Cite this: *Med. Chem. Commun.*,
2019, 10, 1531

Prodrug strategies for targeted therapy triggered by reactive oxygen species

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Increased levels of reactive oxygen species (ROS) have been associated with numerous pathophysiological conditions including cancer and inflammation and the ROS stimulus constitutes a potential trigger for drug delivery strategies. Over the past decade, a number of ROS-sensitive functionalities have been identified with the purpose of introducing disease-targeting properties into small molecule drugs – a prodrug strategy that offers a promising approach for increasing the selectivity and efficacy of treatments. This review will provide an overview of the ROS-responsive prodrugs developed to date. A discussion on the current progress and limitations is provided along with a reflection on the unanswered questions that need to be addressed in order to advance this novel approach to the clinic.

Received 20th March 2019,
Accepted 3rd May 2019

DOI: 10.1039/c9md00169g

rsc.li/medchemcomm

Introduction

Reactive oxygen species (ROS) are transient oxygen intermediates typically produced by successive 1-electron reductions of O_2 .^{1,2} The most relevant ROS include the superoxide anion (O_2^-), hydroxyl radical ($HO^\bullet$), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), and singlet oxygen (1O_2). Under normal physiological conditions, ROS function as important signalling messengers in the regulation of cell growth, proliferation, differentiation, and adhesion properties as well as by participating in oxidative biosynthetic pathways and apoptosis.^{2–8} During immune responses, ROS also partake in the priming and recruitment of immune cells and are directly employed in host defence to eliminate foreign pathogens.^{3,7} Endogenous ROS arise primarily from three sources – the mitochondrial electron transport chain (Mito-ETC), the endoplasmic reticulum (ER) by flavoenzyme Ero1, and NADPH oxidases (NOXs) (Fig. 1).^{1,8}

Redox homeostasis is essential for proper cell function as the unspecific reactivity of ROS can cause oxidative damage to various biomolecules including lipids, proteins, and DNA. Homeostasis is achieved through regulation of ROS production and catabolism along with repair or scavenging of molecules damaged by ROS.⁹ Failure to regulate these pathways can lead to a state of oxidative stress that may cause irreversible damage or impaired cellular function. Indeed, oxidative stress has been linked to the progression of a number of pathophysiological

conditions¹⁰ including cancer,^{8,11,12} autoimmune disorders,^{13,14} inflammation,^{3,15,16} and cardiovascular¹⁷ and neurological diseases.^{18,19} Due to the highly reactive and transient nature of ROS, it has proven difficult to study and accurately quantify physiological concentrations of these species. Nonetheless, studies on H_2O_2 , the most stable ROS, have estimated the extracellular concentrations of healthy cells to be in the range of 0.5–7 μM . In contrast, under pathophysiological conditions, it may be up to 100-fold higher with measurements as high as 1.0 mM.^{6,20–24} This has prompted researchers to

Fig. 1 Simplified schematic illustration of cellular redox homeostasis. Reactive oxygen species (ROS) mainly arise from three major sources – NADPH oxidases (NOXs), the mitochondrial electron transport chain (Mito-ETC), and the endoplasmic reticulum (ER) produced by flavoenzyme Ero1. Superoxide (O_2^-) is the first species formed and can be converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) both intra- and extracellularly. H_2O_2 can be further converted into hypochlorous acid ($HOCl$) by myeloperoxidase (MPO) or in the presence of transition metals such as Fe^{2+} to highly reactive hydroxyl radicals ($HO^\bullet$). Catalase is responsible for degrading H_2O_2 to water.

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Fig. 2 Schematic illustration of the ROS-triggered prodrug concept. The drug is masked with a ROS-sensitive promoiety (PM) and the prodrug will ideally become inactive until exposure to increased levels of ROS which is associated with various pathological conditions.

develop new strategies that target these pathological characteristics in the hope of improving drug selectivity. One approach includes the development of ROS-responsive prodrugs that are locally activated upon stimuli from increased concentrations of ROS. Prodrugs are inactive forms of pharmaceuticals that upon chemical or enzymatic activation *in vivo* release the active drug.^{25,26} Approximately 10% of all marketed drugs are considered prodrugs and it is a common strategy for improving inadequate pharmacokinetic properties of drugs, in particular poor solubility or absorption.²⁵ However, the prodrug concept may also be exploited for improving selectivity through tissue-targeting properties (Fig. 2).

The scope of this review is to give a general overview of the ROS-triggered prodrug strategies for small molecules and biopharmaceuticals developed to date. The review will discuss the current progress of this approach along with limitations and future perspectives in the field. The concept of ROS-responsive groups has also been widely applied to drug delivery platforms and for imaging purposes; however it is beyond the scope of this review to include these areas. Instead, we will refer to recent and comprehensive reviews on drug delivery^{27,28} and imaging²⁹ systems.

