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Glycosidase activated prodrugs for targeted cancer therapy

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In this review glycosidase activated prodrugs that target cancer cells are discussed. Glycosylated prodrugs undergo enzymatic bioconversion, cleaving the prodrug to release the anticancer drug at the desired site of action, hence minimising the toxic side effects associated with many current anticancer drugs. In addition, the presence of the carbohydrate moiety increases the aqueous solubility of the drugs, allowing for a more effective treatment. In the past decade, significant advancements have been made in this field that have led to the development of many novel carbohydrate-based prodrugs – ranging from simple glycoconjugates to complex self-assemblies and materials, which are discussed in detail herein.

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1. Introduction

According to the World Health Organization (WHO), cancer was the second leading cause of death in 2020 worldwide with 19.3 million cases and 10 million deaths.¹ The National Institute for Health (NIH) has anticipated that this number will increase up to 29.5 million and 16.4 million cases and deaths, respectively, by 2040.² Many current therapeutic strategies,

including the drugs used in chemotherapy, usually target cells that are proliferating. Therefore, they are limited in their specificity, targeting not only tumour cells but healthy cells as well. Furthermore, most of the administered drugs remain circulating in the bloodstream, increasing potential side effect on healthy tissue.³ In addition to those drawbacks, current cancer treatments have high systemic toxicity which makes them only applicable in low doses. Hence, there is an urgent need to identify specific cancer-targeting therapies.

Prodrug therapy is one approach to overcome specificity issues involved in chemotherapy and anticancer treatments. Adrien Albert first coined the term ‘prodrug’ or ‘proagent’,⁴ also

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referred to as 'latent drugs', 'bioreversible derivatives', and 'congeners'.^{5,6} This approach has improved cancer drug therapy since the early 1970s.⁷ Usually a prodrug consists of the drug and a chemical moiety that are covalently linked to form the inactive substrate. Upon activation with specific biological media, such as stomach acid as in the case of Aspirin, or in a more targeted way, by an enzyme that carries out a specific biochemical transformation, the active drug is released *in vivo* to exert its therapeutic effect.

Carbohydrates are the most abundant group of macromolecules found in the body, and play pivotal roles in many cellular interactions such as signalling or cell surface receptors.⁸ Due to the rapid proliferation of cancer cells, there exists a high-energy demand. Glucose transporters (GLUTs), which are found to be overexpressed in cancer cells, solve the problem by increasing the uptake of glucose at a higher rate than normal cells, a phenomenon known as the "Warburg Effect".⁹ This effect garnered attention by the science community for the design and development of sugar-based targeted drug delivery.¹⁰ It has also been extensively reported that various glycosidase enzymes are overexpressed in different cancer types (see Table 1). For example, β -glucosidase is upregulated in a number of cancers including breast,¹¹ gastric¹² and liver.¹³ This overexpression may be exploited by using glycosidase activated prodrugs for targeting many different cancers.

The vast majority of carbohydrate-based prodrugs are designed to improve pharmacokinetic properties. They exhibit high solubility in water, low toxicity, and high biocompatibility. It has been shown that several cytotoxic agents *e.g.* glufosfamide, chlorambucil, docetaxel, 3-paclitaxel, *etc.*, (Fig. 1) have been glycosylated and found to be less toxic to noncancer cells than the parental aglycons.³⁵ Tumour-associated carbohydrate antigens (TACAs) are specific targets and therefore considered good biomarkers for cancer detection as well. They are essential for carbohydrate-based cancer vaccines to improve immunological

Table 1 Shows various glycosidase enzymes overexpressed in different cancer types

Glycosidase	Cancer type
β -Glucosidase	Breast, ¹¹ gastric, ¹² liver ¹³
Glucosidase II	Lung, ¹⁴ bladder, ¹⁵ gastric, ¹⁶ melanoma ¹⁷
<i>N</i> -Acetyl- β -D-glucosaminidase	Ovarian, ¹⁸ liver, ¹⁹ leukemia, ¹⁹ thyroid, ²⁰ breast ²¹
α -Glucosidase	Liver, ¹⁹ leukemia ¹⁹
β -Galactosidase	Liver, ¹⁹ ovarian, ²² prostate, ²³ colon, ²⁴ breast, ²⁴ gliomas ²⁵
β -Glucuronidase	Lung, ²⁶ breast, ²⁷ ovarian, ²⁸ pancreatic, ²⁹ colorectal ³⁰
α -Mannosidase	Breast, ³¹ cervical, ³² clear cell renal carcinoma ³³
β -Mannosidase	Liver, ¹⁹ leukemia ¹⁹
α -Fucosidase	Ovarian, ¹⁸ gliomas, ²⁵ colon, ³⁴ pancreatic ³⁴

response.^{36,37} Also, the lysosomal enzyme β -galactosidase is overexpressed in some cancers such as lung and ovarian cancers,^{22,38,39} allowing for the application of galactosidase responsive prodrugs suitable for the treatment of solid tumours. In prodrug monotherapy (PMT), a nontoxic prodrug is administered and cleaved to release the cytotoxic drug upon enzymatic activation, where such enzyme is already overexpressed at the site of action.²⁶ Since in prodrug monotherapy the enzyme is not administered, the selectivity must be achieved through key differences in tumour environments compared to healthy tissue such as the lower pH of cancer tissue, hypoxia or specific enzymes overexpressed in the tumour environment.^{10,40} β -Glucuronidase and β -galactosidase, for instance, are found in high concentration in solid tumours, such as lung, breast or ovarian cancer, which makes it the perfect target for the development of drug carriers.^{26,41} Recent studies have also suggested that mannosidase enzymes are overexpressed in various breast tumors,³¹ cervical cancers³² and clear cell renal carcinoma (ccRCC).³³ Since many glycosidase enzymes are upregulated



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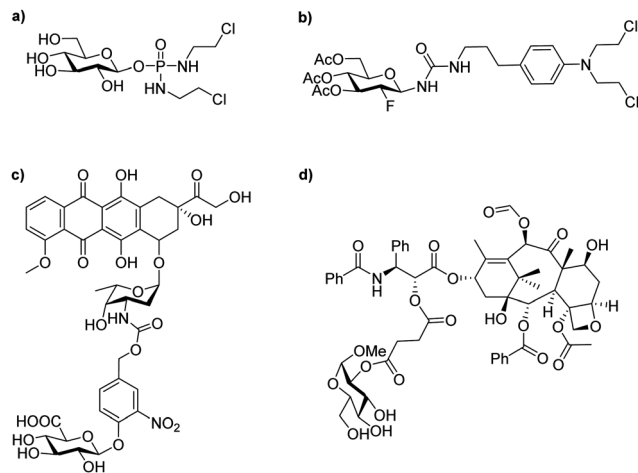


Fig. 1 Examples of typical glycosylated anticancer drugs; (a) glufosfamide (or D-glucose isophosphoramidate mustard); (b) peracetylated 2-fluorodeoxyglucose conjugated chlorambucil; (c) glucuronic acid conjugated doxorubicin; (d) glucose conjugated paclitaxel.

in various cancers, they provide the perfect target to use in glycosidase activated prodrugs for cancer. Extensive research has been reported in the area of sensing glycosidase enzymes intracellularly,⁴² using, for example, fluorescent probes,⁴³ near-IR probes,⁴⁴ and AIE-based glycoclusters.⁴⁵ This allows the identification of glycosidase enzymes that are overexpressed in cancer tissue and hence, allow for the development of prodrugs that would utilise these enzymes for targeted cancer therapy.

In contrast to PMT, directed enzyme prodrug therapies such as ADEPT (antibody-directed enzyme prodrug therapy), GDEPT (Gene-DEPT), MDEPT (Magnetically-DEPT) and VDEPT (Virus-DEPT), all require the administration of an enzyme to the site of action. For example, in ADEPT an enzyme-antibody (Ab) conjugate is administered to the patient. The antibody then binds to the antigen expressed on the surface of the tumour cell

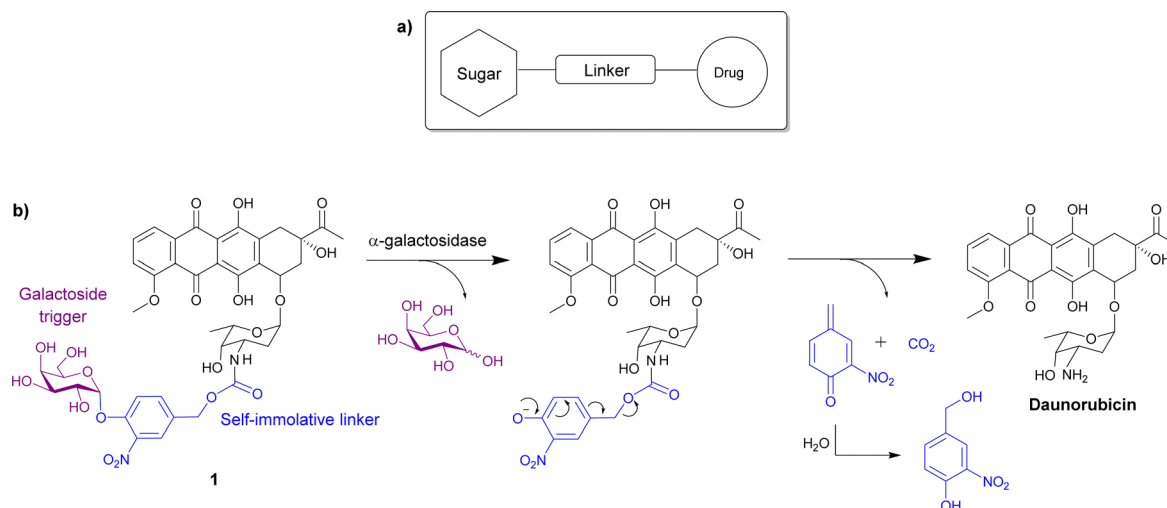
and accumulates in the desired site of action. The second step consists of the injection of a non-toxic prodrug, which will be converted into the active drug once it reaches the tumour cell by a catalyst reaction with the enzyme previously linked to the cell. Thus, the concentration of drug within tumours is higher than that which can be achieved through classical drug therapies.⁴⁶

In this review, some of the most promising recent carbohydrate-based prodrug approaches investigated, such as PMT or DEPT, including ADEPT, GDEPT and VDEPT, will be discussed alongside examples that have been reported in the literature over the past decade. There are many examples of Pt-based cancer prodrugs in the literature, and as these have already been discussed thoroughly in several reviews,^{47–49} the main focus herein will be on featuring “fully organic” prodrugs, that are activated by glycosidase enzymes to exert their anticancer properties. The scope of the review also includes recent developments in supramolecular assemblies and materials as prodrugs.

2. Glycosidase activated prodrugs

2.1 Simple glycoconjugates

Most glycosidase activated prodrugs reported to date consist of a known anticancer drug directly linked *via* a glycosidic bond to a sugar unit, or in most cases a known anticancer drug joined to a sugar unit *via* a linker (Scheme 1a). The linkers are usually self-immolative, meaning they cleave the covalent bond with the active drug after a stimulus.^{50,51} In this case, a glycosidase enzyme will hydrolyse the glycosidic bond, causing the self-immolative linker to collapse, releasing the anticancer drug. Commonly used sugar moieties used include glucose, glucuronic acid and galactose, but other sugars such as rhamnose and xylose have also been utilised. The most popular linkers are based on a phenolic scaffold that upon glycosidic bond hydrolysis, eliminate from the carbamate-substituted benzyl system



Scheme 1 (a) Schematic showing the general structure of glycosidase activated prodrugs; (b) the structure of one of the first daunorubicin prodrugs **1** incorporating an α -galactoside trigger and a self-immolative phenolic linker, and the reaction with the galactosidase that releases the parent drug.



with formation of a reactive methylene quinone moiety along with an unstable carbamic acid which quickly decomposes to give carbon dioxide and the corresponding amine, as shown in the example in Scheme 1b. This system was first used by Monnert, Koch and Gesson who prepared prodrugs of the anticancer drug daunorubicin incorporating an α -galactoside trigger (see prodrug 1 in Scheme 1b).⁵²

Since this first report in 1992, many examples of glycosylated prodrugs have been reported and the topic has been thoroughly reviewed.^{53–55} Hence, this review will mainly focus on some of the more recent developments in this field, which have taken place over the past decade or so. With this in mind, the first section of this overview will focus on the development of simple glycoconjugates consisting of a sugar and an anticancer drug, which have been 'bioconjugated' with and without the use of covalent linkers between the two components. In the second section, advanced glycoconjugates are discussed, which are composed of a sugar, an anticancer drug, a self-immolative linker and an additional component. Beyond the traditional glycosylated prodrug systems, the third section of this review will cover non-traditional supramolecular and macromolecular systems, such as nanoparticles and gels wherein glycosidase activity is required as a triggering step for the drug release.

2.1.1 Self-immolative linkers. Cyclophosphamide is an alkylating agent that is widely used as an anticancer therapeutic. This drug is biotransformed *in vivo* producing a toxic metabolite. To avoid harmful side effects and to enable selective drug targeting, an enzymatically activated prodrug phosphorodiamidic mustard 2 (Table 2) was designed. The prodrug contains a β -galactose unit linked to the drug through a 4-hydroxybenzylcarbonyl moiety. The level of β -galactosidase is often elevated in cancerous tissue and galectins are often present for solid tumour activation. It was confirmed that the prodrug was non-cytotoxic and was hydrolysed by β -galactosidase to release the active drug.⁵⁶

Monomethylauristatin E (MMAE) is a potent inhibitor of tubulin polymerization. It is extremely cytotoxic with subnanomolar activity against numerous cancer cell lines. A glucuronide prodrug of MMAE 3 (Table 2) was developed containing a self-immolative linker, which was found to be significantly less toxic than the parent drug. β -Glucuronidase efficiently releases MMAE with IC_{50} values of 0.16 to 0.52 nM in the presence of various cancer cells – A549 (human lung carcinoma), KB (human squamous carcinoma) and MDA-MB-231 (human breast adenocarcinoma). The antiproliferative activity of the prodrug was also evaluated on primary cultures of patients possessing non-small cell lung cancer. The findings showed that the prodrug did not affect the cell viability when incubated alone at 0.5 or 1 nM, however, addition of the enzyme increased the cytotoxicity, killing about 76% of cells at 1 nM concentration. *In vivo* experiments using C57BL/6 mice with a subcutaneous murine Lewis Lung Carcinoma (LLC) showed that the prodrug caused significant antitumour activity. The MMAE-prodrug 3 demonstrated comparable efficacy to its doxorubicin analogue when administered at a 237-fold lower dose.⁵⁷

Camptothecin is a topoisomerase inhibitor leading to S-phase cell death. However, there are major limitations with

its use as an anticancer agent, including the toxicity, non-selectivity, water insolubility and its inactivation by human serum albumin (HSA). A camptothecin derivative, 5,6-dihydro-4H-benzo[de]quinoline-camptothecin (BQC) was found to have a greater cytotoxicity against cancer cells than other clinically used camptothecin derivatives. A glucuronide prodrug of BQC 4 (Table 2) containing a self-immolative linker had 4000 times greater water solubility and 20–40 times lower cytotoxicity than BQC. Furthermore, the BQC-prodrug was efficiently hydrolysed by β -glucuronidase, leading to release of cytotoxic BQC (IC_{50} = 13 nM), even in the presence of HSA. Treatment of mice bearing human colon cancer xenografts with the BQC-prodrug 4 produced significant antitumour activity. Xenografts with naturally or artificially elevated levels of β -glucuronidase both showed significant reduction in the mean tumour size from day 2 or day 4 after initiation therapy, respectively.⁵⁸

