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## Self-assembled lipid-prodrug nanoparticles for the treatment of severe diseases

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### Abstract:

Combining the distinct advantages of the prodrug strategy with those of nanoparticulate delivery systems has demonstrated several advantages in the treatment of severe diseases such as better diffusion through biological barriers, enzymatic activation of the lipid-conjugate in the diseased area, drug protection, better pharmacokinetic and biodistribution, intracellular delivery and/or targeting properties. Thanks to fundamental advances in supramolecular chemistry and to the fine-tuning of the nature of the lipid (fatty acid, steroid, phospholipid, glyceride etc.), the drug itself (small molecule entity or macromolecule) and the drug-lipid chemical link (ester, amide, disulfide, etc.), it is now possible to develop drug-lipid conjugates capable of self-assembling spontaneously as nanoparticles in water media with unique supramolecular organizations. In other words, apart from using solvents to allow the solubilization of the molecular species, no other excipients are needed. In this review, we offer the reader a deeper understanding of the chemical synthesis, physico-chemistry, structure, and pharmacological activity of lipid-prodrug based nanomedicines and discuss the outlook for translation to clinical practice.

### 1/ Introduction

Nanomedicines<sup>1-3</sup> (i.e., drug encapsulated into liposomes, nanoparticles, polymer micelles, etc.) have gained increasing importance in the treatment of severe diseases, due to the ability of these nanosized devices (i) to protect the loaded drug from degradation, (ii) to favourably modify drug pharmacokinetics and biodistribution, (iii) to improve intracellular delivery, and (iv) to target diseased areas at the cell and tissue level. However, if nanotechnologies have significantly improved the delivery of drugs, the currently available nanomedicines have not yet been applied to many drugs and diseases, due to the poor drug loading (ie. the amount of the transported drug *versus* the amount of the transporter material, generally expressed as %) and the often uncontrolled “burst release” of the loaded drug after administration (Figure 1A). Thus, owing also to regulatory challenges (BOX 1), the need to administer an excessive amount of the transporter material (ie. polymer, lipid etc.) relative to the drug explains the relatively low number of nanomedicines currently on the market, although the use of lipid nanoparticles for mRNA COVID-19 vaccination has recently boosted the field.

On the other hand, the lipid prodrug<sup>4</sup> approach (also named in this review as “lipid-drug conjugate” or “lipid-drug conjugate with a bio-cleavable bond”) may be defined as a pharmacologically active molecule conjugated to a lipid through either a chemical bond or through a spacer, both cleavable by a physicochemical or enzymatic mechanism (Figure 1 B and C). Several lipid prodrugs are already on the market since a long time as depot formulations to extend the pharmacological activity or to improve the drug bioavailability. Injectable formulations such as haloperidol decanoate or testosterone undecanoate, also used for oral administration or lipidated corticosteroids for topical use or local injection are examples of this class of drugs. But conceptually, the prodrug approach may have

additional applications, since tailoring the chemical link allows to control the release of the drug at the right time and the right place in the body and the nature of the lipid (i.e., fatty acids, squalene, cholesterol, triglycerides, or phospholipids) may improve the amphiphilic character of the resulting bioconjugate, thus improving diffusion through various biological barriers. Of note, the lipid prodrugs may also take advantage of the physiological lipid metabolic pathways for allowing the oral absorption of drugs. However, prodrugs suffer from non-specific distribution and rapid clearance, and many of them are non-soluble in the aqueous solvents for intravenous administration (BOX 1).

Combining the distinct advantages of nanoparticulate delivery systems with the prodrug strategy has therefore been considered to achieve more efficient and safer therapies. Considerable efforts have been dedicated to the encapsulation of prodrugs into nanocarriers, either liposomes<sup>5</sup>, polymer micelles<sup>6</sup>, polymer nanoparticles<sup>7</sup>, or solid lipid nanoparticles<sup>8,9</sup>, with some interesting results (Figure 1B). However, the ratio between the amount of the pharmacologically active molecule (i.e., the drug) and the transporter materials (i.e., lipids, polymers, metals, etc.) remains in general rather limited.<sup>7</sup> In other words, in the preparation procedures that use physical means for drug encapsulation (i.e. adsorption, encapsulation, entrapment etc.), the amount of excipient is greater than that of the drug. Interestingly, thanks to fundamental advances in supramolecular chemistry, it is now possible to develop drug-lipid conjugates that can self-assemble as nanoparticles in water media with unique supramolecular organizations (Figure 1C). This approach represents a paradigm change, absence of burst release and ease of preparation. Overall, the use of lipid-prodrugs nanoparticles with self-assembly properties is an emerging and relatively recent technology which has already aggregated interesting proofs of concept at the pre-clinical stage for the treatment of severe diseases, including cancer, infectious diseases, neurological disorders and uncontrolled inflammation. However, as it will be discussed in this review, the translation of this nanomedicine concept towards the clinic is still in its infancy and will deserve additional efforts to better tailor the clinical and preclinical methodologies to the target disease.

The present article will focus on the design of lipid prodrug nanomedicines with self-assembly properties for the treatment of severe diseases. The encapsulation of drugs into solid lipid nanoparticles<sup>10</sup> or liposomes<sup>11</sup>, as well as the self-assembling of polymer<sup>12,13</sup> and peptide drug conjugates<sup>14</sup> are excluded from this review because extensively considered elsewhere. Similarly, detailed information about lipid prodrugs which don't assemble as nanoparticles can be found in several other reviews<sup>15,16</sup>. Finally, nanoassemblies of lipid-based conjugates that are not used for therapeutic purposes such as nucleolipids and glyconucleolipids composed of natural ribo- or deoxyribonucleosides<sup>17,18</sup> will not be addressed too.

## 2/ Lipid prodrugs chemical synthesis

A typical lipid-drug bioconjugate is composed of three main building blocks: (1) the carrier molecule which is a lipid, (2) the drug ranging from small molecule to macromolecule drugs, and (3) the chemical spacer (Figure 2, Figure S1). A slight variation of one of these parameters is enough to modify the physico-chemical properties of the bioconjugate and consequently the in vivo fate of the resulting nanoparticle. The synthesis of lipid-drug bioconjugate is highly dependent on the availability of functional groups and appropriate linkers. Furthermore, by varying the nature of the spacer (hydrophilic or hydrophobic, short or elongated) and of the lipid moiety, the amphiphilicity of the lipid prodrug may be controlled. It is important to note that this modulation strongly influences the supramolecular nanoassembling properties of the prodrug, and therefore the release of the drug. In

addition, the design and synthesis of biodegradable and environment stimuli responsive nanomaterials may improve drug safety and efficacy.

*In that respect*, various strategies have been employed for the chemical design of self-assembled lipid prodrugs: (i) the direct linkage between the lipid moiety (modified or not) and the drug, (ii) the use of a bio-cleavable organic linker, or (iii) the implementation of smart stimuli-responsive lipid conjugates (Figure S1).

### *2.1- Direct drug to lipid conjugation*

Fatty acids (FAs) with a long unsaturated or saturated hydrocarbon chain bearing a free terminal carboxylic acid are commonly used to directly bridge the free hydroxyl or amine functional groups of the drug, resulting in ester or amide bond respectively.<sup>19,20</sup> In that respect, activation of the acid function of FAs is required prior to the conjugation step. The most common activating agents are carbodiimides (EDC or DCC) that activate the acid function into *O*-acylisourea intermediate. More sophisticated, the phosphonium (PyBOP) and uronium (HBTU or HATU) activating agents activate FAs into *O*-acyloxyphosphonium salt and benzotriazol ester, respectively. This acid activation approach is the most common method of linking FAs and has been utilized for the lipidization of many parent drugs, including nucleosides, antiviral or anticancer nucleoside analogues, taxanes, platinum derivatives, topoisomerase inhibitors, NSAIDs, oligonucleotides and others.<sup>19–27</sup> In general, the conjugation of FAs or other lipids to highly hydrophilic drugs (i.e., small molecules, peptides, nucleic acids etc.) is a challenging task due to the limited solubility of both partners in the same solvent (organic or aqueous). Thus, when hydrophilic drugs are used, the polar aprotic *N,N*-dimethyl formamide (DMF) is the privileged solvent which fosters a good match with lipids. Starting with saturated FAs, very few examples of nanoparticles are reported because of the poor self-assembling properties of the resulting bioconjugates. In contrast, the conjugation to unsaturated fatty acids often led to the formation of nanoparticles in aqueous media, especially when polyunsaturated lipids and hydrophilic drugs were used.<sup>19,23</sup> Other linear lipids belonging to the terpene family such as the polyisoprenic derivatives, were also designed to form prodrugs with the ability to self-assemble spontaneously into nanoparticles. In that respect, due to the squalene's molecular flexibility and dynamically folded conformation, tris-nor-squalenic acid, squalenylacetic acid, tris-nor-squalenol, tris-nor-squalenamine, and so on, allowed the direct linkage of the squalene moiety to a huge variety of drug molecules bearing alcohol, amine or acid function through biocleavable ester or amide bond, using the same acid activation approach as for FAs.<sup>23,28–33</sup> Owing to the unique self-assembling capability of polyisoprenoids, another original approach has exploited a polyisoprenoyl moiety itself as a spacer between anticancer drugs (i.e., gemcitabine and paclitaxel) for combined therapy.<sup>34</sup>

### *2.2- Use of a spontaneously releasing spacer*

Using the same acid activation approach, lipids can also be conjugated to the drug molecule through an organic spacer via hydrolyzable bonds (i.e. ester, amide,...). This spacer is used (i) when a direct biocleavable linkage is not possible, (ii) to avoid steric hindrance between the drug and the lipid moiety during the conjugation reaction, (iii) to modulate the release of the drug and/or (iv) to promote the self-assembling properties of the bioconjugates. For example, to prevent aggregation, it was demonstrated that the simple insertion of a dithiodiglycolyl spacer bearing a disulfide bond, between hydrophobic drugs (such as paclitaxel or doxorubicin) and  $\alpha$ -tocopherol, or between hydrophilic drugs (such as fluorouracil or gemcitabine) and stearic acid, led to bioconjugates able to self-assemble as stable nanoparticles, which was not possible when using a simple succinyl linker.<sup>35</sup> To allow bioconjugation of the hydrophilic gemcitabine (through its nucleobase) with fatty alcohols or other lipids bearing an alcohol function (phytanyl alcohol, tris-nor-squalenol, cholesterol,  $\alpha$ -tocopherol),

different spacers such as glutaryl or carbonyl functional groups (providing carbamate moiety) were introduced.<sup>23,36,37</sup> With the aim of promoting the release of hydrophobic paclitaxel from very compact nanoparticles composed of lipophilic bioconjugates, succinyl and more hydrophilic diglycolyl or PEGylated linkers were introduced. These linkers were employed to increase the susceptibility to hydrolysis of the linkage between the hydrophobic paclitaxel (on 2'-hydroxyl group) and the squalene moiety by fostering the water access.<sup>38</sup> A similar approach was applied to link Leu-enkephalin neuropeptide or penicillin G to squalene derivative, using either diglycolyl spacer, or methylene and methylenecarbonyl bridge, respectively, in order to modulate the release of the drug.<sup>28,39,40</sup> Using a nucleoside lipid scaffold, original ruthenium-based octahedral complex nanocarriers were developed as alternative to Pt-based chemotherapeutic drugs. These bioconjugates were made up of one or two oleic acids connected to the ruthenium through a PEGylated nucleoside (thymidine or uridine) used as spacer *per se*. To allow the complexation with Ruthenium, a pyridine-methyl arm was tethered on the nucleobase of the nucleoside lipid.<sup>41</sup>

The lipidization of highly hydrophilic macromolecules such as oligonucleotides was also carried out using FAs. Generally the conjugation occurs at the 5'- or 3'-ends.<sup>42,43</sup> For example, it was demonstrated that conjugation of myristic acid to antisense oligonucleotide through an ApoE cell-penetrating peptide (in order to promote oligonucleotide cell permeability), assisted this hybrid bioconjugate to form a complex by self-assembly. In that respect, myristic anhydride reacted with the N-terminus of resin-bound ApoE peptide bearing C-terminal cysteine. After deprotection and resin cleavage, this latter was then conjugated to the 3'-maleimide-functionalized phosphorodiamidate morpholino oligonucleotide (PMO) via a thioether bond using thiol-maleimide click reaction with a good yield.<sup>44</sup> Of note, the direct conjugation of myristic acid on the secondary amine at 3'-end of the PMO was also possible but with a much lower yield. With respect to siRNA, hetero-Michael addition reaction allowed the thiolated RET/PTC1 siRNA sense strand to react with a diethylene glycol maleimide-derivatized squalene to form a covalent nonreducible thioether bond (55% yield under microwave irradiation in the presence of methanol).<sup>45,46</sup> Another strategy based on the Copper-free click chemistry was also carried out. Thus, the azido-squalene or azido-solanesol reacted through 1,3-dipolar cycloaddition with the dibenzocyclooctyne residue of siRNA. Of note, the TMRSS2-ERG siRNA was linked to dibenzocyclooctyne through a *N*-(hexamethylenyl)-6-oxohexanamide spacer to avoid steric hindrance (synthesis yield up to 95%).<sup>47</sup> In another approach, cholesterol was conjugated to the 3'-end of the sense strand of an apoB siRNA via a *N*-(6-aminohexanoate)-trans-4-hydroxyprolinol linker but regrettably without mention of the final yield.<sup>48</sup>

