

## Review article



# Self-assembled lipid–prodrug nanoparticles

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## Abstract

Nanomedicines suffer from poor drug loading and uncontrolled ‘burst release’ after administration. Combining prodrug strategies with nanostructured carriers can help to overcome these limitations by improving diffusion through biological barriers, enzymatic activation of the lipid conjugate in the diseased area, drug protection, pharmacokinetics and biodistribution, intracellular delivery and/or targeting properties. Owing to fundamental advances in supramolecular chemistry and the fine-tuning of drug, lipid and drug–lipid chemical link modifications, it is now possible to develop lipid–prodrug conjugates that can spontaneously self-assemble into nanoparticles in aqueous media with unique supramolecular organizations and without additional excipients. In this Review, we describe the chemical synthesis, physicochemical properties, structure and pharmacological activity of lipid–prodrug-based nanomedicines and discuss the path towards clinical translation for the treatment of severe diseases.

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### Outlook

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## Key points

- Lipid–prodrug nanomedicines combine the benefits of nanoparticles and prodrugs by modulating drug release, reducing drug metabolism, improving pharmacokinetic and biodistribution and enhancing intracellular delivery.
- A typical lipid–drug bioconjugate is composed of a lipid carrier molecule, a drug and, if required, a chemical spacer, which can be stimulus responsive.
- The supramolecular architectures of lipid–prodrug nanomedicines is a key factor for bioactivation of the prodrug and its pharmacological activity.
- Lipid–prodrug nanomedicines exhibit higher *in vitro* and *in vivo* pharmacological activity than their parent drugs in experimental models of cancer, neurological disorders, infectious diseases or uncontrolled inflammation.
- Clinical translation of lipid–prodrug nanomedicines remains limited owing to difficulties in prodrug chemical synthesis, control of drug release, the lack of predictive preclinical models and the need to improve patient selection.

## Introduction

Nanomedicines<sup>1–3</sup> (drugs encapsulated into materials such as liposomes, nanoparticles or polymer micelles) have gained importance owing to their ability to protect their drug load from degradation, to modulate drug pharmacokinetics and biodistribution, and to improve intracellular delivery to target diseased areas directly at the cell and tissue level. However, currently available nanomedicines suffer from uncontrolled ‘burst release’ of the loaded drug after administration and poor drug loading (that is, the ratio of the amount of transported drug to that of transporter material, generally expressed as a percentage, which is often less than 5%) (Fig. 1a). Overcoming these limitations requires administration of excessive amounts of carrier relative to the drug, such that, in the case of 5% drug loading, each increase in the amount of the drug to be injected requires 20 times more carrier, which might cause toxicity or result in undesired side effects. These are major concerns for regulatory approval, and might also explain the low number of nanomedicines currently on the market.

A promising solution that could extend pharmacological activity and improve drug bioavailability is the lipid–prodrug<sup>4</sup> approach (also referred to as ‘lipid–drug conjugate’ or ‘lipid–drug conjugate with a bio-cleavable bond’), in which a pharmacologically active molecule is conjugated to a lipid through either a chemical bond or a spacer, cleavable by a physicochemical or enzymatic mechanism (Fig. 1b,c). Several lipid–prodrugs are already on the market, including injectable or oral formulations such as haloperidol decanoate (Haldol Decanoas) or testosterone undecanoate (Nebido), or lipidated corticosteroids for topical use or local injection. The prodrug approach, however, could have additional applications: for example, tailoring the chemical link allows for spatiotemporal control of drug release in the body. Furthermore, the nature of the lipid (fatty acids, squalene, cholesterol, triglycerides or phospholipids) could improve the amphiphilicity of the resulting bioconjugate, thus improving diffusion through various

biological barriers. Lipid–prodrugs could also take advantage of physiological lipid metabolic pathways to facilitate the oral absorption of drugs. However, prodrugs suffer from non-specific biodistribution and rapid clearance, and many of them are non-soluble in aqueous solvents for intravenous administration.

To improve efficiency, prodrugs can be encapsulated into nanocarriers such as liposomes<sup>5</sup>, polymer micelles<sup>6</sup>, polymer nanoparticles<sup>7</sup> or solid lipid nanoparticles<sup>8,9</sup> (Fig. 1b). However, drug loading remains generally limited (less than 5%)<sup>7</sup>, particularly when using physical means for drug encapsulation such as adsorption, encapsulation or entrapment. Owing to recent fundamental advances in supramolecular chemistry, it is now possible to develop drug–lipid conjugates that can self-assemble into nanoparticles in water media with unique supramolecular organizations (Fig. 1c). These formulations do not suffer from burst release after administration and they are easy to prepare, thereby holding great therapeutic potential. Moreover, the widely used lipid nanoparticles in mRNA COVID-19 vaccines have recently boosted interest in lipid-based nanocarriers.

This Review 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>, self-assembling polymers<sup>12,13</sup> and peptide–drug conjugates<sup>14</sup>, as well as lipid–prodrugs that do not assemble as nanoparticles<sup>15,16</sup> are excluded as they have been reviewed elsewhere. Similarly, 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 also not be addressed.

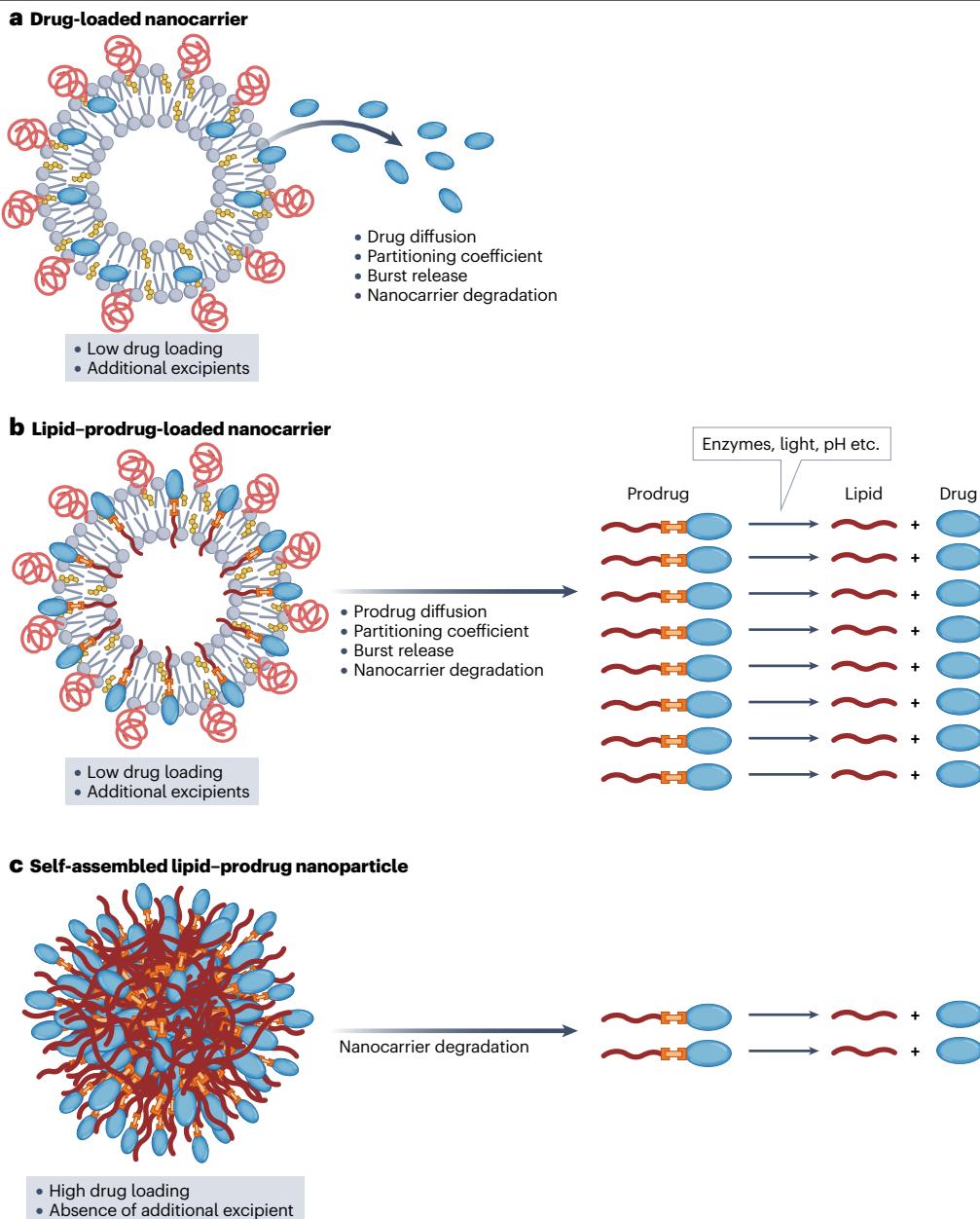
## Lipid–prodrug chemical synthesis

A typical lipid–drug bioconjugate is composed of a lipid carrier molecule, the drug (ranging from small molecules to macromolecular drugs) and the chemical spacer (Fig. 2). Just a slight variation in one of these components is enough to change the physico-chemical properties of the bioconjugate and, consequently, the *in vivo* fate of the resulting nanoparticle. The synthesis of lipid–drug bioconjugates is strongly dependent on the availability of functional groups and appropriate linkers. Furthermore, by changing the properties of the spacer (hydrophilic or hydrophobic, short or elongated) and of the lipid moiety, the amphiphilicity of the lipid–prodrug can be tuned. These modulations influence the supramolecular nanoassembly of the prodrug, and in turn drug release. The design and synthesis of biodegradable and stimuli-responsive nanomaterials could further improve drug safety and efficacy. In that respect, various strategies are used to design self-assembled lipid–prodrugs; for example, by directly linking the lipid moiety (modified or not) and the drug, using a bio-cleavable organic linker or by introducing smart stimuli-responsive lipid conjugates (Fig. 3).

## Direct drug-to-lipid conjugation

Fatty acids (FAs), with their long unsaturated or saturated hydrocarbon chain bearing a free terminal carboxylic acid, are often used to bridge the free hydroxyl or amine functional groups of the drug, resulting in ester or amide bonds, respectively<sup>19,20</sup>. Activation of the acid function of FAs is required prior to the conjugation step, the most common agents being carbodiimides (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl or dicyclohexylcarbodiimide), which activate the acid function into an *O*-acylisourea intermediate. A more sophisticated alternative is to use phosphonium (PyBOP) and uronium (HBTU or HATU) activating agents to activate FAs into *O*-acyloxyphosphonium

