Self-assembling drug-conjugates for anticancer treatment

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Teaser: In this review we summarize the recent advancements in nanoparticles obtained by self-assembling drug-conjugates, a useful tool to improve drug delivery of anticancer compounds.

Abstract: Self-assembling drug-conjugates preparation is a promising approach to improve the activity, the penetration through physiological barriers and to reduce side effects of potent small molecules. Drug-conjugates are able to self-assembly in water to form nanoparticles (NPs) that offer several advantages because: i) these are easy to obtain, ii) these can reach high drug local concentration in tumor tissues and iii) these may reduce the side effects of drugs. All these factors improve drug pharmacokinetic properties. In this article, we have reviewed the scope of nanotechnology-based self-assembling drug delivery approaches focusing on prodrugs able to form NPs by self-assembling and also summarized the current perspective and challenges facing the successful treatment of cancer.

1. Introduction

Many anticancer candidates are not able to show a pharmacological activity despite their potent cytotoxicity because a minimum concentration is not reached at the site of action. In fact, most anticancer drugs have their targets within cells and tissues and the low partition coefficient of drugs can prevent the cell barrier permeation and consequently their cytotoxic effect.

In recent years, nanotechnology has opened new perspectives for biological and biomedical applications to improve the selective delivery of anticancer compounds to their site of action. The use of nanotechnology in cancer treatment has some advantages [1]; in fact, tumour blood vessels are highly disorganized because they are characterized by an inefficient lymphatic network and endothelial cells are less tight compared with normal tissues. NPs can be preferentially delivered to the tumour site because of the enhanced permeation and retention (EPR) effect [2,3]. In addition to that, nanotechnologies can improve drug properties in several ways: by controlling release and distribution, by enhancing drug absorption, and by protecting the drug from degradation. Micelles, liposomes, nanospheres, nanocapsules, nanorods, nanofibers etc. are commonly used to load the drugs in the inner core or into the bilayer. A small but interesting niche inside anticancer nanotechnology is represented by nanosystems spontaneously obtained by self-assembly of conjugates composed by drugs linked to a proper chemical entity. The self-assembly of drug-conjugates into NPs is a process that consists in the formation of an ordered structure by spontaneous organization of building blocks as a consequence of specific local interactions. Assembly can be obtained with different methods and conditions but the key role of the amphilicity of the components is often fundamental.

In recent years, the use of drug-conjugates to obtain NPs has gained considerable attention [4-6]. These conjugates are usually obtained by a covalent coupling of the drug to biocompatible lipid moieties and the resulting molecules are able to form NPs by self-assembling. NPs are usually characterized by different techniques in order to be easily compared to each other and to predict their biological efficiency [7]. Crucial parameters for NPs characterization are the mean diameter, which influences their biodistribution and retention, and the surface charge, which is responsible for the interactions with the environment and physical and chemical stability. Electron microscopy, dynamic light scattering (DLS), NPs tracking analysis (NTA) and disk centrifugation are techniques relied on different physical principles and sample preparation useful for particle size determination. The surface charge of NPs (zeta potential) is generally measured by laser doppler electrophoresis, which evaluates electrophoretic mobility of suspended NPs in the medium.

The aim of this paper is to review the latest literature (2009-2015) about prodrugs able to form NPs by self-assembly with a specific focus on the functionalization of anticancer compounds. We discuss in paragraph 2 the characteristics of self-assembling inducers and in paragraph 3 the drugs modification and NPs biological evaluation.

Figures 1 and 2 represent the structures of the mentioned self-assembling inducers and of the drugs, respectively. In the Table 1 we summarize for the mentioned drugs the linker, the used self-assembling inducer and the corresponding characteristics of the obtained nanostructures together with the cancer cell lines used for the biological evaluation. The table also shows the cases in which the *in vivo* tests were reported.

2. Self-assembling inducers

Self-assembly is a process that is mediated by non-covalent interactions between molecules *via* ionic bonds, hydrogen bonding, hydrophobic interactions, and van der Waals interactions. Different organic molecules, such as biocompatible polymeric chains or endogenous molecules (terpenes or polysaccharides) have been used as self-assembling inducers by covalent linkage to drugs. The drug and the self-assembly inducer can be directly attached or can be connected by a linker, which can be stable in serum but able to release the drug intracellularly. In the literature there are different examples of the use of biocompatible polymeric chains as self-assembling inducers for the formation of NPs.

HPEE (hydrophilic hyperbranched poly(ether-ester)).

The use of biocompatible hydrophilic hyperbranched poly(ether-ester) (HPEE, **1**, Figure 1) for the construction of an amphiphilic copolymer with the hydrophobic drug paclitaxel (**7**, Figure 2) has been reported recently. This conjugate is able to self-assemble in water to form NPs with a diameter of 50-120 nm. The copolymer paclitaxel-HPPE NPs are biodegradable and they can be easily eliminated through excretion pathway *in vivo* because of the presence of many ester groups in the HPPE structure [8].

PEG (polyethylene glycol).