### 2.3- Use of a stimuli-responsive moiety

Recent advances have led to the design of smart stimuli-responsive nano-systems able to control drug biodistribution in response to specific stimuli, either exogenous (variations in temperature, magnetic field, ultrasound intensity, light, or electric pulses) or endogenous (changes in pH, enzyme concentration or redox gradients). In that respect, a stimuli-responsive linkage or spacer was generally inserted between the drug and lipid moiety. Hence, pH-responsive phospholipid-like prodrug formulated into micelles may improve the tumour specificity of doxorubicin when conjugated to 11-mercaptoundecyl phosphorylcholine bearing a hydrazide group *via* a hydrazone linkage.<sup>49</sup> With the aim of facilitating the rapid and selective drug release from hydrophobic oleoylpaclitaxel bioconjugates, an ethyleneglycol-thiodiglycolyl spacer or its dithioether derivative was introduced, to generate original redox dual-responsive prodrug-nanosystems.<sup>50</sup> The synthesis of these prodrugs consisted first in conjugating oleic acid to ethylene glycol and then to thiodiglycolic anhydride or to its dithioether derivative and the resulting oleic acid-linkers were subsequently coupled to paclitaxel. Thus, in cancer cells, these linkers released paclitaxel via two simultaneous phenomena: (i) thiolysis in the presence of the strongly reducing environment of intracellular glutathione (GSH) and (ii)

oxidation of the thioether into hydrophilic sulfoxide or sulfone, because of the overproduction of reactive oxygen species (ROS). Later paclitaxel was conjugated to citronellol using disulfide bond-containing carbon chain linkages of various lengths. It was shown that the position of the disulfide bond in the carbon chain significantly affected the redox dual-responsiveness, but also dramatically influenced tumour-specific drug release and anticancer activity *in vivo*.<sup>51</sup> More sophisticated, photoactivable nanosystems combined lipid-based camptothecin (SN38) prodrug and C18-lipidated protoporphyrin IX (PpIX) photosensitizer. The SN38 prodrug was constituted of two multiple thioether-modified fatty acids connected to SN38 through a thioglyceropropionyl moiety. As regards, the synthesis of the SN38 prodrug, thioether-modified FAs, was conjugated with tert-butyl S-thioglyceropropionate ester. After deprotection of the acid function by TFA, the resulting S-thiodiglyceride propionic acid was activated by oxalyl chloride prior to SN38 conjugation. For the synthesis of C18-PpIX, stearic alcohol was first conjugated to thiodiglycolic anhydride. The resulting C18-thiodiglycolic acid was then coupled to ethylene glycol prior to be conjugated to PpIX, using DCC/DMAP reagents. Therefore, upon laser irradiation, these hybrid co-nanoassemblies underwent the *in situ* oxidization of the multiple thioethers moieties (located within fatty acids and linkers) into hydrophilic sulfones, which resulted in the rapid destruction of the nanoparticle structure, thus triggering fast and targeted drug release.<sup>52</sup> By combining photodynamic therapy and chemotherapy, a new generation of photoactivable nanomedicines was also developed. Thus, cytotoxic cabazitaxel was coupled to  $\alpha$ -linoleic acid via ROS-cleavable S,S(bisethylamino)carbonyl acetone-based thioketal linker and the resulting prodrug was then co-nanoassembled with chlorine e6 (Ce 6) photosensitizer conjugated to  $\alpha$ -linoleic acid through ethylene diamine linker (Figure 2).<sup>53</sup> Then, once in tumour cells, upon activation of endogenous or photosensitizer-induced ROS, the drug was released via intramolecular nucleophilic substitution reaction, while at the same time the released Ce 6 lighted up cancer cells. Other visible-light-triggered prodrug nanoparticles were prepared by conjugation of lipophilic verteporfin photosensitizer and doxorubicin through cathepsin B-cleavable FRRG (Phe-Arg-ArgGly) peptide used as a linker *per se*.<sup>54</sup>

### 3/ Structure and preparation of lipid prodrug nanoparticles

#### 3.1- Preparation and long-term storage

The preparation of prodrug nanoparticles relies on the spontaneous ability of the lipid-prodrug molecules to self-assemble into nanosized particles with or without minimal use of a stabilizer. Lipid prodrug nanoparticles can be prepared in several ways, such as nanoprecipitation<sup>8</sup>, microfluidics<sup>55</sup> and high-pressure homogenization<sup>27</sup>, nanoprecipitation being the most investigated. It is, indeed, an easy procedure that involves dissolving the lipid-prodrug in an organic solvent before addition to an aqueous medium, allowing nanoparticle formation.

The long-term storage and stability of lipid-prodrug nanoparticles are important considerations for clinical translation. Some studies have demonstrated that lipid-prodrug nanoparticles could be lyophilized.<sup>56,57</sup> Of note, the physicochemical properties of the lyophilized formulations, mainly size, polydispersity and zeta-potential, do not change after several months of storage (3-4 months) at 4°C.<sup>56,57</sup> Among different cryoprotectants, trehalose was reported to be the most efficient to allow the maintenance of the physicochemical properties of the formulations. Since nanoparticles accumulate in the liver after intravenous administration, the hepatic toxicity of lyophilized nanoparticles was investigated. It was established that the particles were safe and did not induce toxicity neither *in vitro* on hepatocytes HepG2 cells nor *in vivo* in a mouse model of liver ischemia-reperfusion.<sup>57</sup> Regarding efficacy after lyophilization, it has been reported that lyophilized nanoformulations of squalene-

gemcitabine retained their cytotoxic activity after freeze-drying.<sup>56</sup> Overall, such approach may facilitate the clinical application of freeze-dried lipid-prodrug nanomedicines.

### 3.2- Structure and supramolecular architecture

The creation of a variety of fascinating and complex supramolecular architectures has been reported (Figure 3), including lamellar, hexagonal, cubic, and toroidal forms, ribbons, vesicles, helical strands, spherical or wormlike micelles and amorphous structures.<sup>58-62</sup> Answering the question of what are the typical lipid prodrugs that are capable of self-assembly is rather difficult: it evidently depends on the physicochemical properties of both the lipid and the drug to be conjugated to, but the literature lacks systematic studies involving both partners, drug and lipid. It was reported that the dynamic shapes of amphiphilic molecules decide the morphology of self-assemblies, and the volume ratio of polar heads and hydrophobic tails is the key factor.<sup>63</sup> Even if the amphiphilic character of the drug-lipid is in general preferable, it is not always the case since lipophilic drug-lipid conjugates, like paclitaxel-squalene<sup>38</sup>, doxorubicin-squalene<sup>64</sup>, or even lipophilic prodrugs of dexamethasone<sup>62</sup>, are nevertheless capable to form nanoparticle structures, too. However, the hydrophobicity of lipid-paclitaxel conjugates led to pure hydrophobic nanoassemblies that despite their high accumulation in the tumor tissue, were not able to increase the anti-tumor effect, probably due to lack of accessibility for enzymes to activate the prodrug. However, the insertion of a thio- or dithio-based redox responsive linker, allowed the release of the drug from the nanoparticles within tumor tissues and thus inhibited significantly tumor growth and enhanced the antitumor activity.<sup>50</sup> To identify typical lipid-prodrugs that are capable of self-assembly, a systematic approach has, however, been performed recently using various lipids including terpenoids, sterols, fat soluble vitamins, saturated and unsaturated fatty acids, as well as diglycerides for conjugation to gemcitabine used as a model drug<sup>23</sup>. With some exceptions, conjugates having a hydrophilic-lipophilic balance (HLB) below 8.4 could be formulated as stable nanoparticles and the presence of lateral methyl on the hydrophobic chain represented also a key parameter concerning the ability of the bioconjugates to self-assemble. Another important parameter to consider is the presence of insaturations on the lipid chain. Indeed, with a few exceptions<sup>65</sup>, saturated lipid-based conjugates are generally struggling to spontaneously self-assemble into nanoparticles, probably due to the high degree of flexibility of the lipid chain.

Since the drug release depends on the bioactivation of the prodrug, the supra-molecular architecture is a key factor, often under-estimated, for the pharmacological activity to occur, resulting from the need for the enzymes or other endogenous stimuli (acidity, redox environment etc.) to diffuse into the nanoparticle core. From that point of view, the presence of hydrophilic moieties enabling the creation of aqueous channels in the nanoassemblies is preferred to an amorphous hydrophobic configuration. Even if it is likely that the supramolecular organization of the lipid prodrug nanoparticles may dramatically affect the pharmacological activity, it is rather difficult to draw definitive conclusions since the diversity of the experimental conditions reported in the literature make comparisons risky and because the nanoparticle structure is generally ignored in most of these studies. Nevertheless, if comparing the pharmacological activity of several squalene-based nanodrugs produced under rather comparable experimental conditions, it appears that the absence of a supramolecular structure is clearly detrimental to the therapeutic activity (Figure 3).

The diversity of the designed nanostructures is illustrated by the following examples. For instance, rod-shaped structures of myristoylated cabotegravir, with a particle size of 318 nm, have been used.<sup>27</sup> In another example, nanoparticles of 250 nm composed of doxorubicin conjugated to tocopherol-PEG succinate with a highly ordered lamellar inner structure, were developed.<sup>58</sup> As indicated by the NMR spectra, doxorubicin was located in the particle core while PEG chains and part of the tocopherol were in the corona. Similarly, multidrug nanosystems combining squalenoyl-gemcitabine with edelfosine,

an alkyl-lysophospholipid, displayed a multilamellar concentric organization with a physical resemblance to onion-like multigeometry.<sup>59</sup> The creation of nanoassemblies of hexagonal shape has also been demonstrated. Nanoassemblies of squalene-gemcitabine showed an internal hexagonal structure at the supramolecular scale, made of reticular planes, each particle being surrounded by an external shell.<sup>60</sup> Molecular modelling has suggested the stacking of inverse hexagonal phases, in which the central aqueous core is composed of water, when gemcitabine is surrounded by squalene. Remarkably, it was observed that polyisoprenoyl gemcitabine bioconjugate with 5 isoprene units (gemcitabine-5 isoprene), which differs from squalenoyl gemcitabine only in the position of two methyl groups on the hydrophobic chain, displayed different supramolecular organizations and self-assembled into nanotubes<sup>66</sup>; this was attributed to the higher rigidity of gemcitabine-5-isoprene which hindered the formation of the inverse hexagonal phase observed with squalenoyl gemcitabine nanoparticles. Another study has revealed that subtle chemical differences induced a diversity of structures: when squalene was conjugated to the sugar moiety, the resulting nanoparticles structures turned into a lamellar phase instead of hexagonal, when the grafting was performed onto the nucleobase<sup>67</sup>. Furthermore, it was reported that the simple insertion of a phosphate group between the sugar moiety of gemcitabine and the squalene increased the hydration and hindrance of the polar headgroup leading to the transition of the lamellar phase towards unilamellar vesicles.<sup>22</sup> The complexity of these self-assembled structures results from the interplay among the unique conformational properties of squalene, the packing parameters of the bioconjugates, and possible specific H-bond interactions involving nucleobases. And when gemcitabine was replaced by deoxycytidine or adenosine, spherical nanoparticles were still obtained but with a bi-continuous cubic inner structure, as highlighted by transmission electron microscopy and small-angle X-ray diffraction.<sup>61</sup> Additionally, the nature of the supramolecular structure can even be dramatically modified by the nanoparticle suspending medium, as illustrated with doxorubicin-squalene: in the presence of monovalent salts, the elongation of the cylindrical nanoparticles is obtained<sup>68</sup>, which could be beneficial for prolonging the blood circulation time by hindering the recognition by the Kupffer cells of the liver and the macrophages of the spleen. But divalent anions triggered the lipid-prodrug assembly by means of salt bridges, inducing a transition from individual cylindrical nanoparticles to bilayer tubes. This highlights that the nanoparticles in the test tube and in physiological conditions may be very different, their structure depending on the local *in vivo* environment.

These examples illustrate the extreme complexity of the physicochemical mechanisms involved in the self-assembly of drug-lipid conjugates towards a given supramolecular structure. The involved parameters are numerous: the nature of the drug and the lipid, the nanoparticles concentration, the pH and ionic strength of the nanoparticles suspending medium etc. This makes hard for a given drug to recommend the lipid to be used to obtain a preferred supramolecular organization.