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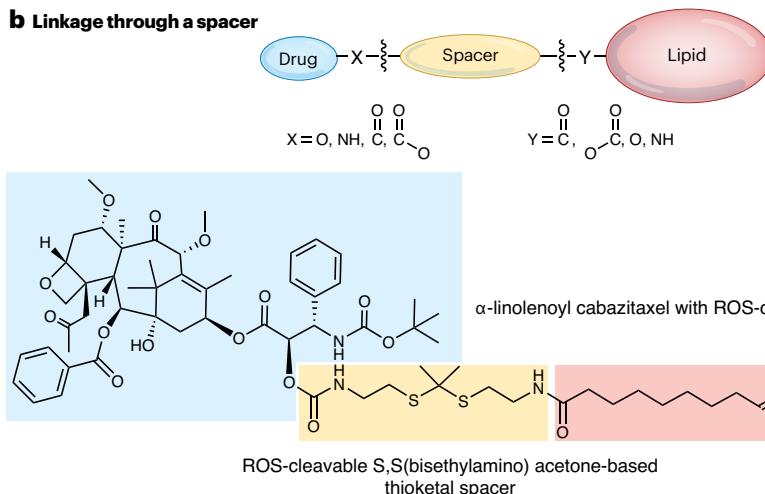
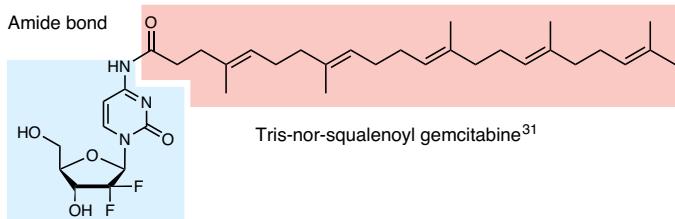
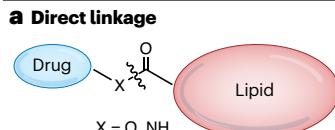
**Fig. 1 | Schematic representation of nanomedicines.** **a**, Nanocarriers such as liposomes, nanoparticles or micelles (here a liposome is shown) release their load of drugs through diffusion, partitioning coefficient, nanocarrier (bio) degradation or burst release from the molecules located on the nanocarrier's surface. **b**, Lipid-prodrugs encapsulated into a nanocarrier (here a liposome is shown) are first released through common mechanisms, after which the drugs are liberated upon activation of the bio-cleavable link (cleaved via enzymes, pH,

reductive or oxidative environment, or an exogenous stimulus such as light). **c**, In self-assembled lipid-prodrug nanoparticles, after dissociation of the nanoparticle's supramolecular structure, the lipid-prodrugs are first released, after which the drugs are liberated upon activation of the bio-cleavable link. As prodrugs<sup>150</sup> and drug-loaded nanocarriers<sup>12,151</sup> have been reviewed elsewhere, this Review focuses exclusively on self-assembled lipid-prodrug nanoparticles.

salt and benzotriazyl ester, respectively. This acid activation approach is the usual method of linking FAs and has been used for the lipidization of many parent drugs, including nucleosides, antiviral or anticancer nucleoside analogues, taxanes, platinum derivates, topoisomerase inhibitors, non-steroidal anti-inflammatory drugs and oligonucleotides, among others<sup>19–27</sup>.

In general, the conjugation of FAs (or other lipids) to strongly hydrophilic drugs (such as small molecules, peptides or nucleic acids) is challenging owing to the limited solubility of both components in the same solvent (organic or aqueous). Therefore, for hydrophilic drugs, using the polar lipid-matching aprotic N,N-dimethyl formamide solvent is preferred. There are very few examples of nanoparticles

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self-assembled from saturated FAs because of the poor self-assembling properties of the resulting bioconjugates. By contrast, conjugation to unsaturated fatty acids often allows nanoparticle formation in aqueous media, especially when polyunsaturated lipids and hydrophilic drugs are used<sup>19,23</sup>. Other linear lipids belonging to the terpene family, such as the polyisoprenic derivatives, have also been proposed. For example, squalene's molecular flexibility and dynamically folded conformation (such as tris-nor-squalenic acid, squalenylacetic acid, tris-nor-squalenol or tris-nor-squalenamine), allow the direct linkage of the squalene moiety to different drug molecules bearing alcohol, amine or acid functions through biocleavable ester or amide bonds using the same acid activation approach as for FAs<sup>23,28–33</sup>. Another original approach consists of using polyisoprenyl moieties, known for their unique self-assembling properties, as a spacer between anticancer drugs (gemcitabine and paclitaxel) for combination therapy<sup>34</sup>.

## Using spontaneously releasing spacers

Following the same acid activation approach, lipids can also be conjugated to drug molecules through an organic spacer using hydrolizable bonds (such as ester or amide). This spacer is used when a direct biocleavable linkage is not possible, to avoid steric hindrance between the drug and the lipid moiety during the conjugation reaction, to modulate the release of the drug and/or to promote the self-assembling properties of the bioconjugates. For example, inserting

a dithiodiglycolyl spacer bearing a disulfide bond between hydrophobic drugs (such as paclitaxel or doxorubicin) and  $\alpha$ -tocopherol, or between hydrophilic drugs (such as fluorouracile or gemcitabine) and stearic acid, prevents aggregation and allows bioconjugates to self-assemble as stable nanoparticles, which is not possible when using a 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) can be introduced<sup>23,36,37</sup>. To promote the release of hydrophobic paclitaxel from highly compact nanoparticles composed of lipophilic bioconjugates, succinyl and the more hydrophilic diglycolyl, polyethylene glycol (PEG) linkers are used instead. These linkers increase the sensitivity of the linkage between the hydrophobic paclitaxel (on 2'-hydroxyl group) and the squalene moiety to hydrolysis by fostering water access<sup>38</sup>. Similarly, linking the Leu-enkephalin neuropeptide or penicillin G to squalene derivatives using either a diglycolyl spacer or a methylene and a methylenecarbonyl bridge, respectively, can help to modulate drug release<sup>28,39,40</sup>. Using a nucleoside lipid scaffold, ruthenium-based octaedric complex nanocarriers can be designed as an alternative to platinum-based chemotherapeutic drugs. These bioconjugates are made up of one or two oleic acids connected to the ruthenium through a PEGylated nucleoside (thymidine or uridine)

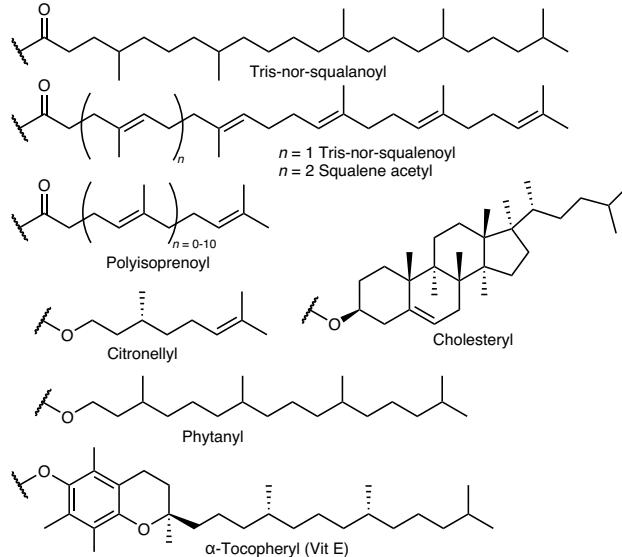
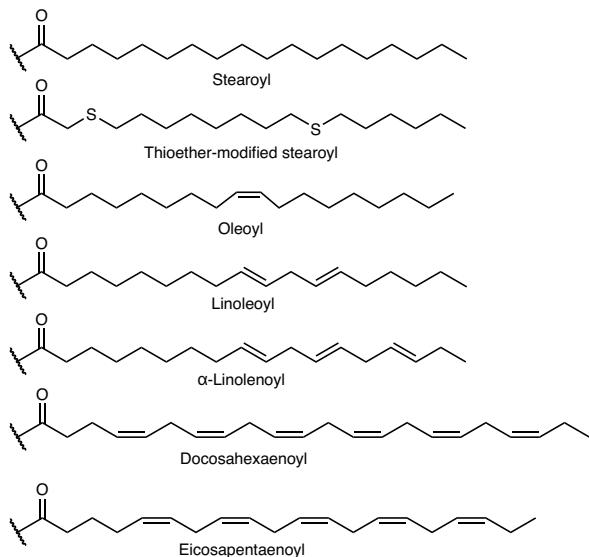
**Fig. 2 | Building blocks of lipid–prodrug conjugates.** Lipid–prodrug conjugates are composed of a drug (blue), a lipid (pink) and a spacer (yellow). Panel **a** shows an example of a lipid–prodrug with direct linkage between the drug and the lipid. Panel **b** shows an example of a lipid–prodrug with a spacer inserted between the drug and the lipid. Data are from refs. 31,53. ROS, reactive oxygen species.

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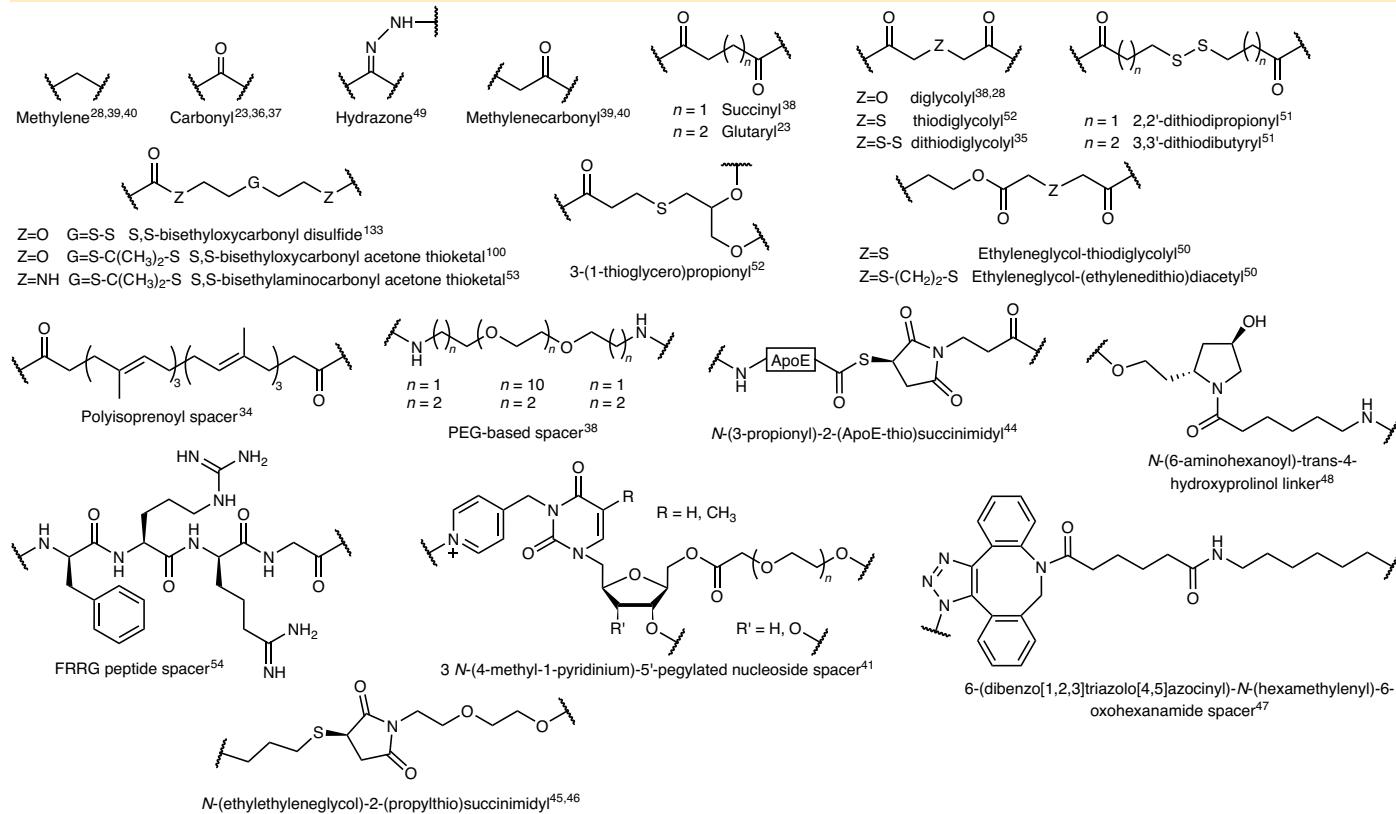
used as spacer itself. To allow complexation with ruthenium, a pyridine-methyl arm is tethered on the nucleobase of the nucleoside lipid<sup>41</sup>.