In another study, a multiarm-polyethylene glycol (PEG, **2**) was employed for the delivery of anticancer drugs by the formation of self-assembled NPs. A folate-eight-arm-PEG-betulinic acid was synthetized by connection of the poor water-soluble drug betulinic acid (**8**) (hydrophobic part), PEG (hydrophilic segment) and folate (target molecule). Also in this case the biocompatibility of the NPs is due to the ester bonds in the conjugate that guarantee the drug release inside the cell. Multiarm-PEGs have some advantages if compared to linear PEG such as the increase in molecular weight (low molecular weight PEG had been reported to be toxic for the kidneys) and the presence of many functional groups useful to improve the drug loading capacity [9].

The use of a linker between the self-assembling inducer and the drug is useful to guarantee the release of the active molecule. In 2010, the covalent conjugation of a Pt(IV) prodrug (**9**) to a self-assembling inducer through an acid-responsive linker was exploited for the formation of NPs to improve the therapeutic efficacy of compound **9**. In this case, a PEG-b-poly(L-lactide) copolymer was used to induce self-assembling because of the advantages of polymeric NPs. These NPs are able to prolong the half-life of drugs in the systemic circulation, limit drugs side effects and release drugs in a controlled manner. The acid-responsive bond favours the control of the drug release in an environmentally sensitive manner. This approach would be useful to minimize the release of the drug in the blood (pH 7.4) and to facilitate the release inside the cells (pH 5-6). The prodrug conjugate, characterized by the presence of an acid-responsive hydrazone bond, was synthetized and it was demonstrated to form NPs by self-assembling via precipitation method [10]. These NPs showed well-controlled drug loading yield, excellent acid-responsive drug release properties and potent cytotoxic activity against ovarian cancer [11].

Recently, two different self-assembling inducers covalently bonded have been used to form NPs: a glycol chitosan chain (**3**) and a PEG block. In addition, the synthetized conjugate consists of a functional 3-diethylaminopropyl isothiocyanate (DEAP) block and a photosensitizing drug, chlorine e6 (**10**). This conjugate self-organized in aqueous solution and some studies revealed that the glycol chitosan and PEG blocks are on the hydrophilic outer shell, and chlorine e6 is within the hydrophobic inner core. The advantages of using these self-assembling inducers are that a polysaccharide-drug conjugate is expected to provide an effective cancer therapy without affecting the safety of normal tissues and a PEG block may improve the stability of the drug conjugate in serum and the penetration into an *in vivo* tumor vasculature. In addition, this conjugate is characterized by the presence of a pH-sensitive functional group that allows the release of the photosensitive drug in acidic condition. In fact, it was previously shown that the pK_b value of a glycol chitosan conjugate with DEAP is near 6.8. In this study it was demonstrated that upon encountering tumor environment, both the diameter and the zeta potential of NPs change because of a variation in the pH value from 7.4 to 6.8. Also the shape of NPs is affected by the pH variation: they are spherical under neutral condition and become disentangled at pH 6.8 [12].

PEG was also used to induce self-assembling in a conjugate with Beclin-1 (**11**), an autophagy-inducing peptide. In particular, the amphiphilic poly(β-amino ester) copolymer used was composed by a hydrophobic monomer 1,6-hexanediol diacrylate, a pH-sensitive monomer 3-(dibutylamino)-1-propylamine and a hydrophilic amino-terminated PEG. This copolymer was synthetized by Michael addition and characterized by ^{1}H-NMR. The amphiphilic PEG-Beclin-1 polymers simultaneously self-assembled during dialysis from dimethyl sulfoxide into water into micelle-like NPs with a mean diameter of 32 nm. The efficacy of the pH-sensitive linker was confirmed by diameter measurements in different pH conditions: the dimension of NPs increase considerably, implying that NPs disassociate and reorganize into larger entities under weakly acidic conditions [13].

Du *et al.* described the use of PEG as a self-assembling inducer in the synthesis of a dual pH-sensitive polymer-doxorubicin (**12**) conjugate. The polyphosphoester was chosen as self-assembling inducer because of its biodegradability and its previous uses in biomedical fields. The parental diblock copolymer monomethoxyl PEG-b-poly-(allyl ethylene phosphate) was easily prepared by ring-opening polymerization. The obtained conjugate self-assembled to give nanostructures in aqueous solution. To prove the efficacy of the pH-sensitive linker, the NPs were incubated at different pH values and the release of the free drug was monitored. Varying the pH from 7.4 to 5, the release of doxorubicin increased markedly indicating the sensitivity of NPs to endo-/lysosomal pH. This suggests also that NPs can reduce premature drug release during circulation but specifically enhance intracellular drug release, which will be a very important feature in cancer treatment [14].

In 2010, the use of a oligomer chain of ethylene glycol (OEG) was reported to form an amphiphilic phospholipid-mimicking prodrug with the anticancer drug camptotecin (**13**). The nature and the dimension (eight repeating units) of the oligomer was selected as the water soluble part to maximize the drug loading content and also to lower the critical vesicle formation concentration. In this case, a linker characterized by the presence of a thioester bond was used because of its ability of being easily hydrolyzed by esterases, which are abundant in cells. This conjugate was demonstrated to form NPs with a diameter of about 180 nm and a negative surface charge. The *in vitro* hydrolysis of the conjugate appeared slow at weakly acidic or neutral pH. Instead, in the presence of esterases, which are abundant in cytoplasm, the conjugate quickly hydrolyzed and released the free drug. Therefore, OEG-camptotecin conjugate was confirmed to act as a prodrug for intracellular release of camptotecin [15].