Prodrugs

(Aryl)boronic acids and esters

Hydrogen peroxide is the most stable ROS and therefore it is found in relevant concentrations *in vivo*. H_2O_2 has been widely employed for chemical transformations as a two-electron oxidant and its reaction with arylboronic acids and esters is well established.³⁰ The mechanism involves oxidation of the B–C bond *via* coordination of H_2O_2 to the boron atom followed by aryl bond migration to form an intermediate boronate (Scheme 1). The boronate rapidly hydrolyses in water to generate a phenol and boric acid/ester, which has proven to be innocuous in humans.³¹ This reaction step already shows the potential use of arylboronates as promoieties to mask phenols in drug molecules. When the phenol is part

of a self-immolative linker such as 4-hydroxybenzyl ether, amine, carbamate, or carbonate (among others),³² it can release the biologically active compound together with quinone methide (QM) *via* 1,6-elimination. QM is rapidly converted into 4-hydroxybenzyl alcohol (HBA) by water (Scheme 1). The overall reaction and the products formed make arylboronates bioorthogonal and chemoselective chemical probes for prodrug approaches.³³ It is worth mentioning that the reactive nitrogen species peroxynitrite (PNT) has been shown to activate arylboronic acid and derivatives *via* the same mechanism but up to a million-fold faster than H_2O_2 .³⁴ This section reviews the use of arylboronic acid derivatives for ROS-activated prodrug strategies.

Boronic acids and esters have been widely investigated as protecting groups activated by H_2O_2 for fluorescent probes and prochelators, but it was not until 2010 when Cohen and co-workers reported their first use in a ROS-activated prodrug strategy.³⁵ In their work, arylboronic esters were coupled to two matrix metalloproteinase inhibitors (MMPis) for use as protective therapeutics following ischemia–reperfusion injury after stroke. By coupling the promoiety to the metal binding group of the MMPis 1,2-HOPO-2 and Py-2, they obtained prodrugs 1 and 2, respectively (Table 1). They were able to show significant attenuation of their inhibitory properties against MMP-9 and MMP-12 in a fluorescent-based assay and the release of the inhibitors as well as efficacy comparable to the parent compounds under oxidative conditions (100 mM H_2O_2 , 10 equiv.).³⁵

Since the seminal publication of ROS-activated prodrugs by Cohen and co-workers, an increasing number of scientific reports have been published.

Alkylating agent prodrugs. In 2011, Peng and co-workers reported the first example of a ROS-activated nitrogen mustard anticancer prodrug.³⁶ An arylboronic acid and an ester were linked to mechlorethamine obtaining prodrugs 3 and 4, respectively (Table 1). The efficient formation of interstrand cross-links (ICLs) in a 49-mer DNA duplex in the presence of H_2O_2 was observed, notably without the generation of QM DNA-alkylation products. Moreover, the cross-linking efficacy of the prodrugs in the presence of 0.5 equiv. of H_2O_2 (from 50 μM to 2.0 mM) was identical to that of the parent nitrogen mustard. The group also achieved an *in vitro* proof-of-concept by showing the cell growth inhibitory properties of the prodrugs in leukemia (SR), lung (NCI-H460), and renal (CAKI-1 and SN12C) cancer cell lines, while being inactive against

Scheme 1 Mechanism for the oxidation of arylboronic acids with H_2O_2 .

human lymphocytes.³⁶ Following the study of the ROS-activated mechlorethamine prodrugs, the same group developed aromatic nitrogen mustard prodrugs such as 5 and 6 (Table 1).^{37,38} The new prodrug strategy consisted of modifying the electronics of the aromatic ring by linking the promoity to the nitrogen mustard *via* an electron-withdrawing carboxamide linker (5) or directly linking the effector boronic acid as the electron-withdrawing group (6). Because the electron density of the aromatic ring is decreased, the formation of the cytotoxic aziridinium intermediate is suppressed. Once activated by H₂O₂, the prodrugs are transformed into electron-rich aromatic nitrogen mustards. The highest *in vitro* cell activity was observed for those prodrugs with neutral linkers and boronic acid triggers (instead of the esters) due to the increased cell permeability and solubility, respectively.^{37,39} A third generation nitrogen mustard prodrug, where the aryl ring was linked to an amino acid sidechain for increased solubility and permeability, was developed and the lead compound 7 (cysteine methyl ester derivative, Table 1) was identified. This prodrug showed impressive

tumour growth inhibition in a breast cancer (MDA-MB-468) mouse xenograft model, achieving an *in vivo* proof-of-concept for the ROS-activated nitrogen mustard prodrug strategy.³⁸

QMs are well known cross-linking agents that Peng and co-workers exploited their use in the ROS-activated arylboronic acid prodrug strategy. They thoroughly evaluated the influence of the leaving group on the formation of QMs in prodrugs 8–10 (Table 1),^{40,41} concluding that the solubility, the leaving group character, and the step-wise formation of the reactive QMs were fundamental for the efficiency of ICLs. Therefore, the most efficient ICL in a 49-mer DNA duplex was achieved with the combination of leaving group properties and solubility of prodrug 10. It was not until 2017 when the second generation QM prodrugs 11–13 (Table 1) were developed, achieving *in vitro* cell growth inhibition in most cancer cell lines of the NCI-60 panel, while displaying no cytotoxicity in lymphocytes from healthy donors.⁴² Their neutral character and the substituents in the aromatic ring improved cell penetration and afforded more efficient ICL. Unfortunately, to date no *in vivo* efficacy has been reported for arylboronic QM-prodrugs.

Table 1 Arylboronic acid based-prodrugs 1–13

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
1, 2	MMPis	Ischemia and reperfusion injury associated with stroke	Inhibition of MMP-9 and MMP-12 enzymes	—	S. M. Cohen	35
3, 4	Mechlorethamine	Cancer	Cell growth inhibition of leukemia (SR), lung (NCI-H460), and renal (CAKI-1 and SN12C) cells	—	X. Peng	36
5–7 ^a	Cross-linking agent aromatic nitrogen mustards	Cancer	Cell growth inhibition against +50 cancer cell lines	Efficacy in a breast (MDA-MB-468) tumour xenograft mouse model; toxicity studies in mice	X. Peng	37–39
8–13	Bis-QM – interstrand cross-linking agent	Cancer	Cell growth inhibition in the NCI-60 panel	—	X. Peng	40–42

^a Stereochemistry was not described in the original reference.