FAs can also be used for the lipidization of highly hydrophilic macromolecules such as oligonucleotides. Generally, the conjugation occurs at the 5'- or 3'-ends<sup>42,43</sup>; for example, conjugation of myristic acid

## a Lipid moieties



## b Spacers



**Fig. 3 | Structure of lipid–prodrug conjugates that can self-assemble into nanoparticles.** **a**, Different structures of lipid moieties composing the lipid-based prodrugs. **b**, Structures of the spacers inserted in the lipid bioconjugates. Data are from refs. 23,28,34–41,44–54,100,133.

to an antisense oligonucleotide through an apolipoprotein E (ApoE) cell-penetrating peptide (to promote oligonucleotide cell permeability), enables this hybrid bioconjugate to form a complex by self-assembly. Specifically, myristic anhydride reacts with the amino-terminus of the resin-bound ApoE peptide bearing carboxy-terminal cysteine. After deprotection and resin cleavage, cysteine is then conjugated to the 3'-maleimide-functionalized phosphorodiamidate morpholino oligonucleotide (PMO) through a thioether bond using a thiol-maleimide click reaction with good yield (65%)<sup>44</sup>. Notably, the direct conjugation of myristic acid on the secondary amine at the 3'-end of the PMO is also possible but with a much lower yield (45%).

For small interfering RNA (siRNA) conjugation, hetero-Michael addition reaction allows the thiolated RET/PTC1 siRNA sense strand to react with a diethylene glycol maleimide-derivatized squalene to form a covalent non-reducible thioether bond with a 55% yield under microwave irradiation in the presence of methanol<sup>45,46</sup>. Another strategy based on copper-free click chemistry consists of azido-squalene or azido-solanesol reacting through 1,3-dipolar cycloaddition with the dibenzocyclooctyne residue of siRNA. Of note, the TMPRSS2-ERG siRNA was linked to dibenzocyclooctyne through a *N*-(hexamethylenyl)-6-oxohexanamide spacer to avoid steric hindrance, with a synthesis yield of up to 95% (ref. <sup>47</sup>). Cholesterol has also been conjugated to the 3'-end of the sense strand of an ApoB siRNA through a *N*-(6-aminohexanoate)-trans-4-hydroxyprolinol linker, but the final yield was not reported<sup>48</sup>.

## Using stimuli-responsive moieties

Smart stimuli-responsive nano-systems can control drug biodistribution in response to either exogenous (variations in temperature, magnetic field, ultrasound intensity, light or electric pulses) or endogenous (changes in pH, enzyme concentration or redox gradients) stimuli. For this purpose, a common approach is to insert a stimuli-responsive linkage or spacer between the drug and lipid moiety. For example, pH-responsive phospholipid-like prodrugs formulated into micelles can improve the KB-tumour specificity of doxorubicin when conjugated to 11-mercaptopundecyl phosphorylcholine bearing a hydrazide group through a hydrazone linkage in nude mice<sup>49</sup>. To facilitate rapid and selective drug release from hydrophobic oleylpaclitaxel bioconjugates, an ethyleneglycol-thiodiglycolyl spacer or its dithioether derivative is introduced to formulate redox dual-responsive prodrug-nanosystems<sup>50</sup>. The synthesis of these prodrugs consists in conjugating oleic acid to ethylene glycol and then to thiodiglycolic anhydride or to its dithioether derivative, after which the resulting oleic acid-linkers are coupled to paclitaxel. In cancer cells, these linkers release paclitaxel by two simultaneous mechanisms: thiolytic as a result of the strongly reducing environment in the intracellular compartment owing to overproduction of the reduced glutathione, and oxidation of the thioether into hydrophilic sulfoxide or sulfone owing to the higher levels of extracellular reactive oxygen species (ROS) compared to normal cells.

Paclitaxel can also be conjugated to citronellol using disulfide-bond-containing carbon chain linkages of various lengths. This bond substantially influences the redox responsiveness and improves tumour-specific drug release and anticancer activity *in vitro* (KB and A549 human carcinoma cells and KT1 mouse cancer cells) and *in vivo* (KB tumour-bearing nude mice)<sup>51</sup>. A more sophisticated approach consists of combining photoactivatable nanosystems with the lipid-based camptothecin (SN38) prodrug and the C18-lipidated protoporphyrin IX (PpIX) photosensitizer<sup>52</sup>. The SN38 prodrug

includes two multiple thioether-modified fatty acids connected to SN38 through a thioglyceropropionyl moiety. To synthesize the SN38 prodrug, thioether-modified FAs is first conjugated with tert-butyl S-thioglyceropropionate ester. After deprotection but prior to SN38 conjugation of the acid function by trifluoroacetic acid, the resulting S-thiodiglyceride propionic acid is activated by oxalyl chloride. To synthesize C18-PpIX, stearic alcohol is first conjugated to thiodiglycolic anhydride. The resulting C18-thiodiglycolic acid is then coupled to ethylene glycol before being conjugated to PpIX using dicyclohexylcarbodiimide and 4-dimethylaminopyridine (DMAP) reagents. Upon laser irradiation, these hybrid co-nanoassemblies undergo *in situ* oxidization of the multiple thioether moieties (located within fatty acids and linkers) into hydrophilic sulfones, resulting in the rapid destruction of the nanoparticle structure, thus triggering fast, targeted drug release.

Photoactivatable nanomedicines can also be developed by combining photodynamic therapy and chemotherapy approaches. For example, coupling cytotoxic cabazitaxel to  $\alpha$ -linoleic acid through a ROS-cleavable S,S(bisethylamino)carbonyl acetone-based thioketal linker results in a prodrug which can be co-nanoassembled with the chlorine e6 (Ce 6) photosensitizer conjugated to  $\alpha$ -linoleic acid through an ethylene diamine linker<sup>53</sup> (Fig. 2). Once in tumour cells, and upon activation of endogenous or photosensitizer-induced ROS, the drug is released by intramolecular nucleophilic substitution reaction, while the released Ce 6 lights up cancer cells. Similar visible-light-triggered prodrug nanoparticles can be prepared by conjugating the lipophilic verteporfin photosensitizer and doxorubicin through the cathepsin B-cleavable FRRG (Phe-Arg-Arg-Gly) peptide used itself as a linker<sup>54</sup>.

## Structure of lipid–prodrug nanoparticles

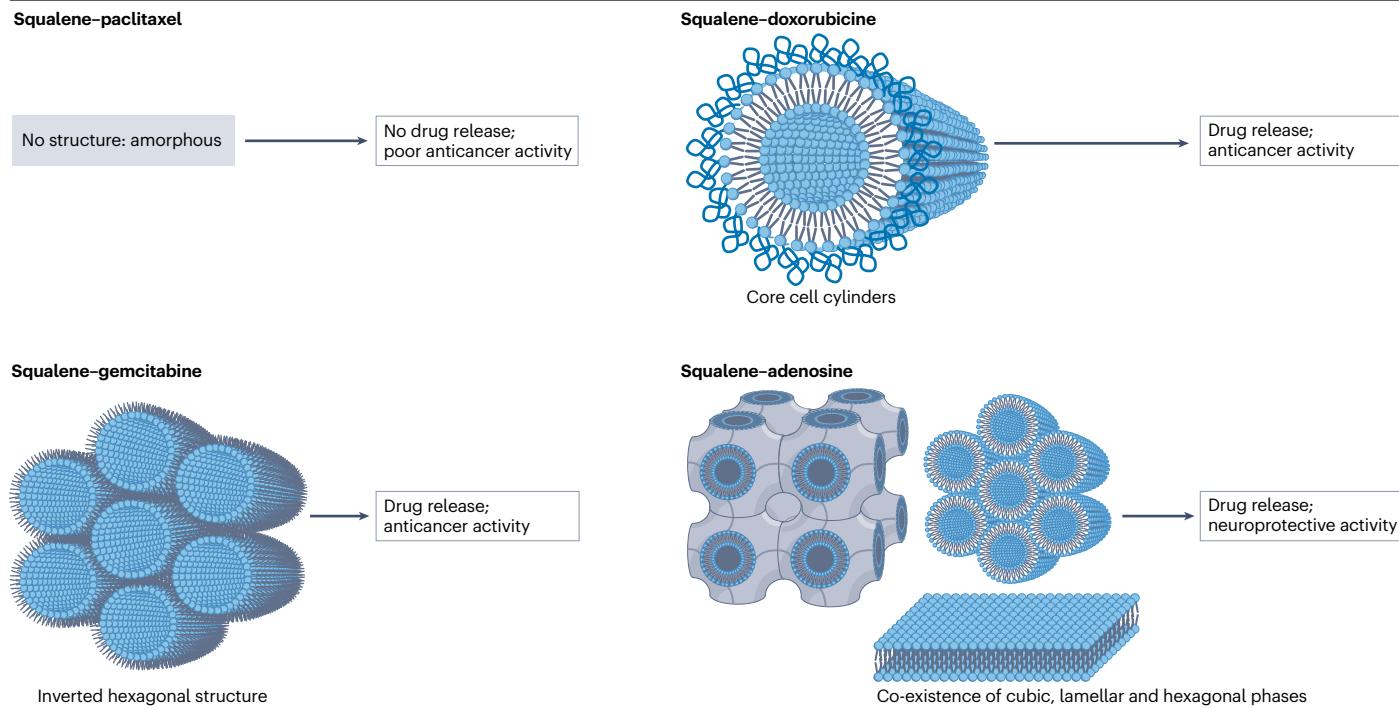
### Preparation and long-term storage

The preparation of prodrug nanoparticles relies on the spontaneous ability of lipid–prodrug molecules to self-assemble into nanosized particles with or without using a stabilizer. For example, nanoprecipitation<sup>8</sup>, microfluidics<sup>55</sup> and high-pressure homogenization<sup>27</sup> are methods of lipid–prodrug nanoparticle preparation. Nanoprecipitation is the best studied owing to its simple procedure, which involves dissolving the lipid–prodrug in an organic solvent before addition to an aqueous medium. Long-term storage and stability of lipid–prodrug nanoparticles are also important factors to consider for clinical translation. Squalene–prodrug nanoparticles can be lyophilized without major changes to their physicochemical properties (size, polydispersity and zeta potential) after 3–4 months of storage at 4 °C, with trehalose being the most efficient cryoprotectant<sup>56,57</sup>. Notably, freeze-dried adenosine–squalene particles did not induce hepatic toxicity *in vitro* on hepatocyte HepG2 cells or in a mouse model of liver ischaemia reperfusion<sup>57</sup>. Furthermore, lyophilized nanoformulations of squalene–gemcitabine retain their cytotoxic activity after freeze-drying<sup>56</sup>. Thus long-term storage through lyophilization could facilitate the clinical application of lipid–prodrug nanomedicines.

