Molecular platforms for targeted drug delivery
Molecular Platforms for Targeted Drug Delivery

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ABSTRACT
The targeted delivery of bioactive molecules to the appropriate site of action, one of the critical focuses of pharmaceutical research, improves therapeutic outcomes and increases safety at the same time; a concept envisaged by Ehrlich over 100 years ago when he described the “magic bullet” model. In the following decades, a considerable amount of research effort combined with enormous investment has carried selective drug targeting into clinical practice via the advent of monoclonal antibodies and antibody-drug conjugates derivatives. Additionally, a deeper understanding of physiopathological conditions of disease has permitted the tailored design of targeted drug delivery platforms that carry drugs, many copies of the same drug, and different drugs in combination to the appropriate site of action in a selective or at least preferential manner. The acquired know-how has provided the field with the design rationale to develop a successful delivery system that will provide new and improved means to treat many intractable diseases and disorders. In this review, we discuss a wide range of molecular platforms for drug delivery, and focus on those with more success in the clinic, given their potential for targeted therapies.
INTRODUCTION

Targeted drug delivery systems represent a rapidly growing class of therapeutics with intense research underway at both academic and industrial levels. The first targeted drug delivery system-related studies focused on developing anticancer therapies, owing to the highly potent but usually non-specific nature of traditional chemotherapeutics (Allen 2002). A given chemotherapeutic or drug can be very selective in blocking a specific cell process, but this process might not be solely present in target cells, thus yielding toxicities that hamper successful therapeutic outcomes. The precise delivery of anticancer drugs to target cells represents one of the best approaches to improve treatment outcomes; however, developing a therapeutic with both enhanced targeting and pharmacological activities remains a difficult task. In this context, antibodies that recognize a specific antigen on the surface of a target cell and induce cell-mediated cytotoxicity represent the best examples.

However, the exploitation of molecular platforms designed for drug delivery and selective targeting may be a more convenient strategy. Proposed molecular platforms include lipid nanocarriers (e.g., liposomes, micelles, and lipid nanoparticles), polymeric nanoparticles, polymer conjugates, and antibody-drug conjugates (ADCs). Selective targeting may take advantage of the intrinsic features of the platform, such as size, charge, or the presence of a specific polymeric component (e.g. a polymer recognized by specific receptors), although targeting can also be achieved via the direct functionalization of the molecular platform surface/backbone with a targeting agent/moiety.

While we can pursue targeted drug delivery via different approaches and at different levels, each strategy must be developed with a defined disease in mind, and then combined with site-selective features, such as site-specific drug release, to increase therapeutic outcome. Historically, the concept of precise therapy dates to 1913, when Ehrlich envisioned the “magic bullet” (Ehrlich 1913). Most research on targeted drug delivery focuses on cancer therapy, although this concept remains important in relation to other diseases. Cancer therapy tends to draw a significant proportion of therapeutic “attention” because of the unfortunately widespread nature of the disease combined with the pharmacological difficulties faced when designing a tumor-cell specific treatment approach. In this field, targeting takes two forms passive targeting and ligand-mediated- or active-targeting; however, many drug delivery systems exploit both targeting modalities. Passive targeting to solid tumors and inflamed tissues takes advantage of anatomical and physiological changes in the tumor blood vessels in these areas, together with the action of mediators regulating blood flow (as will be explained in detail elsewhere in this review).

“Active” targeting often wrongly evokes the idea of a targeting moiety propelling a delivery system to a site of action; instead, targeted systems rely on bodily distribution networks (blood circulation, diffusion, interstitial fluid movements, etc.) for proximal delivery to a specific site, where the targeting moiety then interacts with its partner (ligand-receptor or receptor-ligand) to promote site-specific
retention and accumulation (Fig. 1). Therefore, successful selective targeting requires the combination of a targeting strategy with a tailored delivery strategy that improves biodistribution and extravasation to promote proximity to a site. Only at this stage will the targeting moiety favor the retention/accumulation of a drug at the target site or required cell population.

Figure 1. Schematic representation of nanomedicine selective tumor accumulation by the increased blood vessel permeability and the active cancer cell targeting (EPR = Enhanced permeation and retention)

In anticancer therapy, markers specifically overexpressed on the surface of cancer cells, the tumor vasculature, or the cancer stroma represent possible targets; however, reducing adverse effects of drugs requires the absence or minimal expression of the employed marker by healthy tissues. Targeting moieties described in the literature can be divided in two classes: 1) Antibodies and antibody derivatives (i.e. Fab, scFV, nanobodies, diabodies etc.) (Shahied et al. 2004, van der Meel et al. 2013, Chari et al. 2014), and 2) non-antibody molecules, such as low molecular weight (MW) molecules (e.g. folic acid, galactosamine, etc.)
(Seymour et al. 2002, Xia and Low 2010), peptides (e.g. RGD, GE11, etc.) (Zhang et al. 2012, McGuire et al. 2014, Duro-Castano et al. 2017), aptamers (Wang and Farokhzad 2014, Zhang, Zhang, et al. 2014), proteins (GM-CSF, transferrin, etc.) (Ishida et al. 2001, Frankel et al. 2002), and polymers (e.g. hyaluronic acid) (Platt and Szoka 2008, Zhang, Huang, et al. 2014). Antibodies and antibody derivatives have high affinities towards their antigens and, after the advent of monoclonal antibodies, became the primary choice for effective targeting, yielding derivatives (ADCs) that are in current clinical use. In general, molecules belonging to the second class (non-antibody molecules) suffer from lower binding affinities for their target but have the advantage of lower costs.

Of interest is the consideration of affinity constant value between the ligand and its receptor to ensure effective tumor targeting. Initially, we sought molecules with high affinity to ensure accumulation; however, high-affinity ligands, such as antibodies, display limited tumor penetrance, as the antibody targeted system binds strongly to antigens presented by the first perivascularly-located cancer cells following extravasation and cannot be released from them. This problem is known as the “binding-site barrier” (Saga et al. 1995, Adams et al. 2001, Thurber et al. 2008). Ligands with low affinity can still bind to the target, presenting with a favorable equilibrium in which a fraction of the binding complex dissociates and releases the system, thereby promoting tumor penetration. In this case, the concentration of the target drives tumor accumulation, and therefore, requires time to reach an optimal level. Nevertheless, as ligands with lower affinities display lower selectivity for a specific target, we still expect non-specific accumulation and unwanted toxicity.

Several considerations influence the selection of targeting agents including

i) tumor type (e.g., the easy access of leukemic cells advocates for the application of molecular platforms targeted by a high binding affinity ligand)

ii) therapy type (e.g., antiangiogenic therapies targeting the endothelial cells of tumor vessels require high binding affinity ligands)

iii) the genetic instability of cancer cells (can cause a down-regulation of tumor-associated antigen expression (Loganzo et al. 2016), and consequently requires cancer antigens associated with tumor stroma/vasculature cells to overcome this issue).

While, unfortunately, the success of active targeting has been limited, the exploration of novel targets via omic-based studies might help to underlying the proteins involved in a specific diseases that might be new targets, thus pushing this strategy forward (Matthews et al. 2016, Turanli et al. 2018). However, most current clinically-applied molecular platforms for targeted delivery rely on passive targeting and accumulation at the tumor stroma (taking advantage of the enhanced permeation and retention [EPR] effect), exclusively due to their intrinsic size within the blood pool (Fig. 1) (Baxter and Jain 1989, Jain 2005, Maeda et al. 2016). Circulating plasma concentration, stability, and the plasma half-life of the nanosystem
determines EPR-mediated targeting (Vicent et al. 2009); however, tumor type, tumor region, the presence of intratumoral necrotic or inflamed area, and tumor vascularization also influences the EPR effect (Golombek et al. 2018). Therefore, poorly-vascularized damaged tissues are less suitable for nanomedicine-based treatments (Danquah et al. 2011), demonstrating the need for new therapeutic approaches incorporating targeting moieties.

The positive advantages of EPR effect might be limited by several additional limitations that promote the application of active targeting strategies eventually in combination with passive targeting (Maeda et al. 2013). To exploit EPR effect a drug delivery system should (i) avoid interactions with blood components or blood vessels, for example presenting a weakly negative to near neutral total surface charge; (ii) have a systemic circulation time of several hours/days..

The EPR effect in human patients also presents with patient-to-patient variability, depending on a patient’s pathological and physiological characteristics and clinical condition (Nichols and Bae 2014, Natfji et al. 2017, Tang et al. 2017), and has provoked diverse opinions regarding the real value of the EPR effect. Some studies support the EPR-mediated accumulation of delivery systems within tumors, while others show that the EPR effect depends on the tumor model, suggesting that the EPR effect alone may not provide the entire solution. Maeda himself recognizes this heterogeneity and has developed methods to enhance the EPR effect to overcome the heterogeneity and improve drug delivery to tumors (Maeda 2015, Lehmann et al. 2016).

This review will focus on the analysis of the advantages and limits of different targeted drug delivery platforms and their future perspectives. In particular, the review will discuss the following items divided in sections: polymers conjugates, mainly with low molecular weight drugs, conjugates with unsaturated fatty acids, targeted liposomes and antibody-drug conjugates. The readers are directed to recent and exhaustive reviews for an overview of nanoparticles and micelles as drug delivery systems (Moghimi et al. 2001, Cabral and Kataoka 2014, Nishiyama et al. 2016, Anchordoquy et al. 2017, Parodi et al. 2017, Bennie et al. 2018).

