP1049C

There is a vast number of copolymers that have been used for micelle formation. However, not all of them are biocompatible nor approved by regulatory agencies, such as the FDA, thus reducing the possible materials to be applied in the clinical field. Pluronics® are ternary copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) that gather these requirements. The hydrophobic core is formed by PPO segments, whereas the hydrophilic corona is formed by PEO blocks. Particularly, Pluronics® L61 and F127 have been utilized to develop a 22–27 nm DOX-loaded micellar formulation known as SP1049C, which has reached Phase III clinical trials [92]. This preparation is manufactured by Supratek Pharma (Canada) and has proved to be effective against adenocarcinoma in the esophagus and gastroesophageal junction (GEJ). In a Phase I clinical study of SP1049C performed in 26 patients with histologically proven cancer, the MTD resulted in 70 mg/m^2, whereas the dose-limiting toxicity was myelosuppression at a dose of 90 mg/m^2 [93]. A few years later, in a Phase II clinical trial, 21 patients with advanced adenocarcinoma of the esophagus and GEJ were treated with SP1049C at a dose of 75 mg/m^2 DOX equivalents intravenously every 3 weeks, until appearance of unacceptable toxicity or disease progression. The results showed an objective response rate of 47% (95% CI), a median overall survival of 10 months (95% CI) and a progression-free survival of 6.6 months (95% CI). The researchers observed that neutropenia was the main toxicity of the micellar formulation and that none of the patients exhibited grade 3/4 decrease in their left ventricular eject fraction [94].

NK911

Continuing with PMs, the other well-known micellar formulation that has reached Phase II clinical trials is NK911. In fact, it was the

first micellar preparation that proceeded into clinical evaluations in 2001 [95]. It consists of a DOX-conjugated poly-aspartic acid (ASP)/PEG nanocarrier with a particle size of ~40 nm [96]. In a Phase I clinical study, NK911 was administered intravenously to 23 patients with metastatic or recurrent solid tumors refractory to conventional chemotherapy, at a starting dose of 6 mg/m^2 (DOX equivalent) every 3 weeks, with the aim of defining the MTD, dose-limiting toxicities and evaluating its pharmacokinetic profile in humans. The results showed that grade 3 or 4 neutropenia were observed at doses of 50 and 67 mg/m^2, considering this as a hematological dose-limiting toxicity. Non-hematological toxicities were mild anorexia, stomatitis and alopecia at doses of 67 mg/m^2, thus defining the MTD as 67 mg/m^2 and the recommended Phase II dose as 50 mg/m^2 every 3 weeks. Plasma AUC of NK911 (3.2 μg/h/ml) was higher than free DOX (1.6 μg/h/ml), but significantly lower than that of PEGylated liposomes (902 μg/h/ml) [97]. However, the micellar formulation has proceeded into Phase II clinical studies against metastatic pancreatic cancer but the results have not been yet reported [95].

Ligand-based DOX-loaded nanoformulations

FCE28069/PK2

The different DOX-based nanotechnological approaches previously described were based on a passive drug targeting strategy. However, in recent years, many efforts have been made to actively target antineoplastic drugs to a certain cancer tissue or cell because of specific ligand–receptor interactions [98]. So far, the only DOX-active-targeting-based formulation that has reached clinical trials is known as FCE28069 or PK2. Unlike PK1, PK2 is a targeted DOX–polymer conjugate bound to galactosamine (GAL) residues. These mediate active liver targeting through the asialoglycoprotein receptor (ASGPR) of hepatocytes [99]. As the untargeted preparation, PK2 is a HPMA-based formulation, in which the drug is covalently bound to the copolymer by a peptidyl linker, also manufactured by Pfizer and is currently in Phase II clinical trials [34]. In a Phase I clinical study carried out by Seymour *et al.*, 31 patients with primary or metastatic hepatic cancer were evaluated to determine the toxicity, pharmacokinetic profile and targeting capability of PK2. The scheme consisted of a 1 hour intravenous infusion every 3 weeks, with a dose escalation from 20 mg/m^2 to 160 mg/m^2 DOX equivalents. The results showed that grade 4 neutropenia, grade 3 mucositis and severe fatigue were associated with a dose of 160 mg/m^2; thus defining the MTD. The recommended dose for subsequent clinical trials was established as 120 mg/m^2 DOX equivalents, administered intravenously every 3 weeks. Moreover, 24 hours after the administration, 16.9% of the drug was detected in the liver, whereas the untargeted formulation achieved no hepatic targeting [100].

Concluding remarks and future perspectives

According to a recent study, it is estimated that nanotechnology-based drug delivery systems will account for ~US$136 billion by 2021. Among the studied nanocarriers, it is important to point out that liposomes are expected to reach the leading total addressable market in 2021, representing ~US$15.3 billion (http://www.cientifica.com/wp-content/uploads/downloads/2012/04/NDD-White-Paper-Jan-2012.pdf).

Considering the focus of this review, as we previously described, there is a considerable number of nanotechnology-based DOX formulations in Phase II/III clinical trials and approved by health regulatory agencies, such as the FDA or EMA. Some of the ones that reached clinical studies have shown promising results, even when compared to conventional therapy. However, there are certain issues that should not be ignored.

First, it is not a coincidence that just liposomes among nanotechnology-based DOX formulations are the only nanocarriers with regulatory agency approval. There is no other type of DOX-loaded nanovehicle that has yet been approved by any of these regulatory agencies. This is probably because liposomes are the first nanosystems ever developed as well as commercialized and, hence, present the longest history of research and studies [101].

Second, it is worth mentioning that, currently, there is only one nanotechnological targeted DOX product in clinical studies and it has not even proceeded to Phase III clinical trials. However, the healthcare market has changed. It is clear that a paradigm shift has occurred, from conventional and generalized therapies to a more personalized medicine, in which the patient's genome and immune response are the spinal cord of improved therapeutics, based on targeted and more-effective nanotechnology platforms. Probably, the clinical benefits of these new ligand-based nanotechnological products will not take too long to appear. It seems that 'future medicines' are just around the corner.

Acknowledgements

Authors thank the University of Buenos Aires (Grant UBACyT 20020130200038BA). Maximiliano Cagel and Estefania Grotz are supported by doctoral scholarships of CONICET. Ezequiel Bernabeu, Marcela A. Moretton and Diego A. Chiappetta are partially supported by CONICET, Argentina.

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