### Supramolecular architecture

Supramolecular architectures can take complex shapes including lamellar, hexagonal, cubic and toroidal forms, as well as ribbons, vesicles, helical strands, spherical or wormlike micelles and amorphous structures<sup>58–62</sup> (Fig. 4). The ability of typical lipid–prodrugs to self-assemble depends on the physicochemical properties of the lipid and the drug, but systematic studies investigating both components are lacking. The dynamic shapes of amphiphilic molecules could allegedly decide the morphology of self-assemblies, with the volume ratio

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**Fig. 4 | Relationship between the structure of squalene-based nanoparticles and their pharmacological activity.** Because drug release depends on the bioactivation of the prodrug, the supramolecular architecture is a key factor for allowing the endogenous stimuli (such as enzymes, pH or redox status) to diffuse into the nanoparticle core.

of polar heads to hydrophobic tails being a key factor<sup>63</sup>. However, even if the amphiphilicity of the drug–lipid complex is generally preferable, lipophilic drug–lipid conjugates like paclitaxel–squalene<sup>38</sup>, doxorubicin–squalene<sup>64</sup> or even the lipophilic prodrugs of dexamethasone<sup>62</sup>, can nevertheless form nanoparticle structures. For example, the hydrophobicity of lipid–paclitaxel conjugates leads to pure hydrophobic nanoassemblies that, despite accumulating in the tumour tissue, cannot increase antitumour efficacy in mice bearing human lung A549 tumour, probably because the nanoassembly is inaccessible to the enzymes required to activate the prodrug<sup>65</sup>. Notably, inserting a thio- or dithio-based redox responsive linker allows drug release from the nanoparticles within tumour tissues and inhibits tumour growth<sup>50,66</sup>. A systematic study to identify typical lipid–prodrugs that can self-assemble using different lipids – including terpenoids, sterols, fat-soluble vitamins, saturated and unsaturated fatty acids, and diglycerides – to conjugate with gemcitabine, revealed that, with some exceptions (octacosanoic acid (C28) or Vitamin E linked to gemcitabine), conjugates with a hydrophilic-to-lipophilic balance of below 8.4 can be formulated as stable nanoparticles<sup>23</sup>. Furthermore, the presence of lateral methyl on the hydrophobic chain and insaturations on the lipid chain are also key parameters to ensure self-assembly. Indeed, with a few exceptions (myristoylated abacavir)<sup>67</sup>, saturated lipid-based conjugates generally do not spontaneously self-assemble into nanoparticles, probably owing to the high degree of flexibility of the lipid chain.

Because drug release depends on the bioactivation of the prodrug, the supramolecular architecture is a key factor to ensure diffusion of enzymes or other endogenous stimuli (acidity or a redox environment, for example) into the nanoparticle core. In this regard, hydrophilic moieties that allow the creation of aqueous channels in the

nanoassemblies are preferable to an amorphous hydrophobic configuration. Nonetheless, because of protocol variability between studies and the fact that the nanoparticle's structure is often not investigated, it is difficult to draw definitive conclusions about how the supramolecular organization of lipid–prodrug nanoparticles influences their pharmacological activity. However, by comparing several squalene-based nanodrugs produced under similar experimental conditions, it appears that the absence of a supramolecular structure is detrimental to their therapeutic activity<sup>65</sup> (Fig. 4).

The diversity of the designed nanostructures ranges from rod-shaped structures of myristoylated cabotegravir with a particle size of 318 nm (ref. 27) to doxorubicin conjugated to tocopherol–PEG succinate nanoparticles of 250 nm with a highly ordered lamellar inner structure<sup>58</sup>. In the latter example, NMR measurements revealed doxorubicin to be located in the particle core, whereas the PEG chains and part of the tocopherol were in the corona. Similarly, multidrug nanosystems combining squalenoyl–gemcitabine with edelfosine, an alkyl-lysophospholipid, display a multilamellar concentric organization resembling an onion-like multigeometry<sup>59</sup>. Moreover, nanoassemblies of squalene–gemcitabine show 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 suggests stacking of inverse hexagonal phases, in which the central aqueous core is composed of water, whereas gemcitabine is surrounded by squalene. Remarkably, the polyisoprenoyl gemcitabine bioconjugate with five isoprene units (gemcitabine-5-isoprene, which differs from squalenoyl gemcitabine only in the position of two methyl groups on the hydrophobic chain), self-assembles into nanotubes<sup>68</sup>. This behaviour was attributed to the higher rigidity of gemcitabine-5-isoprene, which

prevents the formation of the inverse hexagonal phase observed with squalenoyl gemcitabine nanoparticles. Similarly, when squalene is conjugated to the sugar moiety and grafted onto the nucleobase, the resulting nanoparticle structure becomes a lamellar phase instead of a hexagonal phase<sup>69</sup>. Furthermore, inserting a phosphate group between the sugar moiety of gemcitabine and the squalene increases the hydration and hindrance of the polar headgroup, leading to the transition of the lamellar phase to unilamellar vesicles<sup>22</sup>.

The complexity of these self-assembled structures results from the interplay between the unique conformational properties of squalene, the packing parameters of the bioconjugates and possible specific H-bond interactions involving nucleobases. When gemcitabine is replaced by deoxycytidine or adenosine, spherical nanoparticles can still be obtained but with a bi-continuous cubic inner structure<sup>61,68</sup>. Moreover, introducing monovalent salts into the suspending medium results in elongated cylindrical nanoparticles of doxorubicin squalene<sup>70</sup>, a shape known to prolong blood circulation time by hindering recognition by Kupffer cells in the liver and macrophages in the spleen<sup>71</sup>. By contrast, divalent anions trigger lipid-prodrug assembly through salt bridges, inducing a transition from individual cylindrical nanoparticles to bilayer tubes<sup>70</sup>. These results highlight that nanoparticles in the test tube and in physiological conditions might behave differently, whereby the local microenvironment influences the final structure of the nanoparticles.

## Lipid-prodrug nanoparticle benefits

### Drug metabolism and biodistribution

Prodrug-based nanoassemblies protect transported biologically active molecules, including metabolically sensitive small molecules and macromolecules. For example, intravenous administration of siRNA conjugated to palmitic acid, cholesterol, squalene derivative or tocopherol decreases degradation in mice<sup>48</sup>. Furthermore, when chemically conjugated to cholesterol or long fatty acid chains, siRNA uptake into cells increases dramatically. Interestingly, lipid-siRNA conjugates strongly interact with blood components in vitro and in vivo, in particular with lipoprotein particles (both high-density lipoprotein (HDL) and low-density lipoprotein (LDL)), lipoprotein receptors and transmembrane proteins. Whereas LDL transports cholesterol-siRNA mainly to the liver, HDL directs lipid-siRNA within the liver, the kidneys, the gut and steroidogenic tissues. Notably, the LDL receptor is necessary for LDL-associated cholesterol-siRNA delivery, as revealed by comparing *Ldlr*<sup>-/-</sup> and wild-type mice<sup>48</sup>. Similarly, the metabolism of antinociceptive neuropeptides (that is, Leu-enkephalin and Met-enkephalin) is hindered after conjugation with squalene, although the association of these bioconjugates with either LDL or HDL is still debated<sup>28</sup>. A similar behaviour is observed with small molecules; for example, when injected intravenously to rats, squalene-gemcitabine and adenosine-squalene nanoparticles rapidly disaggregate owing to their unique hexagonal or cubic supramolecular structures<sup>72</sup>. The released bioconjugates are attached to the LDL as single molecular entities, thereby using LDL as ‘indirect’ endogenous nanocarriers for targeting tumours with hyper-expression of LDL receptors.

One important consideration is whether a nanoparticle formulation is necessary to ensure pharmacological activity or whether a single lipid-prodrug molecule is sufficient. 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 the enhanced permeability and retention (EPR) effect of a nanoparticle is needed to allow the

nanoparticles to diffuse through the endothelial barrier compromised by the disease. However, for those molecules that can take advantage of endogenous particles for precise targeting, the bioconjugate in its molecular form might be sufficient for certain applications (gemcitabine, doxorubicine, adenosine and so on). Nonetheless, in most cases, when a biologically active molecule is conjugated to a lipid, it generally becomes insoluble in water and, for intravenous administration, a nanoparticle formulation remains the only option.

Lipid-prodrugs of gemcitabine have also been synthesized to slow down degradation after intravenous administration. In humans, gemcitabine is enzymatically metabolized by deoxycytidine deaminase into inactive uracil derivatives, which substantially limits its pharmacological efficacy<sup>73</sup>. Intravenous administration of gemcitabine covalently coupled with 1,1,2-trisnor-squalenic acid in mice have prolonged blood half-life, inducing a higher mean residence time owing to a delayed metabolism in difluorodeoxyuridine and reduced urinary elimination compared to the parent drug<sup>74</sup>. Certain lipids could also be used as a targeting ligand for programmed biodistribution of small anticancer molecules; for example, docosahexaenoic acid (DHA), an omega-3 C22 natural fatty acid, conjugated to gemcitabine triggers the uptake of the anticancer compound by tumour cells in vitro (MCF-7 and HepG2 cells) and in vivo (MCF-7- and HepG2-tumour-bearing mice) owing to the phosphatidylethanolamine contents of the cancer cell membranes<sup>75</sup>.

Tuning the supramolecular organization of lipid-prodrugs could also enable modulation of the size and shape of the resulting nanoparticles. For example, doxorubicin-squalene nanoparticles display elongated structures that extend along the blood streamlines after intravenous administration in mice, which results in a prolonged pharmacokinetic profile of doxorubicin with lower cardiac concentration, higher tumour uptake in a mouse pulmonary carcinoma (M109) and in a human xenograft of pancreatic cancer (MiaPaCa-2) and reduced urinary excretion<sup>64</sup>.

### Improving intracellular uptake

Owing to their small size (in general between 50 nm and 150 nm) and lipid nature, prodrug nanoparticles facilitate the intracellular penetration of drugs that would otherwise be unable to diffuse spontaneously through cell membranes. The intracellular uptake of nanoparticles occurs through phagocytosis or clathrin-dependent endocytosis, leading to their intracellular segregation in cellular digestive vesicles such as endosomes or lysosomes<sup>76</sup>. Therefore, prodrugs can be designed to be activated upon exposure to the acidic pH of endosomes or the enzymatic content of late lysosomes. For example, coupling penicillin to squalene promotes intracellular delivery of the resulting nanoparticles, triggering the release of the antibiotic into the endo-lysosomal compartment where resistant *Staphylococcus* bacteria are located<sup>39</sup>. Drug molecules with low molecular weight and appropriate lipophilicity can diffuse through the endo-lysosomal membrane, but for fragile biomacromolecules such as nucleic acids (DNA or RNA), ionizable lipids need to be used as lipid nanoparticle components to allow the export of nucleic acids into the cell cytoplasm<sup>77</sup>.

Although less frequent, caveolae endocytosis and macropinocytosis<sup>78</sup> also mediate nanoparticles’ intracellular delivery<sup>79</sup>. Because cytosolic caveolar vesicles have a neutral pH and do not contain any enzymatic cocktail, this intracellular pathway might be suitable to bypass endo-lysosomal degradation if the carried drug is sensitive to enzymatic degradation (such as peptides, proteins and nucleic acids). However, if the lipoprotein-driven dissociation of the lipid-prodrug nanoparticles occurs extracellularly and is followed by protein-mediated diffusion in the vicinity of the cell, then the prodrug can either insert

into the cell membrane following partition coefficient<sup>80</sup> or be taken intracellularly through specific LDL receptors<sup>81</sup>.

Generally speaking, binding anticancer compounds to polyunsaturated fatty acids improves endocytosis and cytotoxicity<sup>82,83</sup>. For example, linking linoleic acid to the amino group of doxorubicin results in higher cellular uptake through passive plasma membrane diffusion and a two-fold increase in cytotoxicity compared to free doxorubicin *in vitro*<sup>83</sup>. Interestingly, linoleic acid–doxorubicin exhibits higher cellular uptake and cytotoxicity *in vitro* (MCF-7, MDA-MB 231 and HepG2) compared to its palmitic-acid–doxorubicin counterpart, probably because unsaturated fatty acids having better intracellular delivery of drugs than saturated fatty acids<sup>82</sup>.

Anticancer nucleoside analogues such as cytarabine and gemcitabine, instead, can only be taken up by cells through the human concentrative nucleoside transporter (hENT)<sup>84</sup>. Down-regulation of hENT thus results in drug resistance in non-small-cell lung cancer cells<sup>85</sup>, which can be overcome by conjugating anticancer molecules to the fatty elaidic acid, thereby enabling passive diffusion through the cell membrane<sup>86,87</sup>. Similarly, squalene–gemcitabine nanoparticles exhibit three times higher cytotoxicity in murine-resistant leukaemia L1210 10 K cells and in the human leukaemia-resistant cell line CEM-ARAC/8C than free gemcitabine, emphasizing the suitability of lipid–prodrug nanoparticles to circumvent *in vitro* anticancer drug resistance<sup>33</sup>.