#### 4/ Why a lipid prodrug nanoparticle marriage?

##### 4.1- For reducing drug metabolism and improving drug pharmacokinetic and biodistribution

Prodrug-based nanoassemblies make it possible to protect transported biologically active molecules, both metabolic sensitive small molecules and macromolecules. For example, siRNA conjugated to palmitic acid, cholesterol, squalene derivative, or tocopherol resulted in decreased degradation after intravenous administration<sup>48</sup> and it was observed that when chemically conjugated to cholesterol or long fatty acid chains, there was a dramatic increase of siRNA uptake into cells, resulting in gene silencing *in vivo in mice*. Interestingly, a detailed investigation concerning the behaviour of lipid-siRNA conjugates with blood components, both *in vitro* and *in vivo*, has emphasised the occurrence of strong



interactions with lipoprotein particles (both high-density lipoprotein HDL and low-density lipoprotein LDL), lipoprotein receptors and transmembrane proteins. Whereas LDL conveyed cholesterol-siRNA mainly to the liver tissue, HDL directed lipid-siRNA in the liver, the kidneys, the gut, and the steroidogenic tissues. By comparing *Ldlr*<sup>-/-</sup> and wild-type mice, it was found that the LDL receptor was necessary for LDL-associated cholesterol-siRNA delivery *in vivo*.<sup>48</sup> Similarly, the metabolism of antinociceptive neuropeptides (ie., Leu-enkephalin and Met-enkephalin) was hindered after conjugation with squalene but the association of these bioconjugates with either LDL or HDL is still under debate.<sup>28</sup>

A similar behaviour was observed with small molecules. Indeed, when injected intravenously to rats, squalene-gemcitabine and adenosine-squalene nanoparticles rapidly disaggregated due to their unique hexagonal or cubic supramolecular structures and the released bioconjugates were inserted into the LDL as single molecular entities, thus using LDL as a sort of “indirect” endogenous nanocarriers for the targeting of tumours with hyper-expression of LDL receptors.<sup>69</sup> This raises the question if the nanoparticulate formulation is mandatory or if the lipid prodrug as a single molecular entity remains sufficient for the pharmacological activity to occur. The answer to this question depends on both the nature of the drug and the therapeutic application envisaged. Indeed, structuring the prodrug in the form of nanoparticles is preferable when the molecule to be transported is a nucleic acid, a peptide or another fragile biomolecule. The same is true when taking advantage of an EPR effect is needed. On the other hand, in certain applications, when the drug is a more stable small molecule that can take advantage of endogenous particles for a precise targeting, the bioconjugate in its molecular form may be sufficient. However, in most of the cases, when the biologically active molecule is conjugated to a lipid, it becomes generally insoluble in water and, for intravenous administration, a nanoparticle formulation remains the only possible.

Lipid prodrugs of gemcitabine were also synthesized to slow down the degradation after intravenous administration. Indeed, in humans, gemcitabine is enzymatically metabolized by deoxycytidine deaminase into its corresponding inactive uracil derivative which represents a major limitation in the pharmacological efficacy of this anticancer drug.<sup>70</sup> By covalently coupling gemcitabine with the 1,1,2-trisnorsqualenic acid, the resultant nanomedicine displayed in mice a prolonged blood half-life, a greater mean residence time resulting from a delayed metabolism in difluorodeoxyuridine, whereas the urinary elimination was considerably reduced as compared to the parent drug.<sup>71</sup> Apart from reducing the drug metabolism, certain lipids may also be used as a targeting ligand for specific biodistribution of small anticancer molecules. Thus, docosahexaenoic acid (DHA), an omega-3 C22 natural fatty acid, conjugated to gemcitabine was reported to trigger the uptake of the anticancer compound by tumour cells *in vitro* (MCF-7 and HepG2 cells) and *in vivo* (MCF-7 and HepG2 tumor bearing mice), due to the phosphatidylethanolamine contents of the cancer cell membranes.<sup>72</sup> Finally, the fine control of the supramolecular organization of lipid prodrugs may allow tailoring the size and the shape of the resulting nanoparticles. For example, doxorubicin-squalene nanoparticles display elongated structures which extended along the blood streamlines after intravenous administration in mice with xenograft of pulmonary (M109) and pancreatic (MiaPaCa-2) carcinomas, resulting in a prolonged pharmacokinetic profile of doxorubicin with lower cardiac concentration, higher tumour uptake and reduced urinary excretion.<sup>64</sup>

#### 4.2- For improving intracellular capture

Due to their small size and lipidic nature, prodrug nanoparticles may trigger the intracellular penetration of drugs that are unable to diffuse spontaneously through the cell membranes. In general, the intracellular capture of nanoparticles occurs through phagocytosis or clathrin-dependent endocytosis, both pathways leading to the segregation of the nanoparticles into the intracellular cell

digestive vesicles, either endosomes or lysosomes.<sup>73</sup> Chemists have taken advantage of the acidic pH of early endosomes or of the enzymatic content of late lysosomes to design prodrugs that can be activated in this specific intracellular environment for allowing the drug release. For instance, the linkage of penicillin to squalene promoted the intracellular delivery of the resulting nanoparticles, triggering the release of the antibiotic into the endo-lysosomal compartment where resistant staphylococcus bacteria were located.<sup>39</sup> Drug molecules having low molecular weight and appropriate lipophilicity can diffuse through the endo-lysosomal membrane. However, in case of fragile biomacromolecules, especially nucleic acids (DNA or RNA), ionizable lipids need to be used for the construction of lipid nanoparticles to allow the export of the nucleic acids into the cell cytoplasm.<sup>74</sup> Although less frequent, caveolae endocytosis and macropinocytosis<sup>75</sup> are other processes allowing nanosized systems to deliver drugs intracellularly.<sup>76</sup> Since the cytosolic caveolar vesicles display a neutral pH and do not contain enzymatic cocktail, this intracellular pathway may be advantageous to by-pass endo-lysosomal degradation if the carried drug (e.g., peptides, proteins, nucleic acids, etc.) is sensitive to enzymes. But, as discussed above, if the lipoprotein-driven dissociation of the lipid prodrug nanoparticles occurs extracellularly and is followed by the protein-mediated diffusion of the prodrug in the vicinity of the cell, then the prodrug can either insert into the cell membrane following partition coefficient<sup>77</sup> or be taken intracellularly through specific LDL receptors.<sup>78</sup>

In general, it is observed that the linkage of anticancer compounds to polyunsaturated fatty acids improves endocytosis and cytotoxicity.<sup>79,80</sup> For instance, the linkage of linoleic acid to the amino group of doxorubicin resulted in higher cellular uptake by cancer cells (MCF-7, MDA-MB 231 and HepG2) via passive plasma membrane diffusion and a two-fold increased cytotoxicity comparatively to free doxorubicin.<sup>80</sup> Interestingly, linoleic acid-doxorubicin exhibited higher cellular uptake and cytotoxicity on cancer cells (MCF-7, MDA-MB 231 and HepG2) as compared to the palmitic-acid-doxorubicin counterpart. It was concluded that unsaturated fatty acids surpass saturated fatty acid for intracellular delivery of drugs.<sup>79</sup> Anticancer nucleoside analogues, like cytarabine and gemcitabine, can only be taken up by cells via human concentrative nucleoside transporter (hENT). The down-regulation of hENT in non-small lung cancer cells thus results in drug resistance<sup>81</sup>, which can be overcome when the anticancer molecules are conjugated to the fatty elaidic acid, due to the passive diffusion of the lipid prodrug through the cell membrane.<sup>82,83</sup> Similarly, squalene-gemcitabine nanoparticles exhibited, in comparison to gemcitabine, 3-times higher cytotoxicity in murine-resistant leukemia L1210 10K cells and in human leukemia resistant cell line CEM-ARAC/8C<sup>33</sup>, showing that the lipid prodrug nanoparticle represents an interesting approach to circumvent anticancer drug resistance, at least *in vitro*.

#### 4.3- For promoting drug diffusion through biological barriers

Lipid solubility represents a key factor for drug diffusion through biological barriers. Thus, lipid prodrug nanoassemblies have been used to promote drug diffusion through biological barriers, both epithelia and endothelia.

The oral administration of drugs and their diffusion through the intestinal epithelium often represents an important challenge due to various obstacles resulting from the drug's own physico-chemical properties, like poor solubility, low permeability, high molecular weight and/or chemical or metabolic instability in the gastrointestinal (GI) tract. In addition, drug molecules may be the substrate of efflux transporters, or subject to first-pass hepatic metabolism, leading to their elimination. Thus, the conjugation of lipid moieties to pharmacologically active molecules with unfavourable partitioning coefficient has been shown to improve the diffusion through the GI epithelium. If fatty acids, steroids, phospholipids, or glycerides are used for increasing the lipophilicity of the parent drug, this approach has also its downside if the resulting prodrug has restricted dissolution properties. Therefore, the choice of the lipid according to the physicochemical characteristics of the drug must be made with

great caution to avoid over-lipophilicity or an increase in the molecular weight making the lipid prodrug molecule non-diffusible. For instance, in a dose escalation phase I study, patients with advanced solid tumors were treated with gemcitabine esterified at the 5' position with Elaidic fatty acid (trans-9-octadecenoic fatty acid). The prodrug was solubilised in a lipid-based formulation and encapsulated in non-gelatine hard-shell capsules. This study was, however, terminated before the recommended dose had been determined, because of the poor absorption of the drug and its rapid pre-systemic metabolism resulting in accumulation of deaminated metabolite in the liver, thus leading to severe liver toxicity.<sup>84</sup>

On the other hand, taking advantage of the natural physiological absorption pathway of lipids in the GI tract represents a very attractive option for improving the oral bioavailability of poorly diffusible drugs, in particular by forcing hydrophilic molecules to follow the natural lymphatic absorptive route of triglycerides.<sup>85,86</sup> Natural triglycerides are, indeed, hydrolysed in position 1 and 3 by pancreatic lipases and co-lipases, resulting in the production of two fatty acids, together with the corresponding 2-monoglyceride. After absorption into enterocytes, 2-monoglycerides reassemble intracellularly into triglycerides via the monoacylglycerol pathway, allowing the formation of chylomicrons in a highly lipophilic environment.<sup>87</sup> After exocytosis, the chylomicrons are taken by the open capillaries of the mesenteric lymphatic vessel before flowing into the systemic circulation at the junction of left internal jugular and subclavian veins, thus bypassing the portal blood and the liver. The synthesis of triglyceride prodrugs, where the drug is conjugated at the *sn*-2 position of the glyceride, is expected to follow a similar absorption pathway, thus by-passing first-pass hepatic metabolism.<sup>88</sup> A typical example is the conjugation of the immunosuppressant mycophenolic acid (MPA) to triglyceride (TG).<sup>89</sup> After oral administration of the 2-MPA-TG prodrug to Sprague–Dawley rats, a dramatic increase in the lymphatic drug transport was observed, together with a 103-fold higher concentration in lymphocytes, comparatively to MPA administered free.<sup>90</sup> Interestingly, in the mesenteric lymph nodes, the concentration of free MPA released from the prodrug was enhanced 28-fold, when compared to animals orally given MPA as a free drug, thus highlighting the efficient conversion of diglyceride prodrug into the parent molecule. The absorption was even more impressive in greyhound dogs where the prodrug markedly increased (by 288-fold) the lymphatic transport of the immunosuppressive agent, as compared with the control (ie., oral administration of free MPA).<sup>86</sup> The phospholipid prodrugs for their part, are hydrolyzed on the *sn*-2 position by phospholipase A2 enzymes, thus releasing free fatty acid and lysophospholipid. For instance, a phospholipid prodrug of indomethacin administered orally in Wistar rats provided a controlled release of the drug into the plasma following degradation by these enzymes in the gut lumen.<sup>91</sup>

Similarly, lipid prodrugs were also proposed to promote the drug diffusion through the skin epithelium due to either an improved permeation coefficient or the ability to protect the drug from degradation.<sup>92</sup>

After intravenous administration, lipid prodrug nanoparticles may also take advantage of the so-called Enhanced Permeability and Retention effect (EPR) and diffuse through the endothelial barrier where it is compromised by the disease. It has, indeed, been widely recognized that nanoparticles can use the EPR property to specifically disseminate through the endothelium into solid tumours in many pre-clinical experimental rodent models. However, this assumption is challenging and debatable in the clinical reality, due to the diversity and heterogeneity of human cancers. But in other diseases associated with a strong inflammatory process, the use of nanoparticles remains a very attractive option for selective endothelium translocation. For instance, in septic shock<sup>93</sup> or inflammatory pain<sup>28</sup>, drugs were found to be able to bypass the endothelial barrier (EPR-like effect) when administered as lipid prodrug nanoassemblies in rodent models of LPS-induced sepsis and inflammation. In another

study, the ability of conjugated linoleic acid to interact with brain vessels and to prevent new vessel growth in the cerebellum<sup>94</sup> has encouraged the use of this lipid.