Combretastatin A4 (CA4) is a potent tubulin polymerization inhibitor. Microtubule disrupting agents are used for ovarian cancer chemotherapy. Since β -galactosidase activity is enhanced in ovarian cancer, prodrugs of CA4 containing a galactose moiety were developed. In one of the prodrugs the galactose moiety was linked directly to CA4 (structure not shown), while in second prodrug there is a self-immolative benzyl linker between the sugar and drug unit, prodrug 5 (Table 2). Enzymatic investigations confirmed that both prodrugs released the parent drug, however prodrug 5 did so at a quicker rate. In cytotoxicity assays using two ovarian cell lines, prodrug 5 exhibited similar toxicity to the parent drug, while the other prodrug was significantly less cytotoxic. This may be explained by the rapid transformation of prodrug 5 into CA4. This was confirmed by inhibiting intracellular β -galactosidase activity, the cytotoxicity levels of both prodrugs decreased substantially. Using fluorescent dyes and fluorescence confocal microscopy, it was confirmed that the cytotoxicity of these prodrugs to ovarian cancer cells was the results of disruption of the microtubule architecture and subsequent apoptosis.⁵⁹

A small-molecule theranostic prodrug 6 (Table 2) of doxorubicin (DOX) was reported. This prodrug contained a galactose moiety, which was utilised to target asialoglycoprotein receptors on colon cancer cells and to enable enzyme-responsive activation strategies. Following activation by β -galactosidase enzymes in the colon, the parent drug is released leading to a 'turn on' of fluorescence, which allows the monitoring of both localization and the site of action of the drug. The selectivity was confirmed using HT-29, colon adenocarcinoma cell line, and HepG2 cells, a well-known cancer cell line displaying ASGP receptors. These cells showed fluorescence while the negative control (HeLa cells) exhibited little fluorescence. Cytotoxicity assays using HT-29 and HeLa cells showed that Gal-DOX 6 exhibited a 3-fold more potent therapeutic effect in the HT-29 cells than the HeLa cells, demonstrating that not only was cellular uptake improved, but also the enhancement of the anticancer effect. Using a fluorescence imaging system, the tumour-targeting ability of Gal-DOX 6 was confirmed in HT-29 tumour-bearing mice (Fig. 2a and b), along with the therapeutic efficacy (Fig. 2c and d).⁶⁰



Table 2 Prodrugs and the corresponding active chemotherapeutic drugs containing self-immolative linker

Compound	Prodrug	Active anticancer drug	Ref.
2			56
3			57
4			58
5			59
6			60
7a R = NO ₂ 7b R = NH ₂			61
8a R = NO ₂ 8b R = NH ₂			61
9a R = F 9b R = OH			62
10a R = D-Gal 10b R = D-GlcA			63

Hydroxamic acid-based histone deacetylase inhibitors such as Vorinostat and Belinostat have been reported to exhibit antitumour effects, but there have been limitations to its use

as an anticancer agent due to its poor aqueous solubility. To help overcome this problem, β -glucuronic acid-based prodrugs of Vorinostat **7a** and **b** and Belinostat **8a** and **b** (Table 2)



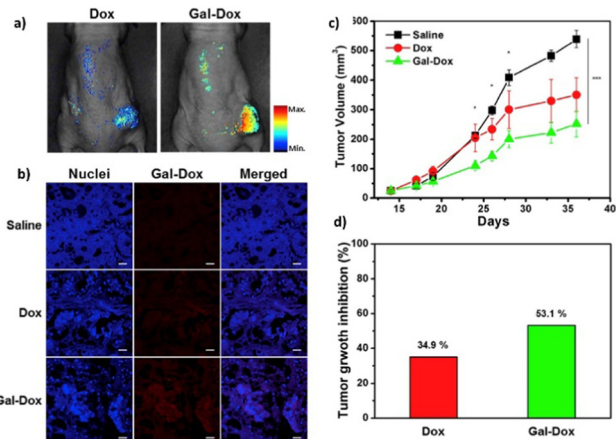


Fig. 2 The antitumour activity of prodrug **6** (Gal-DOX). (a) Fluorescence imaging of Gal-DOX **6** treated mice with HT-29 cancer xenografts; (b) fluorescence images of paraffin-embedded tumour tissue; (c) tumour growth inhibition in mice with HT-29 cancer xenografts, injected with saline, DOX, or Gal-DOX **6** at 3 mg kg⁻¹ four times every two days; (d) tumour growth inhibition after treatment with DOX and Gal-DOX **6** after 36 days. Reprinted from ref. 60 with permission from Elsevier.

were developed, which were 400- to 750-fold more water soluble than the parent drugs. Cytotoxicity assays were then conducted on HT-29 and Hut-78 tumour cell lines. The IC₅₀ values of the Vorinostat derivatives **7a** and **b** were > 10 μM in the absence of β-glucuronidase, however in the presence of the enzyme the inhibitory effects of the prodrugs were comparable to the parent drug, indicating the successful release of the drug upon enzyme-mediated hydrolysis of the glycosidic bond. However, for the Belinostat derivatives **8a** and **b**, no change was observed in the IC₅₀ values, both in the absence or presence of β-glucuronidase. Furthermore, the derivative with an amine functional group on the linker **7b** had relatively higher IC₅₀ values than those with a nitro functional group **7a**.⁶¹

A 2-fluorodeoxyglucose conjugate of the anticancer drug paclitaxel **9a** (Table 2) was synthesised and compared to the parent drug and the glucose conjugate (**9b** with no fluorine) in terms of solubility, stability, cytotoxicity, and selectivity towards cancer cells. A glucose conjugate was selected since most cancers overexpress glucose transporters. The incorporation of fluorine into drugs allows for adjustments of the electronic, lipophilic and steric properties. In previous studies it was found that the 2-deoxyfluoro glucosides had the slowest rate of hydrolysis when compared to other deoxy-glucosides or fluorinated glucosides. It was found that the aqueous solubility of the glucose-prodrug **9b** and the 2-deoxyfluoro glucoside **9a** was drastically improved compared to the parent drug. When analysing the chemical stability of the prodrugs in the presence of β-D-glucosidase, the 2-deoxyfluoro conjugate **9a** exhibited sustained release with less than 50% hydrolysis detected after 12 hours. The non-fluorinated glucose conjugate **9b**, however, was unstable and resulted in spontaneous release of the free paclitaxel. In cytotoxicity assays, the fluorinated prodrug **9a** showed increased cytotoxicity compared to paclitaxel on carcinoma cells of HepG2 and NCI-H460. Both prodrugs showed

excellent nanomolar toxicity to breast cancer cells MCF7. Meanwhile, paclitaxel and the glucose analogue **9b** showed similar cytotoxicity to the normal cell line HUVEC, while the fluorinated analogue showed no obvious cytotoxicity up to 10 μM. This demonstrated that the prodrug could reduce the cytotoxicity of paclitaxel while improving selectivity towards cancer cells over normal cells.⁶²

More recently, we have reported theranostic prodrugs of the toxic anticancer drug Amonafide **10a** and **b** (Table 2). Amonafide exhibited excellent activity in phase II breast cancer clinical trials, however failed the phase III trials due to acute side effects and bone marrow toxicity. Spectroscopic characterization and time-dependent enzymatic release studies showed that the enzyme mediated activation of **10a** and **b** occurs rapidly under physiological conditions. Cellular uptake and cell viability of these glyconaphthalimide compounds showed that upon enzymatic treatment, Amonafide was released and taken up into cells more rapidly than the glycosylated form. The IC₅₀ values were consistent across three cancer cell lines (HeLa, HCT-116 and HepG2), with no cytotoxicity observed in the presence of **10a** or **10b** after 24 hours and low cytotoxicity values observed after incubation with the enzyme. Importantly, release of the therapeutic component was also triggered by the activity of endogenous enzymes that selectively hydrolyse the glycosidic bond, a key finding which was verified using confocal microscopy, whereby emission of the naphthalimide unit was monitored in real-time. This process facilitates selective delivery of the Amonafide to the desired site of action and allows for real-time bioimaging in cancer cell lines; this has also been verified by recording the emission spectra within the cells after delivery, which was red shifted due to enhanced internal charge transfer mechanism in the delivered compounds vs. the prodrug.⁶³ This development was based on some prior work from our laboratory, where in a related study we employed the same principle for the delivery of naphthalimide imaging probes into cells. This system, which we described as a pro-probe (*e.g.* vs. prodrug) became activated only when a glycosidase activation of the glycosylated naphthalimide occurred, which released the fluorescent imaging agent into cancer cells *in vitro*, as was observed using real-time imaging, as only minimal cellular uptake was observed in the absence of the enzyme.⁶⁴

2.1.1.1 Enzyme directed immunostimulants. The glycosidase mediated release strategy has also been widely utilised in the development of enzyme directed immunostimulants. Immunotherapeutics have an ability to instruct the immune system to develop an immune response to tumours. Imidazoquinoline immunostimulants elicit an antitumour immune response when applied to solid tumours. However, they cause severe inflammatory toxicity after systemic administration. To overcome this challenge, a directed enzyme prodrug therapy (DEPT) approach may be utilised using an enzyme-directed imidazoquinoline pro-immunostimulant. Using this methodology, the enzyme-enriched cancer cells generate the immunostimulant and diffusion of this immunostimulant within the cancer micro-environment enables activation of bystander immune cells.



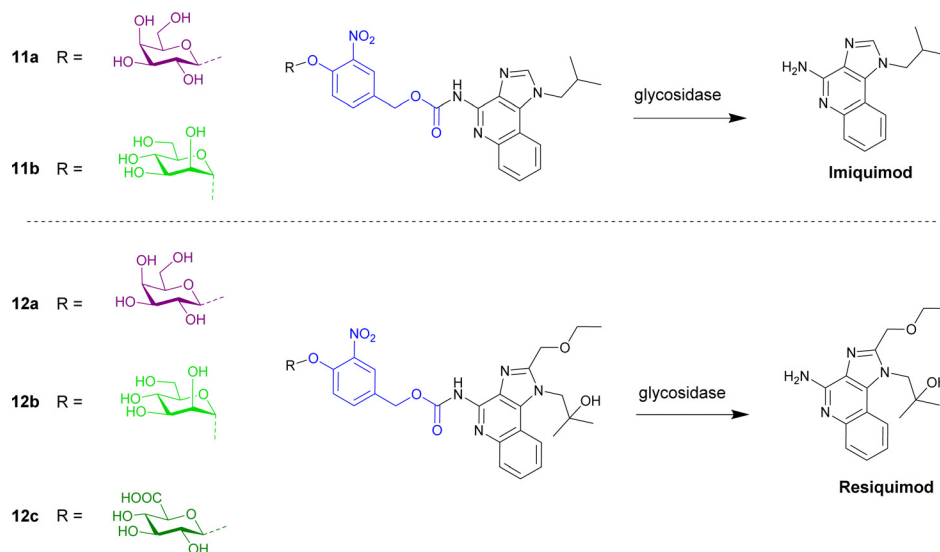


Fig. 3 Structure of enzyme-directed immunostimulants (**11a** and **b** and **12a–c**) explored for cancer therapy.

Imidazoquinoline immunostimulants are agonists for Toll-like receptors 7 and 8 present on innate immune cells, and hence activation of these receptors within the tumour microenvironment results in a robust innate and adaptive antitumour immune response. The imidazoquinoline imiquimod (Fig. 3) was appended with a galactose moiety *via* a self-immolative linker (prodrug **11a**), which yields imiquimod following treatment with exogenous β -galactosidase or β -galactosidase-enriched B16 melanoma cells. The enzyme-directed liberation of the immunostimulant resulted in activation of RAW-Blue macrophage and JAWSII monocyte immune cell lines.⁶⁵

Further work by the Neilsen and Mancini groups reported the first imidazoquinoline-mannoside pro-immunostimulant **11b** that was activated by α -mannosidase.⁶⁶ AT3B-1 prostate cancer cell lines were used since α -mannosidase is reportedly upregulated in multidrug resistant prostate cancers. AT3B-1 cells convert the pro-immunostimulant to imiquimod, leading to efflux of the drug into the extracellular space. Bystander immune cells are subsequently activated.

The same group then synthesised an enzyme-directed immunostimulant prodrug based on another imidazoquinoline, resiquimod (Fig. 3), covalently modified with β -glucuronic acid, β -galactose and α -mannose.⁶⁷ The metabolism of the three prodrugs **12a–c** were examined across three cancer cell lines (B16 melanoma, TC2 prostate, and 4T1 breast cancer). It was found that B16 cells elicited the highest immunogenicity, however the immunogenicity was comparable for a given cell type and independent of the glycosidase substrate in the prodrug or differences in functional glycosidase activity between cell lines. It was also found that the immunogenicity was governed by the efflux potential of cancer cells; this efflux occurring through P-glycoprotein-dependent and P-glycoprotein-independent transport mechanisms.

2.1.2 Other linkers. In addition to self-immolative linkers, other linker types have been explored in the development of

glycosidase activated prodrugs for cancer therapy. These linkers include a hydroxypropionic acid linker and succinate linkers (Fig. 4). Adriamycin (generic name is doxorubicin) is an effective anticancer drug against solid tumours, however its use is limited due to systemic toxicities and multidrug resistance. Adriamycin conjugated with 2-amino-2-deoxy-D-glucose with a succinic acid linker resulted in the formation of compound **13** (Fig. 4), which was designed to target tumour cells through glucose transporter 1 (GLUT 1). Studies showed that this compound had a better inhibition rate to tumour cells and lower toxicity to normal cells.⁶⁸ Although this compound is referred to as a prodrug throughout the paper, no enzymatic release studies were reported to confirm this property.

Docetaxel, an anticancer drug showing cytotoxicity against leukaemia and other cancers such as ovarian, breast and lung, has low water solubility and high toxicity to normal cells. Highly water-soluble sugar conjugates of docetaxel **14a–c** (Fig. 4) were synthesised using a chem-enzymatic procedure, using the relevant enzymes to stereoselectively install the carbohydrate unit onto the hydroxypropionic acid linker, which was subsequently coupled to the docetaxel. Conjugates of glucose, galactose and xylose were synthesised with the glucose conjugate **14a** being 52-fold more water soluble than the parent drug. 7-Propionyl docetaxel 3''-O- β -D-glucopyranoside **14a** and 7-propionyl docetaxel 3''-O- β -D-xylopyranoside **14c** were effectively hydrolysed by glycosidase enzymes releasing docetaxel and showed *in vitro* cytotoxicity against human cancer cell lines. However, the galactose derivative **14b** was not successfully hydrolysed by endogenous enzymes in the KB human cancer cells and exerted low cytotoxicity.⁶⁹

Paclitaxel is a chemotherapy drug used to treat different cancers including breast, ovarian and non-small cell lung cancer. However, due to its low aqueous solubility, the toxic surfactant Cremophor EL (polyethoxylated castor oil) must be used as a formulation vehicle in clinical practices. In this study, two prodrugs of paclitaxel were reported; a single glucose-conjugated paclitaxel **15a** and a double glucose-conjugated



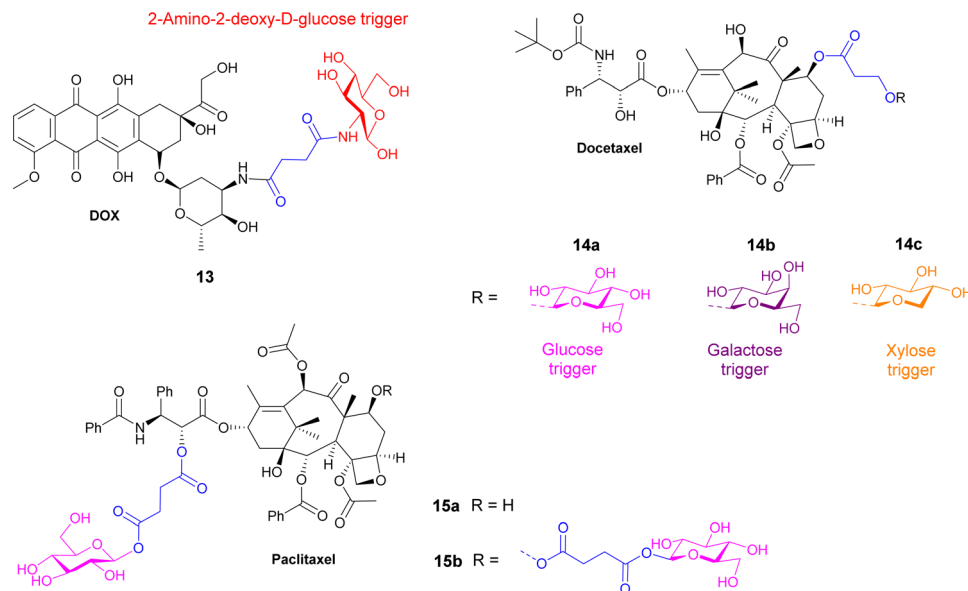


Fig. 4 Structure of prodrugs **13–15** containing hydroxypropionic acid and succinate linkers.

paclitaxel **15b** (Fig. 4), where the glucose moieties were attached *via* succinate linkers.⁷⁰ Both prodrugs showed improved aqueous solubility and drug-like lipophilicity over the parent drug. Of the two, the single glucose conjugate **15a** had a more efficient release in serum, where a high percentage of the parent drug was detected following hydrolysis with β -glucuronidase. This conjugate also exhibited good solubility in a low toxic formulation with no ethanol and lower percentage of Cremophor EL. Both prodrugs maintained effective cytotoxicity against breast cancer cells and apoptosis inducing effect, with the single glucose conjugate **15a**, indeed performing better in both assays.