## Promoting drug diffusion

Lipid solubility is a key factor for drug diffusion, which is why lipid–prodrug nanoassemblies have been used to promote drug translocation through epithelial and endothelial biological barriers. However, oral administration and diffusion of drugs through the intestinal epithelium is often challenged by the poor solubility, low permeability, high molecular weight or chemical or metabolic instability in the gastrointestinal tract of the drug. Moreover, drug molecules might be the substrate of efflux transporters, or subject to first-pass hepatic metabolism, causing their elimination. Therefore, conjugating lipid moieties to pharmacologically active molecules with an unfavourable partitioning coefficient can improve diffusion through the gastrointestinal epithelium. However, if fatty acids, steroids, phospholipids or glycerides are used to increase the lipophilicity of the parent drug, they could also restrict its dissolution properties. Thus, lipids must be carefully selected to match the physicochemical properties of the drug to avoid over-lipophilicity or increasing its molecular weight such that it becomes non-diffusible. For example, in a dose-escalation phase I study (NCT01392976), patients with advanced solid tumours were treated with gemcitabine esterified at the 5' position with elaidic fatty acid (trans-9-octadecenoic fatty acid). The prodrug was solubilized in a lipid-based formulation and encapsulated in non-gelatine hard-shell capsules. However, this study was terminated before the recommended dose had been determined because of poor drug absorption and its rapid pre-systemic metabolism, which resulted in accumulation of deaminated metabolites, leading to severe liver toxicity<sup>88</sup>.

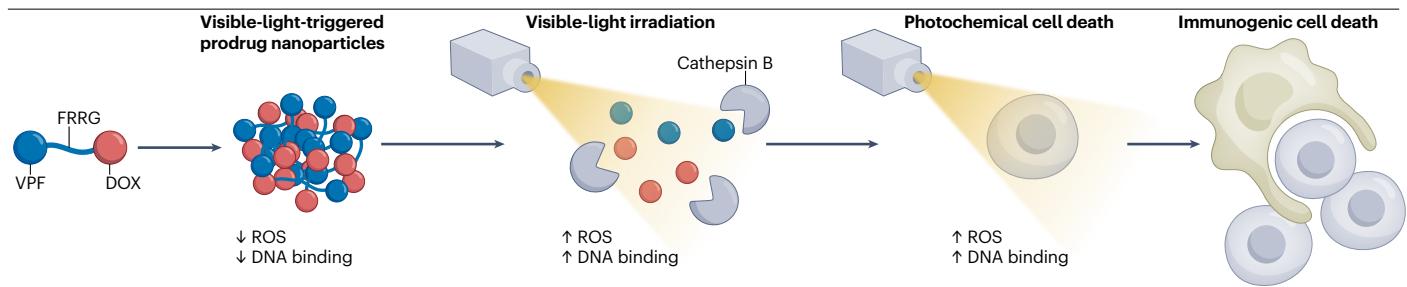
Another attractive strategy is to take advantage of the physiological absorption pathways of lipids in the gastrointestinal tract for improving the oral bioavailability of poorly diffusible drugs; for example, by forcing hydrophilic molecules to follow the lymphatic absorptive route of triglycerides<sup>89,90</sup>. Natural triglycerides are hydrolysed in positions 1 and 3 by pancreatic lipases and co-lipases, which produces two fatty acids and the corresponding 2-monoglyceride. After absorption into enterocytes, 2-monoglycerides reassemble intracellularly into triglycerides through the monoacylglycerol pathway, thereby forming

chylomicrons in a highly lipophilic environment<sup>91</sup>. After exocytosis, the chylomicrons are taken into the open capillaries of the mesenteric lymphatic vessel before flowing into the systemic circulation at the junction of the left internal jugular and subclavian veins, thus bypassing the portal blood and the liver. The synthesis of triglyceride prodrugs, in which the drug is conjugated at the *sn*-2 position of the glyceride, is expected to follow a similar absorption pathway, therefore bypassing first-pass hepatic metabolism<sup>92</sup>. A typical example is the conjugation of the immunosuppressant mycophenolic acid (MPA) to triglyceride (TG)<sup>93</sup>: oral administration of the 2-MPA-TG prodrug in Sprague–Dawley rats increases lymphatic drug transport, with a 103-fold higher concentration in lymphocytes compared to freely administered MPA<sup>94</sup>. Interestingly, the concentration of free MPA released from the prodrug in the mesenteric lymph nodes was 28-fold higher compared to the control, highlighting the efficient conversion of diglyceride prodrug into the parent molecule. Absorption is even higher in greyhound dogs, where the prodrug increases the lymphatic transport of the immunosuppressive agent by 288-fold compared with oral administration of free MPA<sup>90</sup>. Moreover, the phospholipid–prodrugs are hydrolysed on the *sn*-2 position by phospholipase A2 enzymes, thus releasing free fatty acid and lysophospholipid. Therefore, the oral administration of a phospholipid–prodrug of indomethacin provides controlled release into the plasma following degradation by these enzymes in the gut lumen of Wistar rats<sup>95</sup>. Similarly, lipid–prodrugs have also been used to promote drug diffusion through the skin epithelium by either improving the permeation coefficient or by protecting the drug from degradation<sup>96</sup>.

After intravenous administration, lipid–prodrug nanoparticles could also take advantage of the EPR effect, which results from the increased permeability of the blood vessels owing to the disease-associated inflammatory process<sup>12</sup>. However, in oncology, this assumption has been challenged by clinical data, owing to the diversity and heterogeneity of human cancer. Nonetheless, using nanoparticles against inflammatory diseases remains an attractive strategy for selective endothelium translocation. For example, squalene–prodrug nanoassemblies can bypass the endothelial barrier (EPR-like effect) when intravenously administered in rodent models of LPS-induced sepsis<sup>97</sup> and inflammation<sup>28</sup>. The ability of conjugated linoleic acid to interact with brain vessels and to prevent new vessel growth in the cerebellum has also encouraged the use of this lipid<sup>98</sup>. For example, the blood–brain barrier is characterized by endothelial cells with tight junctions and Pgp expression, which hinder delivery of drugs to the brain, especially hydrophilic ones. Lipidation of biologically active molecules can improve diffusion through the blood–brain barrier; for example, supramolecular assembly of paclitaxel–linoleic acid distribute at high concentrations into the brain tissue of glioma-bearing rats, unlike free paclitaxel<sup>99</sup>.

## Controlling drug release

Amide or ester bonds can be used to form versatile drug-to-lipid conjugations that modulate drug release profiles depending on the local enzymatic activity of the target area. Using disulfide or hydrazone bonds instead makes the prodrug nanomedicine responsive to a reductive or low-pH environment<sup>51</sup>. Lipid–prodrug nanoparticles can also be made sensitive to external stimuli; for example, visible-light-triggered doxorubicin–prodrug nanoparticles can turn an immunosuppressive tumour microenvironment into a highly immunogenic one by boosting checkpoint blockade immunotherapy<sup>54</sup>. Specifically, doxorubicin linked to a photosensitizer through a cathepsin-B-specific cleavable



**Fig. 5 | Schematic illustration of a visible-light-triggered prodrug nanoparticles.** Visible-light-triggered nanoparticles based on cathepsin-B-specific cleavable prodrugs of verteporfin (VPF), cathepsin-B-specific cleavable peptide (FRRG) and doxorubicin (DOX) conjugates (VPF–FRRG–DOX) for

combinatorial photodynamic and chemotherapy to boost checkpoint blockade cancer immunotherapy. ROS, reactive oxygen species. Reprinted with permission from ref. 54, American Chemical Society.

peptide is activated upon visible-light irradiation, resulting in the maturation of dendritic cells and their stimulation for cross-presentation of cancer antigens to T cells, resulting in cancer-specific cytotoxicity and immunogenic cell death in CT26-tumour-bearing mice (Fig. 5). Similarly, combining polyunsaturated fatty acid with cabazitaxel through a self-immolation thioketal linkage and lipidated chlorine e6 photosensitizer allows near-infrared irradiation to trigger drug release at the diseased tissue<sup>53</sup>. ROS-activatable heterodimeric prodrugs have also been designed by conjugating camptothecin and 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide photosensitizer through a ROS-sensitive thioketal linker<sup>100</sup>. A light-responsive core-shell self-assembled nanoparticle has been designed using a ROS-responsive oleate prodrug of paclitaxel as the core and the conjugation of lipophilic pyropheophorbide with PEG2000 as the shell<sup>24</sup>.

Stimuli-responsive prodrug nanoparticles open up a new window for designing more specific and selective nanoparticles. However, clinical translation remains limited, owing to regulations demanding perfect reproducibility of drug release after the same stimulus is applied to different patients.

## Therapeutic applications

### Cancer

**In vitro and preclinical data.** Cancer therapeutics is the most extensively investigated application of lipid–drug nanomedicines<sup>101,102</sup> (Table 1). Several well-established anticancer drugs currently used in the clinic – such as gemcitabine<sup>23,60,103</sup>, paclitaxel<sup>51,65,104</sup>, doxorubicin<sup>58,64,105</sup> and other therapeutics such as RNAs<sup>45,47</sup> – have already been turned into lipid–prodrug nanoparticles. The antimetabolite gemcitabine has received the most attention for the formation of lipid–prodrug nanoassemblies because, in its free form, it suffers from short half-life, limited intracellular penetration and drug resistance. To overcome these limitations, gemcitabine has been conjugated with polyunsaturated fatty acids (such as linoleic acid<sup>21</sup> and elaidic acid<sup>87</sup>), squalene derivatives<sup>31,60,106,107</sup> and isoprenic acids<sup>32</sup>, which, once self-assembled into nanoparticles, demonstrate antitumour efficacy in models of pancreatic cancer<sup>21</sup>, leukaemia<sup>33,106,107</sup>, osteosarcoma<sup>108</sup> and glioblastoma<sup>109</sup>.

Of these lipids, the conjugation of gemcitabine to squalene derivatives is the most widely investigated, and its 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>33,59,106,107,109</sup> (Table 2). The most

advanced trial is of the gemcitabine–elaidate prodrug (CP-4126), which has entered clinical trials for the treatment of pancreatic adenocarcinoma (NCT01124786). However, it is questionable why elaidate was used instead of oleate despite its unfavourable metabolic features and potent stress-induction of trans fatty acids compared to oleate<sup>110</sup>.

Paclitaxel has also been conjugated to lipid–prodrug derivatives to form self-assembled nanosystems, albeit investigated to a lesser extent than gemcitabine<sup>65,104</sup>. For example, a lipophilic conjugate of paclitaxel with docosahexaenoic acid shows higher binding to total plasma proteins compared to free paclitaxel and improved antitumour activity in a mouse lung cancer model (M109 model)<sup>111</sup>. In another study, paclitaxel conjugated to tris-nor-squalenic acid resulted in a bioconjugate that self-assembled into nanostructures in an aqueous solution, demonstrating inferior antitumour efficacy but lower subacute toxicity than the parent drug in a human lung carcinoma xenograft model<sup>65</sup>. Similarly, linoleoyl paclitaxel conjugates built as nanoparticles display higher antitumour activity compared to free paclitaxel in MDA-MB-231, B16-F10 and U87-MG tumour cells<sup>104</sup>. Notably, the lipid–prodrug strategy has also been applied for cytarabine by conjugating it to elaidic acid<sup>112</sup> or tris-nor-squalenic acid<sup>113,114</sup>, with promising results against haematological malignancies. Other lipid–prodrug nanoassemblies have focused on overcoming doxorubicin cardiotoxicity<sup>49,58,64,105</sup>, for example, squalenoyl doxorubicin nanoassemblies with a ‘loop–train’ structure exhibit improved antitumour activity against the murine pulmonary (M109) and human pancreatic (MiaPaca) cancers with dramatic reduction in cardiac toxicity, compared to free doxorubicin<sup>64</sup>. Similarly, the tocopherol succinate derivative of doxorubicin nanoparticles with a highly ordered lamellar inner structure demonstrate higher antitumour efficacy than free doxorubicin in a mouse colon cancer model (CT26-tumour-bearing mice)<sup>58</sup>.