On the other hand, the blood-brain-barrier (BBB) is characterized by endothelial cells with tight junctions and Pgp expression which hinder the brain delivery of drugs, especially those with a hydrophilic character. The lipidation of biologically active molecules has been shown to improve the diffusion through the BBE. Hence, the supramolecular assembly of paclitaxel-linoleic acid was shown, unlike to free paclitaxel, to distribute at high concentrations into the brain tissue in glioma bearing rats.<sup>95</sup>

#### 4.4- For controlling drug release

As mentioned earlier, the versatility of the drug to lipid conjugation approaches using amide or esters bonds may allow the fine-tuning of the drug release profile according to the enzymatic content of the target area, and the use of disulfide or hydrazone bonds makes the prodrug nanomedicine responsive to specific reductive or low pH environment.<sup>51</sup> Lipid prodrug nanoparticles may also be sensitive to external stimuli: very innovative in this sense is the conception of visible-light-triggered doxorubicin prodrug nanoparticles able to turn immunosuppressive tumour microenvironment back into high immunogenic tumours by boosting checkpoint blockade immunotherapy. In that respect, doxorubicin was linked to a photosensitizer through a cathepsin B-specific cleavable peptide whose chemistry was detailed in section 2.3 (Figure 4).<sup>54</sup> Cancer-specific cytotoxicity and immunogenic cell death were triggered upon visible light irradiation and maturation of dendritic cells were stimulated for cross-presentation of cancer-antigens to T cells. Among other examples are those nanoparticles, already mentioned before, combining polyunsaturated fatty acid conjugated to cabazitaxel via a self-immolation thioketal linkage and lipidated Ce6 photosensitizer allowing near-infrared irradiation to trigger the drug release at the right place.<sup>53</sup> Using the same approach, ROS-activatable heterodimeric prodrug was conceived associating camptothecin and 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide photosensitizer that were conjugated together via a ROS-sensitive thioketal linker.<sup>96</sup> Innovative light-responsive core-shell self-assembled nanoparticles were also designed by using a ROS-responsive oleate prodrug of paclitaxel as a core and novel photodynamic polymer resulting from the conjugation of lipophilic pyropheophorbide with polyethylene glycol 2000 as a corona.<sup>24</sup>

It is unquestionable that stimuli-responsive prodrug nanoparticles open an interesting new window for further exciting developments towards more specific and selective nanoparticles. But clinical translation remains far away due to regulatory issues related to the need to demonstrate the perfect reproducibility of the drug release after the same stimulus is applied to different patients.

## 5/ Therapeutic applications

### 5.1- Cancer

#### 5.1.1- *In vitro* and *in vivo* preclinical data

Without a doubt, cancer therapeutics remains the most extensively investigated application of lipid-drug nanomedicines (Table S1).<sup>97,98</sup> Many well-established anticancer drugs currently used in the clinic such as gemcitabine<sup>23,60,99,100</sup>, paclitaxel<sup>51,99,101</sup>, doxorubicin<sup>64,102,58</sup> and more innovative therapeutics such as RNAs<sup>45,47</sup>, have been turned into lipid prodrug nanoparticles. This section summarizes selected preclinical studies performed in this area. Upcoming trends in the field such as multidrug nanoparticles, stimuli-responsive particles, and nanoassemblies for theranostics are also discussed.

Among the different anticancer drugs, the antimetabolite gemcitabine has received the greatest attention for the preparation of lipid-prodrug nanoassemblies due to (i) its short half-life, (ii) its limited intracellular penetration and (iii) occurrence of drug resistance. To overcome these limitations gemcitabine has been conjugated to different lipids, such as polyunsaturated fatty acids (i.e., linoleic acid<sup>21</sup>, elaidic acid<sup>83</sup>), squalene derivatives<sup>31,60,103,104</sup> and isoprenic acids<sup>32</sup>, which once self-assembled as nanoparticles have demonstrated efficacy in models of pancreatic cancer<sup>21</sup>, leukemia<sup>33,103,104</sup>, osteosarcoma<sup>105</sup> and glioblastoma<sup>106</sup>. Among these, the conjugation of gemcitabine to squalene derivatives is the most widely investigated. Its high antiproliferative activity has been demonstrated in a large panel of 60 human tumour cell lines performed under the National Cancer Institute's Developmental Therapeutic Program and in several cancer animal models, supporting its candidature for clinical trials.<sup>33,59,103,104,106</sup> The most advanced is, however, gemcitabine-elaidate prodrug (CP-4126), which has entered clinical trials NCT01124786 for the treatment of pancreatic adenocarcinoma (see section 5.1.2). It is questionable why elaidate has been used instead of oleate despite the unfavourable metabolic features and potent stress-inducing character of trans fatty acids in comparison with oleate<sup>107</sup>. To a lesser extent, considerable research has focused on exploiting the wide anticancer spectra of paclitaxel by producing lipid prodrug derivatives to construct self-assembled nanosystems.<sup>99,101</sup> First, a lipophilic conjugate of paclitaxel with docosahexaenoic acid showed higher binding to total plasma proteins compared to paclitaxel alone and enhanced antitumor activity in a mouse lung cancer model (M109 model).<sup>108</sup> In another study, paclitaxel was conjugated to tris-nor-squalenic acid, resulting in a bioconjugate that self-assembled into nanostructures in an aqueous solution, demonstrating comparable antitumor efficacy but much lower subacute toxicity than the parent drug on a human lung carcinoma xenograft model.<sup>99</sup> Similar linoleoyl paclitaxel conjugates built as nanoparticles displayed higher antitumor activity compared to paclitaxel itself in MDA-MB-231, B16-F10 and U87-MG tumour cells.<sup>101</sup> Notably, the lipid-prodrug strategy has also been applied for cytarabine, achieving very significant results in hematological malignancies by conjugation to elaidic acid<sup>109</sup> or tris-nor-squalenic acid<sup>110,111</sup>. Other lipid prodrug nanoassemblies focused on overcoming the challenges arising from doxorubicin cardiotoxicity.<sup>49,58,64,102</sup> Hence squalenoyl doxorubicin nanoassemblies with a "loop-train" structure exhibited enhanced antitumor activity on both experimental murine pulmonary M109 and human pancreatic cancers MiaPaca, also dramatically reducing cardiac toxicity, in comparison to free doxorubicin.<sup>64</sup> In another example, tocopherol succinate derivative of doxorubicin was developed and manufactured into nanoparticles with a highly ordered lamellar inner structure, which demonstrated higher antitumor efficacy than free doxorubicin in a mouse colon cancer model (CT26 tumour-bearing mice).<sup>58</sup>

The versatility of lipid-prodrug nanoparticles has also been demonstrated by their application to more innovative therapeutics such as small interfering RNAs delivery (siRNAs)<sup>45,47</sup>. As already mentioned, these negatively charged molecules are highly unstable under physiological conditions and poorly taken up by cells. Therefore, they require effective delivery systems to function *in vivo*. In one example, squalenoyl moiety was conjugated to siRNA against the junction oncogene RET/PTC1, which is usually found in papillary thyroid carcinoma. The self-assembled nanoparticles demonstrated effective silencing of RET/PTC1 expression and consequently, delayed tumour growth in a mouse xenografted RET/PTC1 experimental model.<sup>45</sup> Another strategy involves clickable siRNA poly-isoprenoid conjugated nanoparticles as a new platform for siRNA delivery in cancer therapy.<sup>47</sup> Nanoparticles made of squalene derivative and solanesol conjugated to two different siRNAs were found to inhibit fusion oncogenes found in prostate cancer and papillary thyroid carcinomas with positive anti-neoplastic activity. These clickable siRNA nanoparticles could be extended to other polyterpenes and other siRNAs, acting as a novel platform for siRNA delivery.

There has been considerable interest in the development of strategies that enable functionalized nanoparticles for targeted drug delivery to cancer cells. As an example, peptide-functionalized

squalenoyl gemcitabine nanoparticles represent the first example of pancreatic cancer-targeted nanoparticles with unique selectivity and multiple mechanisms of action.<sup>112</sup> The conjugation of the peptide (CKAAKN) to squalenoyl gemcitabine nanoparticles allowed the selective targeting of pancreatic cancer cells and angiogenic vessels *in vivo* in tumour-bearing RIP-Tag2 mice. Peptide functionalized nanoparticles were more effective in reducing tumour than non-functionalized nanoparticles and free drug (40% and 60% respectively). From a mechanistic perspective, the therapeutic benefit observed with CKAAKN-functionalized nanoparticles resulted from the dual activity on both cancer cells and tumour vasculature. More recently, self-assembled linoleoyl gemcitabine nanoparticles were decorated with a pancreatic ductal adenocarcinoma (PDAC)-specific peptide and PEG<sub>2k</sub> to achieve targeted cancer cell delivery.<sup>21</sup> The PDAC-peptide facilitated the intracellular uptake of nanoparticles *in vitro* in two pancreatic cancer cells lines (i.e., L3.6pl and BXPC-3). Notably, the intravenous administration of the targeted nanoparticles resulted in a superior inhibition of tumour progression and a lower toxicity than the free drug in two xenograft models (L3.6pl and a patient-derived xenograft model). Several studies used folate receptor 1-targeted porphyrins as photosensitizers for photodynamic therapy showing promise in selectively killing tumour cells in preclinical models of epidermoid carcinoma (KB xenograft) and lung cancer (AE17-sOVA tumor-bearing mice and A549 subcutaneous tumor-bearing mice).<sup>113-115</sup> Upon intravenous administration, folate-targeted porphyrins accumulated, indeed, in tumours and reduced tumour size upon light irradiation, while non-targeted nanoparticles did not, which revealed that nanoparticle uptake and cytotoxicity was clearly folate receptor-dependent. Following the same rationale, porphyrin-high density lipoprotein targeted to the scavenger receptor class B type I expressed in lung cancer demonstrated selective accumulation and photoactivation in the tumour after systemic administration in mice with orthotopic lung cancer<sup>116</sup> (H460 orthotopic lung cancer model). Recently, a bone-targeted nanomedicine based on squalenoyl gemcitabine nanoparticles decorated with squalenyl-hydroxybisphosphonate as bone mineral targeting moiety has been conceived for the treatment of osteosarcoma.<sup>105</sup> These examples have contributed to the development of strategies with higher specificity and affinity towards cancer cells. Of note, the decoration of nanoparticles with specific ligands may affect the assembly and morphology of the resulting nanoparticles. The size and zeta-potential depend on the nature of the ligand and if the functionalization has been performed (or not) through PEG chains. In general, the surface molecular footprint results in a slight increase in the nanoparticles size, whereas the presence of PEG contributes to surface charge reduction towards neutrality. Interestingly, two distinct ligation strategies may be employed for the functionalization of self-assembled lipid prodrug nanoparticles: the ligand can either be directly coupled to the surface of preformed nanoparticles or be linked to the lipid prior to nanoparticle construction. For example, Surface Plasmon Resonance (SPR) analysis of CKAAKN-functionalized nanoparticles has shown that peptide conjugation prior to nanocarrier formation was the most efficient methodology to obtain enhanced binding and avidity for the sFRP-4 receptor<sup>117</sup>. On the other hand, the literature lacks comparisons between the supramolecular structures resulting from functionalized and non-functionalized lipid prodrug nanoparticles. Nevertheless, the functionalization of squalene-gemcitabine nanoparticles by squalenyl-hydroxybisphosphonate resulted in the remodelling of the initial hexagonal structure into a helicoidal and multilamellar onion-type self-assembly which was electrostatically driven by the negatively charged phosphate groups<sup>105</sup>. This illustrates the need to finely characterize the nanoparticles physicochemical parameters which can further impact interactions with biological components and pharmacokinetics.

To overcome drug resistance, synergistic anticancer therapy may be achieved through the simultaneous delivery of two drugs integrated into the same self-assembling nano-cocktail. For instance, two synergistic lipid-prodrugs constructed from cisplatin and 7-ethyl-10-hydroxycamptothecin via conjugation with linoleic acid were integrated into a self-assembling nano-cocktail that exhibited superiority over the free drugs in a cisplatin-resistant tumour-bearing mouse model of

lung cancer (A549 cisplatin-resistant xenograft model).<sup>118</sup> Other strategies focused on the combination of antivascular drugs and cytotoxic agents for more effective anticancer therapies. For example, the lipophilic anti-angiogenic drug iso-combretastatin was combined with squalenoyl gemcitabine and its efficacy was demonstrated in an experimental model of colorectal cancer (human colon carcinoma xenograft model LS174-T).<sup>119</sup> Another multitherapy nanomedicine approach with potential in paediatric osteosarcoma treatment involves the combination of squalenoyl gemcitabine and alkyllysophospholipid edelfosine, which have complementary molecular targets, integrated into the same nanoparticles.<sup>59,120</sup> The above examples illustrate that multidrug therapy based on lipid prodrug nanoassemblies has become a notable trend to provide more effective anticancer treatments.

As already mentioned, among the common stimuli-triggered strategies, light is the most frequently explored. The possibility of remotely activating nanoassemblies of small molecular prodrug cocktails by near-infrared light and achieving synergistic chemo-photodynamic therapy has demonstrated tremendous therapeutic potential against multiple cell-derived and patient-derived xenograft tumour models of melanoma.<sup>53</sup> A chemo-photodynamic approach composed of the light-activable pyropheophorbide-PEG shell and ROS-responsive oleoyl paclitaxel prodrug core enabled high-efficient synergistic cancer therapy in KB tumour-bearing nude mice.<sup>24</sup> In addition to light, acid-triggered drug release is another stimulus that has been investigated, using 11-mercaptoundecyl phosphorylcholine modified doxorubicin linked with an acid-labile hydrazone bond and achieving drug release under the endosomal acidic conditions inside tumour cells (HepG2 liver hepatocellular carcinoma).<sup>49</sup> This lipid prodrug that nano-assembled into core-shell nanomicelles in aqueous solution exhibited much lower cardiotoxicity and better antitumor efficacy than the free drug (KB tumour-bearing mice model). Additional efforts toward redox-responsive approaches led to the development of redox single or dual-responsive prodrug-nanoparticles formed by single thioether-bridged paclitaxel-fatty acid conjugates, designed to respond to the redox-heterogeneity in tumours and releasing the drug rapidly.<sup>50,121</sup> Single-responsive prodrug nanosystems achieved a selective drug release within tumours with elevated intracellular glutathione (GSH) levels while dual-responsive prodrug nanosystems provided selective drug release within tumours that simultaneously overproduced GSH and ROS, resulting in both cases in superior *in vivo* antitumor efficacy in KB-3-1 xenograft tumours.