Gene-directed enzyme prodrug therapy (GDEPT) has also been used to improve cancer drug localization. A recombinant adeno-associated virus (rAAV) was utilised to deliver β -galactosidase; a week later the treatment group received adenocarcinoma cells in the same location of the initial injection (Fig. 5b). Then 1 hour later an inactive prodrug of geldanamycin **16** (Fig. 5a) was administered. Prodrug **16** consists of geldanamycin, a potent antitumour antibiotic from *Streptomyces hygroscopicus*, with a galactose moiety connected *via* an *O*-ethylene linker. It was found that tumour growth was significantly reduced in animals whose muscles were genetically modified to express the enzyme β -galactosidase compared to the control, indicating that the presence of the enzyme releases the parent drug efficiently. Serum assays indicated that there was minimal damage to non-target organs, demonstrating that using this GDEPT technique allows for improved localized activation of chemotherapeutic agents.⁷¹

2.1.3 Carbohydrate-masked drugs. Prodrugs for cancer therapy have also been developed that contain no linker, where the sugar unit is directly bonded to the parent drug. In contrast to the prodrugs containing self-immolative linkers, functionalities such as phenol and amine groups are masked by carbohydrate moieties, that are then exposed after activation

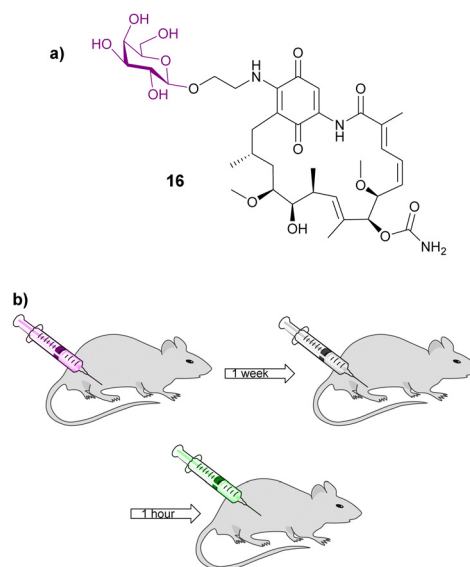
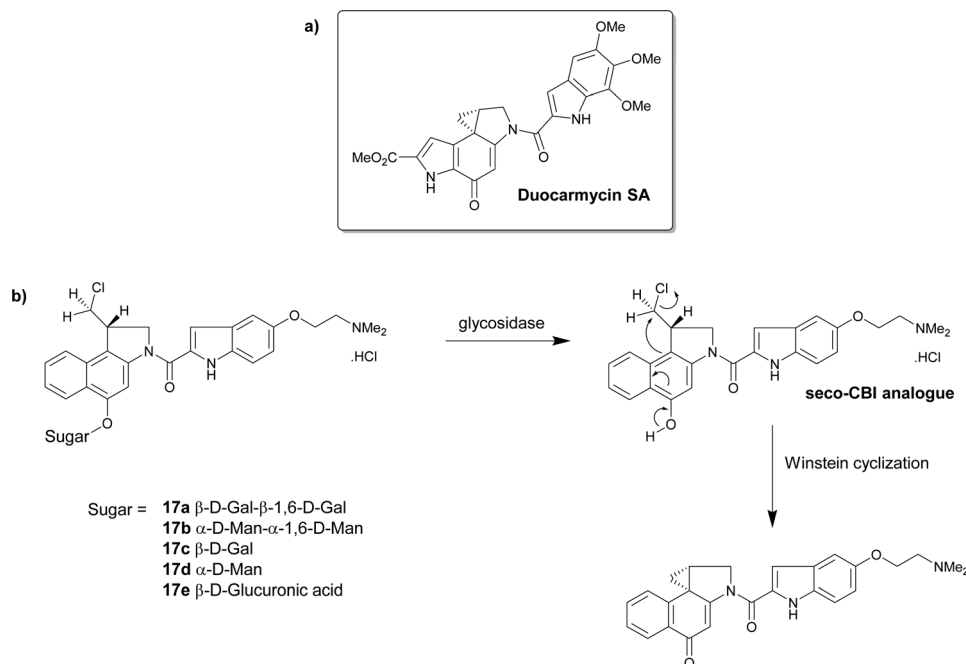


Fig. 5 (a) Structure of galactosylated prodrug of geldanamycin **16**; (b) diagram depicting the GDEPT procedure used in this study. Diagram adapted from ref. 71.

by glycosidase enzymes. The Tietze group, have been active within this area of research, with particular emphasis on developing glycosylated prodrugs and utilising the ADEPT technique. They have previously reported several compounds based on duocarmycin SA (Scheme 2a) that are suitable for ADEPT and PMT.^{72–74} Duocarmycin SA is a natural antibiotic found in *Streptomyces* sp. that is highly potent towards different cell lines ($IC_{50} = 10$ pM), however due to its pronounced myelotoxicity it is not used in chemotherapy. Tietze and co-workers investigated derivatives of the seco-analogue, seco-CBI (cyclopropabenzoindeole) and methyl-seco-CBI, as the pharmacophoric



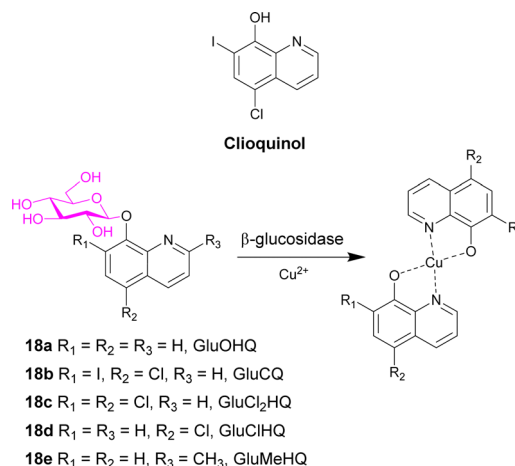


Scheme 2 (a) The structure of duocarmycin SA; (b) the structure of the prodrugs **17a–e** that under hydrolysis give the seco-CBI analogues that finally produce the cytotoxic drug.

units, which undergo rapid Winstein cyclization to produce the final cytotoxic drugs (Scheme 2b). Novel disaccharide prodrugs with a ManMan derivative **17b** and a GalGal derivative **17a** were reported which contain the seco-CBI (cyclopropabenzindole) as the DNA binding unit. The IC_{50} values were found to be higher than for the corresponding monoglycosidic prodrugs (**17c** and **d**), but following enzymatic cleavage of the GalGal prodrug **17a**, it exhibited similar values. The GalGal prodrug **17a** showed the best selectivity with interacting with DNA.⁷⁵

The group further explored the glucuronic acid derivative **17e** as an enzyme prodrug mono-therapeutic. The activation of the prodrug by intracellular and extracellular β -glucuronidase was investigated. The cytotoxicity of the prodrug **17e** was measured against human cell lines that exhibited varying levels of endogenous β -glucuronidase, including β -glucuronidase-deficient fibroblasts and β -glucuronidase knockdown cancer lines displaying low levels of the enzyme. The glucuronide prodrug **17e** was 1000–5000 times less cytotoxic than the parent drug irrespective of the β -glucuronidase levels. Cancer cells expressing membrane-tethered β -glucuronidase were more sensitive to the prodrug indicating that extracellular β -glucuronidase activity was more important than intracellular levels. This glucuronide prodrug **17e** (2.5 mg kg^{-1}) displayed less systemic toxicity and greater antitumour activity than clinically approved anticancer drug carboplatin (50 mg kg^{-1}).⁷⁶

Clioquinol (Scheme 3), a well-known 8-hydroxyquinoline (OHQ), is widely used as an antibiotic. It was also reported as a modulator of metal homeostasis in neurodegenerative disorders such as Alzheimer's disease. This compound also displays anticancer effect both in *in vitro* and *in vivo* preclinical models. It and other 8-hydroxyquinolines must interact with



Scheme 3 Structure of clioquinol and the prodrugs **18a–e** designed in this study. Also shows the complex formed in the presence of copper ions.

Cu^{2+} ions to exert anticancer activity. Cu^{2+} ions are co-factors required for tumour growth and angiogenesis, with high levels of the cation found in many cancer types. To exploit the glucose avidity, the elevated glycolysis of tumour cells, and the over-expression of glucose transporter, new clioquinol glucoconjugates were synthesised (GluOHQA **18a** and GluClQ **18b**). These prodrugs improved the aqueous solubility and since they do not complex Cu^{2+} ions in their glycosylated form, side effects of systemic chelation are prevented. Following hydrolysis with β -glucosidase, the active aglycone is released. However, after studying the antiproliferative activity of the parent compounds and the glycosylated derivatives, the glycoconjugates are less



active than their parent compounds. It was hypothesised that enzyme mediated release of the parent drugs remains incomplete.⁷⁷

The same group then synthesised three new glycoconjugates of OHQs **18c–e**, which demonstrated that the glycosylated compounds had similar activity to the parent compounds, with the exception of the methyl derivative (GluMeHQ **18e**). Computational docking studies of these compounds with human cytosolic β -glucosidase indicate that GluMeHQ **18e** had the best affinity for the binding site, while GluCl₂HQ **18c** showed the lowest affinity. It is suggested that the compounds that are tightly bound to the enzyme are more slowly hydrolysed, and therefore release of the active compound occurs at a slower rate, reducing the cytotoxicity. The presence of Cu²⁺ ions increases their antiproliferative activity by an order of magnitude and the pharmacological profile of the glycoconjugates are dependent on the intracellular β -glycosidase activity.⁷⁸

In another study, galactose was conjugated to the fluorescent dye MPA to evaluate the tumour affinities and hence the cancer targeting ability of galactose. An *in vitro* cell study showed that the galactose-MPA conjugate had higher affinity to tumour cells (HepG2, MCF-7 and A549) than normal liver cells. They then conjugated galacturonic acid onto the anticancer drug doxorubicin (DOX) for targeted tumour therapy. This prodrug conjugate **19** (Fig. 6) had better therapeutic results than DOX suggesting that galactosylated prodrugs may have a potential use in tumour targeted therapy.⁷⁹

Curcumin is a natural polyphenol found in turmeric that was been linked to a lower rate of cancer. However, there is low bioavailability of curcumin due to elimination through faeces and metabolism into inactive glucuronides. Although, the mode of action is not known, it is proposed that curcumin glucuronide **20** (Fig. 6) is an inflammation-responsive prodrug that is hydrolysed back to curcumin at the site of action. It is also known that levels of β -glucuronidase are elevated in some cancers such as breast cancer. In mouse tumour models, high levels of curcumin were generated at the tumour after both oral and intravenous administration. Long-term daily dosing of curcumin resulted in the accumulation of curcumin in tumour tissue and led to the inhibition of tumour growth where high levels of β -glucuronidase were expressed.⁸⁰

The anticancer properties of curcumin were further explored in another study. Colorectal cancer is typically treated with the Pt-complex oxaliplatin. However, it is known that KRAS mutation, common in colorectal cancer patients, hampers the effect of oxaliplatin. NF- κ B activation is an important step in oxaliplatin resistance. This NF- κ B pathway may be inhibited with curcumin. In this study a water-soluble injectable glucuronide-prodrug of curcumin **20** (Fig. 6) was prepared, and its efficacy, safety and pharmacokinetics in a mouse xenograft model was investigated. KRAS/TP53 mutations increased the IC₅₀ values of oxaliplatin in HCT116 cells *in vitro*. The glucuronide-curcumin prodrug **20** exhibited better anticancer effects compared to oxaliplatin in an oxaliplatin-resistant xenograft model, with a combination therapy exhibiting additive effects in these models without increasing the toxicity. Importantly, pharmacokinetic

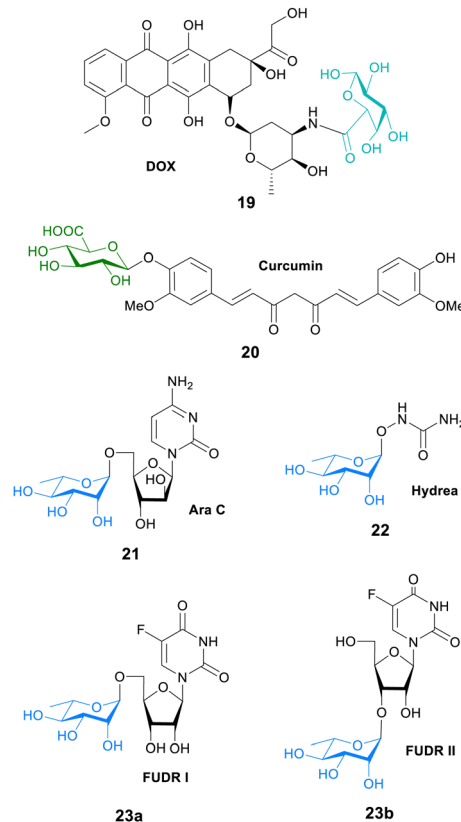
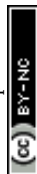


Fig. 6 Structure of prodrugs **19–23**, where the carbohydrate unit is bonded directly to the anticancer agent.

analysis showed high levels of free curcumin in tumour tissue after 48 hours, but was not detected in other organs such as the heart, liver and spleen.⁸¹

In a novel approach to glycosyl prodrug synthesis, an α -L-rhamnosidase from *Alternaria* sp. L1 (RhaL1) was employed to catalyse rhamnosylation through reverse hydrolysis reaction of known anticancer drugs, namely 2'-deoxy-5-fluorouridine (FUDR), cytosine arabinoside (Ara C), and hydroxyurea (Hydreia). The rhamnosylated drugs **21–23** (Fig. 6) exhibited little cytotoxicity, with the toxicity restored only when incubated with exogenous α -L-rhamnosidase. This proves their potential use in enzyme-activated prodrug systems. Also in the study, the cancer-targeting ability of the rhamnose moiety was evaluated by conjugating the monosaccharide to the fluorescent dye rhodamine B. The fluorescent probe displayed higher cell affinity and cellular internalization in oral cancer cell KB and breast cancer cell MDA-MB-231 than that of normal epithelial cells MCF 10A. This suggests that the rhamnose moiety could play a key role in targeting the active therapeutic to cancer cells.⁸²

2.1.3.1 Targeting senescent cells. Senescent cells accumulate during ageing and are associated with cancer, fibrosis and many age-related pathologies. They display elevated activity of the lysosomal β -galactosidase. Different galactose-modified duocarmycin prodrugs preferentially kill senescent cells, in a



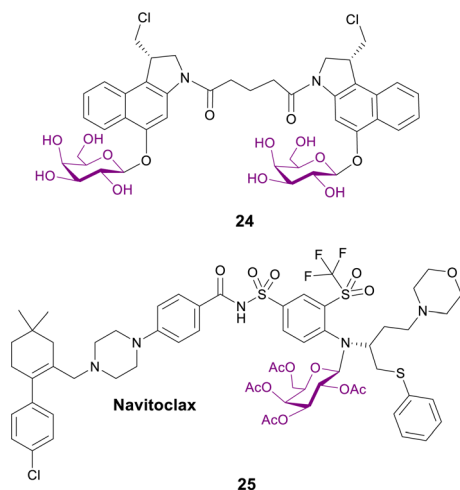


Fig. 7 Structure of prodrugs **24** and **25** that target senescent cells.

lysosomal β -galactosidase-dependent manner. The effects of a galactosylated seco-duocarmycin analogue dimer **24** (Fig. 7) previously described by Tietze⁸³ was analysed on the survival of IMR90 ER:RAS cells (a model of oncogene-induced senescence). This prodrug can selectively eliminate a wide range of senescent cells, including bystander senescent cells. In an adamantinomatous craniopharyngioma (ACP, a pituitary paediatric tumour) mouse model, treatment with prodrug **24** selectively reduced the number of β -catenin-positive preneoplastic senescent cells.⁸⁴

Senescent cells were targeted in another report, where the BCL-2 family inhibitor Navitoclax was conjugated with galactose to exploit the lysosomal β -galactosidase activity of senescent cells. Prodrug **25** (Fig. 7) selectively induces apoptosis of senolytic cells. Interestingly, this prodrug enhances the cytotoxicity of standard cisplatin-induced senescent lung cancer A549 cells. Concomitant treatment with cisplatin and prodrug **25** *in vivo* resulted in the elimination of senescent lung cancer cells and the reduction of tumour growth. Furthermore, galactose conjugation reduces Navitoclax-induced platelet apoptosis in both human and mouse blood samples and reduces thrombocytopenia in mouse lung cancer models.⁸⁵

In summary, numerous anticancer drugs have been conjugated with a carbohydrate moiety, with and without a linker. The resulting 'simple' glycosylated prodrugs exhibit an improvement of their aqueous solubility and their selectivity towards cancer cells with a reduction in the toxic side effects.