HA (Hyaluronic acid).

Xin *et al.* reported the use of hyaluronic acid (HA, **4**) as a self-assembling inducer for the formation of three types of paclitaxel-containing NPs. HA is a linear polysaccharide widely distributed throughout connective, epithelial and neural tissues. It is composed of two alternating units of D-glucuronic acid and N-acetyl-D-glucosamine. HA can be easily used as self-assembling inducer because of its biocompatibility and biodegradability. In addition, it has a strong affinity to cell-specific surface markers overexpressed on the surface of many types of cancer cells, such as glycoprotein CD44 and receptor for hyaluronic acid-mediated motility. Furthermore, in this study different amino acids, such as valine, leucine and phenylalanine, were used as spacers between the drug and the carrier in order to improve the release of paclitaxel from the conjugates. Amino acids are easily used as linkers owing to their bifunctional nature; in fact, they are characterized by the presence of both a reactive carboxylic group to conjugate with the drug via an ester bond and the amino group that can be exploited for the linkage with the carrier. It was demonstrated that these conjugates with different aminoacidic linkers were able to self-assemble to form NPs characterized by a diameter of 275-285 nm [16].

Heparin.

In 2009, the same amino acids were used as spacers in another study in which the self-assembling inducer was heparin (**5**). Heparin is a biocompatible, biodegradable, and water-soluble natural polysaccharide with a structure consisting of a variable sulfated repeating disaccharide units. Heparin has different biological activities including anticoagulant activity, inhibition of tumor development and angiogenesis. The synthesis of paclitaxel-heparin conjugates was reported and it was confirmed that they self-assembled in aqueous solution, forming approximately spherical shaped NPs with a diameter of 140-180 nm composed of a paclitaxel core and carrier shell [17].

Figure 1. Structures of self-assembling inducers used for the formation of the conjugates. The anchor point used for the conjugation reaction with the active compound is highlighted in red.

Squalene.

Squalene is a precursor of cholesterol biosynthesis and it belongs to the terpenoid family. Its inertness and biocompatibility justify the use of squalene derivatives (**6**) [3,6,18-35] and terpenes in general [36-39] for the preparation of self-assembling bioconjugates, as Couvreur reported in several papers. Squalene can be easily functionalized at its terminal double bond due to its compact conformation in very polar solvents. Indeed, the highly coiled conformation leads the internal double bonds sterically shielded whereas the terminal olefinic links is exposed and thus more reactive. So, the terminal double bond can be converted in different functional groups such as aldehyde, carboxylic acid, alcohol or amine.

Recently, Couvreur *et al*. reported the formation of self-assembled NPs obtained by conjugation of squalene with two antiangiogenic multitarget tyrosine kinase inhibitors, semaxanib (**14**) and sunitinib (**15**) [19]. A pH-sensitive linker between the squalene chain and the active compound was introduced because many solid tumors are characterized by an acidic environment and because the intracellular release of the drug is favored by the acidic pH of endolysosomes. It was demonstrated that the bioconjugates self-assembled in NPs with narrow polydispersity in aqueous solution and an average diameter of 120-140 nm.

The introduction of a squalenic tail was further exploited for the derivatization of known anticancer compounds such as paclitaxel, podophyllotoxin (**16**), camptothecin, epothilone A (**17**) and cyclopamine (**18**) with the inclusion of an alternative linker [40-42]. In this study, the squalene moiety and the active compound were connected by a disulfide-containing linker able to guarantee the drug release inside the cell. Indeed, cancer cells express high levels of detoxifying enzymes such as glutathione-S-transferases (GSTs) and glutathione (GSH) to protect themselves from toxic xenobiotics. Testing NPs in the presence of GSH, a progressive slight increase of size was evident and a release, even moderate, of the free drug was observed.

Amphiphilic drug-drug conjugate.

An innovative self-delivery system in which drugs can be delivered by themselves without any carrier was developed by Huang *et al.* An amphiphilic drug-drug conjugate, consisting of the hydrophilic anticancer drug irinotecan (**19**) and hydrophobic anticancer drug chlorambucil (**20**) has been synthetized and demonstrated to be able to self-assemble in water to form NPs with a diameter of about 88 nm. As a result of the presence of an ester bond between the two drugs, the amphiphilic conjugate was able to release both the compounds after the hydrolysis of this bond in the tumor cells [43].

The same strategy was exploited in a recent study in which the hydrophilic gemcitabine (**20**) and the hydrophobic chlorambucil were linked via a hydrolyzable ester bond. The conjugate obtained was used to form NPs following the nanoprecipitation technique [10] and it was demonstrated to form NPs [44].

3. Drugs: modification and biological evaluation

Different anticancer compounds were used for the formation of self-assembled nanostructures by exploiting the presence of the available functional groups for the linkage with the self-assembling inducer.