Moreover, novel nanotheranostic systems based on lipid prodrug nanoparticles have facilitated the combination of therapeutic and imaging properties. For example, magnetite nanocrystals have been included in nanoparticles of squalenoyl gemcitabine to target gemcitabine to solid tumours under the influence of a magnetic field, and to image the targeted cancer nodule.<sup>122</sup> Similarly, T1 Gad3 contrast agent was incorporated in squalene-based nanoparticles of doxorubicin or cisplatin, enlarging the range of application of these theragnostic nanomedicines. In yet another example, apolipoprotein E3 porphyrin-lipid nanoparticles were used for theranostic purposes in glioblastoma.<sup>123</sup> It was observed that when injected intravenously, these nanoparticles accumulated at the tumour site of an orthotopic tumour model, demonstrating *in vivo* glioblastoma targeting. But the theranostic utility of this nano-construction was only demonstrated *in vitro* after photodynamic therapy, using U87 and IdIA7 glioblastoma cells. Following the same rationale, tunable phototheranostic agents have been developed by incorporating porphyrin into biomimetic lipoproteins and their efficacy has been demonstrated in an orthotopic prostate-cancer mouse model.<sup>124</sup> The hydrophobic porphyrin-oleylamide conjugates were useful for photoacoustic imaging, while amphiphilic porphyrin-lipid conjugates enabled fluorescence imaging and photodynamic activity.

Although most of these strategies have demonstrated efficacy following intravenous administration, the oral route has also been explored. For example, the anticancer efficacy of nanoparticles made of squalene-cisplatin bioconjugate has been demonstrated in two different models of intestinal carcinogenesis (ApcMin/+ mice and azomethane-induced).<sup>125</sup> Similarly, squalenoyl gemcitabine

nanoparticles orally administered showed superior anti-tumour activity to the parent drug in leukaemia-bearing rats.<sup>33,126</sup> This system is attractive because squalene is a lipid that is particularly well absorbed orally. Other oral lipid-prodrugs were designed and consisted in lipophilic prodrugs of docetaxel<sup>127</sup>, SN38<sup>128</sup> and cytarabine<sup>129</sup>. Although the formation of nanoparticles was not reported with these conjugates, their spontaneous self-assembly into nanosized supramolecular structures is suspected to occur due to their physico-chemical properties.

Finally, another area of focus is the development of *in situ* antitumor vaccines based on the combination of antitumor drugs and Toll-like receptor agonists (e.g. imiquimod, resiquimod (R848) and CpG) that inhibit tumour growth by stimulating antitumor immune responses. From a mechanistic point of view, the cytotoxic drug induces the release of tumour antigens, whereas the Toll-like receptor agonist promotes dendritic cell maturation to stimulate T-cell responses. Both drugs are often loaded in drug delivery systems to enhance drug retention in the tumour tissue. However, the co-encapsulation of two drugs with different physicochemical properties within the same nanocarrier may be difficult. To address this matter, an interface-sensitized prodrug nanoaggregate was synthesized by co-assembling cationic lipids (DOTAP) with 2 prodrugs consisting of camptothecin and resiquimod linked to oleic acid through a S,S-(bisethoxy)carbonyldisulfide linker.<sup>130</sup>

Inspired by the observation that compounds which bind avidly to serum albumin are capable of lymph node targeting, unique anticancer amphiphile vaccines containing an antigen linked to a lipophilic albumin-binding tail has been developed<sup>131</sup>. These nanomicelles composed of CpG-DNA/peptide amphiphile vaccines exhibited impressive lymph nodes targeting and accumulation comparatively to their parent compounds. This resulted in a dramatic increase in T-cell priming and enhanced anti-tumour efficacy<sup>131</sup>.

Overall, the lipid prodrug nanoassemblies described above were found to exhibit much higher *in vitro* and *in vivo* antitumor activity than their parent drugs in preclinical studies, by improving bioavailability and tumour accumulation, overcoming multidrug resistance, and/or decreasing severe side effects.

### 5.1.2- Clinical trials in oncology

The clinical translation of the prodrug-nanoparticle concept remains, however, disappointing in oncology. Indeed, if the cytarabine-elaidate nanoparticles have shown in phase II clinical trials, a remission rate of 18% *versus* 4% in controls and 5.3 months *versus* 1.5 months survival in controls, there was no significant difference in response rate and relapse-free survival in phase III.<sup>82</sup> Similar results were observed with gemcitabine-elaidate prodrug with the same overall response and toxicity profile as gemcitabine free in phase II clinical trials of pancreatic adenocarcinoma patients.<sup>132</sup> And in contrast to the excellent anti-cancer activity in experimental animal models, DHA-paclitaxel prodrug nanoparticles did not show any overall survival difference in a phase III trial on melanoma, as compared with the standard treatment (dacarbazine).<sup>133</sup> The details of the different clinical trials terminated or ongoing can be found in table 1.

The lack of success associated with prodrug nanoparticles may be explained, in part, by the fact that many experimental animal models in oncology are not predictive enough of the disease in humans. Indeed, pre-clinical pharmacology experiments often use subcutaneously grafted tumours, each tumour and animals having the same genetic background, leading to better reproducibility of the results, which is not the case in human cancers. Transgenic mice models are useful, but they are specific to rodents, whereas animal recipients of xenografted tumours from human origin are immunocompromised. In preclinical models, the size of the tumoral nodule is much smaller than in



humans and this makes an important difference, since to be efficient, the prodrug nanomedicine needs to diffuse deep in the cancer tissue. Additionally, unlike experimental models, many human cancers are difficult to cure because they are poorly perfused, the pancreatic cancer being a typical example. The enhanced penetration and retention (EPR) effect is generally invoked as a way used by the nanoparticles (either prodrug-based or not) to reach the tumour, but it is not well characterized neither for all types of nanoparticles, nor for the different classes of human cancers. Finally, most of the models used at the preclinical stage for the assessment of the anti-cancer efficacy of lipid prodrug nanoparticles did not induce metastasis. Therefore, as discussed below in the outlook, the development of more reliable pharmacological models is urgently needed, while the clinical trials also require a more rigorous selection of patients.

### 5.2- Neurological disorders and pain

A few self-assembled nanomedicines have been developed for the treatment of neurological disorders and pain (Table S1). Since the pioneering article by Jacob *et al.* in 1990<sup>134</sup>, showing that the linkage of GABA and 7-vinyl-GABA to linolenoylglycerol promoted the brain delivery of the two molecules, resulting in depression of the spontaneous locomotor activity of mice, other molecules like L-DOPA<sup>135</sup> or phenytoin<sup>136</sup> have been formulated as pseudoglycerides to increase central nervous system diffusion. However, these articles are now rather old, and it is unclear if these prodrugs assembled as nanoparticles or not. In addition, if lipophilicity increases drug transport through the blood brain barrier (BBB), it may also trigger unexpected toxicity issues, due to either the metabolism of lipid prodrugs by other pathways than the single conversion to the parent molecule or because of the limited ability of the brain tissue to metabolize and eliminate nanoparticles.

Thus, more recently, the design of prodrug nanomedicines capable of acting on peripheral rather than on central receptor targets has been considered. It has been discovered that the linkage of adenosine to squalene derivative and the subsequent construction as nanoassemblies triggered impressive neuroprotective activity in pre-clinical models of brain ischemia and spinal cord injury in rodents.<sup>29</sup> It was demonstrated that adenosine released from nanoparticles interacted with the adenosine receptors located at the surface of the brain micro-vessels, leading to a decrease in the thrombo-embolic events in the brain microcirculation and to a neuroprotective effect on the cells of the neurovascular unit. This was not observed with free adenosine, this molecule being very quickly metabolized with a half-life of 10 seconds.

Along the same line of reasoning, leu-enkephalin an endogenous opioid neuropeptide, has also been conjugated to squalene derivative through various chemical spacers, to design nanoparticles for efficient pain alleviation.<sup>28</sup> Unlike morphine, these nanoparticles spared the brain tissue, acting selectively on peripheral opioid receptors, thus avoiding the detrimental central effects responsible for addiction and tolerance in a rat model of inflammation.

Very recently, it was discovered that intrathecal and intracerebroventricular injection of siRNAs conjugated to 2'-O-hexadecyl moiety displayed pharmacological activity in central nervous system regions and cell types by inducing RNA interference activity for at least 3 months. When the lipidized amyloid precursor protein-targeting siRNA was evaluated in a mouse model of Alzheimer's disease, a significant improvement of physiological and behavioural deficits was observed. In another report, intravitreal administration of hexadecyl-siRNA to the eye or intranasal administration to the lung resulted in potent and durable gene silencing, which opens exciting perspectives for siRNA delivery into extrahepatic tissues.<sup>137</sup>

### 5.3- Infectious diseases

Lipid prodrug nanoassemblies of antiviral compounds have been used to enhance the antiviral potency by slow conversion to parent molecule, leading to prolonged effective plasma concentration (table S1). Since antiretroviral drugs are generally administered at high doses, their direct encapsulation into nanocarriers raises issues regarding dose-volume. Thus, as already discussed before, the much higher drug loading obtained with lipid prodrug nanoparticles, represents an attractive option. For instance, rod-shaped nanoformulations of myristoylated cabotegravir,<sup>27,138</sup> an anti-HIV compound, injected intramuscularly in either mice or rhesus macaque, displayed 4-folds higher plasma concentrations compared to the free drug, together with secondary tissue and immune cells depot. Long-acting effect was also obtained with several other antiretrovirals nano-formulated as lipid prodrugs, including darunavir<sup>139</sup>, lamivudine<sup>140</sup>, abacavir<sup>141</sup> and dolutegravir<sup>26</sup>. Nanoparticles made of dolutegravir conjugated to myristic acid and stabilized by a small amount of Poloxamer 407 were shown to attenuate viral replication and spread in infected CD4+ T cells. After intramuscular administration to mice, dolutegravir blood levels remained at viral inhibitory concentration for 56 days, whereas the drug concentration in tissues (ie., liver, spleen, GALT, lymph nodes, lungs, and kidneys) continued for 28 days. This long-acting nanoformulation resulted in significant protection of HIV-infected humanized mice for two weeks.<sup>26</sup> In another approach, squalenoylated prodrugs of ddl, a nucleoside reverse transcriptase inhibitor, were given orally. Pharmacokinetic and biodistribution investigations in mice have shown that these ddl-loaded nanoparticles acted as a sort of “drug reservoir” in the blood circulation, where they maintained high levels of squalene-ddl, but low amounts of free ddl, triggering higher drug concentrations than after oral administration of the free drug, in cells and tissues, especially those relevant for HIV infection (i.e., liver, spleen, bone marrow, thymus, and brain)<sup>30</sup>. Evidently, oral administration represents a more comfortable treatment for HIV patients with facilitated adherence to drug regimens.

Lipid prodrug nanoparticles were also designed for killing resistant intracellular bacteria, able to resist the inhospitable surroundings of endo-lysosomes to survive and multiply. Thus, various terpenes, including geranyl, farnesyl<sup>40</sup> and squalene derivatives<sup>39</sup> were linked to the antibiotic penicillin via an environmentally sensitive bond allowing them to reduce intracellular replication of pathogen *Staphylococcus aureus in vitro* in Raw 264.7 and J774 macrophages. Although this approach deserves further *in vivo* confirmation, it could be applied to other antimicrobial agents, the nature of the terpene being adapted to the antibiotic to be delivered intracellularly.

#### 5.4- Inflammatory diseases

In this area, the glucocorticoid dexamethasone was successfully linked to palmitate and then formulated as nanoparticles. Of note, those lipid prodrugs did not assemble spontaneously but stabilization was obtained by using distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (DSPE-PEG<sub>2000</sub>).<sup>62</sup> This approach was used to tackle the two main problems observed when glucocorticoids are nanoencapsulated, namely low drug loading and drug crystallization, which produce the destabilization of the nanoparticles. Thus, high drug entrapment efficiency was reported when using this procedure (98% w/w), as well as the absence of drug crystallization. Interestingly, after intravenous administration in healthy mice, enhanced pharmacokinetics and modified tissue distribution of the nanoparticles were observed. In mice suffering from endotoxemia or lethal systemic shock, multidrug nanoparticles were found to efficiently fight out-of-control inflammation by delivering multiple drugs at once (ie., adenosine with anti-inflammatory activity and tocopherol as an antioxidant).<sup>93</sup>

## 6/ Outlook

The prodrug, a non-pharmacologically active molecule that can be activated enzymatically to release the active compound in the diseased cells or tissues, is a rather old concept. Of note, the authors of these ancient studies did not realize currently, that some of their synthesized prodrugs freely self-assembled in a nanosized range. A more recent strategy sought to systematically select specific structures to allow the drug conjugated carrier to form supramolecular nanomedicines in a spontaneous way. This review has shown that a combination of the specific benefits of prodrugs and nanoparticles indeed resulted, in impressive pharmacological activity both *in vitro* and *in vivo*, the oncology field being particularly explored.