2.2 Advanced glycoconjugates

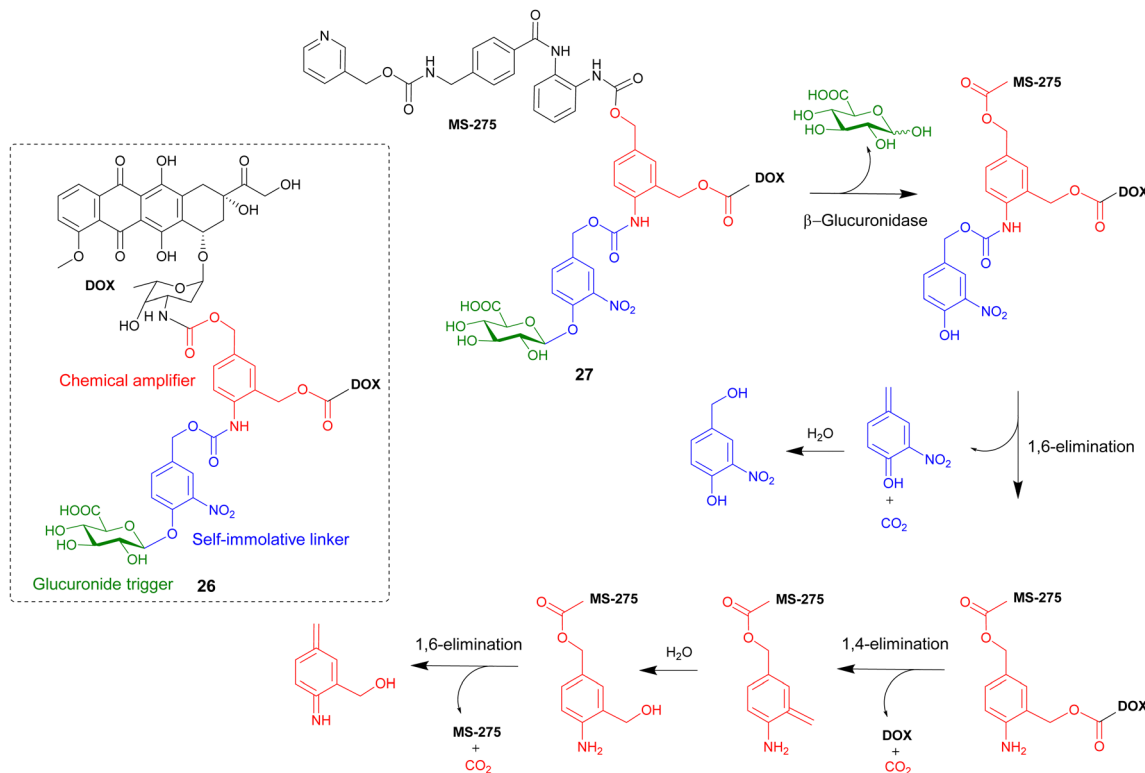
In this section, advanced glycosylated prodrugs are discussed. These prodrugs are composed of a glycosidic trigger, a self-immolative linker, an anticancer drug, and unlike that discussed in the previous section, they all contain an additional component. These additional components include extra drug molecules, hydrophilic chains, targeting ligands and mechanically interlocked systems. The aim of these constructs is to increase the potency, solubility and/or selectivity of the anticancer drugs.

2.2.1 Dual drug release. To increase the potency of these glycosidase activated prodrug systems, Papot and co-workers reported a dendritic glucuronide prodrug of doxorubicin, where two drug units were released following one enzymatic activation step. Prodrug **26** was composed of five units: the enzymatic trigger (glucuronide moiety), a self-immolative linker and a chemical amplifier connected to the two doxorubicin units. Using this design, β -glucuronidase hydrolyses the glycosidic bond resulting in a phenol intermediate, which induces the release of aniline intermediate *via* a 1,6-elimination. The chemical amplifier subsequently releases two doxorubicin units through successive 1,4- and 1,6-eliminations (Scheme 4 shows the similar mechanism for prodrug **27**). HPLC analysis was used to confirm the release of the doxorubicin from the dendritic prodrug in the presence of β -glucuronidase. Prodrug **26** was found to be twice as toxic as its monomeric counterpart, as well as doxorubicin, at the same concentration in antiproliferative assays against H661 lung cancer cell lines.⁸⁶

A similar heterodimeric system was also reported (Scheme 4), where two different anticancer drugs were released upon enzyme activation. The previously used doxorubicin and a well-known histone deacetylase inhibitor MS-275 were incorporated into prodrug **27**. Furthermore, in this study the role of the chemical amplifier was investigated. As well as being responsible for the signal amplification, the amplifier is converted into a highly toxic species that, in combination with the two other drugs, leads to potent cancer tritherapy. In antiproliferative assays against H290 lung mesothelioma cells, confocal microscopy confirmed the β -glucuronidase-mediated release of doxorubicin. Western blot analysis confirmed that enzymatic activation of prodrug **27** also restored the ability of MS-275 to inhibit histone deacetylase. However, since the activated heterodimer **27** was five-fold more toxic than the combination of the two anticancer drugs, it was confirmed that the release of the chemical amplifier as an azaquinone methide unit contributed significantly in the cytotoxicity of prodrug **27**.⁸⁷

2.2.2 Additional hydrophilic chain. Cyclopamine is a natural alkaloid isolated from *Veratrum californicum* which has been identified as an inhibitor of the Hedgehog (Hh) signalling pathway observed in a wide range of cancers such as prostate, breast, lung, gastric and brain. However, cyclopamine can cause serious side effects since somatic stem cells are also Hh-dependent. With this in mind, Papot and co-workers developed a prodrug of cyclopamine incorporating a glucuronide trigger and a self-immolative linker; compound **28a** (Fig. 8). After enzymatic release, it was observed that the resulting phenol intermediate formed a precipitate, limiting the kinetics of the drug release. To circumvent this inherent water insolubility, prodrug **28b** (Fig. 8) was developed containing an additional hydrophilic glycosylated poly(ethylene glycol) side chain. It was confirmed that cyclopamine was released significantly faster from prodrug **28b** than the initial prodrug **28a**. Prodrug **28b** was tested for its antiproliferative activity on U87 glioblastoma cells and when incubated alone, prodrug **28b** did not affect cell viability. Addition of β -glucuronidase in the culture medium induced a significant antiproliferative activity with an





Scheme 4 The β -glucuronidase-mediated drug release mechanism of heterodimeric prodrug **27**. Inset shows the structure of homodimeric prodrug **26**.

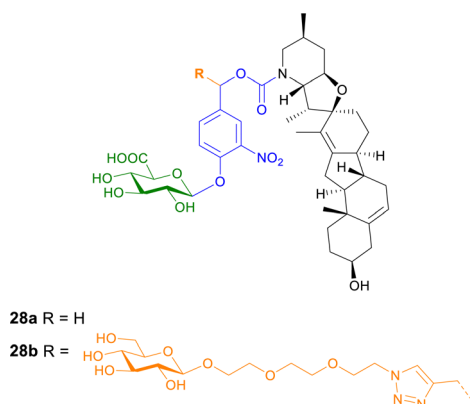


Fig. 8 Structure of prodrugs **28a** and **b** of cyclophosphamide. Prodrug **28b** contains an additional hydrophilic linker to enhance water solubility.

IC_{50} value ($IC_{50} = 24.5 \mu M$) similar to that of the paternal drug cyclophosphamide ($IC_{50} = 16.5 \mu M$).⁸⁸ Prodrug **28b** was then used *in vitro* and *in vivo* toward glioblastoma-initiating cells (GIC). Similarly to before, prodrug **28b** was only active in the presence of β -glucuronidase, which is present in high levels in necrotic areas of glioblastomas, decreasing proliferation and inhibiting the self-renewal of all GIC lines tested. $10 \mu M L^{-1}$ of prodrug **28b** inhibited the Hh pathway, inducing a 99% inhibition of clonogenicity on GICs, similar to the parent drug. Furthermore, in a subcutaneous glioblastoma xenograft model, prodrug **28b** prevented tumour growth with 75% inhibition at 8 weeks,

with the inhibition still significant after 14 weeks. Importantly, unlike the parent drug cyclophosphamide, prodrug **28b** had no detectable toxic side-effects in intestinal crypts.⁸⁹

The cytotoxic properties of prodrug **28b** were also analysed *in vitro*, *ex vivo* and *in vivo* in C6 rat glioblastoma multiforme (GBM) cells, an aggressive primary brain tumour where Hh pathway activation is also observed. The β -glucuronidase activated prodrug was toxic and down regulated the expression of a Hh target gene in cancer cells but not in normal rat astrocytes. When the prodrug was administered to rats bearing fluorescent C6-derived GBM, the tumour density was reduced more efficiently than using cyclophosphamide alone.⁹⁰

2.2.3 Targeting ligands. Using a similar system to previous examples (prodrugs **3** and **6**), Papot and co-workers reported prodrugs incorporating a targeting ligand in addition to the usual components; sugar, self-immolative linker and anticancer drug. Prodrugs **29** and **30** (Fig. 9e), a galactoside triggered doxorubicin⁹¹ or MMAE,⁹² also contain a folate targeting moiety. These prodrugs were designed to be selectively activated by β -galactosidase present in the lysosomes of cancer cells expressing folate receptors, which are overexpressed in several cancer types, while being mainly undetectable in most normal tissues. The principle of tumour targeting involves the following steps: (1) targeting ligand present on the prodrug recognises receptors on cancer cells; (2) receptor-mediated endocytosis occurs; (3) β -galactosidase catalyses drug release; (4) the drug diffuses into the nucleus or the cytoplasm of receptor-positive and surrounding receptor-negative cancer



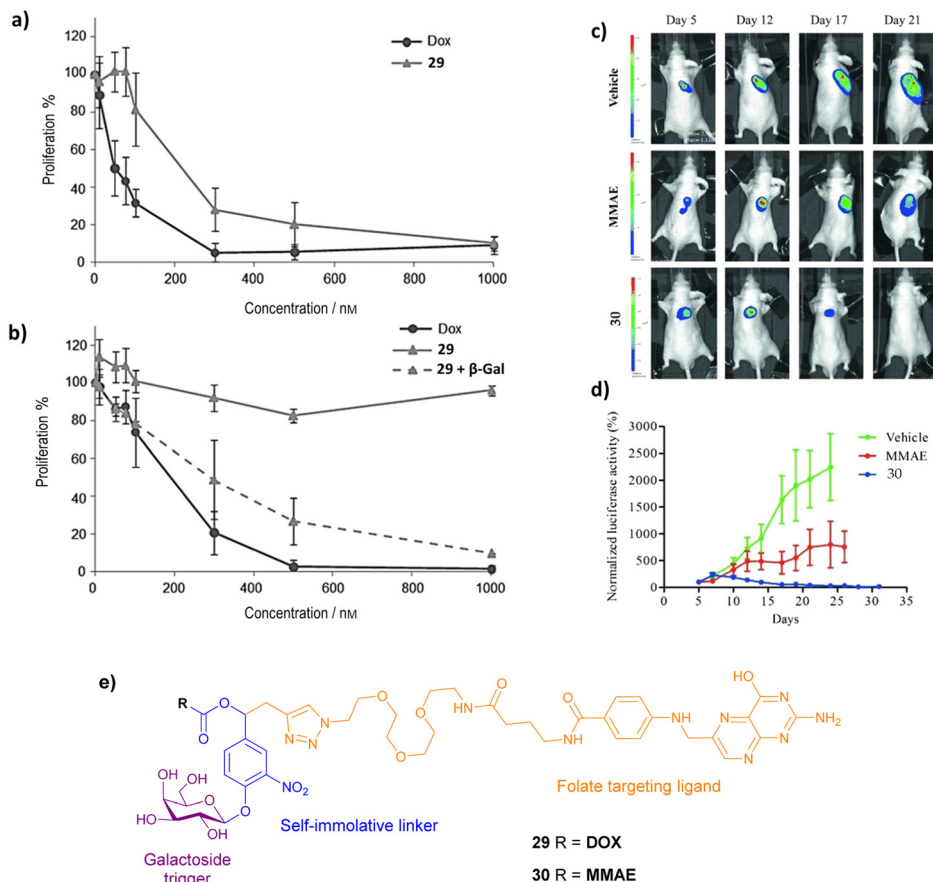


Fig. 9 Proliferative activity of (a) HeLa and (b) A549 cells treated with DOX, prodrug **29** and prodrug **29** + β -galactosidase. (Reprinted from ref. 91 with permission from John Wiley and Sons); (c) bioluminescence imaging of KB xenografts following treatment with vehicle (5% DMSO in PBS buffer), MMAE and prodrug **30**; (d) tumour growth inhibition in the presence of MMAE and prodrug **30**. (Reprinted from ref. 92 with permission from John Wiley and Sons); (e) structure of prodrugs **29** and **30**.

cells, resulting in cell death. In HeLa cells, the targeting prodrug **29** induced a significant antiproliferative effect, while A549 cells were not sensitive to prodrug **29** and free DOX was highly toxic (Fig. 9a and b).⁹¹ Prodrug **30** exhibits a significant antitumour effect when tested against KB xenografts, while treatment with MMAE led to only a moderate inhibition of tumour growth (Fig. 9c and d).⁹²

To advance this strategy, the concept of the dual drug release was combined with the folate-targeting system, creating novel prodrugs (structure not shown) that target the folate receptors as before, and release two anticancer drugs after one enzyme activation step *i.e.* two MMAE molecules,⁹³ or two DOX molecules.⁹⁴ As expected, the dimer prodrugs had superior cytotoxicity towards cancer cells than their monomeric counterparts, demonstrating the increased release of MMAE or DOX within the tumour cells.