Aminoferrocene-based prodrugs. In 2011, Mokhir and co-workers published their first work on ROS-activated prodrugs of aminoferrocene derivatives.⁴³ Under oxidative conditions, aminoferrocenes are transformed into aminoferrocenium ions, which decompose to liberate Fe^{2+} ions. These are efficient catalysts of ROS production and can therefore induce oxidative stress and ultimately cell death. The so-called arylboronic ester

aminoferrocene dual prodrugs are capable of simultaneously inducing catalytic generation of ROS (aminoferrocenium and iron ions) and inhibiting the antioxidant system of cells (glutathione scavenging properties of QMs). The first generation prodrugs 14 and 15 (Table 2) proved their potential in cancer therapy by the study of their cytotoxicity against human promyelocytic leukemia (HL-60) and human glioblastoma-

Table 2 Arylboronic acid based-prodrugs 14–26

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
14–17, 20	QM, aminoferrocenium/ Fe^{2+}	Cancer	Cytotoxicity against human promyelocytic leukemia (HL-60), human glioblastoma-astrocytoma (U373), chronic lymphocytic leukemia (CLL), lymphoma (BL-2), ovarian (A2780) and prostate cancer (LNCaP and DU-145) cells	Efficacy studies on mice carrying L1210 leukemia, in a prostate cancer (LNCaP) xenograft murine model and inhibition of growth of Guerin's carcinoma (T8). Accumulation in ascites of NK/Ly-lymphoma mice Preliminary toxicity evaluation in treated mice, acute toxicity in rats and mice for a 21 day treatment. <i>Ex vivo</i> toxicity in the liver	A. Mokhir	43–49
18–19	QM, aminoferrocenium/ Fe^{2+} and Pt(II) complexes	Cancer	Cytotoxicity against ovarian cancer cell lines A2780 and cisA2780	—	A. Mokhir	50, 51
21–24, 26	4-OHT, endoxifen, and GWK7604	Cancer	Cytotoxicity against breast (MCF-7 and T47D) cancer cells	Efficacy in a breast cancer (MCF-7) xenograft mouse model and acute toxicity in treated animals pharmacokinetic studies in mice	G. Wang	52–54
25	Fulvestrant	Cancer	Inhibition of cell proliferation in MCF-7 and T47D cells	Efficacy in MCF-7 xenograft and patient derived xenograft (PDX) mouse models. Preliminary toxicity evaluation in treated mice metabolism and pharmacokinetic studies in mice	G. Wang	55, 56

astrocytoma (U373) cell lines, while being non-toxic to human fibroblasts. Compound 14 released iron ions, while *N*-alkylated analogues like 15 formed more stable aminoferrocenium products also capable of catalyzing the formation of ROS. Second generation prodrugs like 16 aimed for a stronger inhibition of the antioxidant system of cells by liberating two QM products per aminoferrocene complex (Table 2).⁴⁵ The improved efficacy was shown in HL-60 cells and chronic lymphocytic leukemia (CLL) cells. Moreover, no cytotoxicity was observed in both healthy mononuclear cells and bacterial cells, important cells for normal body function. Prodrug 15 was selected for toxicity and efficacy studies *in vivo* on healthy and L1210 leukemia carrying mice, respectively.⁴⁷ No toxicity was observed and 15 significantly extended the survival rate of leukemic mice. A separate study reported the potential use of 14 and 15 in a prostate cancer (LNCaP) murine xenograft model.⁴⁸ In 2018, the improved membrane permeability and increased solubility of a novel aminoferrocene prodrug 17 demonstrated its potential application as an anticancer prodrug in an *in vivo* xenograft model of Guerin's carcinoma (T8) by suppressing tumour growth (Table 2).⁴⁴

More sophisticated three-component systems were developed in 2017 and 2018 by Mokhir and co-workers. Aminoferrocene arylboronic esters were coupled to Pt(IV) complexes that upon activation released cisplatin or oxaliplatin, prodrugs 18 and 19, respectively (Table 2).^{50,51} After H₂O₂-activation, the resulting aminoferrocenes can donate two electrons to the acceptor Pt(IV) complex, liberating the Pt(II) drugs together with two aminoferrocenium complexes. The prodrugs act synergistically: 1) QMs scavenge the antioxidant system of cells, 2) aminoferrocenes generate ions able to catalyze the generation of ROS, and 3) the locally released Pt(II) complexes exert their cytotoxic activity selectively in targeted cancer cells. Using this strategy, the group proved the efficacy of the prodrugs not only in an ovarian cancer cell line (A2780) but also in a cisplatin-resistant cell line (A2780cis). Importantly, the prodrugs did not display cytotoxicity to human fibroblasts. Further application of aminoferrocene arylboronic esters as prodrugs of *e.g.* delocalized lipophilic cations for targeting mitochondria and lysosomes has been developed and proved efficacious in different animal models. Attachment of fluorescein to an arylboronic ester aminoferrocene prodrug, 20 (Table 2), showed accumulation of the prodrug system in ascites of NK/Ly-lymphoma carrying mice.⁴⁹