POLYMER CONJUGATES

While both synthetic and natural polymers have found applications in various diverse fields, from the material industry to biomedical applications, recent decades have seen their exploration as components of sophisticated drug delivery systems. These developments led to the appearance of a novel class of agents named “polymer therapeutics” (Duncan 2003). These new chemical entities include polymer-drug conjugates (PDCs), polymer-protein conjugates, polymer-aptamer conjugates, polymer-DNA complexes (or polyplexes), and polymeric micelles with drugs covalently tethered to the polymer carrier (Duncan 2014, 2017) (Fig. 2).
Drug delivery systems that rely on physical encapsulation of drugs (such as classical micelles or liposomes) can present certain problems, including the premature release of the active molecule, instability during storage and administration, and poor encapsulation efficiency. Of note, early drug release leads to systemic toxicity and compromises therapeutic outcomes. Polymer therapeutics can overcome these problems thanks to conjugation of drug to the polymeric backbone through cleavable linkers that only trigger drug release under specific conditions (Maeda and Khatami 2018).

The active targeting of these polymer-conjugates can be achieved by incorporating cell-specific ligands, such as peptides, carbohydrates, antibodies or their fragments. These targeting moieties confer a double effect: improved accumulation to the site of action and enhanced uptake of the conjugates through receptor-mediated endocytosis (Muratovska et al. 2001, Tijerina et al. 2003, Nori and Kopecek 2005, Callahan and Kopecek 2006, Cuchelkar et al. 2008). However, to date, the majority of polymer-conjugates rely on the EPR effect for tumor accumulation. Maeda postulated the theory while studying the properties of SMANCS, a styrene-maleic acid copolymer (SMA) conjugated with an antitumoral protein neocarzinostatin (NCS, 12 kDa). The conjugate, developed in 1979, displays an MW of 16 kDa with the two amino groups of NCS (Ala1 and Lys20) derivatized with two polymer chains of 2 kDa (Maeda et al. 1979). SMANCS accumulated more effectively in tumor tissue than in healthy tissue, and in 1994, SMANCS obtained approval in Japan for hepatoma treatment (Maeda et al. 2001).
Typical synthetic polymers employed in the preparation of polymer therapeutics include poly(ethylene glycol) (PEG), N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, poly(vinyl pyrrolidone) (PVP), poly(ethyleneimine) (PEI), poly(acrylic acid) (PAA), polyvinyl alcohol (PVA), poly(N-(2-hydroxypropyl) methacrylamide) (HPMA) copolymers, poly(vinyl pyrrolidone) (PVP), poly(ethylenimine) (PEI), poly(acrylic acid) (PAA), polyvinyl alcohol (PVA), poly(styrene-co-maleic acid/anhydride) (SMA), poly(acryloyl morpholine) (PAcM), divinyl ether maleic anhydride/acid copolymer (DIVEMA) and polyamidoamines. Natural polymers include dextran, HA, chitosan, pullulan, dextrin, and mannan, while pseudosynthetic polymers include poly-L-glutamic acid (PGA), poly(lactide-co-glycolide) (PLGA), polylactide (PLA), poly(L-lysine) (PLL), poly(malic acid) (PMA), poly(aspartamide) (PAA), and poly((N-hydroxyethyl)-L-glutamine) (PHEG).

An explicit model for PDC generation proposed by Ringsdorf in 1975 aimed to improve the therapeutic properties of small hydrophobic drugs by their conjugation to hydrophilic polymers (Ringsdorf 1975). The model outlined the structural components of a PDC: one or more active compounds attached to a polymeric backbone (directly or through a biodegradable linker) and the optional presence of a targeting moiety or a solubilizer group. Following the Ringsdorf model, polymer conjugation of small molecules provides numerous benefits, including improved water solubility of hydrophobic drugs, reduced renal extraction due to a higher hydrodynamic volume of the conjugate, increased protection against chemical and enzymatic degradation, and reduced aggregation, antigenicity, and immunogenicity (Ruth Duncan, 2003). More importantly, polymer conjugation dramatically affects the biodistribution and pharmacokinetics of small molecule drug, permitting cell or tissue-specific delivery via the application of targeted polymers (active targeting) and/or via passive targeting.

The first generation of PDCs exploited generally well-tolerated non-biodegradable polymers such as PEG and HPMA copolymers; however, the persistent nature of the polymer backbone limited the maximum polymer MW applicable, as high MWs risk lysosomal store disease caused by polymer accumulation inside intracellular vesicles (Duncan and Richardson 2012). Biodegradable polymers represent a safe alternative that allows optimized pharmacokinetic profiles and tumor accumulation (due to an enhanced EPR effect) as higher MW polymer can be employed; however, polymer degradation in the bloodstream following systemic administration can compromise the homogeneity and efficacy of PDCs. In general, the application of polymers that do not exhibit non-specific interactions with biological membranes or other body components avoids off-target accumulation.

Importantly, polymeric carriers should not be considered as an inert part of the construct, as various studies have demonstrated their crucial role as mediators of the biological activity of the final compound. Polymer features, including architecture and solution conformation, play essential roles in determining the in vivo fate of conjugates and their interaction with biological systems (Duncan et al. 1981, Veronese et al. 2005, Berna et al. 2006, Nevozhay et al. 2006, Seib et al. 2007, Pasut et al. 2009).

Furthermore, the bioresponsive polymer-drug linkers often employed also drive conjugate therapeutic
output (Arroyo-Crespo et al., 2018; Arroyo-Crespo et al., 2018). The involvement of multiple research teams investigating different polymers and chemical strategies has led to the development of new polymeric structures, including block copolymers (Pechar et al. 2000), dendrimers (Tomalia et al. 1985, Fréchet et al. 1996, Fréchet and Tomalia 2001), star polymers (Mishra & Kobayashi, 1999; Duro-Castaño et al., 2015; Duro-Castaño et al., 2017), and graft polymers (Ferruti et al. 1998, Dautzenberg et al. 2001). These structures aim for high multivalency (Mammen, Choi, & Whitesides, 1998; Conejos-Sánchez et al., 2013; Arroyo-Crespo et al., 2018) and enhanced pharmacokinetics following the conjugation of small drugs.

**Polymer-drug and polymer-protein conjugates relying on Passive Targeting**

The first synthesis of a polymer-drug conjugate involved the attachment of mescaline to PVP using a dipeptide spacer (GlyLeu) (Jatzkewitz 1955). In the 1960s, Ushkoc’s group generated numerous conjugates of PVP and antibiotics (Givental’ et al. 1965, Shumikhina et al. 1966), while in the early 1970s, Kopeček’s group began to develop HPMA copolymers as drug carriers. The first HPMA-drug conjugate reported in 1979 (Obereigner et al. 1979) employed derivatives of the antidiabetic compound N-(4-aminobenzenesulfonyl)-N’-butyl urea.

The first polymer-small drug conjugate that entered clinical trials exploited a natural polymer approved as a plasma expander (dextran) coupled to doxorubicin (Danhauser-Riedl et al. 1993). The conjugate, AD-70, employed a Schiff base between the oxidized dextran (also modified with glycine pendant groups) and the anthracycline. Unfortunately, severe thrombocytopenia and hepatotoxicity in patients stopped a Phase I trial of AD-70 in 13 patients with histological confirmation of a solid tumor that did not respond to any treatment.

An HPMA copolymer-doxorubicin conjugate, also known as PK1, developed by a collaboration between Duncan and Kopecek (Vasey et al. 1999) represents the first conjugate using a synthetic polymer tested in humans (Duncan and Vicent, 2009). Tethering of doxorubicin to a 30 kDa chain of HPMA copolymers through a tetrapeptide spacer (Gly-Phe-Leu-Gly, or GFLG) provided stability during blood circulation and allowed specific cleavage and release of doxorubicin (≈8.5 wt%) thanks to the action of lysosomal cathepsin B after cellular uptake (Duncan, Cable, et al. 1983). HPMA copolymer-doxorubicin progressed until Phase II clinical trials for the treatment of breast, colon, and small-cell lung cancer (Duncan et al. 1998, Vasey et al. 1999, Seymour et al. 2009). A galactosamine-targeted version of HPMA copolymer-doxorubicin, known as PK2, will be discussed in more detail below. Of note, industrial interests inhibited the further clinical progress of HPMA copolymer-doxorubicin and its galactosamine targeted version, perhaps because of a deficient early dosing regimen and the resultant non-compelling results. HPMA copolymer-paclitaxel (Meerum Terwogt et al. 2001) and HPMA copolymer-camptothecin (Schoemaker et al. 2002) also reached clinical trials. A review article from Duncan and Vicent provides an extended view of HPMA copolymers in the clinics (Duncan and Vicent 2010).
HPMA copolymers offer high drug loading capacity due to the reactive pendant groups; however, PEG, one of the most successful polymers in drug delivery (Pasut and Veronese 2012, Pasut 2014), presents only two reactive sites at the polymer end-chain. However, generating branching PEGs or dendronized PEG allows increased drug loading. For example, Etirinotecan pegol (Onzeald), a 4-arm PEG polymer, bears one irinotecan molecule at each extremity of the four chains through a cleavable ester linker (Hoch et al. 2014). The conjugate (nominal MW of 20 kDa) displayed sustained release of irinotecan in plasma and in tumor tissues in preclinical studies in animals, where it is metabolized to form the active compound, SN-38. Despite exhibiting enhanced pharmacokinetics, the conjugate did not improve overall survival when compared to a single-agent standard chemotherapy regimen in patients with advanced breast cancer in Phase III clinical trials (Clinical trial NCT01492101). However, Etirinotecan pegol treatment did prolong survival in a subset of patients who developed brain metastasis and a Phase III clinical study regarding these patients is currently ongoing (Clinical trial NCT02915744).