Gennari and co-workers have explored the conjugation of different anticancer drugs to a peptidomimetic compound cyclo(DKP-RDG), a low nanomolar ligand of integrin $\alpha_v\beta_3$, a transmembrane glycoprotein overexpressed in a variety of cancers (*e.g.* melanoma, glioblastoma, lung, breast, ovary, colon). Two integrin-targeting prodrugs of MMAE containing a

glucuronide trigger and a self-immolative linker were reported. The integrin-targeting ligand are connected to the self-immolative linker *via* two different spacers: (1) a glutaric acid derivative in prodrug **31a**; (2) a triazole and PEG4 spacer in prodrug **31b** (Fig. 10). The cytotoxicity of prodrugs **31a** and **b** were examined by incubating the RDG-MMAE conjugates with $\alpha_v\beta_3$ -expressing cancer cells, in the absence and presence of β -glucuronidase. The anticancer activity of prodrug **31b** increased dramatically upon enzyme activation, with IC_{50} values similar to MMAE in the low nanomolar range. On the other hand, the enhanced anticancer activity of prodrug **31a** was not observed, regardless of β -glucuronidase addition. In this study, the use of a long, hydrophilic PEG4 spacer, connecting the integrin ligand to the linker moiety, was essential for the overall efficiency of the prodrug, which they suggest is most likely due to reduced steric hindrance around the cleavable glycosidic bond.⁹⁵

Antibodies have also been utilised in targeted cancer therapies. Trastuzumab, a monoclonal antibody that recognises growth factor receptors that are overexpressed in some breast cancers, was functionalised using its cysteine residues onto prodrugs of MMAE containing a galactoside trigger and a



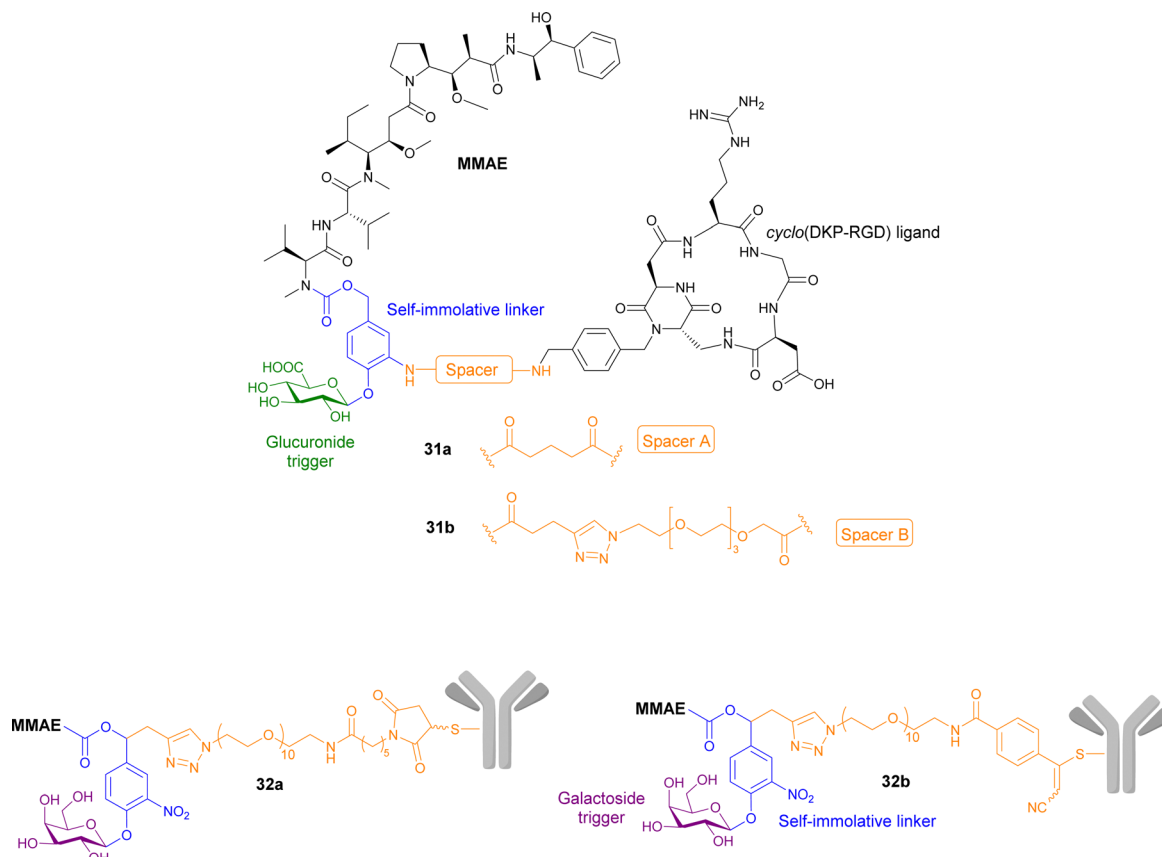


Fig. 10 Structures of prodrugs **31a** and **b**, and **32a** and **b** containing a targeting peptidomimetics and antibodies with two different spacers.

self-immolative linker. Two derivatives were reported containing different spacers between the antibody and the self-immolative linker, both containing a decathylene glycol side chain terminated by (1) a maleimidocaproyl functional group (prodrug **32a**, Fig. 9); and (2) an arylpropionitrile group (prodrug **32b**). These antibody-prodrug conjugates exhibited selective cytotoxicity against HER2+ cancer cell lines with IC_{50} values in the picomolar range. Furthermore, prodrugs **32a** and **b** exhibited a significant antitumour effect *in vivo* after administration of a single 1 mg kg^{-1} dose, both displaying 57–58% reduction of tumour volume 31 days post-injection, indicating that the spacer group used was not significant.⁹⁶

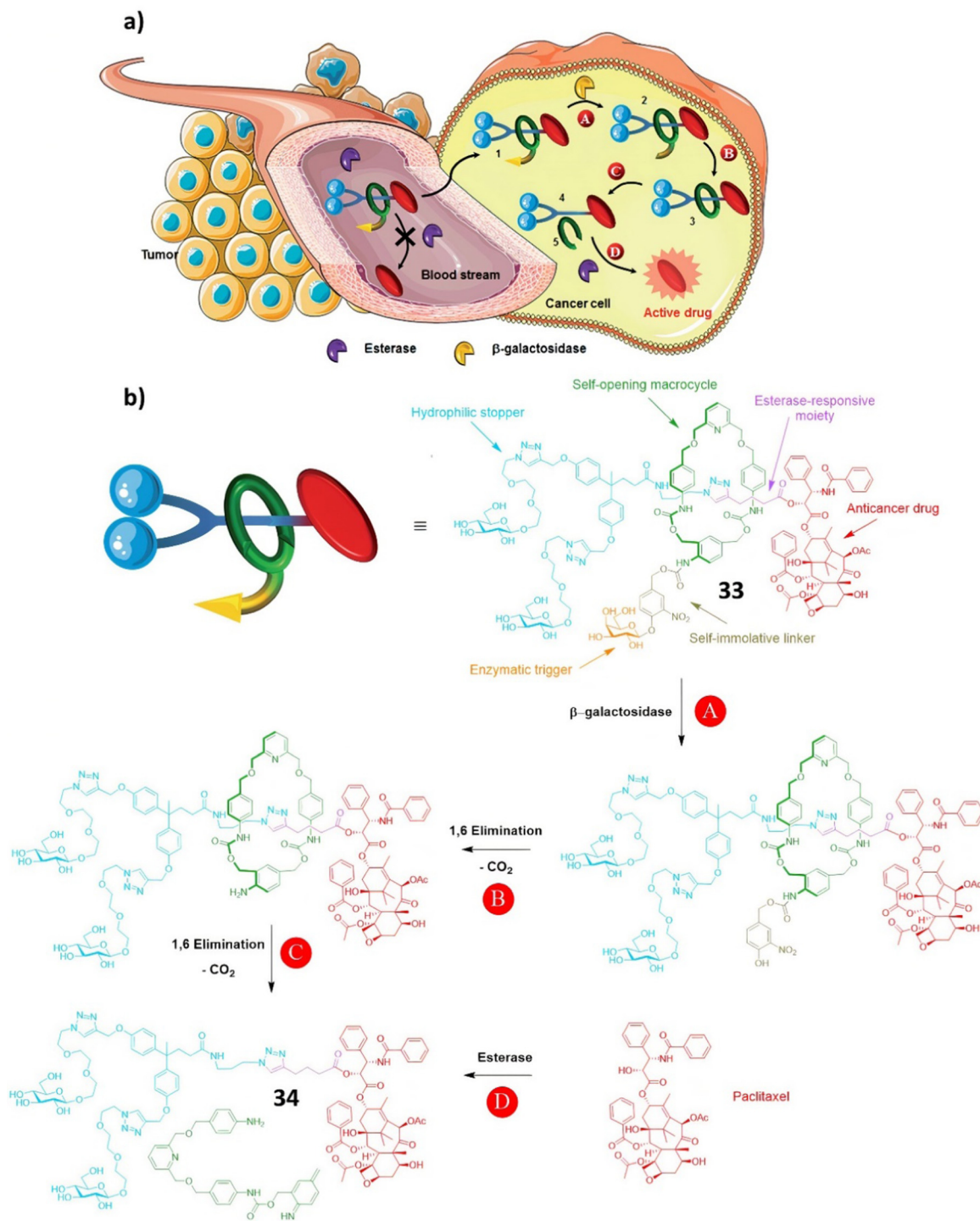
2.2.4 Mechanically interlocked molecular systems. Mechanically interlocked molecular systems designed to operate independently in biological environments have found medicinal applications in recent years. An enzyme-sensitive [2]-rotaxane has been designed to release the potent anticancer drug, paclitaxel, within tumour cells. The system contains a galactoside trigger, a self-immolative linker, a protective macrocyclic ring, a hydrophilic stopper and an esterase-responsive spacer linked to paclitaxel (prodrug **33**, Scheme 5). The macrocyclic ring prevents the untimely release of the drug into the plasma, by shielding the ester bond from hydrolysis. When the system enters the cancer cells, β -galactosidase initiates a sequence of chemical reactions, leading to the opening of the protective macrocyclic ring (Scheme 5). The ester bond becomes exposed

allowing hydrolyses by intracellular esterases, releasing paclitaxel within the cancer cells. The concept was confirmed in rat plasma, where the interlocked system was stable over 48 hours without liberation of the drug. In comparison to the corresponding system with no macrocyclic ring, prodrug **34** was rapidly cleaved to release paclitaxel. Furthermore, the interlocked system increases the aqueous solubility of the inherently insoluble drug, which as mentioned before must be administered in a vehicle containing ethanol and Cremophor EL. Confocal fluorescence microscopy imaging confirmed that rotaxane **33** disturbed the microtubule network of KB tumour cells, confirming the release of paclitaxel from the interlocked system. Rotaxane **33** exhibited high cytotoxicity when incubated with KB, H661 and MDA-MB-231 cells, however, paclitaxel was approximately 4-fold more toxic on the same cancer cells. Nevertheless, the lower antiproliferative properties of rotaxane **33** is reportedly outweighed by the improvement in selectivity for cancer cells overexpressing β -galactosidase.⁹⁷

2.3 Macromolecular and multimolecular systems

In this section, multimolecular systems incorporating glycosidase activated prodrugs are discussed. The main rationale for the development of these multi molecular systems is the enhanced permeability and retention (EPR) effect. EPR is a property of solid tumours, whereby macromolecular drugs and nanoparticles accumulate preferentially in tumour tissues than

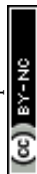


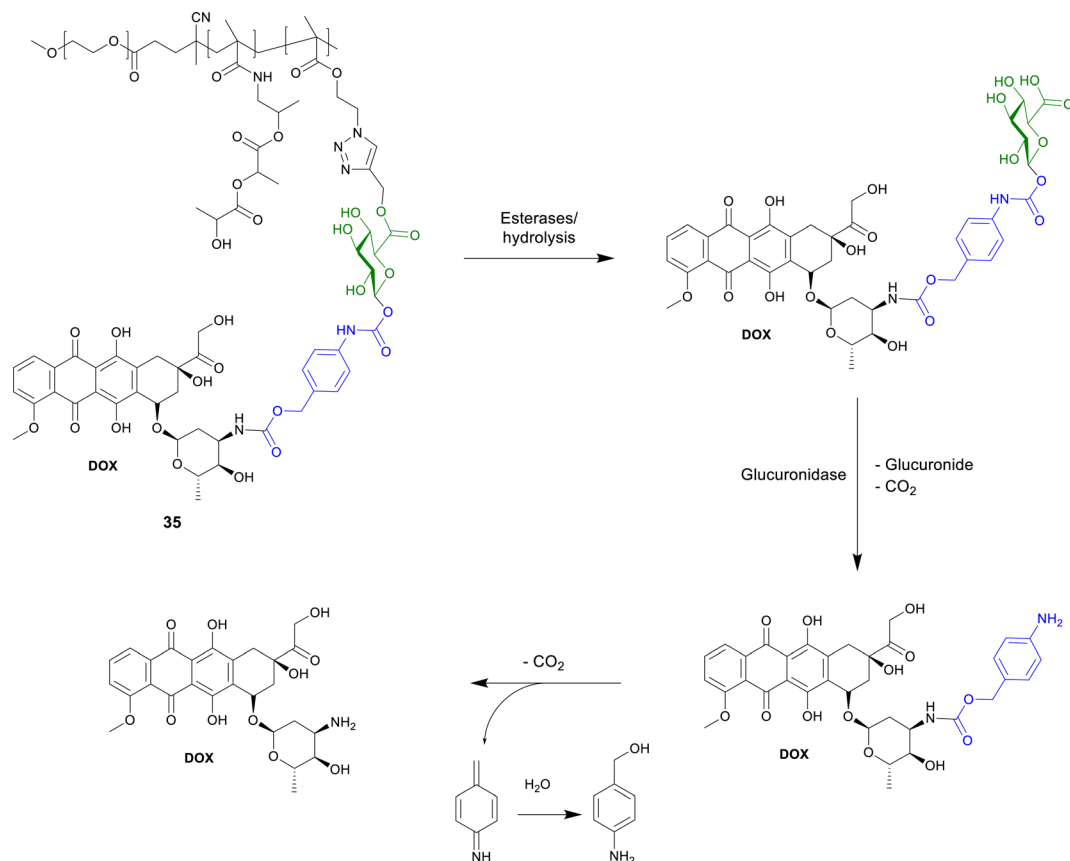


Scheme 5 (a) The principle of drug delivery for the interlocked prodrug **33**; (b) the structure of prodrug **33** and the paclitaxel release mechanism. (Reproduced from ref. 97 with permission from the Royal Society of Chemistry.)

in normal tissue due to increased vascular permeability.^{98,99} To take advantage of the EPR effect, research has focused on developing larger supramolecular and macromolecular systems incorporating the prodrug concept. These include, but are not limited to, polymers, micelles, nanoparticles and protein conjugates.

2.3.1 Polymers. Biodegradable and thermosensitive di-block copolymers have been reported as efficient micelle-forming building blocks for the delivery of hydrophobic drugs. Poly(ethylene glycol)-*b*-poly[*N*-(2-hydroxypropyl)methacrylamide-lactate] (mPEG-*b*-p(HPMAmLacn)) contains a hydrophilic component (mPEG) and a thermosensitive component (p(HPMAmLac_n))





Scheme 6 Structure of the polymer-bound DOX-prop-GA3 **35** and the conversion by chemical hydrolysis and/or by esterases and subsequent release of DOX by β -glucuronidase.

that forms polymeric micelles in aqueous solutions. Hennick and co-workers reported a novel system for enzymatically responsive anticancer therapy.¹⁰⁰ They investigated the covalent coupling of doxorubicin-glucuronide prodrug **35** (Scheme 6) to the micelle core using 'click chemistry'. It was confirmed that the coupling was very efficient and that polymers were obtained at low concentrations. The micelles formed were small (50 nm) and monodisperse. In the presence of β -glucuronidase, 40% of the drug payload was released after 5 days incubation at 37 °C, but less than 5% was released in the absence of the glycosidase enzyme. *In vitro* cytotoxicity experiments using carcinoma cells showed the same cytotoxicity as free doxorubicin only in the presence of β -glucuronidase, also indicating the release of the parent drug.