Estrogen receptor modulator and downregulator prodrugs. Selective estrogen receptor modulators (SERMs) and downregulators (SERDs) like tamoxifen and fulvestrant, respectively, are commonly employed drugs for the treatment of breast cancer. In humans, tamoxifen undergoes *in vivo* metabolism to form 4-hydroxytamoxifen (4-OHT) and endoxifen (*N*-desmethyl-4-hydroxytamoxifen), metabolites with increased potency. In 2012, Wang and co-workers applied boronic acids as a promoiety for prodrugs of 4-OHT, aiming at selective delivery and increased uptake by cancer cells.⁵² The three prodrugs investigated (21–23, Table 2) bore either boron pinacolate (–Bpin), boronic acid, or potassium trifluoroborate

promoieties to mask the hydroxyl group in 4-OHT and endoxifen. They reported the inhibitory cell growth properties of the prodrugs against breast cancer cell lines (MCF-7 and T47D) expressing estrogen receptors (ER+), while being inactive in ER– breast cancer cells (MDA-MB231). Despite the interesting results reported, their following four publications on boronic acid prodrugs of SERMs and SERDs did not mention the goal of selective delivery to cancer cells. Instead, the improved bioavailability compared to the parent drugs was the main focus. The hydroxyl group in 4-OHT, endoxifen, and fulvestrant is susceptible to phase II metabolism to form polar glucuronates. Therefore, masking this functional group with a boronic acid can potentially improve oral bioavailability. The prodrug of endoxifen 24 (Table 2) inhibited cell growth in ER+ breast cancer cells and displayed efficient conversion to endoxifen by oxidative deboronation *in vitro*.⁵³ The metabolic conversion to endoxifen was also efficient *in vivo* when administered to mice. Most interestingly, the prodrug achieved 80-fold increased exposure and 40-times higher plasma concentration over the parent drug. Additionally, 24 retarded tumour growth in a breast (MCF-7) cancer xenograft mouse model. A similar study of a boronic acid prodrug of fulvestrant (25, Table 2) showed efficacy in two mouse xenograft models (MCF-7 and patient derived xenograft) and an improved oral pharmacokinetic (PK) profile,⁵⁵ and demonstrated that fulvestrant was the major oxidative metabolite in mouse and rat oral PK studies.⁵⁶ A similar observation was made for the SERD prodrug 26 in 2016.⁵⁴

Miscellaneous anticancer prodrugs and theranostic agents. In 2014, Kim and co-workers developed the theranostic agent 27 (Table 3), where the antimetastatic metabolite of irinotecan SN-38 was coupled to a boronate ester masked fluorophore (coumarin) as the H₂O₂-triggered unit to monitor prodrug activation. By detection of coumarin release under incubation with H₂O₂, they proved activation of 27 as well as its localization to lysosomes in mouse melanoma (B16F10) and human cervical cancer (HeLa) cells. The prodrug candidate showed antiproliferative activity against the two cell lines and prolonged survival time in a metastatic lung tumour model (B16F10).³⁰ Also worth mentioning is the work by Lu and co-workers, in which the boronic acid was directly attached to SN-38, masking the hydroxyl group of the drug (not shown), proving activity in both *in vitro* and *in vivo* cancer models.⁵⁷ Later in 2014, Kim and co-workers published the more advanced four-component mitochondria-targeting antitumour theranostic prodrug 28 (Table 3). Construct 28 contained a fluorophore (ethidium), two tumour-targeting units (biotin), two ROS-activators (arylboronates) and two drug molecules (5'-DFUR, precursor of 5-FU).²² Interestingly, attachment to the ROS-promoiety by boronate conjugation was possible because of the 5'-deoxy-ribose ring of 5'-DFUR. Preferential uptake and enhanced cytotoxicity in biotin receptor-positive human lung tumour cells (A549) over receptor negative cells (WI-38) were observed, together with *in vitro* localization in target organelles. In an *in vivo* A549-inoculated murine xenograft model, 28 displayed enhanced

Table 3 Arylboronic acid based-prodrugs 27–32

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
27	SN-38	Cancer	Antiproliferative activity in mouse melanoma (B16F10), and human cervical (HeLa) cells	Efficacy in a lung mouse metastasis (B16F10) model	J. S. Kim	30
28	Doxifluridine	Cancer	Cytotoxicity against human lung (A549) tumour cells	Efficacy in a lung (A549) xenograft mouse model, organ distribution, and tumour targeting specificity studies. <i>Ex vivo</i> imaging of tumours	J. S. Kim	22
29, 30	Nitric oxide	Cancer	Cytotoxicity against lung (A549), human cervical (HeLa), and breast (MDA-MB-231) cancer cells	—	H. Chakrapani	58, 59
31	Doxorubicin	Cancer	Cytotoxicity in human cervical (HeLa) cells	—	B. Z. Tang	60
32	Cinnamaldehyde and QM	Cancer	Cytotoxicity against prostate (DU145) and colon (SW620) cancer cells	Efficacy in xenograft models of prostate (DU145) and colon (SW620). <i>Ex vivo</i> tumour analysis for drug accumulation studies	D. Lee	61

fluorescence in tumour tissue and *ex vivo* analysis of tumours demonstrated preferential uptake of the prodrug over other organs. Moreover, impressive tumour growth inhibition was observed in the prodrug-treated mice group.