Despite non-satisfactory clinical data about lipid prodrug nanoassemblies for the treatment of cancers, the more conclusive preclinical results obtained not only in oncology but also in other therapeutic areas should prompt additional efforts toward clinical translation (Figure 5 and Box 2). First, chemical synthetic pathways need further development to avoid the use of toxic reagents. For instance, the oxidation of tris-nor-squalenaldehyde into tris-nor-squalenic acid for bioconjugation of biologically active molecules utilizes chromium trioxide, a toxic compound not accepted by regulatory agencies. The search for safer synthetic reactions and green chemistry is, therefore, becoming an important translational challenge. The fine tuning of the supramolecular structure of the prodrug nanoparticles is another matter for concern. Indeed, because the activation of the prodrug is needed for triggering the pharmacological activity, the ability of the enzymes to diffuse into the core of the particles must be explored. This point, often underestimated, relies on the particle structure which may be determined by advanced methodologies, such as X-ray diffraction, cryo-transmission electron microscopy or even neutron scattering. Interestingly, a better release of the drug may also be obtained through smart design of the linker making it stimuli-responsive or more hydrophilic for a better and more specific enzymatic cleavage. Another important hurdle in the selection of the best nanoformulations relates to the discrepancy between the *in vivo* experimental models and the clinical reality, especially in oncology. Since the drug release from lipid prodrug nanoparticles is often governed by the enzymatic content, and because the enzymatic activity is typically higher in the smaller than in larger animals, a comparison of *in vitro* metabolism across species (often for mouse, rat, dog, monkey and human using plasma, hepatocytes and/or liver microsomes) represents a key study which is lacking in many reports<sup>142</sup>; this may contribute to the failure of these systems in the clinic. The same apply for the interaction with blood components susceptible to interact in the transport and pharmacokinetic of lipid prodrugs, as discussed earlier in this review. Too little information and understanding on nanomedicine exposure and interaction with endogenous enzymes, proteins and lipids in different species may have some contribution to the clinical failure. There is also an urgent need to develop more relevant animal models and tumour 3-D cultures arising from patient biopsies to better understand the nanoparticle/drug diffusion in the tumour tissue. Accordingly, the methodologies used in clinical trials should also move towards more personalized medicine. To this aim, a better selection of the patients and dose regimens according to the patient's genetic background and tumour characteristics must be achieved. Moreover, combination therapy should also be considered to overcome the limitations of a single treatment. From that point of view, the versatility of the lipid prodrug nanoassemblies approach allows for the construction of multidrug nanoparticles, as it was done very recently for the treatment of uncontrolled paradoxical inflammation related to cytokine storm.<sup>93</sup> Finally, although most prodrug nanoparticles were administered intravenously, other routes of administration deserve to be explored (intramuscular, subcutaneous, oral) and some local applications could also be considered.

In addition to applications in therapeutics, disease prevention through vaccination today opens exciting new prospects. If there is currently no vaccine in either pre-clinical or clinical development based on self-assembled lipid bioconjugates, the use of ionizable lipids for mRNA delivery into cells

has, on the other hand, revolutionized the field during the COVID crisis. As discussed in this review, the chemical linkage of nucleic acids to lipids has laid the groundwork for possible future developments of biomolecules that when coupled with ionizable lipids, could produce, after cellular internalization, increased RNA export from cell endosomes towards cytoplasm, thus eventually promoting better vaccinal response or *in vivo* production of therapeutic proteins.

In conclusion, the translation of lipid prodrug nanoassemblies towards the clinic deserves additional efforts through improvements in the chemical synthesis of prodrugs (by minimising the presence of organic and inorganic impurities and the formation of by-products), deeper physicochemical characterization of nanoparticles, and development of new pre-clinical experimental models, which should open broad research avenues for chemists, bioengineers, pharmacists, as well as medical doctors.

Boxes:

**Box 1: Regulatory considerations:**

Because the drug loading (ie. the amount of the transported drug *versus* the amount of the transporter material, generally expressed as %) into nanocarriers is usually less than 5%, the quantity of the drug administered is often not enough to reach a pharmacologically active concentration in the target cells or tissues. With a drug loading of 5%, each increase in the amount of the drug to be injected will necessitate 20-times more transporter material to be administered. From a regulatory point of view, it may represent a major concern because, if the amount of the required carrier material is too great, toxicity or undesirable side-effects may occur.

The prodrug approach has also its own regulatory hurdles to be overcome: the enzymatic activation must be shown to be reproducible for a controllable drug release and an efficient pharmacological activity to occur and many lipidic prodrugs need the use of non-aqueous solvents which also raises toxicological issues.

**Box 2: Translational consideration:**

The clinical application of lipid prodrug nanoparticles needs to overcome several hurdles: (i) The synthesis of the prodrug generally occurs with low yield, too many synthesis steps and/or non-regulatory reagents. It is recommended to develop new synthetic pathways based on green chemistry; (ii) the biologically active compound is sometimes not or poorly released from the nanostructure, which can be overcome by investigating the nanoparticle supramolecular structure, by using a more hydrophilic drug-lipid link or by developing novel stimuli-responsive approaches; (iii) it is suggested to develop new more predictive *in vitro* and *in vivo* experimental models to break the discrepancy between pre-clinical data and clinical efficacy, and (iv) the clinical outcomes could be improved by screening more targeted patients, also considering other routes of administration, examining combination chemotherapy, and improving dosing regimens.

Figures captions :

- *Figure 1*: Schematic representation of nanomedicines made of (A) drug-loaded nanocarrier (liposome, nanoparticle, micelle etc., here a liposome is represented). The drug release may occur through various mechanisms (diffusion, partitioning coefficient, nanocarrier

(bio)degradation or burst release for those drug molecules located on the nanocarrier surface). (B) lipid prodrugs encapsulated into a nanocarrier (liposome, nanoparticle, micelle etc., here a liposome is represented). The release of the lipid prodrugs may occur through various mechanisms (diffusion, partitioning coefficient, nanocarrier (bio)degradation or burst release, for those prodrug molecules located onto the nanocarrier surface), before drug liberation after activation of the bio-cleavable link. (C) Self-assembled lipid prodrug nanoparticle. After dissociation of the nanoparticle supramolecular structure, the lipid prodrugs are released as single molecular entities, leading to the drug liberation after activation of the bio-cleavable link by either an endogenous (enzymes, pH, reductive or oxidative environment etc.) or an exogenous (light) stimulus. Of note, since prodrugs<sup>143</sup> and drug-loaded nanocarriers<sup>144,12</sup> have been already reviewed earlier, the present review focuses exclusively on self-assembled lipid prodrug nanoparticles.

- *Figure 2:* Main building blocks composing the lipid-drug conjugates able to self-assemble into nanoparticles. The drug is represented in red (anticancer, antibiotics, antiviral or anti-inflammatory small molecules, neuropeptides, siRNA, etc.), the lipid in blue and the spacer in green. A) Example of lipid prodrug with direct linkage between the drug and the lipid. B) Example of lipid prodrug with a spacer inserted between the drug and the lipid.
- *Figure 3:* Relationship between the structure of several squalene-based nanoparticles and their resulting pharmacological activity. Since the drug release depends on the bioactivation of the prodrug, the supra-molecular architecture is a key factor for allowing the endogenous stimulus (enzyme, pH, redox status etc.) to diffuse efficiently in the nanoparticle core.
- *Figure 4:* Schematic illustration of visible-light-triggered prodrug nanoparticles (NPs) combining chemotherapy and photodynamic therapy to boost checkpoint blockade cancer immunotherapy. These NPs were prepared based on cathepsin B-specific cleavable prodrug of verteporfin (VPF), cathepsin B-specific cleavable peptide (FRRG), and doxorubicin (DOX) conjugates (VPF-FRRG-DOX). Reprinted with permission from Jiwoong Choi et al. 2021<sup>54</sup> Copyright© 2021, American Chemical Society.
- *Figure 5:* Regulatory and translational considerations. Schematic representation highlighting the key regulatory and translational considerations to bring lipid prodrug nanoassemblies from the laboratory to the clinic and the possible solutions to accelerate clinical translation. The figure includes original elements from Servier Medical Art.
- *Figure S1:* General structure of lipid-drug conjugates able to self-assemble into nanoparticles. The drug is represented in red (anticancer, antibiotics, antiviral or anti-inflammatory small molecules, neuropeptides, siRNA, etc.), the lipid in blue and the spacer in green. A) and B) Main building blocks composing the lipid-drug conjugates able to spontaneously self-assemble into nanoparticles. C) Different structures of lipid moieties composing the lipid-based prodrugs. D) Structures of the spacers inserted in the lipid bioconjugates.

Tables legends:

*Table 1:* Lipid drug conjugates in clinical trials as anticancer agents.

*Table S1:* Self-assembled lipid prodrug nanoparticles and their pharmacological application as published in the last 5 years.

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**Table 1: Lipid drug conjugates in clinical trials as anticancer agents**

INDICATION	TYPE OF STUDY	STATUS	CT IDENTIFIER	OUTCOME
<b>Gemcitabine-5'-elaidate (CO-1.01)</b>				
Advanced Solid Tumor	Phase I	Completed	NCT01392976	No results available
Metastatic Pancreatic Adenocarcinoma	Phase II	Completed	NCT01233375	No results available
Metastatic Pancreatic Adenocarcinoma	Phase II	Completed	NCT01124786	Study did not meet the primary outcome. The development of CO-1.01 was stopped and secondary endpoints were not analyzed. <sup>132</sup>
Solid Tumor Non-small-cell Lung Cancer Lung Cancer	Phase I	Terminated	NCT01641575	No results available
<b>Docosahexaenoic acid (DHA)-Paclitaxel (Taxopresin®)</b>				
Non-Small Cell Lung Cancer	Phase III	Completed	NCT00243867	No results available
Cancer of the Liver	Phase II	Terminated	NCT00422877	No results available
Metastatic Melanoma	Phase II	Completed	NCT00249262	No results available
Metastatic Melanoma	Phase II	Completed	NCT00244816	No results available
Pancreatic Cancer	Phase II	Unknown	NCT00024375	No results available
Prostate Cancer	Phase II	Unknown	NCT00024414	No results available
Colorectal Cancer	Phase II	Unknown	NCT00024401	No results available
Kidney Cancer	Phase II	Unknown	NCT00024388	No results available
Malignant Melanoma	Phase III	Completed	NCT00087776	DHA–paclitaxel was not superior to dacarbazine. <sup>133</sup>
<b>Elacytarabine (CP 4055)</b>				
Interventional (healthy volunteers)	Phase I	Completed	NCT01783964	No results available
Relapsed/Refractory Acute Myeloid Leukemia	Phase I	Completed	NCT01258816	No results available
Acute myeloid leukemia	Phase II	Completed	NCT01035502	No results available
Acute Myeloid Leukemia	Phase III	Completed	NCT01147939	No significant differences in overall survival between elacytarabine or any of the seven alternative treatments investigated doi: 10.1200/JCO.2013.52.8562
Hematologic Malignancies Acute Myeloid Leukemia	Phase I/II	Completed	NCT00405743	No results available
Advanced Colorectal Cancer Colorectal Cancer	Phase II	Completed	NCT00498407	No results available
Ovarian Cancer	Phase I/II	Completed	NCT00831636	No results available
Malignant Melanoma	Phase II	Completed	NCT00498836	No results available
Malignant Melanoma Neoplasm Metastasis	Phase II	Completed	NCT00232726	No results available

Terminated: The study has stopped early and will not start again.

Completed: The study has ended normally, and participants are no longer being examined or treated (that is, the last participant's last visit has occurred).

Unknown: A study on ClinicalTrials.gov whose last known status was recruiting; not yet recruiting; or active, not recruiting but that has passed its completion date, and the status has not been last verified within the past 2 years.