Cyclodextrins are cyclic oligosaccharides comprising (α-1,4)-linked-α-D-glucopyranose units arranged in supramolecular structures with lipophilic cone-shaped cavities and hydrophilic shells (Uekama et al. 1998, Challa et al. 2005). Because of their peculiar structure and physic-chemical properties, cyclodextrins interact with a large variety of molecules, forming non-covalent inclusion complexes. The three natural CDs (α-, β-, and γ-cyclodextrins) and their synthetic derivatives have primarily found use as building blocks for the construction of drug delivery systems. To date, CRLX101 (formerly IT-101) represents the most significant example of a cyclodextrin-based nanopharmaceutical, produced for the delivery of the topoisomerase I inhibitor camptothecin (Schluep et al. 2006, Davis 2009, Young et al. 2011). Drug attachment to a linear copolymer of β-cyclodextrin and PEG (MW = 3400 Da) through an ester bond, led to the production of self-assembled nanoparticles of ≈ 30 nm size with a slightly negative zeta potential (≈ -2 mV) in PBS pH 7.4. The polymeric backbone is obtained by coupling a diamino acid-β-cyclodextrin monomer with a difunctionalized PEG and has an MW of ≈ 70 kDa with PDI of ≈ 2. Camptothecin is then functionalized at the 2′-hydroxyl group with a glycine linker to preserve the lactone form of the drug (Cheng et al. 2003). The derivative is then covalently attached to the cyclodextrin blocks of the cyclodextrin-PEG copolymer providing a final drug loading of ≈ 10 wt%. As for other hydrophobic small drugs, camptothecin forms inclusion complexes with cyclodextrins via intra- and inter-molecular interactions that induce the self-assembly of several cyclodextrin-PEG copolymer strands into nanoparticles. The lactone form of camptothecin is fundamental for anticancer activity; however, in physiological conditions, the lactone ring spontaneously undergoes reversible hydrolysis to form the inactive carboxylate form (Potmesil 1994). The linker strategy applied in CRLX101 “freezes” the lactone ring in the closed, active form. Also, the overall nanoparticle architecture additionally protects against premature hydrolysis and the action of metabolic enzymes. Consequently, CRLX101 behaves as a depot that allows the controlled and sustained release of pure camptothecin over the time at the tumor site,
where it accumulates by the EPR effect. Furthermore, upon camptothecin release, nanoparticles disassemble, re-forming the original individual polymer strands that undergo renal clearance because of their small size (< 10 nm). Preclinical studies discovered favorable pharmacokinetics and biodistribution profiles in rats and tumor-bearing mice (Schluep et al. 2006), encouraging scientists to proceed with human clinical trials. In the combined Phase I/IIa study, treatment of patients with advanced solid tumor malignancies uncovered low levels of toxicity and promising antitumor activity (Weiss et al. 2013). CRLX101 is currently being investigated in different Phase II trials for the treatment of solid tumors (non-small cell lung cancer, rectal cancer, renal cell carcinoma, and recurrent ovarian/tubal/peritoneal cancer) as a monotherapy or in combination with anticancer drugs (as capecitabine and bevacizumab) [Clinical trials: NCT00333502, NCT01380769, NCT01625936, NCT01652079, NCT02010567, NCT02187302, NCT02389985].

Although PEG remains the most frequently used polymer for biomedical applications, many recent developments in controlled polymerization techniques have provided alternatives to PEG-based systems, including those with a biodegradable structure (Barz et al., 2011). Polypeptide-based materials (Zagorodko et al, 2017) have been explored for drug conjugation (Li 2002, Bajaj and Singhal 2011, Ogunleye et al. 2015), with PGA, an anionic, biodegradable, and non-toxic polymer formed by naturally occurring L-glutamic acid units with pendant y-carboxylic groups, being of considerable interest. The conjugation of PGA with PTX to produce paclitaxel polyglumex (PPX - previous trade name Xytotax®, now known as Opaxio®) (Singer 2005, Chipman et al. 2006, Zhao et al. 2018) by CTI BioPharma generated a product with an average MW of 38.5 and a drug loading of around 36-37% w/w (1 drug molecule every 11 PGA monomers). Drug conjugation through an ester linkage between the y-carboxylic acid group of the polymer and the 2’ hydroxyl group of PTX led to the formation of a relatively stable conjugate. A 24 h incubation in human plasma prompted the release of less than 14% of PTX, proving stability in the presence of plasma esterases. Following cellular internalization, PPX metabolism requires lysosomal enzymes (in particular cathepsin B) to generate monoglutamyl-PTX, which spontaneously degrades releasing free PTX, and diglutamyl-PTX. While Opaxio entered clinical trials in the mid-2000s, the poor therapeutic outcome in patients reduced the initial enthusiasm generated by the animal studies. Results in non-small-cell lung carcinoma patients treated with single-agent PPX presented with a marginal reduction of PTX toxicity with non-significant improvements in survival and disease control. However, a subsequent patient stratification analysis correlated response rate to the hormone status of the patients: pre-menopausal women responded, while post-menopausal women and men did not. This differential behavior occurred due to heightened estrogen levels in pre-menopausal women, with estrogen levels positively correlated with cathepsin B activity. This was the first example of the use of a biomarker for nanomedicine clinical trial patient selection (Atkinson et al. 2018). In 2012, Opaxio received orphan drug designation for the treatment of glioblastoma multiforme (GBM) in combination with radiotherapy and temozolomide by the US Food and Drug Administration (FDA) and belonged to the few formulations that reached advanced
Phase III clinical trials. Despite the discovery of an enhanced safety profile in all trials compared to free PTX, the inability to display significant improvement over current standard care led to the discontinuation of Opaxio in 2016 (Zhao et al. 2018). Overall, the development of PGA-based and other polypeptide-based conjugates as single agents and in combination therapy is in exponential growth, thereby demonstrating the enormous potential of these polymers as targeted carriers in advanced therapeutics (Klinker and Barz 2015, Zagorodko et al. 2017, Leong et al. 2018).

Hyaluronic acid (HA) is an interesting example of a natural polymer that possesses unique intrinsic targeting features and biological properties. Formed by repeating units of D-glucuronic acid and N-acetyl-D-glucosamine disaccharide, HA is naturally present in human body, mostly in the skin, but also as a component of extracellular matrix (ECM), synovial fluid of joints, cartilage, and the vitreous body of the eye (Laurent et al. 1995). Notably, there exists an abundance of HA in tumor and inflamed tissues; the first evidence of the correlation between HA and tumor invasion reported in the late 1970s by Toole et al. established the higher concentration of HA present in tissues surrounding invasive tumors (Toole et al. 1979). From these studies, it became evident that high levels of HA may represent an indicator of tumor presence and invasiveness (Ropponen et al. 1998, Setälä et al. 1999, Auvinen et al. 2000).

HA displays multifunctionality, as it interacts with different cellular receptors, such as CD44, RHAMM, LYVE-1, IVd4 and LEC receptors (Sherman et al. 1994), activates kinase pathways (Hall et al. 1995, Nelson et al. 1995), and regulates angiogenesis in the tumor environment (Rooney et al. 1995). Other important roles for HA include regulation of cell migration and proliferation and cell-cell aggregation, and the promotion of angiogenesis. Among HA receptors, CD44 is the most studied due to its involvement in cancer progression and its overexpression in various solid cancers (Marhaba and Zöller 2004). The signaling pathway activated by CD44 is implied in cell survival/death and regulates the adhesion and rolling of lymphocytes and CD44 is ultimately involved in cell migration during morphogenesis, angiogenesis, and tumor invasion and metastasis. HA specificity for CD44 improves drug distribution in tumor tissues that overexpress CD44 receptor (Sneath and Mangham 1998) and for this purpose, the degree of substitution (ratio between the number of substituted reactive groups and the number of disaccharide units) must be less than 25% to preserve the ability of HA to target CD44 receptors.

As a polymeric carrier, HA presents multiple exploitable functional groups in its backbone (hydroxyl and carboxylic acid) for drug conjugation and several HA-drug conjugates have been tested for cancer treatment, with payloads including butyric acid, paclitaxel, SN38, doxorubicin, cisplatin, 5-fluorouracil, and methotrexate (Coradini et al. 2004, Campisi and Renier 2011, Lee et al. 2012, Venable et al. 2012, Dong et al. 2013, Oommen et al. 2014, Yang et al. 2014, Tripodo et al. 2015). HA has also been conjugated with several anti-inflammatory drugs for intra-articular arthritis treatment, such as dexamethasone, hydrocortisone, fluorocortisone, betamethasone, corticosterone, prednisone, and prednisolone (Pouyani
and Prestwich 1994) and with peptides, including epidermal growth factor, salmon calcitonin, Exendin, anti-FLT1 peptide (Kong et al. 2010, Mero et al. 2014) and protein, such as interferon alpha, hGH, insulin, ovalbumin, and antibodies (Ferguson et al. 2010, Lee et al. 2012, Mero et al. 2013, 2015, Friedrich et al. 2014, Montagner et al. 2016).