Since nitric oxide is associated with nitrosative stress and cell growth inhibition, Chakrapani and co-workers developed the nitric oxide donor arylboronic ester prodrug 29 (Table 3). In their initial report in 2014, they proved prodrug activation under incubation with H_2O_2 as well as intracellular activation

in bacterial cells.⁵⁸ In 2017, they coupled the same NO-donor to a fluorophore with a masked phenol as an arylboronic ester to form 30. This NO-donor prodrug/theranostic agent underwent activation under oxidative conditions as well as preferential activation in lysosomes when applied to H_2O_2 -pretreated HeLa cells and catalase knock-down HeLa cells. Cytotoxicity in A549, MDA-MB 231, and HeLa cells was reported while there was significantly lower toxicity towards human fibroblasts.⁵⁹

Early in 2018, Tang and co-workers developed a three-component theranostic prodrug 31 (Table 3) based on carboxylated tetraphenyl-ethene (TPE) as the fluorescent probe, benzylboronic ester as the trigger unit, and doxorubicin as the drug cargo.⁶⁰ *In vitro* cytotoxicity as well as accumulation of carboxylated-TPE in the cytoplasm of HeLa cells following chemically induced H₂O₂ generation was observed, accompanied by detection of doxorubicin in the nucleus. In 2015, Lee and co-workers developed a hybrid anticancer agent combining the effects of cinnamaldehyde as a ROS inducer and the reaction of QM with the antioxidant glutathione (GSH). The prodrug candidate 32 (Table 3) induced increased ROS concentrations in cancer cells after exposure as well as decreased GSH levels. Positive *in vitro* growth inhibition against prostate (DU145) and colon (SW620) cancer cells and a lack of cytotoxicity for non-malignant fibroblasts encouraged efficacy studies in two xenograft models of the cancer cell lines. Reduced tumour volumes after treatment with 32 in the two models were observed.

In 2018, two studies on prodrugs of the histone deacetylase inhibitors (HDACis) belinostat and vorinostat, prodrugs 33 and 34 (among other analogues), were developed by Wang and co-workers and Qin and co-workers, respectively (Table 4).^{62,63} In both studies, the hydroxamic acid zinc-binding domain of HDACis was masked with an arylboronic ester/acid as the ROS-selective promoiety. Cytotoxicity studies against breast (MDA-MB-231), lung (A549 and NCI-H460), and skin (SK-MEL-28) cancer cells using 33 showed generally lower efficacies than the parent drug belinostat.⁶² This was explained as a result of partial activation of 33 to form belinostat *in vitro*. Previous studies from the same group demonstrated the improved oral bioavailability of boronic acid prodrugs, encouraging the evaluation of the efficacy in a mouse breast xenograft model (MCF-7). Both belinostat and 33 inhibited tumour growth in the *in vivo* assay but more importantly, the animals treated with prodrug 33 achieved tumour remission after two weeks, unlike belinostat treated mice. Measurement of the concentrations of belinostat and the prodrug in tumour tissue proved the accumulation of the prodrug at the target site as well as a higher concentration of belinostat in the 33-treated group. These results supported the improved biocompatibility and bioavailability of 33 over the parent drug. In the study by Qin and co-workers on 34 (and analogues), activation of the prodrug by both H₂O₂ and PNT was confirmed *in vitro*, exhibiting a faster activation when using the RNS in agreement with previous studies.⁶³ The antileukemic activity in AML cells (U937 and MV4-11) was confirmed and attributed to vorinostat release with a positive contribution from the generated QMs.

The application of arylboronic acid promoieties to bio-pharmaceuticals was first explored by Xu and co-workers in 2014. Promoieties were linked *via* a carbamate bond to lysine residues in protein ribonuclease A (RNase A), an enzyme that cleaves RNA and induces cytotoxic effects.⁶⁴ This pro-RNase A 35 (Table 4) bore an average of seven promoieties per enzyme

as determined by mass spectrometry, masking lysine residues essential for its biological activity. Reduced enzymatic activity and improved cytotoxicity against skin melanoma (B16F10) cancer cells when chemically stimulated to produce H₂O₂ were among the results obtained.

Arylboronic acid and ester prodrugs for other indications. In 2018, Raines and co-workers published the only other reported biopharmaceutical prodrug 36 (Table 4), a prodrug of angiogenin, the enzyme compromised in amyotrophic lateral sclerosis (ALS) patients.⁶⁵ Using recombinant DNA methods, they replaced Lys40 by a cysteine and functionalized the latter for attachment of the boronic acid trigger unit. Exposure to H₂O₂ re-established enzymatic activity and induced endothelial cell proliferation *in vitro* as well as oxidative stress neuroprotection in astrocytes.

p-Hydroxybenzyl alcohol (HBA) is the major component of the herb *Gastrodia elata*, which is commonly used for the treatment of inflammatory diseases in traditional medicine.⁷¹ This alcohol, the main product of the reaction of *para*-QMs with water, is an antioxidant with an important protective role against oxidative stress-associated diseases like ischemic brain injury and coronary heart disease. Lee, Kang, and co-workers synthesized and evaluated HBA-prodrug 37 (Table 4).⁶⁶ They proved the activation of the prodrug and its ability to inhibit ROS production in LPS- and H₂O₂-activated macrophages (RAW264.7). Cellular protection from H₂O₂ in rat cardiomyocytes was also demonstrated *in vitro*. The anti-inflammatory properties of 37 were confirmed by measuring reduced levels of the pro-inflammatory cytokine TNF- α . The study of the efficacy in a mouse model of hepatic and cardiac ischemia reperfusion (I/R) injury showed the strong protective effects (anti-inflammatory and antiapoptotic activities and reduced levels of H₂O₂) of 37 as compared to HBA. The prodrug accumulated in diseased tissues over other organs and demonstrated an excellent safety profile in mice at therapeutic doses.