**Table S1: Self-assembled lipid prodrug nanoparticles and their pharmacological application as published in the last 5 years**

DRUG	LIPID	FUNCTIONALIZATION/COMPOSITION	IN VITRO/IN VIVO MODEL	THERAPEUTIC OUTCOMES	REF
<b>CANCER</b>					
<b>Gemcitabine</b>					
Gemcitabine (Gem)	Linoleic acid (LA)	Plectin-1 receptor expressed by human PDAC / Gem-LA, DSPE-PEG2k, DSPE-PEG2k-plectin-1 targeting peptide	Cytotoxicity against PDAC Cells Efficacy in PDAC cell-derived xenograft models, one of which is a patient-derived xenograft model	PDAC-targeting peptide increases the intracellular uptake and increase its cytotoxic activity Inhibition of tumor progression and alleviation of systemic toxicity	[21]
Gemcitabine	Tris-nor-squalenic acid (SQ)	No / Gem-SQ self-assembling	Cytotoxicity on RNK-16 LGL leukemia cells Absorption and biodistribution studies after oral administration in rats	Greater accumulation and retention of nanoassemblies in RNK-16-LGL cells More potent after oral administration due to resistance to deamination, improved pharmacokinetics, and increased accumulation in the lymphoid organs	[126]
Gemcitabine	Tris-nor-squalenic acid	No / Gem-SQ self-assembling	Proliferative assays using a commercial (U2-OS) and a patient-treated derived (531) osteosarcoma cells	Half-life enhancement via squalenylation and improved NP bone retention	[105]
Gemcitabine and Edelfosine (Edf)	tris-nor-squalenic acid	No / Gem-SQ and Edf-SQ self-assembling	Antiproliferative effect on c-Fos overexpressing P1.15 and primary osteosarcoma cells (p53+/- OSs) Pharmacokinetic, tolerability, antitumor efficacy, and safety profile in an orthotopic osteosarcoma tumor model (P1.15 OS cells)	Multidrug nanoassemblies display better anticancer profile <i>in vitro</i> in P1.15 cells than in p53+/- OSs cells Better tolerability and lower toxicity profile in mice Multidrug nanoassemblies decreased primary tumor growth kinetics and reduced the number of lung metastases.	[59] [120]
<b>Paclitaxel</b>					
Paclitaxel (PTX)	Citronellol (Cit)	Pegylated /PTX-Cit, DSPE-PEG <sub>2k</sub>	Cytotoxicity in human oral epidermoid carcinoma cells KB, human pulmonary carcinoma cells A549 and mouse breast cancer cells 4T1 Pharmacokinetics and biodistribution Antitumor efficacy in KB-tumor-bearing nude mice	Redox dual-responsive drug release The position of disulfide bonds in the carbon chain linkage has impacts on the redox dual responsiveness and on the anti-tumor efficiency, being $\alpha$ -paclitaxel-SScitronellol NPs the most effective	[51]
Paclitaxel	Oleic acid (OA)	Pegylated and light activatable./ PTX-OA, DSPE-PEG <sub>2k</sub> , pyropheophorbide a (PPa)-PEG	Synergistic cytotoxicity on human epidermoid carcinoma cell line (KB), non-small cell lung cancer cell line (A549), mouse breast cancer cell line (4T1) Pharmacokinetics Synergistic cytotoxicity on KB tumor-bearing nude mice	Synergistic antitumor efficacy of chemotherapy and PDT <i>in vitro</i> .  Synergistic chemo-photodynamic therapy was obtained under laser irradiation <i>in vivo</i>	[24]
<b>Doxorubicin</b>					
Doxorubicin (Dox)	Tris-nor-squalenic acid	No / Dox-SQ self-assembling	Intracellular distribution in murine lung carcinoma M109 and human breast carcinoma MDAMB-231 cells	Intracellular distribution of SQ-DOX was higher than doxorubicin Nanoparticles were localized into both cell cytoplasm and nucleus	[102]
<b>siRNA</b>					
siRNA against TMPRSS2-ERG	Azido-Squalene and azido-solanesol	No / siRNA-SQ self-assembling	VacP cells and papillary thyroid carcinoma cell lines (BHP 10-3 and TPC-1) Subcutaneous xenograft VacP tumor model in mice Biodistribution	Nanoformulations decrease the oncogene and oncoprotein expression <i>in vitro</i> . <i>In vivo</i> : two nanoformulations showed anti-neoplastic activity.	[47]
<b>Porphyrin</b>					
Phorphyrin (Ppy)	Fatty acid (FA)	Folate Receptor 1 / Ppy-FA (pyropheophorbide-lipid), cholesterol, PEG2000-DSPE, Folate-	Cellular uptake and fluorescence activation in lung cancer cells (FOLR1-positive A549, H647, H460 and SBC5 were used as the positive cell lines, while FOLR1-negative DFC1024 cell was the negative control) Mouse lung orthotopic tumor models	Enhanced therapeutic efficacy <i>in vitro</i> Tumor preferential accumulation of nanoassemblies. Folate-porphysomes mediated PDT successfully inhibited tumor cell proliferation and activated tumor cell apoptosis.	[114]

		PEG2000-DSPE			
Phorphyrin (Ppy)	Fatty acid	Folate Receptor 1 / Ppy-FA (pyropheophorbide-lipid), cholesterol, PEG2000-DSPE, Folate-PEG2000-DSPE	MPM cell lines. Murine (AE17, AE17-sOVA, AK7, AB12 and RN5) and human (H28, H226, H2052 and H2452) Orthotopic mesothelioma model was established in Nu/nu mice	Efficacy of FP-enabled PDT for the treatment of FOLR1-positive MPM in preclinical models <i>in vitro</i> and <i>in vivo</i> The combined therapeutic effect of pretreatment with EGFR-TKI	[115]
Porphyrin	Porphyrin-HDL	scavenger receptor class B type I (SR-BI) / Porphyrin-HDL, DMPC, cholesteryl oleate, 18-amino acid apolipoprotein A-I mimetic peptide	Human cancer cell lines used were large-cell lung carcinomas NCI-H460 and H460SM Antitumor efficacy in orthotopic lung cancer xenograft model Biodistribution and pharmacokinetics	Porphyrin-HDL is taken by the SR-BI receptor, highly expressed in subtypes of lung tumor Selective accumulation and photoactivation in the tumor after systemic administration	[116]
Porphyrin	Oleic acid (OA)	LDLR / Porphyrin-OA, DMPC, cholesteryl oleate, ApoE3	Human U87 glioblastoma and chinese hamster ovary IdIA7 cells, with high and low levels of LDLR expression Orthotopic U87-GFP tumour-bearing animals	4-fold higher uptake of nanoassemblies by LDLR-expressing U87 glioblastomas cells compared to minimally expressing IdIA7 cells Cell-specific potent PDT sensitization <i>in vitro</i> Selective uptake of porphyrin by malignant tissue and detection of tumour-localized porphyrin fluorescence	[123]
Porphyrin	Oleylamine	Scavenger receptor class B type I (SR-BI) / Porphyrin-oleylamide (Porphyrin-HDL-Like), DMPC, cholesteryl oleate,	Fluorescence microscopy and <i>in vitro</i> PDT using CHO, Idl(mSR-BI), IdIA-7, human KB, HT1080 and PC-3M-luc-C6 cells In vivo fluorescence imaging, photoacoustic imaging and PDT in tumor xenografts mice (Idl(mSR-BI) and IdIA-7 cell)	porphyrin moiety as an oleylamide conjugate leads to a highly stable J aggregate with strong photoacoustic contrast, while incorporation as an amphiphilic lipid moiety into the lipid shell yields an effective fluorescent and photodynamic agent	[124]
<b>Others</b>					
Cisplatin (CisPt) and SN38	Linoleic acid	Pegylated / CisPt-LA, SN38-LA, DSPE-PEG <sub>2k</sub>	Human NSCLC A549 and cisplatin-resistant A549 <sup>cisR</sup> cells A549 <sup>cisR</sup> cells tumor-bearing mice Toxicity assessment in mice	Preferred synergism in resistant cancer cells Higher reduction of tumor growth and tumor inhibition rate Low systemic toxicity	[118]
SN38 and protoporphyrin IX	Thioether-modified fatty acids (FA) and stearyl alcohol (SA)	No / SN38-SA, Ppy-modified FA self-assembling	Cytotoxicity on CT26 colonic cancer cells	Upon laser irradiation, oxidization of the multiple thioethers by photosensitizer generated singlet oxygen could rapidly destroy the NA structure, resulting in faster drug release and higher cytotoxicity	[52]
Cabazitaxel (CTX), photosensitizer chlorine e6 (Ce6)	$\alpha$ -linolenic acid ( $\alpha$ LA)	Photoactivatable (laser inducible) / CTX- $\alpha$ LA, Ce6- $\alpha$ LA self-assembling	<i>In vitro</i> cytotoxic activity induced by photodynamic/chemotherapy in A375 cells Efficacy in A375 xenograft-bearing mice and orthotopic patient-derived xenograft model of melanoma	Synergistic effects of the combined photodynamic/chemotherapy in A375 cells Eradication of human melanoma tumor in both animal modes of melanoma after treatment with nanoassemblies followed by laser irradiation	[53]
Cisplatin	Squalenamane	No / CisPt-SQ self-assembling	Toxicity and efficacy in a panel of human cancerous epithelial cell lines (HT-29, Colo 320HSR, Caco-2, SW620, LoVo colon cells, HeLa cervix and PC-3 prostate cell lines Mouse models of colorectal cancers (C57Bl/6J-Apc <sup>Min/+</sup> and Azomethane-induced)	Improvement of cisplatin oral bioavailability allowing the delivery of higher doses of cisplatin compared to the free drug	[125]
Docetaxel (Dtx)	Triglyceride(TG)	No / Dtx-TG self-assembling	Cytotoxicity and drug release in 4T1 cells, KB cells, and L02 cells Pharmacokinetic and biodistribution Anti-tumor effect in 4T1 tumor bearing mice	High accumulation in tumors due to improved oral absorption Excellent tumor suppression effect and significantly less gastrointestinal toxicity compared with oral and intravenous docetaxel solution	[127]
<b>PAIN</b>					

Leu-enkephalin LENK	Tris-nor-squalenic acid	No / LENK-SQ self-assembling	Carrageenan induced pain model using a thermal nociception test (Hargreaves) to assess hyperalgesia Biodistribution of NPs was investigated in mice and toxicological study in rats	Antihyperalgesic effect Nanoassemblies act through peripherally located opioid receptors NPs target the body area where inflammation and nociception occur.	[28]
<b>INFECTIOUS DISEASES</b>					
Cabotegravir (Ctb)	Myristic acid (MA)	No/ Ctb-MA, Poloxamer P407	Monocyte-derived macrophages from HIV-1/2 and hepatitis B seronegative donor blood cells Pharmacokinetics in mice and monkey and biodistribution in mice (i.m. administration) Viral restriction in humanized adult lymphocyte mice	Enhanced antiretroviral activity <i>in vitro</i> . Improve biodistribution and viral clearance <i>in vivo</i> Sustained plasma drug concentrations above the PA-IC90 for four months in monkeys. A 2.5-fold extension in drug half-life and a 1.6-fold increase in area under the concentration-time curve was observed in monkeys	[27] [138]
Darunavir (Drv)	Fatty acids (FA)	No/ Drv-FA, Poloxamer P407	Cytotoxicity and cell viability and antiretroviral activity in human monocyte-derived macrophages and CEM-SS CD4 <sup>+</sup> T cells Pharmacokinetics in mice	Improved intracellular and tissue drug accumulation and sustained antiretroviral activities	[139]
Lamivudine (Lmd)	Docosanol (Doco) (ProTide Technology)	No/ Lmd-Doco, Poloxamer P407	Effects on cell viability in primary monocyte-derived macrophages and CEM-CD4 <sup>+</sup> T cells. Pharmacokinetics in rats	Improved drug uptake, retention, intracellular 3TC triphosphates and antiretroviral activities in MDM and CD4 <sup>+</sup> T cells. PK tests of Sustained prodrug and drug triphosphate levels in blood and tissues for 30 days	[140]
Abcavir (Abv)	Docosanol (ProTide Technology)	No / Abv-Doco, PLGA or poloxamer	Antiretroviral activity in human monocyte-derived macrophages	Sustained intracellular carbovir-triphosphate and antiretroviral activities for up to 30 days.	[141]
Dolutegravir (Dlg)	Myristic acid (MA)	No / Dlg-MA, poloxamer	Antiretroviral activity in HIV infected macrophages Protection against HIV-1 challenge in CD34 <sup>+</sup> humanized mice	Improved antiretroviral activities up to 30-fold. PK and pharmacodynamics significantly improved over a native drug formulation (5.3-fold extension in drug apparent half-life, broad tissue distribution, and increased antiretroviral efficacy)	[26]
<b>INFLAMMATORY DISEASES</b>					
Dexamethasone (Dxm)	Palmitic acid (PA)	No / Dxm-PA, DSPE-PEG <sub>2k</sub>	- <i>In vitro</i> : raw 264.7 - <i>In vivo</i> : Pharmacokinetics in healthy mice	Anti-inflammatory effects in macrophages activated with LPS	[62]
Adenosine (Ade) Vit E	Squalenyl acetic acid	No / Ade-SQ, Vit E	- Oxidative insult in H9c2 cardiomyocytes and LPS-induced inflammation model in Raw 264.7 - Biodistribution in two different models: local acute inflammation and systemic inflammation. - Efficacy in endotoxemia LPS model in mice	Efficient control of inflammation and enhanced survival in models of endotoxemia	[93]
<b>OTHERS</b>					
Camptothecin (Cpt) and resiquimod (R848)	Oleic acid (OA)	No / Cpt-OA, R848-OA, DOTAP	Cellular uptake and apoptosis in CT26 cells Drug release in tumor cells, multicellular spheroids and tumors In vivo antitumor efficacy in CT26 tumor-bearing BALB/C mice	Accelerated intracellular drug release due to the increased redox-responsiveness Higher antitumor activity and more efficient to stimulate antitumor immune responses at the tumor site	[130]