The most advanced example of an HA-conjugate with targeting properties is probably ONCOFID™-P developed by Fidia. In this conjugate, esterification of the hydroxyl group of PTX with 4-bromobutyric acid precedes conjugation to HA (200 kDa) through a second ester bond, providing a final drug loading of 20% w/w (Campisi and Renier 2011). The internalization of the conjugate by CD44 expressing cells and the subsequent intracellular release of PTX led to an inhibitory effect on bladder cancer cells overexpressing CD44 (RT-4 and RT-112/84). Indeed, active internalization provided for stronger antitumor activity of the conjugate when compared to the free drug (IC50 values approximately 800 and 120 times higher in RT-4 and RT-112/84 cell lines, respectively). However, ONCOFID™-P requires intravesicular administration due to a plasma half-life of only 10 minutes. Other studies reported that circulating HA chains are mainly removed from the bloodstream by HA receptor for endocytosis (HARE)-mediated clearance by liver sinusoidal endothelial cells (Zhou et al. 2000). Pharmacokinetic studies revealed retention of the conjugate in the bladder for at least two hours after intravesicular administration, probably due to the mucoadhesive properties of the long hyaluronan chains. After displaying promising results in mouse xenograft models with intraperitoneal implants of ovarian cancer cells, ONCOFID™-P is now undergoing Phase II clinical trials in six European countries for the treatment of refractory bladder cancer (EudraCT: 2009-012274-13).

In a recent report, Montagner et al. exploited HA to target the delivery of a therapeutic protein, interferon-α2a (INFα2a) (Montagner et al. 2016). Interferons are a family of cytokines with antitumoral and antiviral properties that are employed to treat different pathologies, including hepatitis B/C and various cancers (e.g., B- and T-cell lymphomas, melanoma, and renal carcinoma) (Ferrantini et al. 2007). A Phase I/II study for the treatment of ovarian cancer (Marth et al. 2006) provided promising results for an interferon-based combination therapy; however, the potential for toxic effects limit the potential for Interferons as therapeutic agents, especially for type I Interferons like INFα2a. As ovarian cancer overexpresses CD44, Montagner et al. created an HA-INFα2a bioconjugate as a strategy to permit the targeted delivery of the cytokine.

The synthetic scheme included the derivatization of HA (200 kDa) carboxylic groups with an acetal moiety that converted to aldehyde after mild acidic hydrolysis (4% mol modification). Tethering of the N-terminal group of IFNα2a to the modified HA generated a final protein loading of 28% (w/w), with the unreacted aldehyde groups quenched with glycine. Site-selective conjugation approaches like protein N-terminal modification allow the preservation of protein biological activity and avoid the risk of cross-linking, and HA-INFα2a maintained a high level of antiviral and anti-proliferative capabilities when compared to the
unmodified cytokine. Biodistribution studies in mouse models demonstrated that when intravenously injected, HA-IFNα2a rapidly targeted the liver, while following intraperitoneal injection, the conjugate remains confined in the peritoneal cavity for a significant interval of time (maximum peak at 2 h that decreased after 24 h). Of note, both administration routes lead to the rapid removal of free interferons from the body. Therefore, HA-IFNα2a ensures a prolonged cytokine release in the peritoneum, thereby improving IFNα2a bioavailability. Histological analysis revealed no alterations to the intraperitoneal and peripheral organs. To evaluate the in vivo antitumoral activity, the study employed treatment of SCID mice carrying the intraperitoneally inoculated HCT-15 cells presenting a low or high expression of the CD44 receptor with HA-IFNα2a or IFNα2a. While the unmodified cytokine had no effect, HA-IFNα2a exhibited therapeutic efficacy against HCT-15 tumors with high CD44 expression but had no effect against HCT-15 tumors with low CD44 expression. These results strongly underscore the requirement of HA targeting for the successful outcome of the therapy.

**Actively Targeted Polymer-drug Conjugates**

HPMA copolymer-doxorubicin was further improved by coupling N-acetyl galactosamine residues as targeting moieties to promote liver accumulation via an interaction with asialoglycoprotein receptors on the cell surface of hepatocytes (Duncan, Kopecek, et al. 1983). Galactosamine-targeted HPMA copolymer-doxorubicin featured an HPMA copolymer of 25 kDa MW, a Doxo content of around 7.5 wt%, and a galactosamine content of 1.5-2%. Galactosamine-targeted HPMA copolymer-doxorubicin became the first targeted polymeric conjugate to enter clinical trials (Seymour et al. 2002) for the treatment of primary and secondary liver cancer. In a Phase I clinical trial, galactosamine-targeted HPMA copolymer-doxorubicin treatment of 31 patients with primary (n=25) or metastatic (n=6) liver cancer investigated toxicity, pharmacokinetics, and targeting efficacy (Seymour et al. 2002). The results suggested a recommended dose of 120 mg/m², administered every three weeks by intravenous infusion over 1 h, with a maximum tolerated dose of 160 mg/m². 16.9% ± 3.9% of the injected doxorubicin dose localized to the liver 24 h following administration, with 3.3% ± 5.6% of the dose delivered to hepatoma cells. Interestingly, the conjugate lacking galactosamine residues did not adequately target the tumor but it also targets normal hepatocytes. Unfortunately, as in the case of HPMA copolymer-doxorubicin, industrial business influences stopped the progression of the galactosamin-targeted version, which remains as the only targeted PDC that has reached clinical evaluation.

However, many researchers have devoted their efforts to move active targeting forward as a means to enhance conjugate therapeutic output. For example, Satchi-Fainaro’s group in Tel Aviv, Israel targeted an HPMA copolymer-paclitaxel with alendronate (ALN), a bisphosphonate with high affinity for the hydroxyapatite of bones that displays antiangiogenic activity, with the aim of treating bone metastasis (Miller et al. 2009). Derivatization of the polymeric backbone with a GFLG-p-nitrophenol (GFLG–ONp) linker permitted the direct conjugation of ALN and paclitaxel (PTX) previously coupled with a Phe-Lys-p-
amino benzyl carbonate (FK–PABC) spacer. Cathepsin B, overexpressed and secreted by tumor endothelial and epithelial cells, cleaves both the GFLG and FK spacers to release the drugs. Enzymatic cleavage of the FK dipeptide leads to the formation of an amine intermediate of PTX, which, in turn, spontaneously releases free PTX through 1,6-elimination and decarboxylation. The application of this coupling strategy derived from the failure of HPMA copolymer-PTX (PNU166945) in clinical trials due to conjugate neurotoxicity and neuropathy (Meerum Terwogt et al. 2001). In PNU166945, the ester bond employed to tether PTX to the polymer suffers from instability under physiological conditions, which promotes premature releases of the drug. Testing of the capacity of HPMA copolymer-PTX-ALN to bind bone tissue through the ALN moieties employed hydroxyapatite as a model mineral while anti-cancer efficacy used the human prostate cell line PC3 and the human MDA-MB-231 and the murine 4T1 breast cancer cell lines. The evaluation of antiangiogenic properties employed various in vitro essays: cell proliferation assays, capillary-like tube formation assays, and endothelial cells migration assays of conjugates (Miller et al. 2009, 2011). The overall results established the conjugate as an anticancer and antiangiogenic agent that can bind bone tissue. Previous studies had reported the selective accumulation of HPMA copolymer-ALN and PEG-ALN in bone (Wang et al. 2003, Pan et al. 2008). Wang et al. labeled the conjugates with fluorescein isothiocyanate and injected the derivatives into BALB/c mice, with subsequent fluorescent microscopic analysis of the femurs and tibias establishing bone targeting of the conjugates. Another study monitored the biodistribution of injected HPMA copolymer-PTX-ALN in a xenograft mice model in which mCherry-labeled mammary adenocarcinoma cells were inoculated into the tibia (Miller et al. 2011). The conjugate inhibited tumor growth to a greater degree than free PTX or a combination of free PTX plus ALN and displayed a reduction in toxicity due to an improved pharmacokinetic profile.

With the continuing desire to increase the drug loading capacity of PEG, Pasut’s lab functionalized one end group of a linear heterobifunctional PEG chain with a dendron structure, using bicarboxylic amino acids (beta glutamic or amino adipic acids) as branching units. The authors employed this PEG-dendron polymeric platform, with two or four reacting carboxylic groups at one end, to investigate the effect of targeting agent/drug ratio (Santucci et al., 2006, 2007; Pasut et al., 2008; Pasut et al., 2009; Canal et al., 2010). Following the promising example of the HPMA copolymer-PTX-ALN, the synthesis of PTX-PEG-ALN employed a heterobifunctional PEG dendron as a polymeric platform (Clementi et al. 2011, Miller et al. 2013). The starting polymer possessed four carboxylic groups and one amino group for ALN and PTX coupling, respectively. In contrast to the HPMA copolymer predecessor, the PEG conjugate displayed a high degree of homogeneity. The structure of PTX-PEG-(ALN)₄, comprised of the hydrophobic PTX and the hydrophilic ALN at the opposite extremities of the polymer chain, induced the formation of micelles (∼190 nm, PBS pH 7.4) with PTX located in the internal core and ALN on the outer shell, where it is available for binding to hydroxyapatite. PTX attachment to the polymer used the formation of an ester bond following the introduction of a small succinimidyl spacer. The formation of micelles with PTX located in the inner core
may have the advantage of protecting against blood esterases and stabilizing the system. Similar drug release in plasma and PBS pH 7.4, with 50% of PTX released within 1 h and the remaining released within 24 h, suggested cleavage of the ester bond by hydrolysis without a significant contribution of esterases. With the synthesis of PTX-PEG-ALN, the authors hoped to obtain stronger bone tropism and faster drug release when compared to HPMA copolymer-PTX-ALN. Both high ALN loading (11 % w/w) and a micelle conformation that promotes ALN exposure on the outer surface supported the strong bone affinity observed for PTX-PEG-ALN; in comparison, multivalent polymers such as HPMA copolymer tend to embed ALN groups in a PTX cluster, thereby reducing the targeting properties of the conjugate. Furthermore, rapid bone accumulation also allowed the application of an ester bond and hydrolytic cleavage, rather than the slower enzymatic cleavage of a PTX linker mediated by cathepsin B. In vitro experiments demonstrated that PTX-PEG-ALN displayed a similar IC\textsubscript{50} to free PTX and that possessed anti-angiogenic properties. Additionally, the conjugate selectively accumulated in tumor tissues and exhibited elevated antitumoral activity in mouse models.