Paclitaxel (**7**) is a powerful antitumor agent mainly used for the treatment of breast and ovarian cancer. It inhibits cell replication in the late G2/M phase of the cell cycle by interfering with the depolymerization of microtubules. However, its poor water solubility and toxicity are disadvantages in the use of paclitaxel in cancer treatment. Paclitaxel has been functionalized at the 2' position forming an ester bond, able to be easily cleaved to release the free drug. NPs obtained from the conjugate of paclitaxel with HPPE [8] were biologically evaluated on

MCF-7 (breast cancer) and Tca8113 (oral squamous carcinoma) cell lines and they showed potent cellular growth inhibition abilities (Table 1). This result indicated that NPs are able to enter the cell and it is conceivable that the acidic pH and the enzymes inside the cell would lead to a slow release of paclitaxel which is responsible for the therapeutic effect. *In vivo* evaluation was made using two malignant tumor cell lines of MCF-7 and Tca8113 to establish tumor models. These evaluations showed that NPs exhibit antitumor effect with a lower toxic effect with respect to the free drug. When paclitaxel was linked to HA using different aminoacidic linkers [16], the obtained NPs were evaluated *in vitro* on MCF-7 cell line. It was demonstrated that NPs are more cytotoxic than free paclitaxel and flow cytometry analysis showed that NPs significantly enhance the extent of apoptosis-induced cell death. The same authors reported the formation of a conjugate with paclitaxel and heparin [17]. Also in this case, they demonstrated the activity of NPs on MCF-7 cell line showing a better activity for the NPs than for the free drug. *In vivo* evaluation demonstrated that NPs show a similar ovarian tumor growth inhibition than paclitaxel and induce no body weight loss. The conjugation of paclitaxel with a squalenic tail through a disulfide-containing linker leaded to the formation of NPs that were biologically evaluated on MCF-7 cell line [40]. It was demonstrated that the conjugation to squalene decreased the cytotoxicity of the drug toward MCF-7 cells but it was confirmed that the disulfide bond improved the release of the toxic moiety.

Figure 2. Structures of anticancer compounds used as building blocks for the formation of the conjugate compounds. The anchor point used for the conjugation reaction with the self-assembling inducer is highlighted in red.

Cisplatin has been widely used in the clinic to treat a variety of cancers such as ovarian, breast and small cell lung cancer because of its potent activity to cross-link DNA upon entering the cells. However, cisplatin is vulnerable to attack by plasma proteins which deactivate the drug, leading to less therapeutic efficacy, and accounted for some severe side effects of cisplatin therapy. To improve the therapeutic index of cisplatin while minimizing its adverse side effects, cisplatin analog Pt(IV) prodrugs have been synthesized. Recently was reported the functionalization of cisplatin analog (**9**) with a PEG-polylactide copolymer which was covalently bonded to the hydroxylic groups of compound **9** [11]. *In vitro* evaluation on A2780 human ovarian carcinoma cell line showed an approximate 7-fold cytotoxicity increase for NPs respect to cisplatin analog **9** and this result could be due to the burst drug release in the acidic intracellular environment.

Chlorine e6 (**10**) is an attractive photodynamic therapy drug candidate because of its high absorption in the red spectral region and its cheapness compared to other porphyrin-based photodynamic therapy drugs. Other advantages of chlorine e6 are its long lifetimes in photoexcited triplet states and high molar absorption in the red region of the visible spectrum. Chlorine e6 was recently used for the synthesis of a biocompatible conjugate by the linkage with a polysaccharide exploiting a pH-sensitive linker for the release [12]. Biological evaluation of NPs on HeLa (cervical adenocarcinoma) cells demonstrated that higher levels of apoptosis are induced at pH 6.8 and 6.4 than at pH 7.4, while no noticeable difference in cell apoptosis with changes of pH values was observed in the presence of free chlorine e6.

Beclin-1 (**11**) is the peptide encoded by the human gene BECN1 and participates in autophagy regulation. It has been suggested that the overexpression of beclin-1 could inhibit tumor development but this peptide has some drawbacks. In fact, beclin-1 has a low chemical stability *in vivo* and it is characterized by a non-specific biodistribution in tissues. To overcome these disadvantages, beclin-1 was engineered to have a thiol group at the N-terminal in order to be easily conjugated with a pH-sensitive polymer [13]. The obtained NPs were biologically evaluated on MCF-7 cell line and they showed a significant cytotoxicity. It was also proved that NPs strongly induced autophagy and potentially led to autophagic cell death. *In vivo* evaluation on MCF-7 cancer cells xenografted tumor nude mice model demonstrated that the tumor size of tumor-bearing nude mice treated with NPs increased significantly more slowly than that of the control group, demonstrating that NPs can kill tumor cells synergistically and effectively showing no extreme toxicity.

Doxorubicin (**12**), a widely used anticancer drug, was involved in the formation of a dual pH-sensitive polymer conjugate [14]. Doxorubicin was linked onto PEG through an acid-labile hydrazone bond. SK-3rd, a cancer stem cell line, was chosen to evaluate the efficacy of doxorubicin NPs and it was demonstrated that NPs enhanced the cellular internalization, subsequently released the free drug in the cells in response to the endo/lysosomal pH and so inhibited the progression of cancer stem cells. Moreover Song *et al.* described the synthesis of a disulfide-linked doxorubicin drug-drug conjugate [45]. The *in vitro* cytotoxicity of doxorubicin NPs was weaker than that of the free drug but the antitumor efficacy of NPs evaluated in MCF-7 bearing mice was demonstrated to be higher than that of free doxorubicin.