LPS: lipopolysaccharide, PDAC: pancreatic ductal adenocarcinoma, PDT: Photodynamic therapy, DMPC: 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine, DOTAP : 1,2-dioleoyl-3-trimethylammonium-propane, DSPE-PEG 2k: h 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)2000], SN38 7-ethyl-10-hydroxy-camptothecin, PLGA: poly lactic-co-glycolic acid



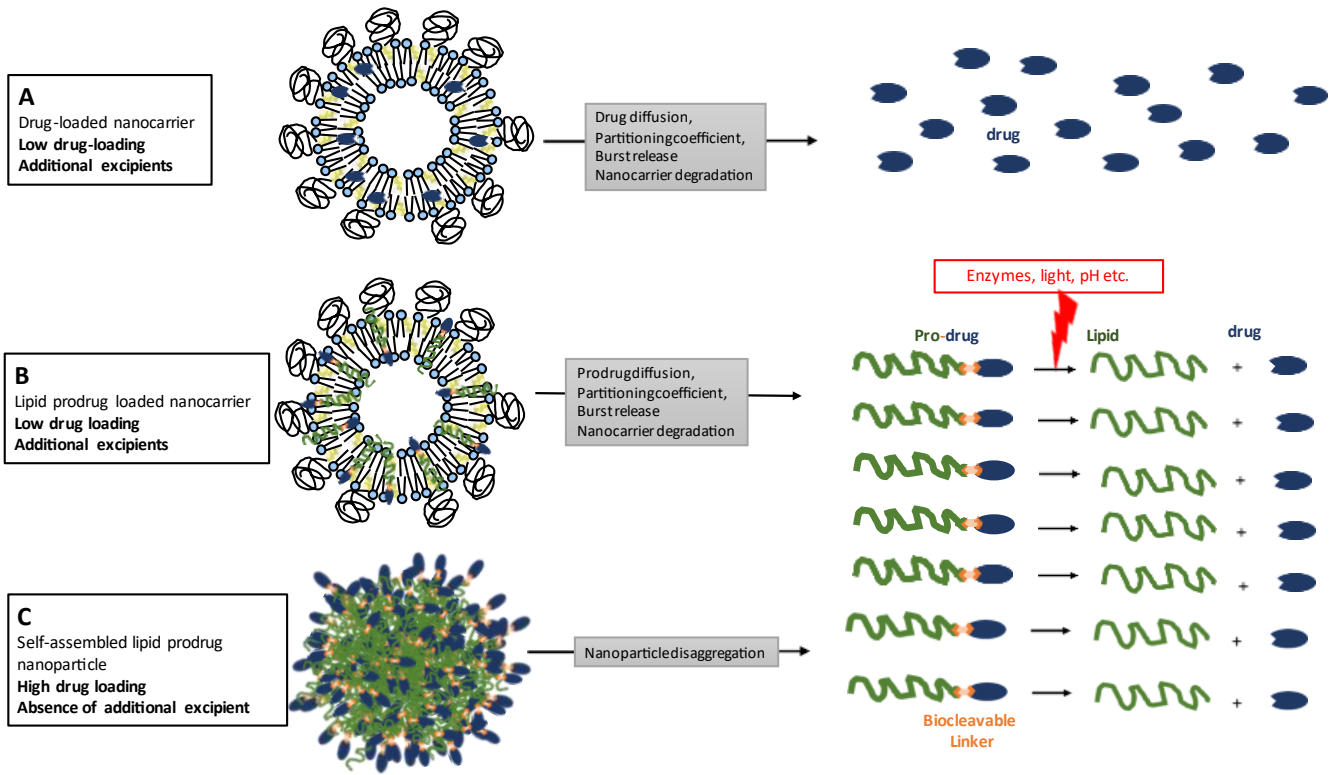


Figure 1

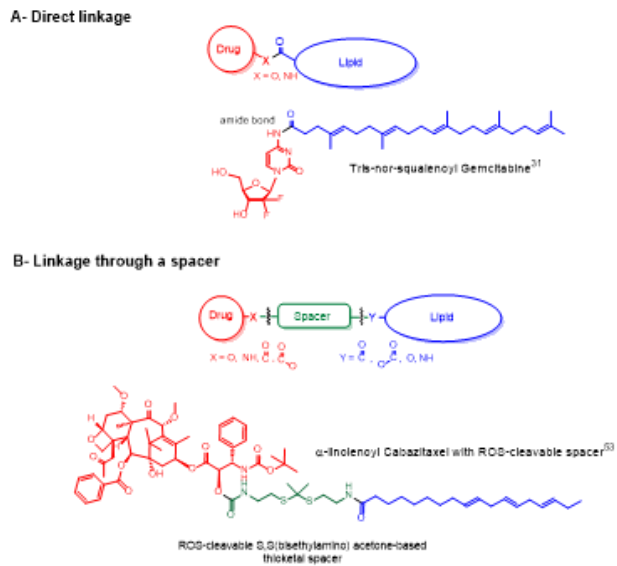


Figure 2

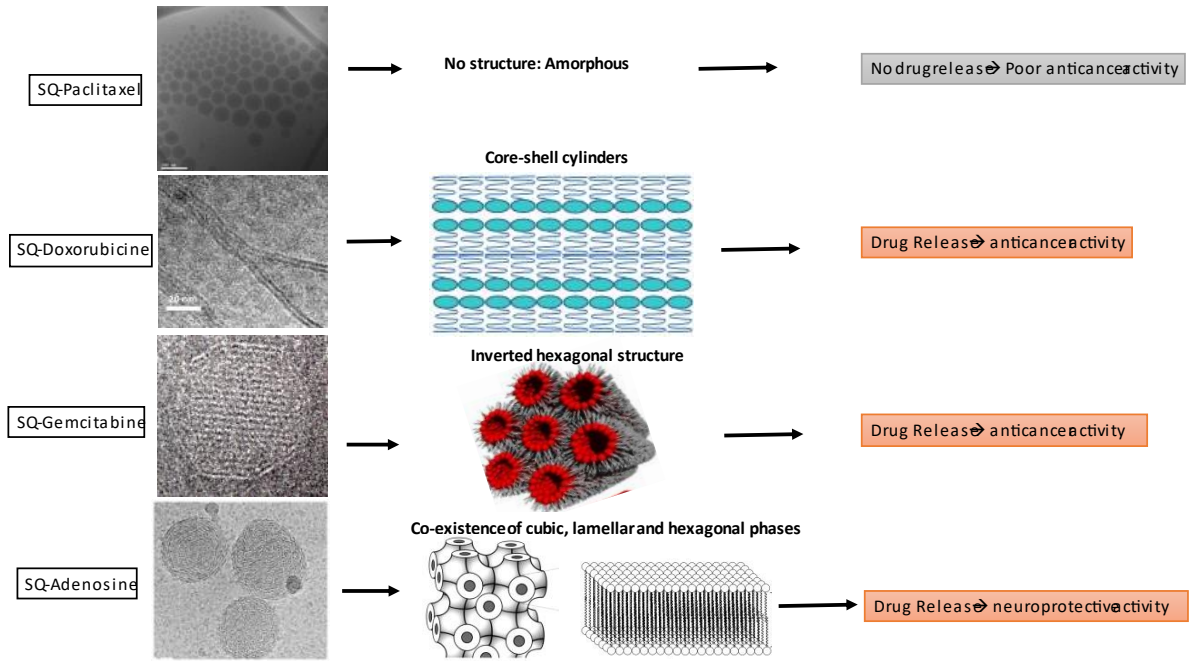


Figure 3

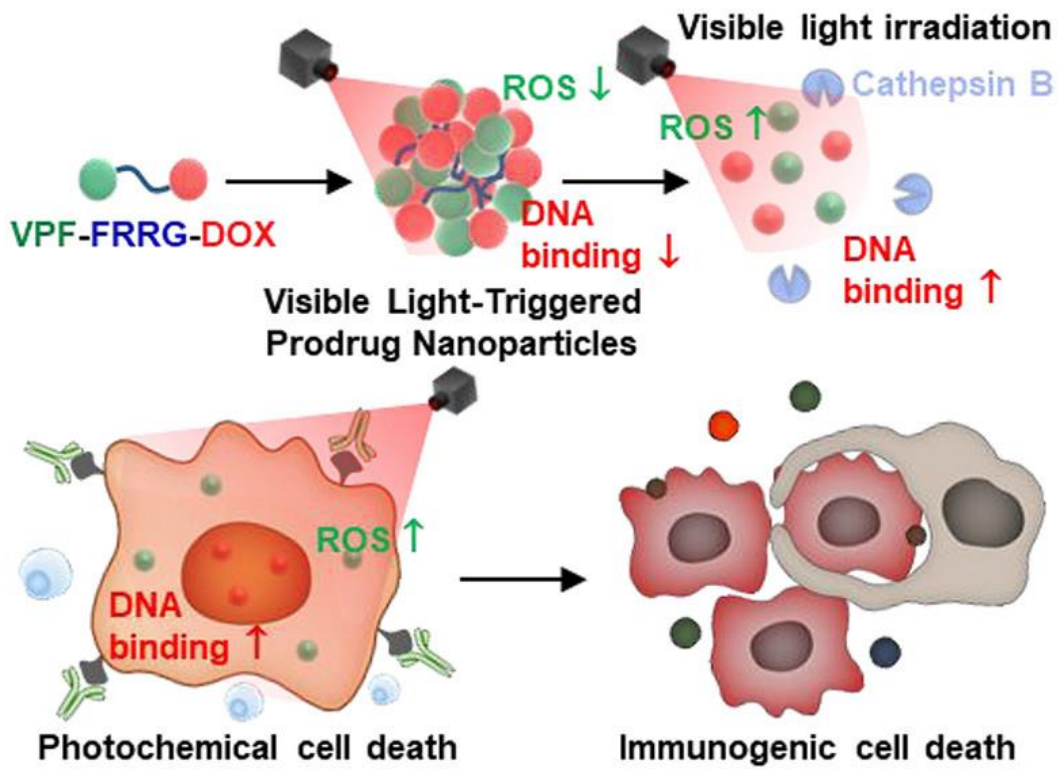


Figure 4

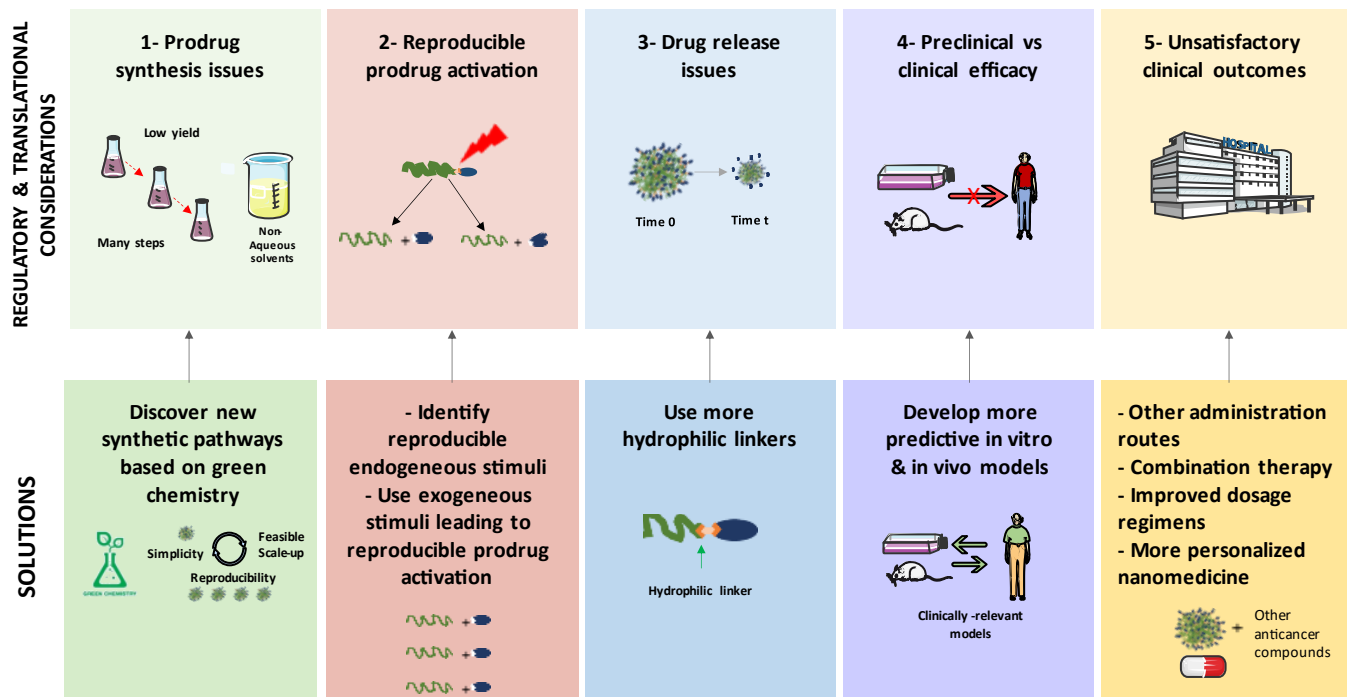


Figure 5