Further studies with PEG-epirubicin conjugates, having increasing amounts of FA molecules per polymer chain, evaluated the cytotoxicity, cellular uptake, and internal trafficking in cell lines with different levels of FA expression, and established that conjugate biological behavior depended on the amount of targeting units and receptor expression levels: as the targeting molecule number or FA receptor expression increased, conjugate internalization rate increased. In another study, Pasut’s group compared FA-targeted PEG-gemcitabine conjugates (with two different drug loading of active drug) to non-targeted conjugates (Pasut et al. 2008), using a bicarboxylic amino acid (amino adipic acid) as a branching unit to double drug loading and allow tethering of FA to the PEG amino group through its γ carboxylic group. Overall, in vitro studies demonstrated that the FA targeting agent increased affinity towards the cells overexpressing the receptor by two to three times.

Giammona’s group (Cavallaro et al. 2006) investigated an FA/gemcitabine combination through another polymeric platform - α-β-Poly(N-2-hydroxyethyl)-DL-aspartamide or PHEA, a protein-like polymer with biocompatible properties. The study synthesized and linked two derivatives of gemcitabine - succinyl gemcitabine and diglycolyl-gemcitabine - to the polymeric backbone via two different hydrolyzable ester bonds. FA functionalization with ε-aminocaproic acid as a spacer (80% of the modification at the γ-carboxylic acid and 20% at the α-carboxylic acid) occurred before tethering to the polymer. Again, FA played a fundamental role in the biological activity of the conjugate by promoting cellular uptake of the therapeutic system; further evidenced by the lack of biological activity of non-targeted systems.

Antibodies represent exciting targeting agents; nevertheless, the application of antibodies to polymer therapeutics requires additional considerations. The relatively large dimensions of antibodies can limit tumor infiltration, while antibody tethering must ensure Fab region exposure to the external
environment to allow interaction with its target while keeping the Fc portion internal to inhibit undesired interactions with the reticuloendothelial system. These technical issues, combined with the high costs of monoclonal antibodies (mAb), have promoted the application of peptides and peptidomimetics as alternative targeting moieties as these small molecules can be easily produced in large quantities at relatively low costs and display a very high affinity for the desired particular target.

Peptides can be natural (such as somatostatin and bombesin) or synthetic, identified through screening combinatorial libraries or derived from the chemical modification of natural ligands. RGD (Arg-Gly-Asp)-based peptides that bind the αβ3 integrin represent well-known examples of targeting peptides. The RGD sequence, shared by natural ligands of the αβ3 integrins, such as fibrinogen, plasminogen, prothrombin, and MMP-2, binds to receptors restricted to angiogenic tumoral blood vessels. However, the overexpression of RGD by certain cancers, such as neuroblastoma, melanoma, ovarian cancer, and breast cancer, permits tumor-targeting opportunities. Interestingly, changing the structure of RGD peptides can alter binding specificity and stability; for example, cyclization confers higher rigidity and enhances stability, leading to heightened targeting capacity when compared to linear RGD peptides. Furthermore, different studies have established that the enhanced binding affinities of multimeric RGD peptides for αβ3 integrins translates into enhanced tumor retention in vivo (Janssen et al. 2002, Dijkgraaf et al. 2006).

Mitra et al. demonstrated that RGD-based polymer conjugates achieve specific targeting to the tumor neovasculature (Mitra et al. 2005). They produced an HPMA copolymer–RGD4C conjugate (≈30 kDa) labeled with technetium-99m and injected in SCID mice bearing a prostate tumor. RGD4C is a doubly cyclized peptide with four cysteine residues arranged into two disulfide bridges to conformationally restrain the RGD motif, thus increasing the affinity for αβ3. Following conjugate biodistribution, the authors observed a sustained accumulation over the time in the tumor mass. A collaboration between Dr. Vicent and Prof. Satchi-Fainaro led to the development of a PGA-PTX-E-[c(RGDfK)2] conjugate that aimed to selectively deliver PTX to tumor cells and the surrounding tumor endothelial cells (Eldar-Boock et al. 2011) via passive accumulation and active targeting afforded by c(RGDfK)2. The synthetic scheme employed PTX coupling to PGA (17.8 kDa) via an ester bond followed by targeting moiety coupling to PGA through a peptidic linker. The scheme provided a PTX loading of 8.6 ± 0.3 mol%, a peptide loading of 4.8 ± 0.4 mol%, and a hydrodynamic diameter of the final PGA-PTX-E-[c(RGDfK)2] conjugate of ~7.7 nm as measured by DLS. PGA-PTX-E-[c(RGDfK)2] proved to be stable in plasma, with cathepsin B-dependent degradation allowing controlled drug release. Besides acting as a targeting agent, E-[c(RGDfK)2] preserved its inhibitory effects on adhesion of endothelial cells to fibrinogen following polymer conjugation. In vitro assays with PGA-PTX-E-[c(RGDfK)2] demonstrated an antiangiogenic effect on human umbilical vein endothelial cells overexpressing αβ3 integrin, attributed to the pharmacological action of PTX. Assessments of the anti-proliferative effects of αβ3 employed integrin-expressing U87-MG cells from human glioblastoma discovered that PGA-PTX-E-[c(RGDfK)2] displayed improved growth inhibition when compared to free PTX.
and confocal microscopy analysis of cells samples established that the conjugate accumulated in the cytoplasm. *In vivo* evaluations of targeting ability, via the injection of RGD-bearing conjugates into mice bearing sub-cutaneous U87-MG or MG-63 (human osteosarcoma) tumors, demonstrated tumor accumulation of only the targeted conjugate. Further analysis in a murine breast cancer model (4T1) found that the targeted conjugate exhibited improved antitumoral effects and lowered toxic effects when compared to free PTX or the non-targeted conjugate. Of note, as 4T1 cells express low levels of α_vβ_3 integrin, the authors attributed the antitumoral activity of PGA-PTX-E-[c(RGDfK)]_2 to its anti-angiogenic effects on the angiogenic endothelial cells surrounding the tumor.

**UNSATURATED FATTY ACIDS CONJUGATES**

Studies on tissue-isolated hepatomas with a single arterial inflow and a single venous outflow demonstrated the keen uptake of certain natural fatty acids by cancer cells, probably for use as energy sources and biochemical precursors (Sauer and Dauchy 1990, 1992). Following this evidence, researchers hypothesized that conjugating natural fatty acids to anticancer drugs might represent an effective tumor targeting strategy. Furthermore, certain unsaturated fatty acids are biologically active both *in vivo* and *in vitro*; for example, linoleic and linolenic acids inhibit cancer cell proliferation, while oleic, linoleic and palmitoleic acids prolong survival in Ehrlich ascites carcinoma-bearing mice (Fujiwara et al. 1987, Bégin et al. 1988, Igarashi and Miyazawa 2000).

Docosahexaenoic acid, a ω-3 C22 natural fatty acid with six *cis* double bonds, forms part of cell membranes in the brain and is present in human milk as a precursor for biochemical and metabolic paths. Bradley et al. (Bradley et al. 2001) covalently linked docosahexaenoic acid to PTX at the 2'-hydroxyl position, creating an inactive O-acyl conjugate prodrug that displayed improved anticancer properties in a mouse lung tumor model when transformed into the active compound upon cellular metabolism. Docosahexaenoic acid-PTX displayed reduced toxicity in mice, rats, and dogs compared to free PTX (3 to 4.4 times less toxic on a molar basis) and the conjugate exhibited stability in plasma and enhanced antitumoral activity in M109 murine lung carcinoma tumor-bearing mice. The pharmacokinetic studies established a prolonged circulation time (240 h docosahexaenoic acid-PTX versus 16 h PTX) together tumor accumulation. Interestingly, pharmacokinetic analyses found a 21-fold higher conversion of docosahexaenoic acid-PTX to PTX in tumor tissue compared to plasma. DHA-PTX has now been developed by Protarga Inc under the name Taxoprexin® and has shown efficacy in Phase II clinical trials against prostate, breast, pancreatic, gastric, esophageal, and lung cancers (Hennenfent and Govindan 2006, Jones et al. 2008). A Phase III trial comparing docosahexaenoic acid-paclitaxel with single-agent dacarbazine for first-line treatment of metastatic malignant melanoma is currently undergoing (Clinical trial NCT00087776), as well as a Phase III trial comparing Taxoprexin® plus carboplatin to paclitaxel plus carboplatin for the treatment of advanced lung cancer (Clinical trial NCT00243867).
Another docosahexaenoic acid conjugate synthesized by Wang et al. linked the fatty acid to 10-hydroxy camptothecin through a piperazine linker (docosahexaenoic acid-hydroxy camptothecin) (Wang, Li, et al. 2005). The selection of piperazine aimed to increase aqueous solubility, as it is expected to be protonated at physiological pH, and provide additional stability when facing circulating carboxylate esterases through the introduction of a carbamoyl bond. Docosahexaenoic acid-hydroxy camptothecin testing in three different mouse tumor models (L1210 leukemia, Lewis lung carcinoma, and colon 38 adenocarcinoma) proved the higher efficacy of the conjugate when compared to the parental free drug (hydroxy camptothecin). The authors suggested that, as for other conjugates of docosahexaenoic acid and anticancer drugs, the fatty acid modification created a prodrug, in which the fatty acid moiety prolonged the drug half-life and increased tumor accumulation; however, the study did not report the data of these improvements but focused on the enhanced therapeutic efficacy. Of note, the S-phase-specific nature of hydroxy camptothecin requires prolonged exposure for heightened therapeutic activity.