Another interesting strategy is to develop functionalized nanoparticles for targeted drug delivery to cancer cells. For example, conjugating the peptide CKAAN to squalenoyl–gemcitabine nanoparticles selectively targets pancreatic cancer cells and angiogenic vessels in tumour-bearing RIP-Tag2 mice<sup>115</sup>. Peptide-functionalized nanoparticles are more effective at reducing tumour size than non-functionalized nanoparticles and the free drug (40% and 60% respectively). Mechanistically, the therapeutic benefit observed with CKAAN-functionalized nanoparticles results from its dual activity against both cancer cells and tumour vasculature. Similarly, intravenous administration of self-assembled linoleoyl gemcitabine nanoparticles decorated with a pancreatic ductal adenocarcinoma (PDAC)-specific peptide and PEG2000 inhibits tumour progression with lower toxicity than the free

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**Table 1 | Pharmacological application of self-assembled lipid–prodrug nanoparticles**

Drug	Lipid	Functionalization / composition	In vitro/in vivo model	Therapeutic outcomes	Refs.
<b>Cancer</b>					
<b>Gemcitabine</b>					
Gemcitabine	LA	Plectin-1 receptor expressed by human PDAC / Gemcitabine-LA, DSPE-PEG <sub>2000</sub> , DSPE-PEG <sub>2000</sub> -plectin-1 targeting peptide	Cytotoxicity against PDAC cells; Efficacy in PDAC cell-derived xenograft models, one of which is patient-derived	PDAC-targeting peptide increases the intracellular uptake and its cytotoxic activity; Inhibition of tumour progression and alleviation of systemic toxicity	21
Gemcitabine	SQ	No / Gemcitabine-SQ self-assembling	Cytotoxicity on RNU-16 LGL leukaemia cells; Absorption and biodistribution studies after oral administration in rats	Greater accumulation and retention of nanoassemblies in RNU-16-LGL cells; More potent after oral administration owing to resistance to deamination, improved pharmacokinetics and increased accumulation in lymphatic organs	129
Gemcitabine	SQ	No / Gemcitabine-SQ self-assembling	Proliferative assays using a commercial (U2-OS) and patient-treated derived (531) osteosarcoma cells	Half-life enhancement through squalenoylation and improved nanoparticle bone retention	108
Gemcitabine and edelfosine	SQ	No / Gemcitabine-SQ and edelfosine-SQ self-assembling	Antiproliferative effect on c-Fos overexpressing P1.15 and primary osteosarcoma cells (p53 <sup>+/−</sup> ); Pharmacokinetic, tolerability, antitumour efficacy and safety profile in an orthotopic osteosarcoma tumour model (P1.15 osteosarcoma cells)	Multidrug nanoassemblies display stronger anticancer activity in P1.15 cells than in p53 <sup>+/−</sup> osteosarcoma cells in vitro; Improved tolerability and lower toxicity profile in mice; Multidrug nanoassemblies reduce primary tumour growth kinetics and the number of lung metastases	59,123
<b>Paclitaxel</b>					
Paclitaxel	Citronellol	PEGylated / Paclitaxel-citronellol, DSPE-PEG <sub>2000</sub>	Cytotoxicity in human oral epidermoid carcinoma cells (KB), human pulmonary carcinoma cells (A549) and mouse breast cancer cells (4T1); Pharmacokinetics and biodistribution; Antitumour efficacy in KB-tumour-bearing nude mice	Redox dual-responsive drug release; The position of disulfide bonds in the carbon chain linkage influences the redox dual responsiveness and antitumour efficiency α-paclitaxel-SScitronellol nanoparticles are the most effective	51
Paclitaxel	OA	PEGylated and light activatable / Paclitaxel-OA, DSPE-PEG <sub>2000</sub> , PPa-PEG	Synergistic cytotoxicity on human epidermoid carcinoma cell line (KB), non-small-cell lung cancer cell line (A549) and mouse breast cancer cell line (4T1) pharmacokinetics; Synergistic cytotoxicity on KB-tumour-bearing nude mice	Synergistic antitumour efficacy of chemotherapy and PDT in vitro; Synergistic chemo-photodynamic therapy upon laser irradiation in vivo	24
<b>Doxorubicin</b>					
Doxorubicin	SQ	No / Doxorubicin-SQ self-assembling	Intracellular distribution in murine lung carcinoma M109 and human breast carcinoma MDAMB-231 cells	Intracellular distribution of SQ-DOX higher than free doxorubicin; Nanoparticles localized in both cell cytoplasm and nucleus	105
<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 VCaP tumour model in mice; Biodistribution	Nanoformulations decrease the oncogene and oncoprotein expression in vitro; Two nanoformulations showed anti-neoplastic activity in vivo	47
<b>Porphyrin</b>					
Porphyrin	FA	FOLR1 / Porphyrin-FA (pyropheophorbide-lipid), cholesterol, PEG2000-DSPE, folate-PEG2000-DSPE	Cellular uptake and fluorescence activation in lung cancer cells (FOLR1-positive A549, H647, H460 and SBC5 used as the positive cell lines, FOLR1-negative DFC1024 cell was the negative control); Mouse lung orthotopic tumour models	Enhanced therapeutic efficacy in vitro; Nanoparticles preferentially accumulate in tumours; Folate-porphysomes-mediated PDT inhibit tumour cell proliferation and activates tumour cell apoptosis	117

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**Table 1 (continued) | Pharmacological application of self-assembled lipid–prodrug nanoparticles**

Drug	Lipid	Functionalization / composition	In vitro/in vivo model	Therapeutic outcomes	Refs.
<b>Porphyrin (continued)</b>					
Porphyrin	FA	FOLR1 / Porphyrin-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 established in Nu/nu mice	Efficacy of FP-enabled PDT for the treatment of FOLR1-positive MPM in preclinical in vitro and in vivo models; Combined therapeutic effect of pre-treatment with EGFR-TKI	118
Porphyrin	Porphyrin-HDL	SR-BI / Porphyrin-HDL, DMPC, cholestryloleate, 18-amino acid apolipoprotein A-I mimetic peptide	Human large-cell lung carcinomas NCI-H460 and H460SM; Antitumour 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 tumour; Selective accumulation and photoactivation in the tumour after systemic administration	119
Porphyrin	OA	LDLR / Porphyrin-OA, DMPC, cholestryloleate, ApoE3	Human U87 glioblastoma and Chinese hamster ovary ldlA7 cells, with different 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 ldlA7 cells; Cell-specific strong PDT sensitization in vitro; Selective uptake of porphyrin by malignant tissue and detection of tumour-localized porphyrin fluorescence	126
Porphyrin	Oleylamine	SR-BI / Porphyrin-oleylamide (porphyrin-HDL-like), DMPC, cholestryloleate	Fluorescence microscopy and in vitro PDT using CHO, LDL(msR-BI), LDLA-7, human KB, HT1080 and PC-3M-luc-C6 cells; In vivo fluorescence and photoacoustic imaging, PDT in ldl(msR-BI and ldlA-7 cell tumour xenografts of mice	Porphyrin moiety as an oleylamine conjugate results in stable J-aggregate with strong photoacoustic contrast, whereas incorporation as an amphiphilic lipid moiety into the lipid shell results in an effective fluorescent and photodynamic agent	127
<b>Others</b>					
Cisplatin and SN38	LA	PEGylated / Cisplatin-LA, SN38-LA, DSPE-PEG <sub>2000</sub>	Human NSCLC A549 and cisplatin-resistant A549 <sup>cisR</sup> cells; A549 <sup>cisR</sup> cells tumour-bearing mice; Toxicity assessment in mice	Preferred synergism in resistant cancer cells; Reduction of tumour growth and tumour inhibition rate Low systemic toxicity	121
SN38 and protoporphyrin IX	Thioether-modified FA and stearyl alcohol	No / SN38-stearylalcohol, porphyrin-modified FA self-assembling	Cytotoxicity on CT26 colonic cancer cells	Upon laser irradiation, oxidization of the multiple thioethers by photosensitizers generate singlet oxygen that can rapidly destroy the nanoparticles structure, resulting in faster drug release and higher cytotoxicity	52
Cabazitaxel, photosensitizer chlorine e6	αLA	Photoactivatable (laser inducible) / Cabazitaxel-αLA, chlorine e6-αLA self-assembling	In vitro 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 in both mouse models of melanoma after treatment with nanoassemblies followed by laser irradiation	53
Cisplatin	Squalenamine	No / Cisplatin-SQ self-assembling	Toxicity and efficacy in a panel of human cancerous epithelial cell lines (HT-29, Colo 320HRS, Caco-2, SW620, LoVo colon cells, HeLa cervix and PC-3 prostate cell lines); Mouse models of colorectal cancer (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	128
Docetaxel	Triglyceride	No / Docetaxel-triglyceride self-assembling	Cytotoxicity and drug release in 4T1 cells, KB cells, and LO2 cells Pharmacokinetic and biodistribution; Antitumour effect in 4T1-tumour-bearing mice	High accumulation in tumours owing to improved oral absorption; Excellent tumour suppression effect with lower gastrointestinal toxicity compared with oral and intravenous docetaxel solution	130

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**Table 1 (continued) | Pharmacological application of self-assembled lipid–prodrug nanoparticles**

Drug	Lipid	Functionalization / composition	In vitro/in vivo model	Therapeutic outcomes	Refs.
<b>Pain</b>					
Leu-enkephalin	SQ	No / Leu-enkephalin-SQ self-assembling	Carrageenan-induced pain model using a thermal nociception test (Hargreaves) to assess hyperalgesia; Biodistribution of nanoparticles investigated in mice and toxicological study in rats	Antihyperalgesic effect; Nanoparticles act through peripherally located opioid receptors; Nanoparticles target the location where inflammation and nociception occur	28
<b>Infectious diseases</b>					
Cabotegravir	MA	No / Cabotegravir-MA, Poloxamer P407	Monocyte-derived macrophages from HIV-1/2 and hepatitis B seronegative donor blood cells; Pharmacokinetics in mice and monkey; Biodistribution in mice (intramuscular administration); Viral restriction in humanized adult lymphocyte mice	Enhanced antiretroviral activity in vitro Improved biodistribution and viral clearance in vivo 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 the area under the concentration-time curve for monkeys	27,145
Darunavir	FA	No / Darunavir-FA, Poloxamer P407	Cytotoxicity, 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 with sustained antiretroviral activities	146
Lamivudine	Docosanol (ProTide Technology)	No / Lamivudine–docosanol, 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 Sustained prodrug and drug triphosphate levels in blood and tissues for 30 days	147
Abacavir	Docol (ProTide Technology)	No / Abacavir–docosanol, PLGA or poloxamer	Antiretroviral activity in human monocyte-derived macrophages	Sustained intracellular carbovir-triphosphate and antiretroviral activity for up to 30 days	148
Dolutegravir	MA	No / Dolutegravir-MA, poloxamer	Antiretroviral activity in HIV infected macrophages; Protection against HIV-1 challenge in CD34 <sup>+</sup> humanized mice	Improved antiretroviral activity up to 30-fold pharmacokinetics and pharmacodynamics substantially better than 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	Palmitic acid	No / Dexamethasone-palmitic acid, DSPE-PEG <sub>2000</sub>	Raw 264.7 macrophages; Pharmacokinetics in healthy mice	Anti-inflammatory effects in macrophages activated with LPS	62
Adenosine Vit E	Squalenyl acetic acid	No / Adenosine-SQ, Vit E	Oxidative insult in H9c2 cardiomyocytes and LPS-induced inflammation model in Raw 264.7 macrophages; Biodistribution in two different models of local acute inflammation and systemic inflammation; Efficacy in endotoxaemia; LPS model in mice	Efficient control of inflammation and enhanced survival in models of endotoxaemia	97
<b>Others</b>					
Camptothecin and resiquimod (R848)	OA	No / Camptothecin-OA, R848-OA, DOTAP	Cellular uptake and apoptosis in CT26 cells; Drug release in tumour cells, multicellular spheroids and tumours; In vivo antitumour efficacy in CT26 tumour-bearing BALB/C mice	Accelerated intracellular drug release owing to increased redox-responsiveness; Higher antitumour activity and better at stimulating antitumour immune responses at the tumour site	133