Hydrogen sulfide (H₂S) is an important cellular signaling molecule that protects cells against ROS-associated damage in inflammation.⁷² In 2016, Pluth and co-workers developed ROS-triggered H₂S-donors based on thiocarbamate functionalized arylboronates, compounds 38–40 (Table 4).⁶⁷ Upon incubation with H₂O₂, the arylboronates released carbonyl sulfide (COS) which is transformed by carbonic anhydrase (CA, a ubiquitous enzyme in cells) to generate H₂S. The H₂S-donors were activated in cell cultures both by exogenous H₂O₂ in HeLa cells and *via* chemically induced endogenous generation of H₂O₂ in macrophages. The prodrugs also exhibited cytoprotective effects against ROS-damage in HeLa cells treated with cytotoxic concentrations of H₂O₂. In a later report, the same group performed a detailed study on the kinetics of carbonyl sulfide donors based on thiocarbamates, thiocarbonates, and dithiocarbonates. Among them was the carbamothioate compound 41 (Table 4), which proved its potential as a H₂S-donor prodrug.⁶⁸ Chakrapani and co-workers further developed analogues of 41, including the attachment of the anti-inflammatory drug mesalamine (not shown),

Table 4 Arylboronic acid based-prodrugs 33–43

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
33, 34	Belinostat and vorinostat	Cancer	Cytotoxicity against breast (MDA-MB-231), lung (A549 and NCI-H460), skin (SK-MEL-28) and AML cells (U937 and MV4-11)	Efficacy in a breast cancer (MCF-7) xenograft model and preliminary toxicity in treated mice	Z. Qin and G. Wang	62, 63
35	RNase	Cancer	Cytotoxicity against skin melanoma (B16F10) cells	—	Q. Xu	64
36	Angiogenin	Amyotrophic lateral sclerosis	Endothelial cell proliferation and neuroprotection of astrocytes	—	R. T. Raines	65
37	HBA	Ischemia-reperfusion injury	Anti-apoptotic, antioxidant, and anti-inflammatory properties in macrophages (RAW264.7) and cardiomyocytes	Efficacy in mouse models of cardiac and hepatic I/R injury. Cumulative toxicity studies. <i>Ex vivo</i> analysis for accumulation of the drug in the liver	D. Lee and P. M. Kang	66
38–41	Carbonyl sulfide and hydrogen sulfide	Cytoprotection under ROS-induced damage conditions	Cytoprotection against ROS-induced damage in human cervical (HeLa) cells and macrophages	—	M. D. Pluth and H. Chakrapani	67–69
42, 43	Aminopterin (AMT) and methotrexate (MTX)	Rheumatoid arthritis	Cytotoxicity in lung (NCI-H460) and breast (MCF-7) cancer cells	Efficacy in a collagen-induced arthritis (CIA) murine model and preliminary toxicity in treated mice	M. H. Clausen	70

additionally demonstrating the cytoprotective use of this class of prodrugs.⁶⁹

In 2018, Clausen and co-workers coupled the disease modifying antirheumatic drugs (DMARDs) aminopterin (AMT) and methotrexate (MTX) to arylboronic acid promoieties to form prodrugs 42 and 43, respectively (Table 4). The different linker (N–C bond *vs.* carbamate) and leaving group properties of the released drugs were shown to influence the rate of H₂O₂-mediated prodrug activation. *In vitro* cell viability in

breast (MCF-7) and lung (NCI-H460) cancer cell lines was used as a proof-of-concept for the efficacy of the prodrugs, since AMT and MTX are also used as antifolates in cancer therapy. Evaluation of the solubility as well as chemical and metabolic stability of the lead compounds encouraged the study of their anti-rheumatic properties in a collagen-induced arthritis (CIA) mouse model. This study reported the comparable efficacy of the prodrugs in addition to an improved safety profile when compared to the parent drugs.⁷⁰

Prochelators

Metal ions are essential for a wide range of physiological processes, but if their homeostasis is not properly regulated they can be implicated in a range of different diseases, from cancer to neurodegenerative disorders.⁷³ Metal ions like iron, copper, and zinc are capable of redox cycling and can catalyze Fenton-like reactions leading to ROS formation, in particular, highly reactive hydroxyl radicals generated from H₂O₂ (eqn (1) and (2)).⁷³

Previously described aminoferrocene drugs and prodrugs (boronic acids and esters, *vide supra*) exploit this mechanism to induce oxidative stress and cell death with potential application in oncology. From an opposite approach, metal-chelating agents, or chelators, are ligands that coordinate to metals by multiple points of attachment and afford a metal-ligand complex with high thermodynamic affinity. They afford protection against oxidative damage by inhibiting the metal-catalyzed generation of hydroxyl radicals. Despite this potential, their administration carries risks of indiscriminate metal chelation, causing unintended systemic metal depletion with associated toxicity. A prodrug strategy allows for their conversion to the corresponding chelators only under specific stimuli. Stimulus-responsive prochelators have been quite extensively studied, and over the years different strategies have been reported.^{73,74}