In 2010, the hydrophobic anticancer drug camptotecin (**13**) was involved in the formation of an amphiphilic conjugate with a short ethylene glycol [15]. In addition, these NPs were loaded with the water soluble drug doxorubicin to obtain a synergic effect. The cytotoxicity of these NPs was evaluated on SKOV-3 ovarian and MCF-7 breast cancer cell lines. Camptotecin-NPs was shown to be as cytotoxic as the free drug on both the cell lines but doxorubicin-loaded NPs showed a higher activity than camptotecin-NPs, free camptotecin and doxorubicin demonstrating an additive or synergic anticancer activity of the two drugs involved in the NPs. *In vivo* evaluations were performed using athymic mice bearing intraperitoneal tumors and camptotecin-NPs were demonstrated to have a strong *in vivo* anticancer activity.

Betulinic acid (**8**) is an anticancer compound that has some drawbacks such as poor water solubility, severe side effects for healthy tissues, rapid blood clearance and low tumor selectivity. However, the formulation of betulinic acid as NPs can overcome these problems. In particular, the conjugation with PEG induced the formation of NPs which were also loaded with the anticancer drug hydroxycamptotecin (**22**) [9]. The cytotoxicity of these NPs was evaluated on LLC (Lewis lung carcinoma) and A549 (human lung cancer) cell lines and it resulted considerably higher than the one of free betulinic acid. The combination therapy of NPs containing betulinic acid and hydroxycamptotecin results in a better *in vitro* activity than the two free drugs, suggesting a significant synergic effect of NPs by co-delivering of the two drugs. *In vivo* efficacy of NPs was evaluated in xenografts models of lung tumor: tumor-bearing mice treated with NPs showed a clear survival advantage compared with the control treated mice. The antitumor effect of NPs was higher than the one of both free betulinic acid and hydroxycamptotecin, and no signs of systemic toxicity were observed by monitoring mice body weight. Moreover, the authors reported the preparation of NPs with the addition of a target molecule (folate). These NPs were demonstrated to be even more active both *in vitro* and *in vivo* with respect to the NPs with no target molecules, confirming that these NPs could be delivered into cells more easily because of the presence of the folate.

Table 1. Properties of the described NPs: anticancer drug used as building block, linker, self-assembling inducer, diameter, Z potential, cell lines used for the *in vitro* evaluation, available *in vivo* studies and ref. number.

Semaxanib (**14**) and sunitinib (**15**) are multitarget tyrosine kinase inhibitors but they have some side effects as cardiotoxicity, severe cutaneous toxicities and hematotoxicity. The NPs obtained by the covalent linkage of these compounds with squalene were biologically evaluated on HUVEC (umbilical vein endothelial) cell line, demonstrating that squalenoyl–sunitinib conjugate NPs showed notable cytotoxicity whereas semaxanib NPs were not active [19]. In addition, sunitinib NPs showed moderate cytotoxic activity against Mia Paca2 pancreatic cancer cells.

The water soluble drug irinotecan (**19**), a camptotecin derivative and a potent DNA topoisomerase I inhibitor, and the water insoluble chlorambucil (**20**), a DNA alkylating anticancer drug, were used to form an amphiphilic conjugate by an esterification reaction [43]. The NPs obtained were biologically evaluated using MCF-7 and HeLa cell lines. The cytotoxic activity of the drug-drug conjugate was demonstrated to be strongly dependent on its concentration: if the concentration is lower than the CAC value, the cytotoxic activity is worse than the mixture of the two drugs, but if the concentration is higher than the CAC value, NPs showed a much better activity than the free drugs mixture. This result suggested that the NPs enter in the tumor cells and that the released irinotecan and chlorambucil play a synergic action. Furthermore, MCF-7 tumor-bearing mice were intravenously injected with irinotecan, chlorambucil, irinotecan/chlorambucil mixture and irinotecan-chlorambucil NPs: at the end of the experiments, the tumor volumes in mice treated with NPs were much smaller than the tumor volumes of all other mice, demonstrating that NPs produce a better tumor growth inhibition than the free drugs and their mixture. Moreover the hydrophilic drug irinotecan was recently used with the hydrophobic combretastatin A-4 to form drug-drug conjugate. The obtained NPs were evaluated on MCF-7 and HUVEC cell lines and they showed a reduced cell survival ratio [46].

The use of chlorambucil in combination with another hydrophilic anticancer compound (gemcitabine, **21**) was also reported in 2015 [44]. *In vitro* evaluation of NPs toward a number of cancer cell lines (SMMC-7721 (human hepatoma), HeLa, MCF-7, SGC-7901 (gastric cancer), U87 (glioblastoma)) confirmed an increased cytotoxic activity with respect to the free drugs. Imaging studies were performed and it was demonstrated that NPs are internalized in the cell. The *in vivo* anticancer activity of NPs was evaluated in the SMMC-7721 tumor bearing mouse model and it was reported that NPs have a high anticancer efficacy probably due to the tumor targeting via EPR effect. No significant body weight loss was observed, showing that NPs are well tolerated at the tested dose level.