The data in this table are from the past 5 years. LPS, lipopolysaccharide; PDAC, pancreatic ductal adenocarcinoma; PDT, photodynamic therapy; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOTAP, 1,2-dioleyl-3-trimethylammonium-propane; DSPE-PEG<sub>2000</sub>, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)2000]; SN38, 7-ethyl-10-hydroxy-camptothecin; PLGA, poly lactic-co-glycolic acid; FOLR1, folate receptor 1; PPa, pyropheophorbide a; SR-BI, scavenger receptor class B type I; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; GFP, green fluorescent protein; FA, fatty acid; LA, linoleic acid; αLA, alpha linolenic acid; OA, oleic acid; SQ, tris-nor-squalenic acid; MA, myristic acid.

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**Table 2 | Lipid–drug conjugates in clinical trials as anticancer agents**

Indication	Type of study	Status	Clinical trial identifier	Outcome
<b>Gemcitabine-5'-elaídate (CO-1.01)</b>				
Advanced solid tumour	Phase I	Completed	NCT01392976	N/A
Metastatic pancreatic adenocarcinoma	Phase II	Completed	NCT01233375	N/A
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 analysed <sup>137</sup>
Solid tumour, non-small-cell lung cancer, lung cancer	Phase I	Terminated	NCT01641575	N/A
<b>DHA-paclitaxel (Taxopresin)</b>				
Non-small-cell lung cancer	Phase III	Completed	NCT00243867	N/A
Cancer of the liver	Phase II	Terminated	NCT00422877	N/A
Metastatic melanoma	Phase II	Completed	NCT00249262	N/A
Metastatic melanoma	Phase II	Completed	NCT00244816	N/A
Pancreatic cancer	Phase II	Unknown	NCT00024375	N/A
Prostate cancer	Phase II	Unknown	NCT00024414	N/A
Colorectal cancer	Phase II	Unknown	NCT00024401	N/A
Kidney cancer	Phase II	Unknown	NCT00024388	N/A
Malignant melanoma	Phase III	Completed	NCT00087776	DHA-paclitaxel was not superior to the gold standard dacarbazine <sup>138</sup>
<b>Elacytarabine (CP 4055)</b>				
Interventional (healthy volunteers)	Phase I	Completed	NCT01783964	N/A
Relapsed/refractory acute myeloid leukaemia	Phase I	Completed	NCT01258816	N/A
Acute myeloid leukaemia	Phase II	Completed	NCT00405743	Remission rate and survival improved
Acute myeloid leukaemia	Phase III	Completed	NCT01147939	No significant differences in overall survival between elacytarabine or any of the seven alternative treatments investigated <sup>136</sup>
Haematologic malignancies, acute myeloid leukaemia	Phase I/II	Completed	NCT00405743	N/A
Advanced colorectal cancer, colorectal cancer	Phase II	Completed	NCT00498407	N/A
Ovarian cancer	Phase I/II	Completed	NCT00831636	N/A
Malignant melanoma	Phase II	Completed	NCT00498836	N/A
Malignant melanoma, neoplasm metastasis	Phase II	Completed	NCT00232726	N/A

N/A, no results available; DHA, docosahexaenoic acid.

drug control in two xenograft models (L3.6pl and a patient-derived xenograft model)<sup>21</sup>.

Folate-receptor-1-targeted porphysomes have also been used as photosensitizers for photodynamic therapy to selectively kill tumour cells in preclinical models of epidermoid carcinoma (KB xenograft) and lung cancer (AE17-sOVA tumour-bearing mice and A549 subcutaneous tumour-bearing mice)<sup>116–118</sup>. Following the same rationale, porphyrin–HDL particles targeted at the scavenger receptor class B type I expressed in lung cancer accumulate in the tumour and are photoactivated after systemic administration in mice with H460 orthotopic lung cancer<sup>119</sup>. Similarly, squalenoyl–gemcitabine nanoparticles decorated with squalenyl-hydroxybisphosphonate as the bone-mineral-targeting moiety have been designed to treat osteosarcoma *in vitro*<sup>108</sup>.

Of note, decorating nanoparticles with specific ligands could affect the assembly and morphology of the resulting nanoparticles. The size and zeta-potential depend on the nature of the ligand and whether functionalization has been performed through PEG chains. In general, the surface molecular footprint results in a slight increase in the size of the nanoparticles, whereas PEG contributes to surface charge reduction.

Two distinct ligation strategies can be used for functionalizing self-assembled lipid–prodrug nanoparticles: the ligand can either be directly coupled to the surface of preformed nanoparticles or linked to the lipid prior to nanoparticle construction. Surface plasmon resonance analysis of CKAAN-functionalized nanoparticles revealed that peptide conjugation prior to nanocarrier formation

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is the most efficient method of enhancing binding and avidity for the sFRP-4 receptor<sup>120</sup>. However, the literature lacks direct comparisons between the supramolecular structures resulting from functionalized and non-functionalized lipid–prodrug nanoparticles. One example of squalene–gemcitabine nanoparticles functionalized by squalenyl-hydroxybisphosphonate revealed remodelling of the initial hexagonal structure into a helicoidal and multilamellar onion-type self-assemblage, which was electrostatically driven by the negatively charged phosphate groups of hydroxybisphosphonate<sup>108</sup>.

To overcome cancer drug resistance, synergistic anticancer therapy could be achieved through simultaneous delivery of two drugs integrated into the same self-assembling nano-cocktail. For example, integrating two synergistic lipid–prodrugs, namely cisplatin and 7-ethyl-10-hydroxy-camptothecin conjugated with linoleic acid, has higher antitumour activity than the free drugs in a A549 cisplatin-resistant tumour-bearing mouse model of lung cancer<sup>121</sup>. Similarly, the lipophilic anti-angiogenic drug iso-combretastatin combined with squalenoyl–gemcitabine is more effective than the free drugs in a mouse model of colorectal cancer<sup>122</sup>. Another multitherapy nanomedicine against *in vitro* and *in vivo* paediatric osteosarcoma combines squalenoyl–gemcitabine and alkyl-lysophospholipid edelfosine, which have complementary molecular targets, into the same nanoparticles<sup>59,123</sup>.

Nanotheranostic systems based on lipid–prodrug nanoparticles can also facilitate the combination of therapeutic and imaging properties. For example, combining magnetite nanocrystals with nanoparticles of squalenoyl–gemcitabine allows the targeted delivery of gemcitabine to mice with subcutaneously implanted solid L1210 tumours, when the tumour nodule is under the influence of a magnetic field. In addition, the magnetic nanocrystals permit imaging of the targeted cancer nodule<sup>124</sup>. Similarly, the T1Gad3 contrast agent can be incorporated in squalene-based nanoparticles of doxorubicin or cisplatin<sup>125</sup>. ApoE3 porphyrin–lipid nanoparticles have also been used for theranostic purposes<sup>126</sup>; when injected intravenously, these nanoparticles accumulate at the tumour site of a mouse orthotopic model of glioblastoma. However, the theranostic utility of this nano-construction was only demonstrated *in vitro* after photodynamic therapy in U87 and ID1A7 glioblastoma cells. Following the same rationale, tunable phototheranostic agents have been developed by incorporating porphyrin into biomimetic lipoproteins in an orthotopic prostate cancer mouse model<sup>127</sup>. The hydrophobic porphyrin–oleylamide conjugates allowed photoacoustic imaging, whereas the amphiphilic porphyrin–lipid conjugates enabled fluorescence imaging and photodynamic activity.

Lipid–prodrug nanoparticles can also be tuned for oral administration; for example, squalene–cisplatin or squalenoyl–gemcitabine nanoparticles display higher antitumour efficacy than their parent drugs in mouse models of intestinal carcinogenesis (*Apc*<sup>Min/+</sup> mice and azomethane induced) and leukaemia-bearing rats<sup>33,128,129</sup>. These systems are attractive because squalene is a lipid which is particularly well absorbed orally. Other oral lipid–prodrugs have been designed using lipophilic prodrugs of docetaxel<sup>130</sup>, SN38<sup>131</sup> and cytarabine<sup>132</sup>. However, nanoparticle formation was not reported for these conjugates, although spontaneous self-assembly into nanosized supramolecular structures is expected to occur owing to their physico-chemical properties.

Another focus area is to develop *in situ* antitumour vaccines by combining antitumour drugs and Toll-like receptor agonists (for example imiquimod, resiquimod (R848) and CpG). The cytotoxic drug triggers 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 into drug carriers to enhance drug retention in the tumour tissue; however, co-encapsulation of two drugs with different physicochemical properties within the same nanocarrier is difficult. To address this issue, a cationic surface-sensitized prodrug nanoaggregate was synthesized by co-assembling cationic lipids (DOTAP) with two prodrugs consisting of camptothecin and resiquimod linked to oleic acid through a S,S-(bisethoxy) carbonyldisulfide linker. This multidrug nanomedicine triggers a strong anticancer effect in CT26 colorectal tumour-bearing BALB/C mice by accelerating the glutathione-responsive drug release at the tumour site<sup>133</sup>.

Inspired by the observation that compounds that bind avidly to serum albumin can target the lymph node<sup>134</sup>, anticancer amphiphile vaccines containing an antigen linked to a lipophilic albumin-binding tail have been developed<sup>135</sup>. These nanomicelles composed of CpG–DNA/peptide amphiphile vaccines exhibit strong lymph-node targeting and accumulation compared to their parent compounds, resulting in a substantial increase in T cell priming and enhanced antitumour efficacy on TC-1 tumours (expressing the E7 oncoprotein from HPV) and B16F10 melanomas<sup>135</sup>.

**Clinical trials.** Despite promising preclinical results, clinical translation of prodrug-nanoparticles for oncological applications remains limited (Table 2). In a phase II clinical trial (NCT00405743), cytarabine-elaideate nanoparticles have shown a remission rate of 18% and 5.3 months survival in acute myeloid leukaemia patients versus 4% and 1.5 months in controls. However, no substantial difference in response rate and relapse-free survival was observed at phase III (NCT01147939)<sup>136</sup>. Similarly, the gemcitabine–elaideate prodrug had the same overall response and toxicity profile as free gemcitabine in a phase II clinical trial (NCT01124786) of patients with pancreatic adenocarcinoma<sup>137</sup>. Moreover, and in contrast to its excellent preclinical anti-cancer activity, DHA–paclitaxel prodrug nanoparticles did not show any overall survival difference in a phase III trial (NCT00087776) for melanoma compared with the standard treatment (dacarbazine)<sup>138</sup>.