You et al. prepared a library of unsaturated fatty acid esters of 4′-demethyldeoxypodophyllotoxin to test antitumoral activity (You et al. 2003). 4′-demethyldeoxypodophyllotoxin, a derivate of Podophyllotoxin, is a natural cyclolignan with marked cancer cell cytotoxicity due to the inhibition of both tubulin polymerization and DNA topoisomerase-II activity. 4′-demethyldeoxypodophyllotoxin exhibits strong cytotoxic effects on S180 murine Sarcoma cancer cells in vitro (IC50 = 0.03 μM) but fails to demonstrate in vivo activity. In an attempt to boost in vivo activity, the study esterified the hydroxyl group of 4′-demethyldeoxypodophyllotoxin with different unsaturated fatty acids, finding lower cytotoxicity for esters with long-chain acids (C16-C-22) when compared to short-chain acids (C2-C-6) in A549 lung carcinoma cells and SK-MEL-2 human melanoma cells. Also, alkenic acid esters displayed higher cytotoxicity than alkanoic acid esters with the same number of carbon atoms: the cytotoxicity tends to increase as the number of double bonds increase. However, despite lower in vitro activity compared to the unconjugated drug, the fatty acid conjugated forms of 4′-demethyldeoxypodophyllotoxin displayed increased in vivo antitumor activity, using VP-16, a derivate of Podophyllotoxin that preserves in vivo activity, as a positive control. Of note, the ester of all-cis-11,14-eicosadienoic acid displayed elevated in vivo efficacy when compared to the positive control.

Sasaki et al. demonstrated that the antitumoral properties of a daunomycin-arachidonic acid conjugate (DM-C20:4) in rats previously inoculated with α-fetoprotein-producing rat hepatoma cell line AH66 (Sasaki et al. 1984). α-fetoprotein is abundant in plasma during fetal development and is produced by the liver. However, high levels of this fetal glycoprotein in adults are connected to several pathological conditions, included malignancies. Malignant hepatocytes produce α-fetoprotein, leading to an increase in the serum concentrations of α-fetoprotein in the majority of patients (Hirohashi et al. 1983). α-fetoprotein is considered a useful biomarker for hepatocellular carcinoma (HCC) and an index of tumor aggressivity associated with vascular invasion, metastasis, and poor differentiation (Abelev 1971). Evaluation of α-
fetoprotein levels for early detection of HCC and subclinical recurrence or metastasis represents a critical approach to improve the survival of HCC patients. Interestingly, α-fetoprotein strongly binds large amounts of arachidonic (C20:4) and docosahexaenoic (C22:6) acids (Carlsson et al. 1978, Parmelee et al. 1978). Daunomycin was coupled to unsaturated C20:4 and saturated C20:0 fatty acids through the formation of a peptide bond with the amino group of drug lyxose residue. Intravenous administration of daunomycin- C20:4 in Donryu rats with an intraperitoneally injected AH66 cell line demonstrated diminished in vivo toxicity when compared to free daunomycin and superior antitumoral activity than both free drug and daunomycin-C10:0 (Sasaki et al. 1984).

LIPOSOMES

Liposomes emerged as sustained release systems for drugs that can increase the delivery of a therapeutic agent to the tumor site and reduce the off-target toxicity of the anticancer drug. The first use of liposomes as drug carriers in 1973 by Gregoriadis (Gregoriadis 1973) established that drug entrapment led to considerable improvements in pharmacokinetics, a slow rate of drug clearance, and altered biodistribution of the liposomes, displaying a minor tendency to localize in normal tissues when compared to the free drug.

Liposomes are phospholipid-based vesicles with a diameter range of 50-200 nm (common size range for in vivo parenteral injection uses), in which the circular bilayer, formed by the lipids, creates an aqueous core. This structure offers the possibility to entrap both hydrophilic molecules, hosted in the aqueous space, and hydrophobic molecules, inserted within the lipid bilayer.

Non-targeted liposomes

Overall, liposomes display biocompatibility, self-assembling characteristics, and versatility with regards to the transport of drugs. Their composition, surface charge and functionalization, size, and other physicochemical characteristics can be tightly controlled in order to modulate biological outcomes (e.g., drug release, circulation time, and biodistribution) (Gregoriadis 1995, Allen 1998). Liposomes represent a solution to the poor solubility of certain drugs and degradation by enzymatic or harsh pH environments, thus preserving drug stability. Uptake by the mononuclear phagocyte system, in particular in the liver and spleen where they display high non-specific accumulation, represents the principal mechanism of liposomal clearance from the bloodstream. The inclusion of hydrophilic polymers (especially PEG) to form a coating on the surface of the liposomes creates a barrier against the adherence of opsonins that can enhance phagocytosis. So-called Stealth® liposomes that display steric stabilization and low opsonization suffer from reduced uptake and clearance by the mononuclear phagocyte system (Čeh et al. 1997). The resulting liposomes circulate for more extended periods, thereby representing an efficient carrier that avoids the mononuclear phagocyte system uptake in favor of the delivery of therapeutic drug to cells or tissues (Mori et al. 1991, Allen et al. 1995). Long-circulating liposomes function through sustained drug release and
passive targeting, providing accumulation in areas of increased capillary permeability, thereby reducing exposure to healthy tissues, in agreement of EPR concept.

Prolonged circulation and improved therapeutic efficacy of drug-loaded stealth liposomes have been observed in animal models (Needham and Rudoll 1993, Unezaki et al. 1996, Gabizon et al. 1997); overall, encapsulated drugs display lower toxicity, and the therapeutic index of the liposomal formulation is higher than that of the free drug. Increasing the strength of the interaction of PEG chains with the phospholipid bilayer can further prolong the half-life of a stealth liposome. PEG chains bearing two or four phospholipids per polymer chains can generate doxorubicin- or cisplatin-loaded liposomes with increased circulation time when compared to classic stealth liposomes in which PEG interacts with the liposomal surface through a single phospholipid unit per polymer chain (Pasut et al. 2015, Catanzaro et al. 2018).

The improved patient outcomes helped PEGylated liposomes reach widespread clinical acceptance, with Doxil, a doxorubicin-loaded PEGylated liposome formulation, entering the clinic in 1995. While initially approved by the Food and Drug Administration (FDA) in the USA for treatment of Kaposi’s sarcoma, Doxil also found use in the treatment of ovarian cancers in 2005, for multiple myeloma in 2008, and metastatic breast cancer in 2012 (Northfelt et al. 1996, Barenholz 2012). However, despite a reduced adverse effect profile and a slight increased patient response to therapy, Doxil treatment did not lead to statistically significant improvements in overall survival, time to disease progression, or treatment failure in advanced breast cancer in comparison to doxorubicin.

Recently, a pH-sensitive liposomal formulation for controlled cell up-take was developed by decorating liposomes with a 4 mol% synthetic non-peptidic oligo-arginine cell-penetration enhancer (CPE), quenched by mPEG5 kDa-SDM8 (methoxy-PEG5 kDa-polymethacryloyl sulfadimethoxine), that acts as a reversible stealth polymer (Barattin et al. 2018). The CPE (Arg4-DAG) has a dendron structure ending with 1,2-distearoyl-3-azidopropane for liposome bilayer insertion and exposure on one side, and four arginine units on the other. At pH 7.4, the mPEG5 kDa-SDM8 shields the Arg4-DAG liposomes, resulting in liposomes with neutral zeta potential. Interestingly, PEG dissociates at a pH similar to that of the tumor environment (pH 6.5), leading to Arg4-DAG exposure and subsequently higher cancer cell association when compared to non-decorated liposomes. Testing in HeLa cells demonstrated liposomal uptake in around 99% of cells at pH 6.5, but little uptake at pH 7.4. Overall, this alternative system for the targeted controlled intracellular delivery of model macromolecules and small molecules loaded in the liposome-based on local microenvironmental activation showed promising results that must be validated in vivo.

**Targeted liposomes**

Subsequent studies investigated ligand-targeted liposomes as a strategy to increase interactions between liposomes and target cells and to enhance drug delivery (Allen and Moase 1996). While non-targeted liposomal drug formulations display consistent therapeutic improvements in term of systemic
toxicity when compared to free drug treatment (Safra et al. 2000), selective targeting of cells via ligand-directed liposomes will improve tumor drug accumulation and uptake, resulting in elevated anti-tumor effects (Park et al. 2002).