Franz and co-workers focused on multifunctional chelating agents for neurodegenerative diseases such as Alzheimer's and Parkinson's, both associated with elevated levels of metal ions (iron, copper, and zinc) and high levels of oxidative stress.⁷⁵ In this context, the strategy for designing prochelators responsive to ROS relies on the peroxide-mediated transformation of arylboronates to phenols,⁷⁶ following the same mechanism previously described in Scheme 1. The group described a first-generation prochelator **44**, in which a boronic pinacol ester masks a phenolic oxygen, the key donor atom of salicylaldehyde isonicotinoyl hydrazone (SIH), which is a well-studied arylhydrazone iron chelator (Table 5). Employing this strategy, the group was able to modulate the iron affinity and the reaction rates with H₂O₂ through modifications at the aryl ring of the chelators and at the boron-containing ring of the prochelators.⁷⁷ In a cultured cell model for retinal pigment epithelium (ARPE-19), prochelator **44** protected the cells against H₂O₂-induced cell death.⁷⁸

Unfortunately, **44** failed to achieve full conversion to SIH under oxidative conditions because of its hydrolytic susceptibility in aqueous solutions causing conversion to its hydrolysis product isoniazid.⁸² Several modifications to **44** improved its hydrolytic stability while maintaining low cytotoxicity,⁸³ including the second-generation prochelator **45** with increased hydrolytic stability in plasma.^{84,85} Prochelator **45** did not induce iron deficiency in cultured retinal pigment epithelial cells and it showed cardioprotective effects against catecholamine-induced oxidative cellular injury.⁸⁶ More recently, Franz and co-workers worked on the release of the

Table 5 Prochelators 44–48

<p>44, R¹ = H; R² = </p> <p>45, R¹ = CH₃; R² = </p> <p>46, R = </p> <p>48, R = </p> <p>47, </p>						
Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
44, 45	Prochelators SIH and HAPI	Wilson's, Alzheimer's, Parkinson's, Huntington's, and transfusion-related iron overload diseases and cancer	Cytotoxicity in human retinal pigment epithelial (ARPE-19) cells and cervical cancer (HeLa) cells	—	K. J. Franz	73
46, 48	Prochelator 8HQ and (for 48) also umbelliferone (Umb)	Alzheimer's disease	Aβ aggregate formation	—	K. J. Franz	73, 79, 80
47	Prochelator deferaseriex (ICL670A)	Iron overload	Cytotoxicity in the human retinal pigment epithelial cell line ARPE-19	—	K. J. Franz	73, 81

chelating agents 8-hydroxyquinoline (8HQ) and deferasirox (ICL670A) from the corresponding hydrolytically stable prochelator derivatives **46**, a pinanediol boronic ester derivative of 8HQ, and **47**, a boronate triazol prochelator for peroxide-triggered tridentate metal binding (Table 5).^{73,81} In particular, the boronic ester derivative **46** found application for the prevention of metal-induced amyloid beta (A β) aggregation upon activation in experiments designed to mimic Alzheimer's disease pathology *in vitro*.⁷⁹ New coumarin fluorophores were introduced into prochelator **46** through stable *cis*-cinnamate precursors, providing the stimulus-responsive prochelator **48** that released the chelating agent 8HQ and the fluorophore umbelliferone (Umb) after oxidation and intramolecular nucleophilic substitution (Table 5).⁸⁰

The aforementioned studies suggest that the described prochelators could potentially constitute promising candidates for neuronal and retinal protection, with applications for neurodegenerative disorders, ischemia/reperfusion injury, and cardiovascular diseases. While *in vitro* experiments and cell culture studies showed promising results, challenges for the future include the increase in selectivity for specific metal ions and the elucidation of the efficacy and cytotoxicity of these agents in tissues and animal models.

Other reactive linkers and strategies

Organochalcogen – selenium and sulphur. Selenides are very sensitive to oxidation by ROS and most oxidizing agents convert selenides to selenoxides, selenols to seleninic acids, and hydrogen selenide to selenium dioxide.⁸⁷ Selenium is an essential trace element in humans; however, at elevated concentrations it can cause severe toxicity.⁸⁸ Nevertheless, several pharmacological applications of organoselenium compounds for the treatment of different diseases have been recently reported *e.g.* various anticancer and anti-inflammatory drug candidates.^{89,90} In 2017, Wang and co-workers reported a strategy for the preparation of organoselenide prodrugs that can selectively release carbon monoxide (CO) in response to ROS.⁹¹ CO-Releasing prodrugs are considered promising therapeutic agents against several diseases, including cancer, bacterial infection, and inflammation.⁹¹ The group synthesized two CO prodrugs, **49** and **50**, using phenylselenium as the ROS-sensitive moiety (Table 6). In the presence of ROS, the resulting selenoxide readily undergoes *syn*-elimination to form a C5–C6 alkene. The intermediate subsequently releases CO through a cheletropic extrusion. Prodrugs **49** and **50** showed a promising activation profile *in vitro*, as both were stable in aqueous solution and activated by HOCl, ¹O₂, and O₂^{•−}, with HOCl being the most reactive trigger. On the contrary, other oxidants such as H₂O₂ (1 mM), HO[•] (500 μ M), *tert*-butyl hydroperoxide (TBHP, 500 μ M), and *tert*-butoxy radical (tBuO[•], 500 μ M) were not effective activators. Moreover, delivery of CO to HeLa (cervical cancer) and RAW264.7 (macrophages) was demonstrated using the known fluorescent CO probe **1** (COP-1). Additionally, CO prodrugs were reported to be not cytotoxic to heart/myocardium cells (H9c2)

and to be beneficial for the treatment of cancer by sensitizing cancer cells to some chemotherapy treatment (*e.g.* doxorubicin).