5-Fluorouracil is a clinical anticancer drug that suffers from poor bioavailability. Many chemically modified derivatives of this drug have been reported, however there are still difficulties in targeting particular cancers. Negi and co-workers have reported a xylan-5-fluorouracil-1-acetic acid conjugate **36** (Fig. 11) as a colon specific prodrug.¹⁰¹ Xylan was used since it remains intact in the upper gastrointestinal tract and it degraded by xylanases and β -xylosidase enzymes present in the colon. *In vitro* drug release profiles in the presence of rat's gastrointestinal contents, showed that small amounts of the drug were released in the gastric and small intestine contents, while 53–61% were released in the cecum and colonic contents.

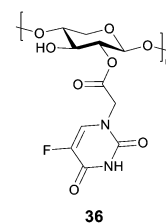
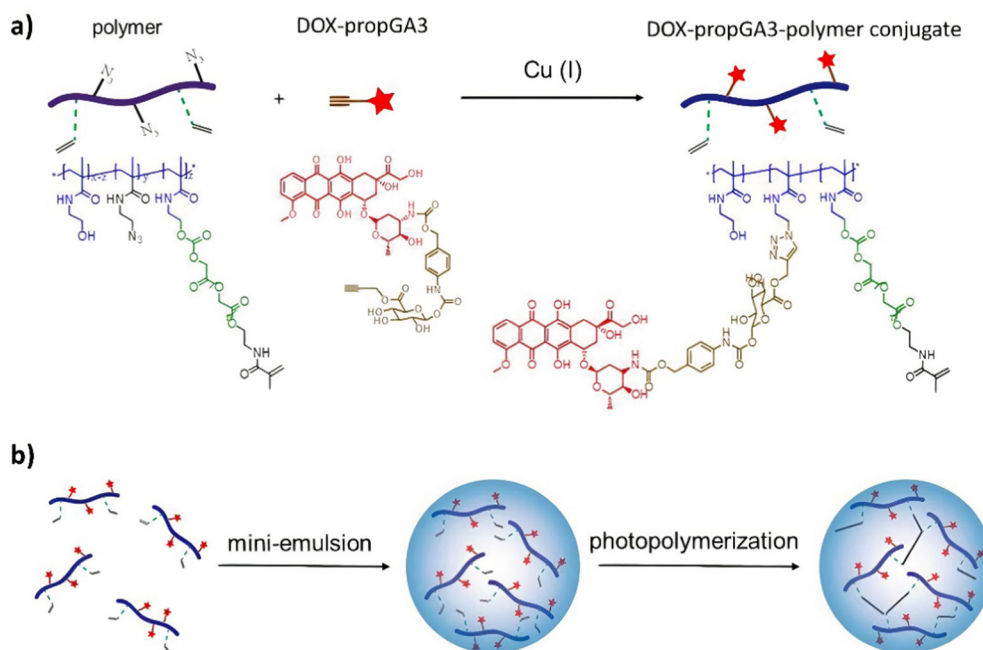


Fig. 11 Structure of the xylan-5-fluorouracil-1-acetic acid conjugate **36**.

The ester linkage of the conjugate is resistant to ester hydrolysing enzymes in the upper GI tract due to the steric hindrance of the polymeric xylan chain. In a human colorectal cancer cell line (HTC-15 and HT-29), the conjugates were more cytotoxic than the parent drug.

2.3.2 Nanogels. To improve the pharmacokinetics and selectivity of anticancer drugs, Deckers and co-workers have developed a doxorubicin-glucuronide prodrug nanogel.¹⁰² Nanogels are nano-sized hydrogels that are composed of hydrophilic polymer chains that are cross-linked and can be loaded with drugs or conjugated with pharmacologically active agents. First, the doxorubicin-glucuronide prodrug was conjugated to a polymer (p(HEMAM-co-AzEMAM)-Gly-HEMAM) using copper-catalysed 'click chemistry' (Scheme 7a). The polymer was subsequently used to prepare the nanogels by mini-emulsion





Scheme 7 (a) The synthesis of the doxorubicin–glucuronide prodrug (DOX-propGA3)–polymer using click chemistry; (b) shows the preparation of the nanogels using inverse mini-emulsion photopolymerization. Reprinted from ref. 102 with permission from MDPI.

photopolymerisation, where the methacrylamide side-chains of the polymers were cross-linked under UV (Scheme 7b). 4T1 cells, a breast cancer cell-line, were then exposed to HIFU (high-intensity focused ultrasound) and it was confirmed that the supernatant contained large quantities of β -glucuronidase. Microscopic images of untreated cells were round with a smooth surface, however those that were exposed to HIFU contained cell debris with no viable cells present. The supernatant of the exposed 4T1 cells could convert the nanogels completely into doxorubicin in less than 48 hours. In the absence of either bovine β -glucuronidase or β -glucuronidase liberated from cells exposed to HIFU, the nanogels showed little cytotoxicity.

2.3.3 Albumin-binding. Another method that has been used to utilise the EPR effect, is to conjugate the drug onto a protein and hence increase the overall molecular weight of the specimen. Albumin, the most abundant protein in the blood, is most widely used for this approach. The first β -glucuronidase-responsive albumin-binding prodrug consisted of a glucuronide trigger, the anticancer drug doxorubicin and a self-immolative linker bearing a poly(ethylene glycol) side chain terminated by a maleimide functional group (compound 37, Fig. 11).¹⁰³ In the presence of albumin, the maleimide reacts *via* Michael addition with the thiol of the cysteine at position 34. This forms the macromolecular drug carrier, which prevents the rapid renal clearance observed by other β -glucuronidase-responsive prodrugs. Biological studies have shown that: (i) this conjugate did not affect the cell viability even at high doses of 2 μ M; (ii) addition of β -glucuronidase induced antiproliferative effects similar to the parent drug; (iii) this conjugate is 4-fold less toxic than the HMR-1826 derivative (same with no PEG-maleimide function) in the

absence of β -glucuronidase; (iv) *in vivo* studies of a murine lung carcinoma model proved the good antitumour response in the presence of this conjugate (better than that with maleimide) and (v) this conjugate does not exhibit nephrotoxicity like the parent drug doxorubicin.

The protein-conjugate approach was next utilised to administer another anticancer drug, monomethylauristatin E (MMAE), a highly toxic anticancer therapeutic that can only be administered when conjugated onto a monoclonal antibody (compound 38, Fig. 12). Using the same design as the previous example, this compound covalently binds to cysteinyl residues on serum albumin through Michael addition, facilitating the accumulation of the prodrug in tumours where extracellular β -glucuronidase triggers the release of the drug. Once again, it was confirmed that the parent drug was released in the presence of the glycosidase, becoming cytotoxic only after the drug was released. This prodrug had remarkable therapeutic efficacy on orthotopic triple-negative mammary and pancreatic tumours in mice, where there was a large reduction or disappearance of tumours, *e.g.* 50% and 33% of mice with respective tumours, were cured. Importantly, treatment with this drug delivery system did not induce any side effects.¹⁰⁴

To increase the efficacy of the targeting strategy, a dimeric β -glucuronidase-responsive albumin-binding prodrug of MMAE, compound 39, was developed. Compared to the previously mentioned monomeric MMAE conjugate, the dimeric prodrug contained a chemical amplifier which upon enzymatic activation releases two molecules of the anticancer drug.¹⁰⁵ This study represented the first *in vivo* studies of drug delivery systems containing these molecular constructs. The antitumour efficacy of the dimeric prodrug was tested in Balb/c athymic mice



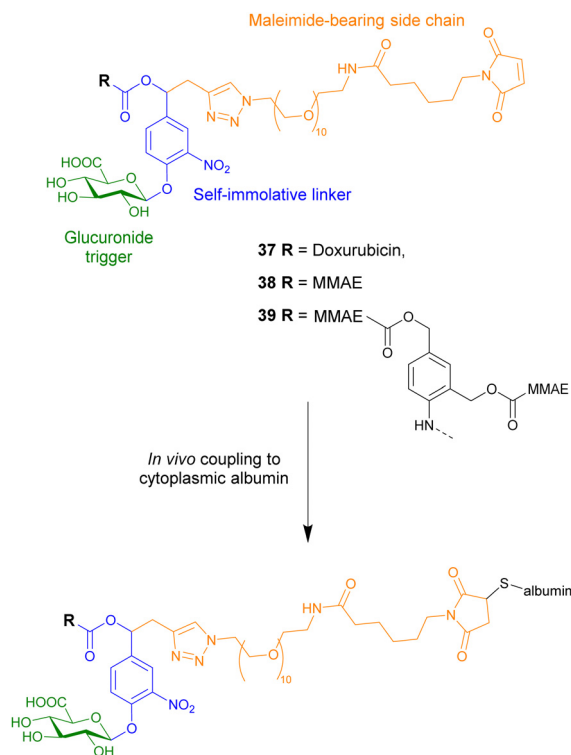


Fig. 12 Structure of three β -glucuronidase-responsive albumin-binding prodrugs **37–39**, showing how they couple to albumin.

bearing subcutaneous LS174T colorectal adenocarcinoma xenografts, a model known to exhibit reduced levels of β -glucuronidase. Although the tumour volumes decreased, the dimeric prodrug did not exhibit improved efficacy compared to the monomeric prodrug **38**.

To increase the potency of the monomeric conjugate **38**, a triple-loaded albumin conjugate (compound **40**, Fig. 13)

was investigated.¹⁰⁶ In line with previous studies, following intravenous administration, the maleimide unit of the prodrug reacted with the cysteine-34 of the plasmic albumin. The albumin conjugate then accumulated in malignant tissue where β -glucuronidase triggered the release of the drug. In this case, the enzymatic processes release three MMAE molecules into the extracellular space. The drugs then penetrate into surrounding cancer cells where they exerted their cytotoxicity. In the absence of β -glucuronidase, prodrug **40** was non-cytotoxic. However, in the presence of β -glucuronidase, prodrug **40** was more cytotoxic than the previously reported MMAE conjugate **38**. This drug delivery system was then administered to mice bearing intra-pancreatic MIA PaCa-2 tumours at 2.16, 4.32 or 6.48 mg kg⁻¹ (corresponding to 0.35, 0.7 or 1.1 μ mol kg⁻¹) per injection. The tumour volumes were recorded as a function of time by 3D echography. It was found that this compound had antitumour activity at all tested doses, without weight loss or side effects. A large reduction in tumour size was observed in animals treated at the two higher doses, with 1/8 and 3/8 mice, respectively, experiencing complete remission. When compared to the previous MMAE conjugate, the trimer **40** resulted in an 18-fold better reduction in tumour size compared to the monomer **38**.¹⁰⁶

Zelikin and co-workers also explored enzyme prodrug therapies by exploiting the enzyme-rich microenvironment of the tumour.¹⁰⁷ They set out to maximize the prodrug delivery to the tumour by engineering molecular, macromolecular, and supra-molecular prodrugs of the anticancer drug monomethyl auristatin E (MMAE). All prodrugs incorporated the *o*-substituted *p*-hydroxybenzyl alcohol scaffold, since it contains three suitable reactive sites at which to append the effector molecule (MMAE), the trigger for drug release (glucuronic acid) and a protraction arm. The protraction arm varied from PEG groups, such as 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE, compound **41**, Fig. 14), 4-(*p*-iodophenyl)butyric

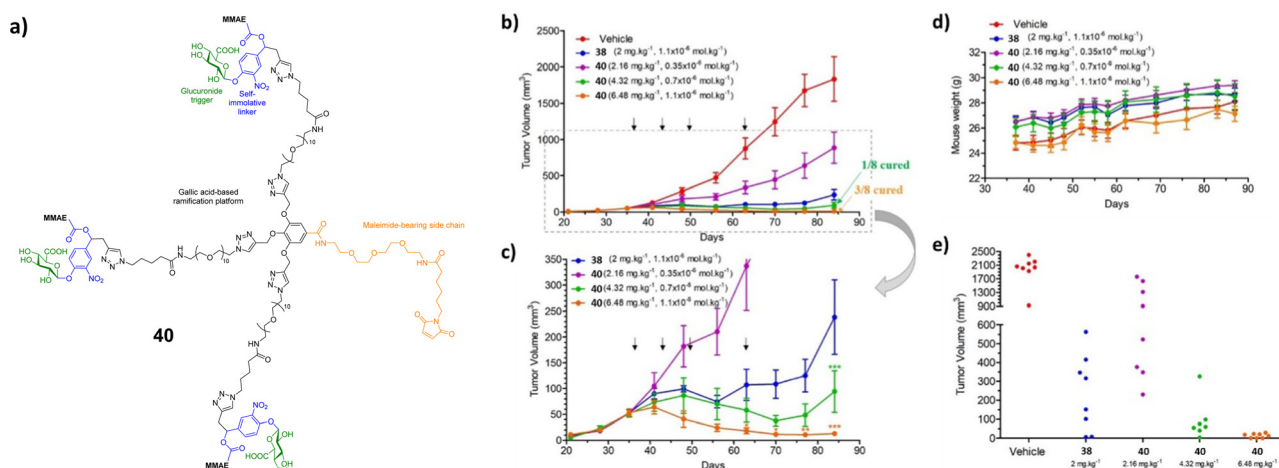


Fig. 13 (a) Structure of the triple-loaded albumin target **40**; (b) antitumour activity of the β -glucuronidase-responsive albumin-binding prodrug **40** on MIA PaCa-2 orthotopic models. MIA PaCa-2 tumour growth inhibition under therapy with vehicle, **38** (2.16, 4.32 and 6.48 mg kg⁻¹) and **40** (2 mg kg⁻¹). (c) MIA PaCa-2 tumour growth inhibition under therapy with **38** and **40**. (d) Mean body weights of each group of mice bearing MIA PaCa-2 xenografts. (e) Tumour volumes at day 84 of mice bearing MIA PaCa-2 xenografts treated with vehicle, **38** and **40**. Reprinted from ref. 106 with permission from Elsevier.



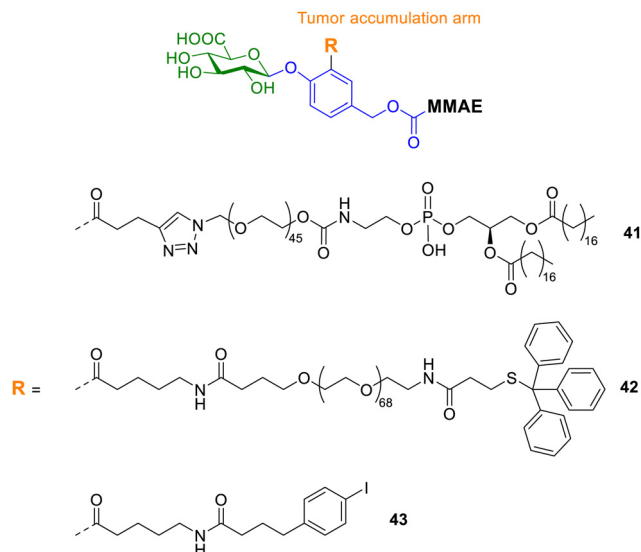


Fig. 14 Structure of supramolecular prodrugs **30–32** containing albumin-binding moieties.

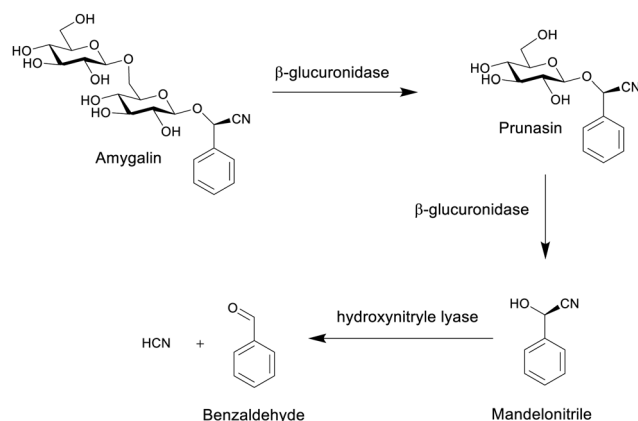
acid (AlbuTag, compound **43**), and the trityl group (Tr, compound **42**). All of the reported prodrugs masked the toxicity of the parent drug, which was released upon enzymatic bioconversion. However, only one prodrug exhibited significant anticancer effects *in vivo*. The Tr-PEG glucuronide prodrug **42** displayed statistically significant suppression of tumour growth, while a close analogue without an albumin-binding group did not. Conversely, prodrugs containing the other albumin-binding groups (DSPE and AlbuTag, compounds **41** and **43**) were not active; indicating the albumin affinity alone is not sufficient for success. Pharmacokinetic studies revealed that tumour accumulation was the only parameter that showed correlation with the *in vivo* anticancer effects.