Park and collaborators used Fab’ fragments of recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) as a ligand, to create immunoliposomes (ILs) for HER2-positive cancer treatment, with the aim of increasing intracellular delivery of the encapsulated drug through receptor-mediated internalization (Park et al. 1997). These liposomes employed PEG-mediated steric stabilization and rhuMAbHER2 Fab’-mediated targeting via covalent links to PEG, leading to the generation of doxorubicin-loaded liposomes displaying 50-100 Fab’ fragments. Testing of anti-HER2 ILs in SK-BR-3 breast cancer cells after encapsulation of a fluorescent probe or gold particles demonstrated rapid uptake followed by elevated accumulation in cell cytoplasm, proving elevated surface binding and receptor-mediated endocytosis when compared to the non-targeted formulation. Interestingly, empty anti-HER2 ILs inhibited SK-BR3 cell growth, thereby providing an additional intrinsic antiproliferative activity to the antitumor effect of doxorubicin. In vivo, the formulation exhibited stability, with no evidence of drug leakage or dissociation and demonstrated prolonged pharmacokinetics. The in vivo assessment of tumor localization and active targeting in a HER2-overexpressing tumor xenograft models found that intravenously administered anti-HER2 gold-loaded ILs accumulated in the tumor interstitium, where they localized to the cytoplasm of cancer cells. In comparison, control gold particles without Fab’ appeared in the extracellular tumor region. Studies in HER2-overexpressing xenograft models of human breast cancer discovered specific enhanced antitumor cytotoxicity of doxorubicin-loaded anti-HER2 ILs with a decrease in systemic toxicity when compared to the free drug or non-targeted doxorubicin-loaded liposomes. Finally, the authors tested their system for the intracellular delivery of nucleic acid, finding that anti-HER2 cationic ILs that form complexes with DNA molecules achieved specific transfection of target cells in vitro. In another formulation for gene delivery, the authors labeled liposomes with rhodamine and oligonucleotides with FITC and visualized the accumulation of the liposomes and the oligonucleotides in the cytosol and the nucleus, respectively. In conclusion, this study suggests that anti-HER2 ILs suppose a valuable strategy for the targeted intracellular delivery of therapeutic agents.

In another example of targeted liposomes, Daniel et al. designed a formulation for the treatment of B-cell lymphoma with the aim of selectively eradicating malignant B cells from the blood of patients, meanwhile preserving T cells and the progenitor population (Lopes de Menezes et al. 1998). This strategy exploited the CD19 receptors exclusively expressed on malignancies affecting B-cell types and absent on hematopoietic stem cells. The study assessed doxorubicin-loaded anti-CD19 liposomes in a CD19+ human B-cell lymphoma model, finding three-fold higher specific binding, uptake, and cytotoxicity when compared to an untargeted formulation. The loss of these improvements upon addition of a free anti-CD19 highlighted the importance of the targeting strategy while the specific recognition of anti-CD19 liposomes
for B cells was demonstrated in a mixture of B and T cells. In vitro analyses established that the targeted liposomes have specific B cell cytotoxicity and in vivo they improved the mean survival time of the mice by selectively eliminating circulating malignant B cells.

A more recent study preclinically evaluated a HER2-targeted liposomal formulation of doxorubicin (MM-302) as in combination with trastuzumab (an mAb used in the treatment of HER2-positive breast cancer) against HER2-overexpressing breast and gastric xenograft models (Espelin et al. 2016). Targeted liposomes display single-chain anti-HER2 antibodies (scFv), with around 45 scFv conjugated to the liposomal surface. Both MM-302 and trastuzumab target the HER2 receptor, but on different non-interfering domains, thereby providing a simultaneous combinatorial therapy. However, coadministration revealed that trastuzumab increased the amount of MM-302 that reached the tumor, resulting in synergistic antitumor efficacy in human xenograft models of breast and gastric cancer.

In a recent work aimed to the treatment of glioblastoma (GBM), Jhaveri and co-workers from the Torchilin laboratory synthesized PEGylated liposomes for the delivery of resveratrol (RES), thus solving problems related to poor solubility and stability (Jhaveri et al. 2018). Active targeting of these liposomes employed transferrin, due to the known upregulation of transferrin receptors in GBM. Rhodamine-labeled transferrin-modified liposomes demonstrated a higher association with cancer cells compared to human astrocytes, with a more significant internalization in cancer cells when compared to non-targeted liposomes. RES-loaded transferrin-modified PEGylated liposomes (transferrin-RES-Liposomes) displayed significantly higher cytotoxicity, providing a higher level of apoptosis when compared to free RES or RES-loaded PEGylated liposomes. In a subcutaneous xenograft mouse model of GBM, transferrin-RES-Liposomes inhibited tumor growth to a greater level than other treatments and improved survival in mice. In conclusion, the application of transferrin-RES-Liposomes represents a valuable strategy for the treatment of GBM, with promising preliminary data.

So far, clinically approved liposome drug formulations do not include specific active-targeting strategies (Doxil®/Caelyx™, Daunoxome®, Myocet®, Marqibo®, Onivyde®, Vyxeos™); however, a doxorubicin-loaded immunoliposome targeted with Cetuximab (mAb epidermal growth factor receptor (EGFR) inhibitor) Fab fragments is in Phase II clinical trials for the treatment of advanced triple-negative EGFR-positive breast cancer (Clinical trial NCT02833766). Future directions may include the targeting of multiple tumor cell subtypes; as heterogeneity characterizes many tumors, targeting two or more different tumor cell receptors or cell types the by the functionalization of liposomes with multiple ligands may improve antitumor activity. Some strategies may employ the co-administration of two targeted formulations or the combination of two drugs within a single targeted carrier. However, several critical obstacles to clinical application must also be appreciated, including the characterization of formulations and problems associated with industrial scale up.
ANTIBODY DRUG CONJUGATES

Specific drug targeting employing antibodies, especially mAbs, can decrease non-specific drug uptake and increase specific interaction and internalization within targeted cells. Coupling an anti-cancer drug to an mAb with high affinity to a cancer cell-specific antigen can improve the therapeutic index (Wu and Senter 2005a). Consequently, mAbs take center stage in an intense area of studies, either as a drug in themselves or as targeting agents. Unmodified mAbs used as an anticancer agent can block a cell activation cascade and/or activate antibody-dependent cell cytotoxicity; however, mAbs usually suffer from a limited therapeutic potential against cancer, requiring their application as part of a combination with more classic pharmacological therapies to improve outcomes (Carter 2001, Wu and Senter 2005b). mAbs can also be armed with cytotoxic drugs, to create antibody-drug conjugates (ADCs), to target the specific delivery of drugs to the precise site of action (Weiner et al. 2010, Casi and Neri 2012, Gébleux and Casi 2016).

Forty-five years after the birth of the “magic bullet” concept, the first ADC materialized in a study that linked the cytotoxic antitumoral drug methotrexate to a polyclonal leukemia cell-targeting antibody in an attempt to target diseased tissue and spare healthy tissue (Mathe et al. 1958). Early studies focused on the application of polyclonal antibodies conjugated with the radionuclide $^{131}$I (Ghose et al. 1967) or the nitrogen mustard chlorambucil (Ghose et al. 1972), but this area of research attracted more attention following the advent of mAbs (Köhler and Milstein 1975) and especially humanized mAbs to avoid human of anti-mouse antibody response (Goldstein et al. 1986). This intense development has led to the current appearance of four ADCs in clinical use, with many others passing through clinical development and nine ADCs presently in Phase III clinical trials (Kaplon and Reichert 2018).

i. Mylotarg (Hills et al. 2014) - Gemtuzumab ozogamicin targeting the CD33 antigen was approved in 2000 for treatment of acute myeloid leukemia, but then withdrawn in 2010 upon FDA request as it failed to meet the efficacy targets required in post-marketing follow-up clinical trials as a condition of its accelerated approval. Mylotarg was re-introduced in 2017 with new specifications, including a lower recommended dosage, a different schedule (alone or in combination with chemotherapy), to treat newly-diagnosed CD33-positive acute myeloid leukemia.

ii. Adcetris (Senter and Sievers 2012) - Brentuximab vedotin targeting the CD30 antigen for the treatment of relapsed or refractory Hodgkin lymphoma and systemic anaplastic large cell lymphoma

iii. Kadcyla (Ballantyne and Dhillon 2013) - Trastuzumab emtansine targeting the HER2/neu receptor for the treatment of HER2-positive metastatic breast cancer

iv. Besponsa (Kantarjian et al. 2016) - Inotuzumab ozogamicin targeting the CD22 antigen used for the treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia
The first attempts to develop ADCs moved from the application of approved anticancer drugs with low cytotoxicity, such as doxorubicin and methotrexate, (Yang and Reisfeld 1988), to the application of more potent drugs, such as calicheamicin (Hinman et al. 1993) or maytansine (Chari et al. 1992) and their derivatives (Anderl et al. 2013, Koehn 2013). This shift permitted the generation of ADCs with $IC_{50}$ values in the range of 0.01 - 0.4 nM.

The first clinical trial with an ADC, conducted in 1983 by Ford and colleagues, employed an anti-carcinoembryonic antigen-antibody conjugated to vindesine (a vinca alkaloid) in patients with advanced metastatic colorectal or ovarian carcinoma (Ford et al. 1983). Five of the eight treated patients displayed clear localization of the ADC at the tumor site determined by detecting radioactivity from the $^{131}$I labeled ADC. For six out of the eight patients, this study was conducted by evaluating ADC tumor accumulation after injecting only the $^{131}$I labeled ADC while the last two patients were firstly treated with the unlabeled ADC and then with the iodinated unconjugated antibody. In these last two cases, we might expect that pretreatment with an unlabeled ADC would prevent tumor accumulation of the labeled antibody because all or most of the anti-carcinoembryonic antigen was blocked by the previous injection of unlabeled ADC.