It is worth to highlight the possibility to obtain hetero-NPs by mixing different drugs functionalized with the same self-assembling inducer. Couvreur *et al.* reported the formation of hetero-NPs by co-self-assembly of three squalene-based conjugates: gemcitabine(**21**)-squalene (therapeutic component), rhodamine-squalene (fluorescent moiety), and biotin-squalene (targeting agent) [26]. These hetero-NPs demonstrated improved internalization in MCF7, M109 (murine lung cancer) and HeLa cell lines, which overexpress biotin receptors, and a stronger cytotoxic activity than non-biotinylated nanoparticles.

Our research group studied the formation of hetero-NPs by co-self-assembly of two squalene-based conjugates with the drugs paclitaxel (**7**) and cyclopamine (**18**) [42]. Biological evaluation of hetero-NPs on OVCAR5 (ovarian cancer), #83 and #110 (ovarian tumor-initiating) and U251 (glioblastoma) cell lines demonstrated a combined efficacy in apoptosis induction. Furthermore, we reported the formation of fluorescent hetero-NPs by co-self-assembly of the above-mentioned drug-squalene conjugates with a tetramethylrhodamine-squalene conjugate. The demonstration of cell internalization was accomplished by the application of multiple fluorescence microscopy methods: three-dimensional confocal microscopy allowed the identification of fluorescent NPs in the cytoplasm of the cells and super-resolution microscopy (dSTORM) permitted to quantify the diameter of the bright particles.

4. Summary

This review highlighted the last progresses in the field of prodrug conjugates able to form NPs by self-assembly. The simple functionalization of known anticancer compounds and the easy formation of NPs make this strategy simple to apply for different types of drugs and biological targets. This approach could improve drug biocompatibility and delivery efficacy.

In addition, the functionalization of different known drugs with a cleavable linker and a proper lipophilic chain that induces self-assembly and the generation of hetero-NPs could be exploited for personalized treatment of different types of diseases [42-44].

The same strategy can be used for the preparation of fluorescent hetero-NPs that can help follow the dynamic of the delivery and it may be possible to trace these NPs in the cells by imaging [26,40].

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Figure1

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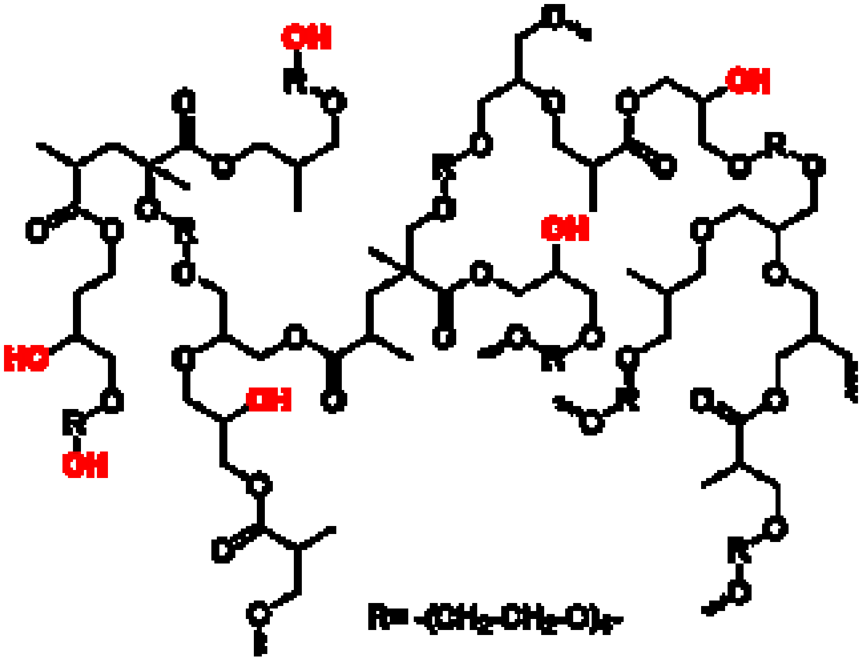

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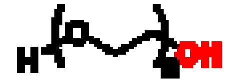

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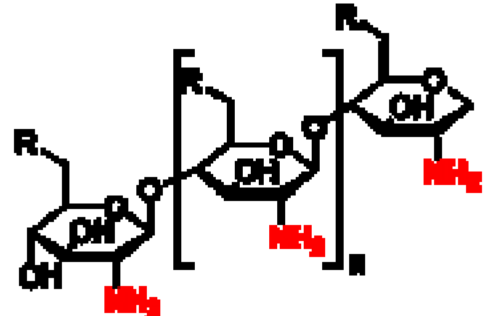

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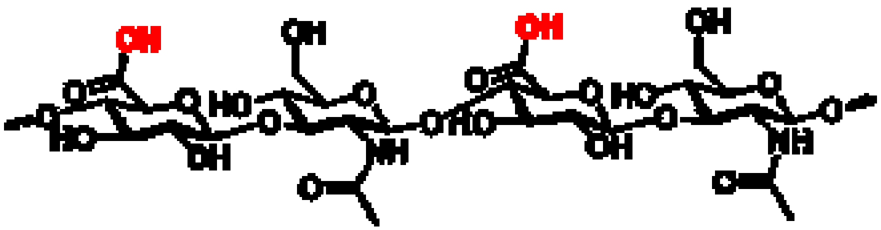