2.3.4 Nanoparticles. Magnetic nanoparticles (MNP) can be utilized in magnetically directed enzyme/prodrug therapy (MDEPT). Starch-coated magnetic nanoparticles were conjugated with β -glucosidase (β -Glu) and polyethylene glycol (PEG). In these surface functionalised systems the β -glucosidase can be used to activate amygdalin (structure in Scheme 8), a natural cyanogenic glycoside that releases a toxic cyanide ion¹⁰⁸ that can kill prostate cancer cells. When the nanoparticles were subjected to an external magnetic field, they accumulated in targeted tumour tissue, preventing the accumulation in the liver and spleen, which would in normal circumstances lead to dangerous side effects. Using the combination therapy of PEG- β -Glu-MNP/amygdalin targeted activation of amygdalin was achieved and tumour growth in C57BL/6 mice bearing RM1 xenografts were inhibited.¹⁰⁹

To improve the activation of prodrugs *in vivo*, Guo and co-workers developed an innovative strategy for synthesising ketal glycoside prodrugs that have dual-activation properties. Glucosyl acetone-based ketal-linked glycoside prodrugs of etoposide (ETP) **44a–d** (Fig. 15a), a topoisomerase II inhibitor, underwent self-assembly into glucose-decorated nanoparticles. These

could then be internalized into tumour cells containing enzyme-rich acidic organelles, where they could subsequently be activated through hydrolysis of the glycosidic linkage by glycosidase activity or through hydrolysis of the ketal linkage by acid. This triggers the self-immolative hydrolysis of the other linkage, concomitantly releasing the drug, with the formation of both glucose and acetone as by-products (Fig. 15b). Four prodrugs were reported; the two α -linked isomers **44a** and **b**, and two β -linked isomers **44c** and **d** to the 2'' and 3''-hydroxyl groups of the ETP molecule. An A549 xenograft tumour model was used to assess the intratumoural accumulation and antitumour activity of the prodrugs. It was found that the pH and β -glucosidase sensitivity depended on the anomeric nature of the prodrugs, whereby the α -linked prodrug NPs only underwent acid-activated hydrolysis, whereas the β -linked prodrugs underwent hydrolysis by both acid and β -glycosidase. *In vivo* biodistribution studies showed that prodrug **44c** (3''-positional β -anomeric prodrug) NPs exhibited greater tumour accumulation than free ETP. Also, the NPs were successfully hydrolysed in tumour tissue and exhibited better efficacy in reducing tumour size than free ETP, which was confirmed using hematoxylin and eosin (H&E) and Ki67 immunohistochemical staining (Fig. 15c).¹¹⁰

As outlined earlier in Section 2.1.2, gene-directed enzyme prodrug therapy (GDEPT) has been utilised in successful cancer treatment to reduce harmful side effects and to improve pharmacokinetics. Using this approach, cancer cells are transfected with a plasmid containing the bacterial gene *lacZ*, which promotes the expression of β -galactosidase (Fig. 16). Dendrimer-like mesoporous silica nanoparticles (DMSNs) were loaded with a prodrug consisting of doxorubicin conjugated to a galactose residue through a self-immolative linker (structure similar to prodrug **6**). The DMSNs were then capped with a disulfide-containing polyethyleneglycol gatekeeper. It was confirmed that the prodrug remained in the nanoparticles until after the addition of glutathione (GSH), which cleaved the disulfide bonds and released the prodrug, which could then be activated by β -galactosidase. The combined treatment of β -galactosidase transfected cells and the DMSNs-prodrugs resulted in a more effective therapy than that observed for the prodrug alone.¹¹¹



Scheme 8 The formation of hydrogen cyanide from the hydrolysis of amygdalin.⁸⁰



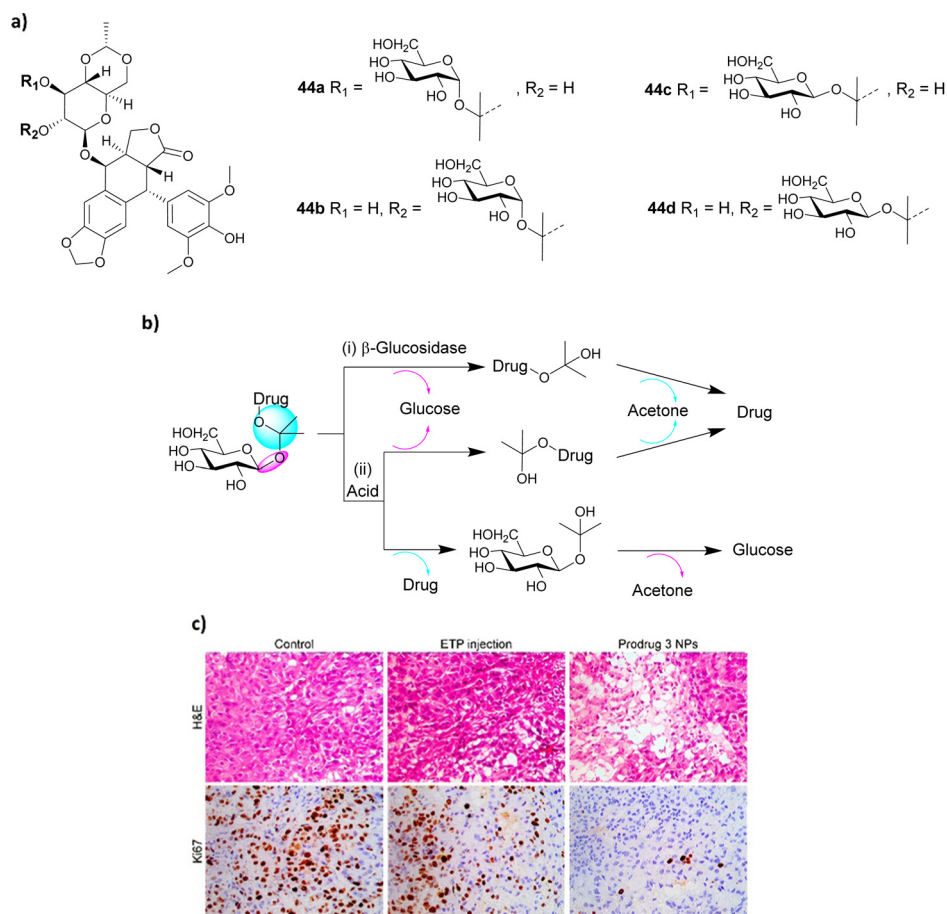


Fig. 15 (a) Structure of four prodrugs **44a–d**; (b) dual glycosidase- and acid-catalyzed self-immolative hydrolysis of ketal glycoside prodrugs; hydrolysis of the glycosidic or ketal linkage triggers hydrolysis of the other linkage, resulting in spontaneous self-immolation; (c) staining of cancer cells. Reprinted from ref. 110 with permission from the American Chemical Society.

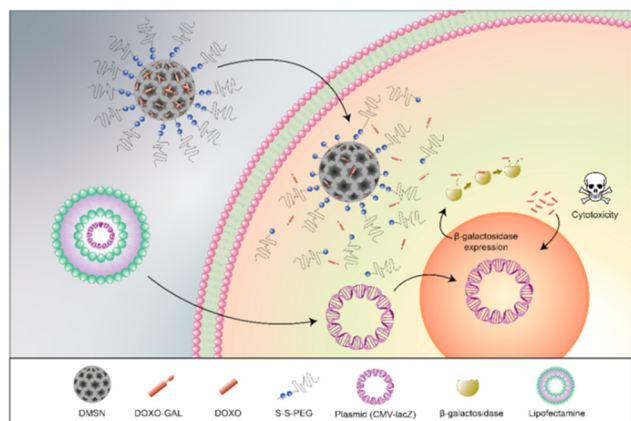


Fig. 16 Mechanism of action of the GDEPT system. Reprinted from ref. 111 with permission from MDPI.

3. Conclusion

The glycosidase activated prodrug approach has been utilised in many ways in recent years, incorporating various sugars, linkers and known anticancer drugs. The most common sugars

used in this strategy are glucuronic acid and galactose residues, as it is known that their corresponding glycosidases are highly expressed in the tumour microenvironment, allowing the release of the anticancer drugs at the desired site of action. Additional functionality has been incorporated into the simple glycoconjugates, including hydrophilic linkers and targeting ligands to aid in their aqueous solubility and their selectivity for cancer cells. More sophisticated approaches are now being developed that incorporate the prodrugs into supramolecular and macromolecular systems such as gels and nanoparticles. Also, to exploit the EPR effect, albumin-binding prodrugs have been developed that are retained in solid tumours, exerting their anti-cancerous effects in the desired site of action and reducing cytotoxic side effects. Herein, we have featured some select examples of such designs which demonstrate their importance and potential applications. The future direction of this area will undoubtedly continue to develop novel prodrugs incorporating more diverse anticancer agents. Utilising the ADEPT concept, more complex glycosides will also be incorporated into the prodrug design. New strategies will certainly be developed to target cancers more effectively thereby eliminating dangerous side effects.



Conflicts of interest

There are no conflicts to declare.

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Notes and references

- World Health Organisation, *Cancer*, <https://www.who.int/news-room/fact-sheets/detail/cancer>, accessed 20/01/2022, 2022.
- National Cancer Institute at the National Institutes of Health, *Cancer Statistics*, <https://www.cancer.gov/about-cancer/understanding/statistics>, accessed 20/01/2022, 2020.
- S. N. Khleif, O. Rixe and R. Skeel, *Skeel's Handbook of Cancer Therapy*, Wolters Kluwer Health, 2016.
- A. Albert, *Nature*, 1958, **182**, 421–423.
- V. J. Stella, W. N. A. Charman and V. H. Naringrekar, *Drugs*, 1985, **29**, 455–473.
- A. A. Sinkula and S. H. Yalkowsky, *J. Pharm. Sci.*, 1975, **64**, 181–210.
- H.-K. Han and G. L. Amidon, *AAPS PharmSci.*, 2000, **2**, 48–58.
- B. K. Brandley and R. L. Schnaar, *J. Leukocyte Biol.*, 1986, **40**, 97–111.
- O. Warburg, *Science*, 1956, **123**, 309–314.
- W. A. Denny, *Cancer Invest.*, 2004, **22**, 604–619.
- X. Zhou, Z. Huang, H. Yang, Y. Jiang, W. Wei, Q. Li, Q. Mo and J. Liu, *Biomed. Pharmacother.*, 2017, **91**, 504–509.
- Z. Li, D. Xu, X. Tong and C. Shan, *Clin. Res. Hepatol. Gastroenterol.*, 2021, **45**, 101456.
- Y. Zhang, K. Zhu, X. Miao, X. Hu and T. Wang, *Biomarkers*, 2016, **21**, 249–256.
- B. Suradej, S. Pata, W. Kasinrerker and R. Cressey, *Oncol. Rep.*, 2013, **30**, 2511–2519.
- Q. Lin, L. Pei, Z. Zhao, X. Zhuang and H. Qin, *BMC Cancer*, 2022, **22**, 817.
- Y. Qin, L. Zhao, X. Wang, D. Tong, C. Hoover, F. Wu, Y. Liu, L. Wang, L. Liu, L. Ni, T. Song and C. Huang, *Glycobiology*, 2017, **27**, 306–317.
- Z. Boussadia, J. Lamberti, F. Mattei, E. Pizzi, R. Puglisi, C. Zanetti, L. Pasquini, F. Fratini, L. Fantozzi, F. Felicetti, K. Fecchi, C. Raggi, M. Sanchez, S. D'Atri, A. Carè, M. Sargiacomo and I. Parolini, *J. Exp. Clin. Cancer Res.*, 2018, **37**, 245.
- S. K. Chatterjee, M. Bhattacharya and J. J. Barlow, *Cancer Res.*, 1979, **39**, 1943–1951.
- R. J. Bernacki, M. J. Niedbala and W. Korytnyk, *Cancer Metastasis Rev.*, 1985, **4**, 81–101.
- A. Krzeslak, L. Pomorski and A. Lipinska, *Int. J. Mol. Med.*, 2010, **25**, 643–648.
- C. Slawson, J. Pidala and R. Potter, *Biochim. Biophys. Acta, Mol. Basis Dis.*, 2001, **1537**, 147–157.
- X. Li, Y. Pan, H. Chen, Y. Duan, S. Zhou, W. Wu, S. Wang and B. Liu, *Anal. Chem.*, 2020, **92**, 5772–5779.
- J. Wagner, N. Damaschke, B. Yang, M. Truong, C. Guenther, J. McCormick, W. Huang and D. Jarrard, *PLoS One*, 2015, **10**, e0124366.
- H. B. Bosmann and T. C. Hall, *Proc. Natl. Acad. Sci. U. S. A.*, 1974, **71**, 1833–1837.
- P. Wielgat, U. Walczuk, S. Szajda, M. Bień, L. Zimnoch, Z. Mariak and K. Zwierz, *J. Neuro-Oncol.*, 2006, **80**, 243–249.
- K. Bosslet, R. Straub, M. Blumrich, J. Czech, M. Gerken, B. Sperker, H. K. Kroemer, J. P. Gesson, M. Koch and C. Monneret, *Cancer Res.*, 1998, **58**, 1195–1201.
- N. Albin, L. Massaad, C. Toussaint, M.-C. Mathieu, J. Morizet, O. Parise, A. Gouyette and G. G. Chabot, *Cancer Res.*, 1993, **53**, 3541–3546.
- P. H. J. Houba, E. Boven, I. H. V. D. Meulen-Muileman, R. G. G. Leenders, J. W. Scheeren, H. M. Pinedo and H. J. Haisma, *Br. J. Cancer*, 2001, **84**, 550–557.
- B. Sperker, U. Werner, T. E. Mürdter, C. Tekkaya, P. Fritz, R. Wacke, U. Adam, M. Gerken, B. Drewelow and H. K. Kroemer, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 2000, **362**, 110–115.
- S. Feng and J. D. Song, *World J. Gastroenterol.*, 1997, **3**, 251–252.
- K. Fujita, M. Kamiya, T. Yoshioka, A. Ogasawara, R. Hino, R. Kojima, H. Ueo and Y. Urano, *ACS Cent. Sci.*, 2020, **6**, 2217–2227.
- C. R. F. Silveira, M. Cipelli, C. Manzine, S. H. Rabelo-Santos, L. C. Zeferino, G. Rodríguez Rodríguez, J. B. de Assis, S. Hebster, I. Bernadinelli, F. Laginha, E. Boccardo, L. L. Villa, L. Termini and A. P. Lepique, *PLoS One*, 2019, **14**, e0213184.
- C. Li, L. Guo, F. Chen, W. Yu, T. Rao and Y. Ruan, *Oncol. Res. Treat.*, 2020, **43**, 264–275.
- S. D. Szajda, N. Waszkiewicz, A. Stypułkowska, J. Dadan and K. Zwierz, *Folia Histochem. Cytobiol.*, 2010, **48**, 351–357.
- E. C. Calvaresi and P. J. Hergenrother, *Chem. Sci.*, 2013, **4**, 2319–2333.
- P. Rosenbaum, C. Artaud, S. Bay, C. Ganneau, M. Campone, S. Delaloge, C. Gourmelon, D. Loirat, J. Medioni, F. Pein, M.-P. Sablin, O. Tredan, A. Varga and C. Leclerc, *Cancer Immunol. Immunother.*, 2020, **69**, 703–716.
- S. Nishat and P. R. Andreana, *Vaccines*, 2016, **4**, 19.
- E.-J. Kim, R. Kumar, A. Sharma, B. Yoon, H. M. Kim, H. Lee, K. S. Hong and J. S. Kim, *Biomaterials*, 2017, **122**, 83–90.
- T. Wirth, K. Schmuck, L. F. Tietze and S. A. Sieber, *Angew. Chem., Int. Ed.*, 2012, **51**, 2874–2877.
- F. M. de Groot, E. W. Damen and H. W. Scheeren, *Curr. Med. Chem.*, 2001, **8**, 1093–1122.
- B. Y. Lee, J. A. Han, J. S. Im, A. Morrone, K. Johung, E. C. Goodwin, W. J. Kleijer, D. DiMaio and E. S. Hwang, *Aging Cell*, 2006, **5**, 187–195.