Interestingly, the results of the study outlined another critical point in the selection of the tumor antigen: the risk of antigen shedding in the bloodstream. Anti-carcinoembryonic can be detected in blood, and the patient with the highest pretreatment level of anti-carcinoembryonic in the blood showed a high liver uptake of the iodinated ADC, likely due to the interaction of the ADC with the circulating anti-carcinoembryonic; obviously this ADC/anti-carcinoembryonic circulating complex lost its targeting selectivity against the tumor.

An optimal tumor antigen should remain localized within the tumor to avoid unspecific accumulation of the ADC at non-tumor sites. Scott et al. detailed cancer antigens and antibodies used against tumors in an interesting review (Scott et al. 2012), while the tumor stroma and vasculature express antigens not present in the healthy tissues (Scott et al. 2003, Sato et al. 2007, Deckert 2009, Neri and Schliemann 2010, Matsumura 2012).

Among the most studied antigens are:

i. anti-carcinoembryonic - a highly glycosylated 180 kDa protein overexpressed in about 95% of pancreatic and gastrointestinal cancers and other tumors (Gold and Freedman 1965)

ii. prostate-specific membrane antigen (PSMA) - a transmembrane receptor expressed only in prostatic epithelium and prostate cancer cells (Henry et al. 2004)

iii. A33 - a transmembrane glycoprotein expressed in normal human colonic and small bowel epithelium and >95% of human colon cancers (Heath et al. 1997)

iv. human epidermal growth factor receptor-2 (HER2) - a transmembrane kinase protein overexpressed in some breast cancers (Dawood et al. 2010)
v. CD20 - a glycosylated transmembrane phosphoprotein targeted in the treatment of all B cell lymphomas and leukemias

vi. the extra-domain B (EDB) of fibronectin, arising from alternative splicing of fibronectin mRNA, present in the extracellular matrix surrounding the newly formed blood vessel of solid tumors (Neri and Schliemann 2010).

Most ADCs developed towards cancer antigens ensured rapid ADC internalization after binding, thus promoting drug release inside tumor cells via selective release through a specific linker that is efficiently cleaved after ADC internalization thanks to the unique conditions found in endosomes/lysosomes (Fig. 3) (Dubowchik et al. 2002, Rudnick et al. 2011, McCombs and Owen 2015, Xu 2015). This approach has led to encouraging preclinical (Blanc et al. 2011, Petril et al. 2012) and clinical (Barginear et al. 2012, Ogura et al. 2012) results, although the process of internalization may also hamper ADC efficacy. In fact, mAbs usually display a slow rate of diffusion into solid tumors, with respect to small drugs, for several reasons such as the binding-site barrier, active internalization processes, and high mass (Saga et al. 1995, Adams et al. 2001, Thurber et al. 2008). The combination of these factors limits ADC tumor penetration to cancer cells located in the perivascular space, while the genetic instability of cancer cells can cause down-regulation of tumor-associated antigens expression, thus representing an important mechanism of resistance to ADC (Loganzo et al. 2016). Targeting cancer antigens associated with tumor stromal or tumor vasculature cells may overcome these issues, while the genomic stability of these cells may also prevent mutation-mediated drug resistance. Targeting an ADC to the tumor neovasculature will block the supply of nutrients and oxygen to tumor cells (Fig. 3) (Jain 2005, Minchinton and Tannock 2006), while the elimination of stromal cells will reduce the expression of growth factors that are essential for cancer cell growth (Mahadevan and Von Hoff 2007, Shin et al. 2013). Alternately, non-internalizing ADCs may overcome reduced tumor penetration of ADCs - in this case, the applications of linkers sensitive to extracellular conditions present in the vicinity of solid tumors allow the release of drugs, which then penetrate the tumor mass (Fig. 3). Linkers include disulfide bonds or specific peptides, where glutathione released in the intercellular space by dying cancer cells (Perrino et al. 2014) or proteases (Dal Corso et al. 2017) promote cleavage.

From a conjugation point of view, the approach employed for drug/linker coupling to mAbs plays a crucial role in the successful development of an ADC. The development of many ADCs presently under investigation use random chemical conjugation methods involving lysines or cysteines amino acids of the mAbs. These sites display appropriate reactivity, although drawbacks include the requirement for protocols that yield heterogeneous isomer mixtures, varying the number of drugs attached per mAb unit and the positions of coupling (Wang, Amphlett, et al. 2005). Thiol conjugation, although generating more homogenous ADC isomers mixtures, remains a suboptimal choice - cysteines can be obtained by partial reduction of mAb’s disulfide bonds, interchain disulfides are usually reduced under milder conditions with respect to intrachain disulfides, but a certain degree of reduction of the latter cannot be avoided. The same
chemistry can be coupled with the genetic insertion of cysteines in the precise position of the mAbs, thus allowing the generation of an ADC with the desired drug-antibody ratio (DAR) and structure (Junutula et al. 2008, Bhakta et al. 2013). This approach requires mild steps of reduction/re-oxidation to form the reduced and reactive inserted cysteine, as, during mAb expression, cysteines tend to be oxidized with glutathione or another cysteine. Research into new site-selective methods for the synthesis of homogeneous ADCs continues to be very active and with certain studies now exploiting genetic engineering and enzymatic methods (Sochaj et al. 2015).

Indeed, genetic engineering approaches represent the basis for the next generation of ADCs. The genetic code expansion method exploits an orthogonal amber suppressor tRNA/aminocyl-tRNA synthetase pair to incorporate an unnatural amino acid (Wang et al. 2001, Liu et al. 2007), with an orthogonal chemical reactivity with respect to the classic 20 amino acids, in precise positions by the insertion of an amber nonsense codon in the mAb gene. This technique allows the synthesis of chemically defined ADCs with perfect control over the DAR and the site of drug conjugation (Axup et al. 2012). The low yield of expression, especially for proteins containing multiple amber nonsense codons, supposes the main limitation of the approach (Wals and Ovaa 2014).
Enzymatic methods for drug conjugation to ADC are based on the use of glycosyltransferases, transglutaminases, sortase-A, or formyl glycine-generating enzyme. Antibodies are glycosylated with two N-linked glycans in the Fc region, one per heavy chain, at asparagine 297. The sialic acid of glycans can then be oxidized under mild conditions by periodate to generate aldehyde groups suitable for conjugation with molecules containing aminoxy- or hydrazide- groups. A two-step enzymatic approach for IgG glycan conjugation employs β1,4 galactosidase to remove the galactose residues from the glycan termini and β1,4-galactosyltransferase to transfer galactose residues carrying an orthogonal reactive group at the C2 position, such as a keto- or azide- group, which in turn can be exploited for conjugation (Boeggeman et al. 2009, Zhu et al. 2014). Transglutaminases catalyze the transfer reaction between the acyl moiety of a protein-bound glutamine residue (acyl donor) and a primary linear amine (acyl acceptors) (Mero et al. 2016). IgGs display a substrate of transglutamininase (glutamine 295 in the Fc region), although the glycans located on Asn297 sterically hinder any reaction, thereby requiring IgG deglycosylation by PNGase F to expose the Gln295 and allow the conjugation of molecules containing a primary amino group. Jeger and coworkers investigated this approach with the genetic replacement of Asn297 with a Gln to yield the ADCs with up to four drug molecules per antibody (Jeger et al. 2010). Alternatively, a TGase specific substrate, like the pentapeptide LLQGA, can be inserted at the desired position in the IgG sequence allowing a better design of the final ADC (Farias et al. 2014). Sortase-A, a transpeptidase that recognizes LPXTG motifs (where X is any amino acid) at the C-terminus of proteins, catalyzes the replacement of the Gly residue of a motif with molecules containing a GGG tripeptide. One study employed this approach to generate anti-CD20 conjugates with monomethyl auristatin E (Pan et al. 2017). Formyl glycine-generating enzyme converts the cysteine of the motif CXPXR (where X is serine, threonine, alanine, or glycine) inserted at desired position into the IgG sequence, into a Cα-formylglycine, which can be exploited for selective conjugation with molecules containing an amino-oxy- or hydrazide-group (Rabuka et al. 2012).

In conclusion, other than the selection of the starting mAb, the present approaches of ADC preparation present critical points that need to be addressed by the future ADC generation:

I. random conjugation of a drug to a mAb is the main exploited approach, although several proposals of site-selective conjugation have been proposed based on genetic engineering and/or enzymatic methods (Junutula et al. 2008, Axup et al. 2012, Farias et al. 2014)

II. linkers should be improved to allow better tuning of DAR and to balance the hydrophobicity of the drug
III. The limited predictability of the preclinical models - in animals, the selected human cancer epitope is tumor-specific, while in humans it is tumor-associated with potential expression in other cell types.

Future developments in this area will offer tailored ADC design by expanding the different formats of mAbs and their moieties, e.g., Fab, scFV, diabody, nanobody (Frenzel et al. 2013).

CONCLUSIONS

From the first early attempts to the systematic studies of recent years, the generation of selectively targeted drug delivery systems has made impressive progress. The availability of optimized mAbs and their second-generation derivatives, together with the improved design of specific drug delivery systems, will propel the therapeutic capabilities of targeted molecular platforms of drug delivery. Owing to the different characteristics of each disease, there remain important obstacles and unaddressed needs that must be solved, such as overcoming the mechanisms of drug resistance. A single overarching platform is unlikely to arise; instead, we foresee the development of individual highly-specialized and complex platforms for a precise application. Furthermore, these platforms will also venture further from cancer to applications in other diseases. The continuous advances in this field will change current treatment approaches toward more precise and safer medicines.

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