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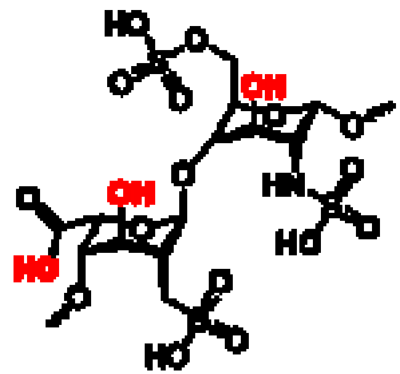

6a X= CHO

6b X= CO_2H

6c X= CH_2OH

6d X= CH_2NH_2

Figure2

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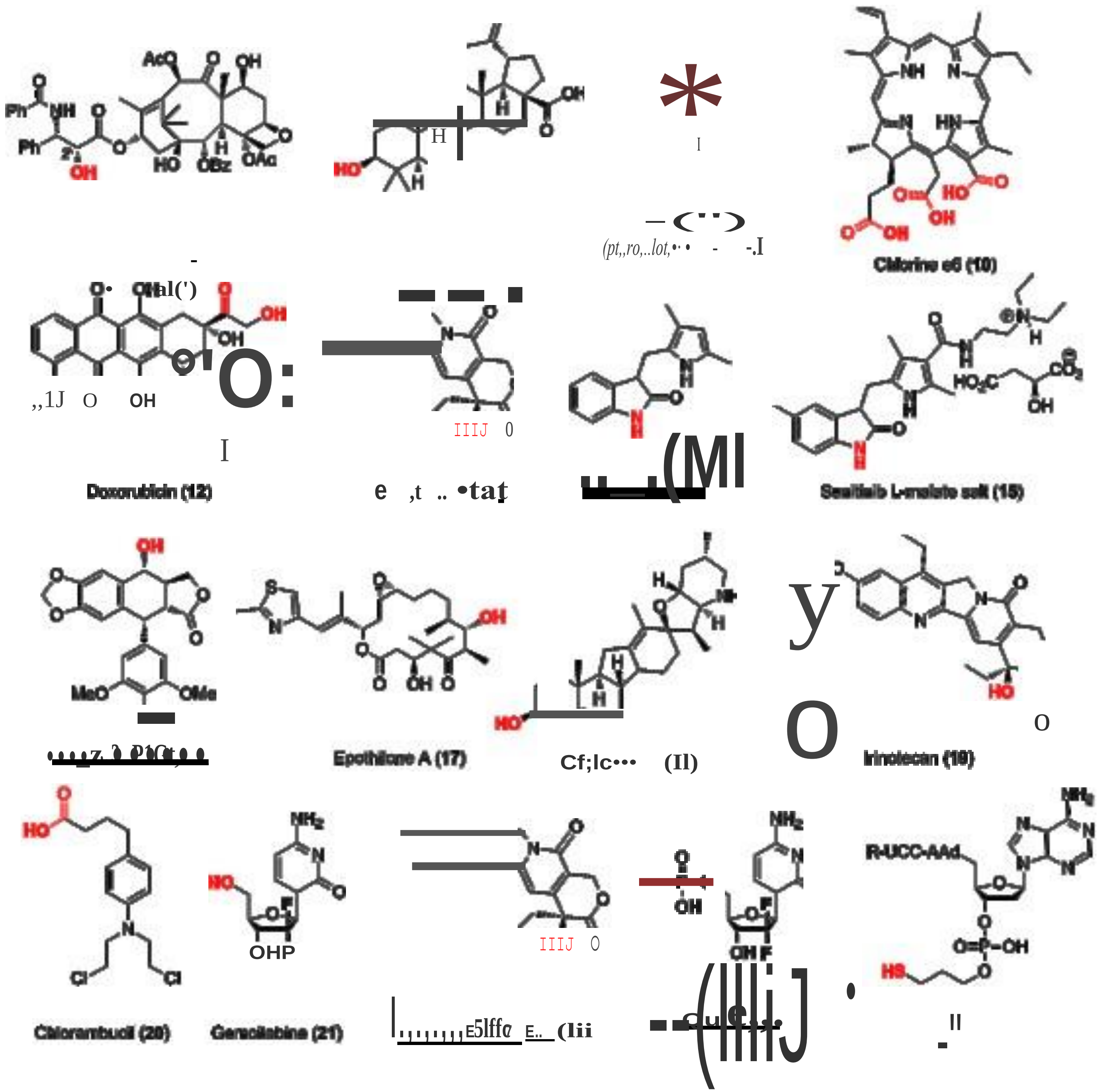
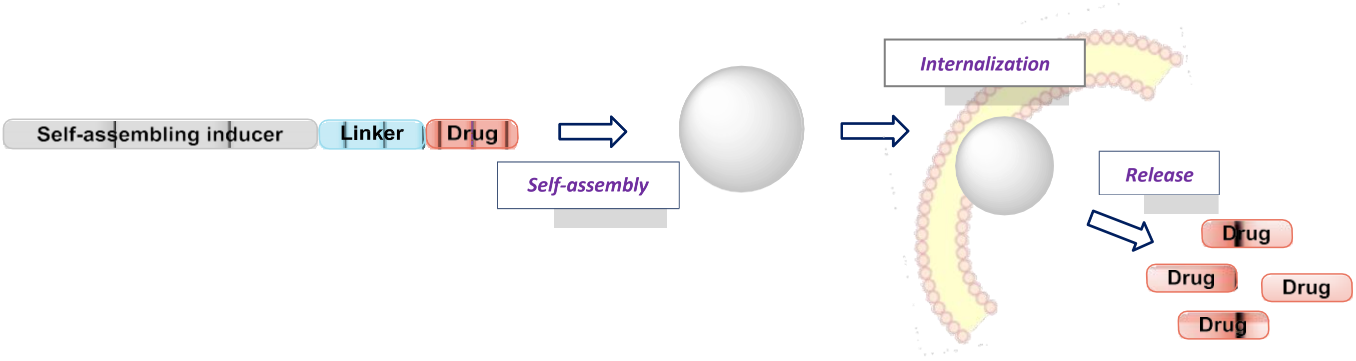