- 42 H.-W. Liu, L. Chen, C. Xu, Z. Li, H. Zhang, X.-B. Zhang and W. Tan, *Chem. Soc. Rev.*, 2018, **47**, 7140–7180.
- 43 G. Jiang, G. Zeng, W. Zhu, Y. Li, X. Dong, G. Zhang, X. Fan, J. Wang, Y. Wu and B. Z. Tang, *Chem. Commun.*, 2017, **53**, 4505–4508.
- 44 T.-C. Cheng, S. R. Roffler, S.-C. Tzou, K.-H. Chuang, Y.-C. Su, C.-H. Chuang, C.-H. Kao, C.-S. Chen, I. H. Harn, K.-Y. Liu, T.-L. Cheng and Y.-L. Leu, *J. Am. Chem. Soc.*, 2012, **134**, 3103–3110.
- 45 L. Dong, M.-Y. Zhang, H.-H. Han, Y. Zang, G.-R. Chen, J. Li, X.-P. He and S. Vidal, *Chem. Sci.*, 2022, **13**, 247–256.
- 46 K. D. Bagshawe, *Expert Rev. Anticancer Ther.*, 2006, **6**, 1421–1431.
- 47 C. Fei Chin, D. Yuan Qiang Wong, R. Jothibas and W. Han Ang, *Curr. Top. Med. Chem.*, 2011, **11**, 2602–2612.
- 48 D. Gibson, *ChemMedChem*, 2021, **16**, 2188–2191.
- 49 Z. Wang, Z. Deng and G. Zhu, *Dalton Trans.*, 2019, **48**, 2536–2544.
- 50 P. L. Carl, P. K. Chakravarty and J. A. Katzenellenbogen, *J. Med. Chem.*, 1981, **24**, 479–480.
- 51 R. V. V. S. Edupuganti, D. A. J. Tyndall and B. A. Gamble, *Recent Pat. Anti-Cancer Drug Discovery*, 2021, **16**, 479–497.
- 52 S. Andrianomenjanahary, X. Dong, J. C. Florent, G. Gaudel, J. P. Gesson, J. C. Jacquesy, M. Koch, S. Michel, M. Mondon, C. Monneret, P. Petit, B. Renoux and F. Tillequin, *Bioorg. Med. Chem. Lett.*, 1992, **2**, 1093–1096.
- 53 F. L. Tietze and T. Feuerstein, *Curr. Pharm. Des.*, 2003, **9**, 2155–2175.
- 54 L. F. Tietze and K. Schmuck, *Curr. Pharm. Des.*, 2011, **17**, 3527–3547.
- 55 L. F. Tietze and B. Krewer, *Chem. Biol. Drug Des.*, 2009, **74**, 205–211.
- 56 R. Adidala, H. Devalapally, C. Srivari, K. Devarakonda R and A. Raghuram Rao, *Drug Dev. Ind. Pharm.*, 2012, **38**, 1047–1053.
- 57 T. Legigan, J. Clarhaut, B. Renoux, I. Tranoy-Opalinski, A. Monvoisin, C. Jayle, J. Alsarraf, M. Thomas and S. Papot, *Eur. J. Med. Chem.*, 2013, **67**, 75–80.
- 58 Z. M. Prijovich, P.-A. Burnouf, H.-C. Chou, P.-T. Huang, K.-C. Chen, T.-L. Cheng, Y.-L. Leu and S. R. Roffler, *Mol. Pharmaceutics*, 2016, **13**, 1242–1250.
- 59 T. Doura, K. Takahashi, Y. Ogra and N. Suzuki, *ACS Med. Chem. Lett.*, 2017, **8**, 211–214.
- 60 A. Sharma, E.-J. Kim, H. Shi, J. Y. Lee, B. G. Chung and J. S. Kim, *Biomaterials*, 2018, **155**, 145–151.
- 61 J. Li, Y. Zhu, M. Xie, Q. Zhang and W. Du, *Chem. Biol. Drug Des.*, 2019, **94**, 1760–1767.
- 62 X. Meng, X. Lian, X. Li, Q. Ya, T. Li, Y. Zhang, Y. Yang and Y. Zhang, *Carbohydr. Res.*, 2020, **493**, 108034.
- 63 E. Calatrava-Pérez, L. A. Marchetti, G. J. McManus, D. M. Lynch, R. B. P. Elmes, D. C. Williams, T. Gunnlaugsson and E. M. Scanlan, *Chem. – Eur. J.*, 2022, **28**, e202103858.
- 64 E. Calatrava-Pérez, S. A. Bright, S. Achermann, C. Moylan, M. O. Senge, E. B. Veale, D. C. Williams, T. Gunnlaugsson and E. M. Scanlan, *Chem. Commun.*, 2016, **52**, 13086–13089.
- 65 J. D. Hantho, T. A. Strayer, A. E. Nielsen and R. J. Mancini, *ChemMedChem*, 2016, **11**, 2496–2500.
- 66 A. J. Burt, J. D. Hantho, A. E. Nielsen and R. J. Mancini, *Biochemistry*, 2018, **57**, 2184–2188.
- 67 A. T. Ryan, A. J. Pulukuri, M. Davaritoucheae, A. Abbasi, A. T. Hendricksen, L. K. Opp, A. J. Burt, A. E. Nielsen and R. J. Mancini, *Acta Pharmacol. Sin.*, 2020, **41**, 995–1004.
- 68 J. Cao, S. Cui, S. Li, C. Du, J. Tian, S. Wan, Z. Qian, Y. Gu, W. R. Chen and G. Wang, *Cancer Res.*, 2013, **73**, 1362–1373.
- 69 K. Shimoda and N. Kubota, *Molecules*, 2011, **16**, 6769–6777.
- 70 Y. Mao, Y. Zhang, Z. Luo, R. Zhan, H. Xu, W. Chen and H. Huang, *Molecules*, 2018, **23**, 3211.
- 71 K. H. Carruthers, G. Metzger, M. J. During, A. Muravlev, C. Wang and E. Kocak, *Cancer Gene Ther.*, 2014, **21**, 434–440.
- 72 H. J. Schuster, B. Krewer, J. M. von Hof, K. Schmuck, I. Schuberth, F. Alves and L. F. Tietze, *Org. Biomol. Chem.*, 2010, **8**, 1833–1842.
- 73 L. F. Tietze, H. J. Schuster, B. Krewer and I. Schuberth, *J. Med. Chem.*, 2009, **52**, 537–543.
- 74 L. F. Tietze, H. J. Schuster, K. Schmuck, I. Schuberth and F. Alves, *Bioorg. Med. Chem.*, 2008, **16**, 6312–6318.
- 75 L. F. Tietze, K. Schmuck, H. J. Schuster, M. Müller and I. Schuberth, *Chem. – Eur. J.*, 2011, **17**, 1922–1929.
- 76 K.-C. Chen, K. Schmuck, L. F. Tietze and S. R. Roffler, *Mol. Pharmaceutics*, 2013, **10**, 1773–1782.
- 77 V. Oliveri, M. L. Giuffrida, G. Vecchio, C. Aiello and M. Viale, *Dalton Trans.*, 2012, **41**, 4530–4535.
- 78 V. Oliveri, M. Viale, G. Caron, C. Aiello, R. Gangemi and G. Vecchio, *Dalton Trans.*, 2013, **42**, 2023–2034.
- 79 Y. Ma, H. Chen, S. Su, T. Wang, C. Zhang, G. Fida, S. Cui, J. Zhao and Y. Gu, *J. Cancer*, 2015, **6**, 658–670.
- 80 G. Liu, V. Khanna, A. Kirtane, A. Grill and J. Panyam, *FASEB J.*, 2019, **33**, 9453–9465.
- 81 H. Ozawa-Umeta, A. Kishimoto, A. Imaizumi, T. Hashimoto, T. Asakura, H. Kakeya and M. Kanai, *Cancer Sci.*, 2020, **111**, 1785–1793.
- 82 L. Xu, X. Liu, Y. Li, Z. Yin, L. Jin, L. Lu, J. Qu and M. Xiao, *Appl. Microbiol. Biotechnol.*, 2019, **103**, 7997–8008.
- 83 L. F. Tietze, J. M. von Hof, M. Müller, B. Krewer and I. Schuberth, *Angew. Chem., Int. Ed.*, 2010, **49**, 7336–7339.
- 84 A. Guerrero, R. Guiho, N. Herranz, A. Uren, D. J. Withers, J. P. Martínez-Barbera, L. F. Tietze and J. Gil, *Aging Cell*, 2020, **19**, e13133.
- 85 E. González-Gualda, M. Pàez-Ribes, B. Lozano-Torres, D. Macías, J. R. Wilson Iii, C. González-López, H.-L. Ou, S. Mirón-Barroso, Z. Zhang, A. Lérída-Viso, J. F. Blandez, A. Bernardos, F. Sancenón, M. Rovira, L. Fruk, C. P. Martins, M. Serrano, G. J. Doherty, R. Martínez-Mañez and D. Muñoz-Espín, *Aging Cell*, 2020, **19**, e13142.
- 86 M. Grinda, J. Clarhaut, B. Renoux, I. Tranoy-Opalinski and S. Papot, *MedChemComm*, 2012, **3**, 68–70.
- 87 M. Grinda, J. Clarhaut, I. Tranoy-Opalinski, B. Renoux, A. Monvoisin, L. Cronier and S. Papot, *ChemMedChem*, 2011, **6**, 2137–2141.
- 88 B. Renoux, T. Legigan, S. Bensalma, C. Chadéneau, J.-M. Muller and S. Papot, *Org. Biomol. Chem.*, 2011, **9**, 8459–8464.



- 89 A. Balbous, B. Renoux, U. Cortes, S. Milin, K. Guilloteau, T. Legigan, P. Rivet, O. Boissonnade, S. Martin, C. Tripihana, M. Wager, R. J. Bensadoun, S. Papot and L. Karayan-Tapon, *Mol. Cancer Ther.*, 2014, **13**, 2159–2169.
- 90 S. Bensalma, C. Chadeneau, T. Legigan, B. Renoux, A. Gaillard, M. de Boisvilliers, C. Pinet-Charvet, S. Papot and J. M. Muller, *J. Mol. Neurosci.*, 2015, **55**, 51–61.
- 91 M. Thomas, J. Clarhaut, P.-O. Strale, I. Tranoy-Opalinski, J. Roche and S. Papot, *ChemMedChem*, 2011, **6**, 1006–1010.
- 92 T. Legigan, J. Clarhaut, I. Tranoy-Opalinski, A. Monvoisin, B. Renoux, M. Thomas, A. Le Pape, S. Lerondel and S. Papot, *Angew. Chem., Int. Ed.*, 2012, **51**, 11606–11610.
- 93 J. Alsarraf, E. Péraudeau, P. Poinot, I. Tranoy-Opalinski, J. Clarhaut, B. Renoux and S. Papot, *Chem. Commun.*, 2015, **51**, 15792–15795.
- 94 M. Grinda, T. Legigan, J. Clarhaut, E. Péraudeau, I. Tranoy-Opalinski, B. Renoux, M. Thomas, F. Guilhot and S. Papot, *Org. Biomol. Chem.*, 2013, **11**, 7129–7133.
- 95 P. López Rivas, C. Müller, C. Breunig, T. Hechler, A. Pahl, D. Arosio, L. Belvisi, L. Pignataro, A. Dal Corso and C. Gennari, *Org. Biomol. Chem.*, 2019, **17**, 4705–4710.
- 96 S. Kolodych, C. Michel, S. Delacroix, O. Koniev, A. Ekhkirch, J. Eberova, S. Cianférani, B. Renoux, W. Krezel, P. Poinot, C. D. Muller, S. Papot and A. Wagner, *Eur. J. Med. Chem.*, 2017, **142**, 376–382.
- 97 R. Barat, T. Legigan, I. Tranoy-Opalinski, B. Renoux, E. Péraudeau, J. Clarhaut, P. Poinot, A. E. Fernandes, V. Aucagne, D. A. Leigh and S. Papot, *Chem. Sci.*, 2015, **6**, 2608–2613.
- 98 J. Fang, H. Nakamura and H. Maeda, *Adv. Drug Delivery Rev.*, 2011, **63**, 136–151.
- 99 J. Y. Yhee, S. Son, S. Son, M. K. Joo and I. C. Kwon, in *Cancer Targeted Drug Delivery: An Elusive Dream*, ed. Y. H. Bae, R. J. Mersny and K. Park, Springer New York, New York, NY, 2013, pp. 621–632.
- 100 M. Talelli, K. Morita, C. J. F. Rijcken, R. W. M. Aben, T. Lammers, H. W. Scheeren, C. F. van Nostrum, G. Storm and W. E. Hennink, *Bioconjugate Chem.*, 2011, **22**, 2519–2530.
- 101 Sauraj, S. U. Kumar, P. Gopinath and Y. S. Negi, *Carbohydr. Polym.*, 2017, **157**, 1442–1450.
- 102 H. C. Besse, Y. Chen, H. W. Scheeren, J. M. Metselaar, T. Lammers, C. T. W. Moonen, W. E. Hennink and R. Deckers, *Pharmaceutics*, 2020, **12**, 536.
- 103 T. Legigan, J. Clarhaut, B. Renoux, I. Tranoy-Opalinski, A. Monvoisin, J.-M. Berjeaud, F. Guilhot and S. Papot, *J. Med. Chem.*, 2012, **55**, 4516–4520.
- 104 B. Renoux, F. Raes, T. Legigan, E. Péraudeau, B. Eddhif, P. Poinot, I. Tranoy-Opalinski, J. Alsarraf, O. Koniev, S. Kolodych, S. Lerondel, A. Le Pape, J. Clarhaut and S. Papot, *Chem. Sci.*, 2017, **8**, 3427–3433.
- 105 B. Renoux, L. Fangous, C. Hötten, E. Péraudeau, B. Eddhif, P. Poinot, J. Clarhaut and S. Papot, *MedChemComm*, 2018, **9**, 2068–2071.
- 106 R. Châtre, J. Lange, E. Péraudeau, P. Poinot, S. Lerondel, A. Le Pape, J. Clarhaut, B. Renoux and S. Papot, *J. Controlled Release*, 2020, **327**, 19–25.
- 107 M. T. Jarlstad Olesen, R. Walther, P. P. Poier, F. Dagnæs-Hansen and A. N. Zelikin, *Angew. Chem., Int. Ed.*, 2020, **59**, 7390–7396.
- 108 E. Jaszczak-Wilke, Ż. Polkowska, M. Koprowski, K. Owsianik, A. E. Mitchell and P. Bałczewski, *Molecules*, 2021, **26**, 2253.
- 109 J. Zhou, J. Hou, J. Rao, C. Zhou, Y. Liu and W. Gao, *Int. J. Nanomed.*, 2020, **15**, 4639–4657.
- 110 N. Yu, T. Liu, X. Zhang, N. Gong, T. Ji, J. Chen, X.-J. Liang, D. S. Kohane and S. Guo, *Nano Lett.*, 2020, **20**, 5465–5472.
- 111 V. Candela-Noguera, G. Vivo-Llorca, B. Díaz de Greñu, M. Alfonso, E. Aznar, M. Orzáez, M. D. Marcos, F. Sancenón and R. Martínez-Mañez, *Nanomaterials*, 2021, **11**, 1298.