This lack of success might be explained, in part, by the fact that experimental animal models in oncology are not sufficiently predictive of human diseases. Preclinical experiments often use subcutaneously grafted tumours with tumour and animals having the same genetic background to ensure reproducibility, an assumption which does not hold for humans. Transgenic mouse models are useful, but are specific to rodents, and animal recipients of xenografted tumours from human origin are immunocompromised. Moreover, in preclinical models, the size of the tumoral nodule is much smaller than in humans; this is a major difference, because prodrug nanomedicines, to be efficient and effective, need to diffuse deep into the tumour tissue. Furthermore, unlike in experimental models, human cancers are often difficult to treat because they are poorly perfused and characterized by the tumour microenvironment which hinders drug diffusion and perfusion, pancreatic cancer being a typical example<sup>139</sup>. The EPR effect is generally invoked as a mechanism used by nanoparticles (whether prodrug-based or not) to reach the tumour, but is neither well characterized for all types of nanoparticle, nor for the different classes of human cancer. Moreover, most preclinical models do not induce metastasis; therefore, developing more reliable pharmacological models is urgently needed. Finally, clinical trials also require a more rigorous selection of patients who have a better chance of responding to the treatment; for example, performing preliminary imaging with ultrasmall iron oxide particles or

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nanotheranostics could identify patients with nanoparticle-diffusible tumours (such as those characterized by an EPR effect).

## Neurological disorders and pain

Self-assembled nanomedicines have also been developed for the treatment of neurological disorders and pain (Table 1). Since the pioneering work of Jacob et al. in 1990 (ref. 140) showing that linking  $\gamma$ -aminobutyric acid (GABA) and 7-vinyl-GABA to linolenoylglycerol promotes delivery to the brain, resulting in depression of the spontaneous locomotor activity of mice, several other molecules such as L-3,4-dihydroxyphenylalanine (L-DOPA)<sup>141</sup> or phenytoin<sup>142</sup> have been formulated as pseudoglycerides to increase diffusion to the central nervous system. However, it is unclear whether these prodrugs assemble as nanoparticles; moreover, if lipophilicity increases drug transport through the blood–brain barrier, it might also trigger unexpected toxicity owing to either the lipid–prodrugs being metabolized by pathways other than single conversion to the parent molecule, or because of the limited ability of the brain tissue to metabolize and eliminate nanoparticles.

Therefore, designing prodrug nanomedicines that can act on peripheral rather than on central receptor targets has been investigated. For example, binding adenosine to squalene derivatives to form nanoassemblies triggers strong neuroprotective activity in preclinical models of brain ischaemia and spinal cord injury in rodents<sup>29</sup>. When released from nanoparticles, adenosine interacts with the adenosine receptors located at the surface of brain microvessels, decreasing thrombo-embolic events and inducing a neuroprotective effect on the cells of the neurovascular unit. These effects are not observed with free adenosine, which is quickly metabolized with a half-life of 10 seconds. Similarly, Leu-enkephalin, an endogenous opioid neuropeptide, has been conjugated to squalene derivatives using various chemical spacers, to design nanoparticles for pain alleviation<sup>28</sup>. Unlike morphine, these nanoparticles spared the brain tissue, acting selectively on peripheral opioid receptors, thus avoiding the detrimental central nervous system effects responsible for addiction and tolerance in a rat model of inflammatory pain.

Recently, intrathecal and intracerebroventricular injection of siRNAs conjugated to the 2'-O-hexadecyl moiety revealed long-lasting (at least three months) pharmacological activity in central nervous system regions. Conjugating an amyloid precursor protein-targeting siRNA to a hexadecyl moiety improved physiological and behavioural deficits in a *Tg-hAPPswDI/mNos2<sup>-/-</sup>* transgenic mouse model of Alzheimer's disease. Similarly, intravtrial administration of siRNA-hexadecyl towards transthyretin or intranasal administration of Sod1 mRNA towards superoxide dismutase 1 to the lung results in potent and durable gene silencing, which opens up exciting perspectives for siRNA delivery into extrahepatic tissues<sup>143</sup>. In another study, conjugating squalene to Pmp22-targeting siRNA, a protein involved in Charcot–Marie–Tooth disease type 1A, revealed Pmp22 levels normalization, allowing remyelination and regeneration of the axons with substantial restoration of motor and neuromuscular activities in two mouse models of Charcot–Marie–Tooth disease type 1A<sup>144</sup>.

## Infectious diseases

Lipid–prodrug nanoassemblies of antiviral compounds have also been used to enhance antiviral potency by slowing conversion to the parent molecule, resulting in prolonged effective plasma concentration (Table 1). Moreover, because antiretroviral drugs are generally administered at high doses, their direct encapsulation into nanocarriers

raises issues regarding dose volume. Therefore, the higher drug loading obtained with lipid–prodrug nanoparticles represents an attractive option. For example, intramuscular injection of rod-shaped nanoformulations of myristoylated cabotegravir<sup>27,145</sup>, an anti-HIV compound, in either mice or rhesus macaques, increases plasma concentration 4-fold compared to the free drug, including deposition in secondary tissue and immune cells. Long-lasting effects have also been obtained using other antiretrovirals formulated as lipid–prodrugs, such as darunavir<sup>146</sup>, lamivudine<sup>147</sup>, abacavir<sup>148</sup> and dolutegravir<sup>26</sup>. For example, intramuscular administration of nanoparticles made of dolutegravir conjugated to myristic acid and stabilized by small amounts of poloxamer 407, attenuate viral replication and spread to infected CD4<sup>+</sup> T cells in mice<sup>26</sup>. Moreover, dolutegravir blood levels remained at viral inhibitory concentration for 56 days, whereas drug concentration in tissues (that is, liver, spleen, gut-associated lymphoid tissue, lymph nodes, lungs and kidneys) lasted for 28 days, which protected HIV-infected humanized mice for two weeks. Similarly, orally administered squalenoylated prodrugs of 2'-3'-dideoxyinosine (ddI), a nucleoside reverse transcriptase inhibitor, act as a sort of 'drug reservoir' while circulating in the blood of mice, with high levels of squalene–ddI but low amounts of free ddI detected in cells and tissues relevant for HIV infection (that is, liver, spleen, bone marrow, thymus and brain)<sup>30</sup>. A major benefit of these formulations is their ability to be administered orally, which could facilitate HIV patients' adherence to drug regimens.

Lipid–prodrug nanoparticles have also been designed to kill intracellular bacteria that can resist the hostile environment of endolysosomes, survive and multiply. Various terpenes, including geranyl, farnesy<sup>140</sup> and squalene derivatives<sup>39</sup> linked to penicillin through an acyl environmentally sensitive bond reduce intracellular replication of *Staphylococcus aureus* in vitro in Raw 264.7 and J774 macrophages. Further in vivo validation is needed, but this approach could potentially be extended to other antimicrobial agents.

## Inflammatory diseases

Lipid–prodrug nanoparticles could also help to treat inflammatory diseases; for example, the glucocorticoid dexamethasone has been linked to palmitate and then formulated into a nanoparticle. Of note, these lipid–prodrugs do not self-assemble into nanoparticles spontaneously but need to be stabilized using distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (DSPE–PEG<sub>2000</sub>)<sup>62</sup>. Stabilization solves two of the main problems of glucocorticoid nanoencapsulation, namely poor drug loading (entrapment efficiency was improved to 98% w/w) and drug crystallization (which destabilizes the nanoparticles). Interestingly, intravenous administration of these nanoparticles in healthy mice substantially improved the drug pharmacokinetic profile because the release of dexamethasone was controlled for up to 18 h, whereas freely injected dexamethasone was rapidly eliminated from the blood stream. Moreover, intravenous administration of multidrug nanoparticles of squalene conjugated to adenosine as the anti-inflammatory agent and tocopherol as the antioxidant resolved out-of-control inflammation in mice suffering from endotoxaemia or lethal systemic shock<sup>97</sup>.

## Outlook

Enzymatic activation of a non-pharmacologically active molecule to enable the release of an active compound within diseased cells or tissues, as happens in prodrugs, is an old concept. However, whether the synthesized prodrugs freely self-assemble into nanostructures had never been considered in the original studies<sup>140,141</sup>. In this Review, we show that systematically and selectively combining drugs and lipid

carriers may allow for spontaneous supramolecular formation of nanostructured prodrugs with promising preclinical pharmacological activity. However, thus far, oncological clinical trials using lipid–prodrug nanoassemblies have not been satisfactory, prompting further efforts towards clinical translation.

First, chemical synthesis procedures must avoid the use of toxic reagents by minimizing the presence of organic and inorganic impurities and prevent the formation of harmful byproducts. For example, the oxidation of tris-nor-squalenaldehyde into tris-nor-squalenic acid for the bioconjugation of biologically active molecules makes use of chromium trioxide, a toxic compound not accepted by regulatory agencies. Searching for safer synthetic and ‘green’ reactions is becoming more important. The fine-tuning of the supramolecular structure of the prodrug nanoparticle is another matter for concern. Indeed, because activation of the prodrug is necessary to trigger pharmacological activity, the ability of the enzymes to diffuse into the core of the particles must be ensured, a requirement which is often underestimated. Therefore, the structure of the particles should be determined using advanced techniques such as X-ray diffraction, cryo-transmission electron microscopy or even neutron scattering. Improved drug release can also be obtained through smart design of the linker by making it stimuli-responsive or more hydrophilic for efficient and specific enzymatic cleavage.

Another important consideration when selecting the most suitable formulations is the discrepancy between *in vivo* experimental models and clinical reality, especially for oncological applications. Because drug release from lipid–prodrug nanoparticles is often governed by enzymatic activity, which is typically higher in smaller than in larger animals, a comparison of *in vitro* metabolism across species (for mouse, rat, dog, monkey and human using plasma, hepatocytes and/or liver microsomes) is a key metric that is lacking in many studies<sup>149</sup>, and that may be contributing to the clinical trial failures of these systems. The same applies for the interaction of lipid–prodrugs with blood components (such as endogenous enzymes, proteins and lipids in different species), which might influence the pharmacokinetic and pharmacodynamic profiles of the compound. There is also an urgent need to develop more relevant animal models and personalized tumour 3D cultures from patient biopsies to better understand lipid–prodrug diffusion in the tumour tissue. For clinical trials, patients and dose regimens should be chosen according to the patient’s genetic background and tumour characteristics. Moreover, lipid–prodrug nanoassemblies allow for multidrug nanoparticle construction, a feature which could prove useful for combination therapies, as has been shown for the treatment of uncontrolled paradoxical inflammation related to cytokine storm<sup>97</sup>. Finally, although most prodrug nanoparticles are administered intravenously, other routes of administration should also be explored (intramuscular, subcutaneous, oral) and some local applications could also be considered.

In addition to therapeutic applications, disease prevention (through vaccination, for example) is also a promising research area. Despite the lack of a pre-clinical or clinical study of vaccines based on self-assembled lipid bioconjugates, the use of ionizable lipids for mRNA delivery into cells during the COVID19 pandemic has revolutionized the field. In particular, the chemical linkage of nucleic acids to lipids has laid the groundwork for the development of future biomolecules that, when coupled with ionizable lipids, could boost RNA export from cell endosomes to the cytoplasm after cellular internalization, eventually improving vaccine response or *in vivo* production of various therapeutic proteins.

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## Author contributions

P.C. organized the writing of the Review, provided the first drafts of the text and the boxes, drew some figures, discussed with the co-authors and answered the reviewer and editor queries. S.L.-M. collected the data and wrote the lipid–prodrug chemical synthesis and drew the figures with the chemical structures and was involved in replying to the reviewers. E.G. and M.J.B.-P. collected data and wrote parts of the Review, drew some figures and tables, and were involved in replying to the reviewers.

## Competing interests

S.L.-M. and P.C. have non-financial competing interests as inventors of patents related to squalene-based nanoparticles; M.J.B.-P. and E.G. declare no competing interests.

## Additional information

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