Table 1

Drug	Linker	Self-assembly Inducer	Diameter (nm)	Z potential (mV)	Cell line	*In vivo* evaluation	Ref.
9	hydrazone bond	2	~86	-33	A2780		[11]
Beclin-1 (**11**)	3-(dibutylamino)-1-propylamine	2	~32	+24	MCF-7	✓	[13]
Betulinic acid (**8**) Hydroxycamptothecin (**22**)	-	2	108-125	+5.3 - +86	LLC or A549	✓	[9]
Camptotecin (**13**)	β-thioester	2	~186	-4	SKOV-3 MCF-7	✓	[15]
Camptotecin (**13**)	disulfide	6	~195	-41	MCF-7		[40]
Chlorambucil (**20**) Gemcitabine (**21**)	-	-	~160	+15.7	SMMC-7721 Hela MCF-7 SGC-7901 U87	✓	[44]
Chlorambucil (**20**) Irinotecan (**19**)	-	-	~88	+3.4	MCF-7 HeLa	✓	[43]
Chlorine e6 (**10**)	3-diethyl aminopropyl isothiocyanate	2+3	~150	-8	HeLa	✓	[12]
Cyclopamine (**18**)	disulfide	6	~105	+37	U251		[42]
Cyclopamine (**18**) Paclitaxel (**7**)	disulfide	6	~229	+26	U251		[42]
Doxorubicin (**12**)	-	6	~130	+35	Mia PaCa-2 M109	✓	[39]
Doxorubicin (**12**)	hydrazone bond	2	~27	n.a.	SK-3rd		[14]
Doxorubicin (**12**)	disulfide	-	~89	n.a.	MCF-7	✓	[45]
Epothilone A (**17**)	disulfide	6	~161	-56	MCF-7		[40]
Gemcitabine (**21**)	-	6	~130	n.a.	MCF-7 KB3-1 L1210 10K	✓	[23]
Gemcitabine (**21**)	-	6	~149	-25	HeLa M109 MCF7		[26]
Gemcitabine (**21**)	-	6	130-170	-6	pancreatic	✓	[35]
Gemcitabine (**21**)	-	6	~104	-6	L1210 10K		[18]
Gemcitabine monophosphate (**23**)	-	6	50-150	n.a.	L1210 10 K		[21]
Irinotecan (**19**)	azobenzene bond	-	~196	n.a.	MCF-7 HUVEC	✓	[46]
Paclitaxel (**7**)	Val, Leu, Phe	4	275-285	+0.17 - +0.43	MCF-7		[16]
Paclitaxel (**7**)	Val, Leu, Phe	5	140-180	-21 - -31	MCF-7	✓	[17]
Paclitaxel (**7**)	-	1	50-120	n.a.	MCF-7 Tca8113	✓	[8]
Paclitaxel (**7**)	disulfide	6	~103	-37	MCF-7		[40]
Paclitaxel (**7**)	cis,cis-1,4-dienic	6	90-150	-19 - -26	A549 HT-29 KB 3.1	✓	[22]
Paclitaxel (**7**)	ester bond	6	118-293	-44 - -36	M109		[25]
Paclitaxel (**7**)	-	6	149-225	n.a.	A549		[41]
Podofillotoxin (**16**)	disulfide	6	~184	-11	MCF-7		[40]
Semaxanib (**14**)	hemiaminal	6	~141	-40	HUVEC		[19]
siRNA (**24**)	maleimide-sulfhydryl	6	~165	-26	TPC-1 BHP 10-3	✓	[29]
Sunitinib (**15**)	hemiaminal	6	~123	+23	HUVEC Mia Paca2		[19]

Graphical Abstract (for review)



Highlights

Many anticancer compounds are unable to show a pharmacological activity because they do not reach the therapeutic concentration at the site of action.

Nanotechnology is a useful tool to improve the selective delivery of anticancer compounds to their site of action.

A very interesting application of anticancer nanotechnology is represented by nanostructures spontaneously obtained by self-assembly of conjugates of drugs linked to a proper chemical entity.

The generation of hetero-NPs could be exploited for personalized combined treatment of different types of diseases.