Sulfides, disulfides, and thioethers share similar mechanisms of activation under oxidative conditions. Likewise, thioketal protecting groups react rapidly and selectively with ROS to release ketone products. Taking advantage of their oxidation-mediated transformations, various ROS-responsive structures such as nanoparticles and vesicles have been synthesized in recent years.^{27,28} Despite the great promise of such moieties in the biomedical field, the corresponding applications for prodrugs are rare. Recently, Xu and co-workers have reported paclitaxel (PTX) prodrug **51** using a bio-sensitive thioether linkage with potent *in vivo* efficiency in a mouse xenograft model for breast cancer (Table 6).⁹² The synthesized prodrug contains a maleimide group, which rapidly conjugates with albumin *in vivo*, serving as a vehicle to deliver more prodrug to tumours. The thioether linkage facilitates PTX release in the ROS-rich tumour microenvironments through facilitated hydrolysis following oxidation.⁹²

By manipulating the biosynthesis of leinamycin in *Streptomyces atroolivaceus* S-140, Shen and co-workers were able to isolate the intermediate leinamycin E1 (**52**, Table 6). Oxidative activation of the LNM E1 thiol to sulfenic acid by cellular ROS leads to an episulfonium ion that alkylates DNA, thereby causing DNA cleavage and eventually cell death.⁹³ Compound **52** showed potent *in vitro* cytotoxicity against two prostate cancer cell lines (LNCaP and DU-145) after treatment with ROS inducers.⁹⁴

Sulfonate esters. Fluorescent probes incorporating sulfonate groups have shown use in detection of ROS, liberating a sulfonic acid and a fluorescent dye upon exposure to certain ROS.^{95–97} These probes are specific for nucleophilic ROS due to their mechanism of activation, which involves a nucleophilic attack (Scheme 2). Following this line of research, Cohen and co-workers used sulfonate esters for the development of matrix metalloproteinase inhibitor prodrugs **53** and **54** (Table 6).⁹⁸ After treatment with H₂O₂, the sulfonate esters **53** and **54** were proved to be activated compared to the parent drug, resulting in increased inhibition of matrix metalloproteinase MMP-12 in a fluorescence based assay. Unfortunately, nearly complete hydrolysis of the sulfonate esters **53** and **54** was observed in HEPES buffer (pH 7.5) after 24 h. In contrast, their corresponding self-immolative boronic ester version, **1**, reported by the same group was entirely stable under identical conditions (Table 1).³⁵

Even though the sulfonate ester group shows promising results in terms of prodrug activation with H₂O₂, further development is necessary to suppress non-specific hydrolysis and to investigate the activation of such prodrugs by other ROS, reductants (reductases), and nucleophiles such as GSH, as well as esterase.

Thiazolidinones. Another approach for developing ROS activated prodrugs relies on the use of thiazolidinone (TZ) masking groups for carboxylic acids. In the presence of ROS, the promoiety is hydrolyzed under nucleophilic conditions to

Table 6 Miscellaneous prodrugs 49–60

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
49, 50	Carbon monoxide (CO)	Bacterial infections, inflammation and cancer, among others	Cytotoxicity in cervical cancer (HeLa) and rat heart/myocardium (H9c2) cells	—	B. Wang	91
51	Paclitaxel (PTX)	Cancer	Cytotoxicity and cellular uptake in human prostatic carcinoma (PC-3), human oral epidermoid (KB), and mouse breast cancer (4T1)	Efficacy and organ distribution in a xenograft model of mouse breast cancer (4T1)	Y. Xu	92
52	Episulfonium ion of the intermediate leinamycin E1 (LNM E1)	Cancer	Cytotoxicity in androgen sensitive prostate cancer (LNCap) and androgen insensitive prostate cancer (DU-145)	—	B. Shen	94
53, 54	Matrix metalloproteinase inhibitors (MMPis)	Arthritis, cancer, and cardiovascular disease	Inhibition of MMP-12 enzymes	—	S. M. Cohen	98
55, 56	Ibuprofen/MMPI	Analgesic and anti-inflammatory/cancer and ischemic reperfusion injury	Inhibition of COX-1 or MMP-2 enzyme Cytotoxicity assay for the TZ moiety in fibroblast (NIH3T3) cells	—	S. M. Cohen	99
57	Methotrexate (MTX)	Rheumatoid arthritis	—	Efficacy in a collagen-induced arthritis (CIA) murine model	M. H. Clausen	100
58–60	DNA modifying agents (Hq ^{ox})	Cancer	Cytotoxicity in several cancer cell lines, focus on renal carcinoma (786-O) and acute myeloid leukemia (AML)	Toxicity in <i>Drosophila melanogaster</i>	E. J. Merino	101–104

generate the thiazolidinone leaving group and the free carboxylic acid of the drug (Scheme 3).

Cohen and co-workers applied this prodrug approach to ibuprofen and a well-studied MMi, to obtain prodrugs 55 and

56, respectively.⁹⁹ Despite being activated by nucleophilic attack, they achieved molecules with good stability in aqueous buffer, GSH stability, and a fast rate of H₂O₂-mediated release. Nevertheless, they lack further *in vitro* and *in vivo*

Scheme 2 Mechanism for the activation of sulfonate esters with H_2O_2 .

Scheme 3 Mechanism for the activation of thiazolidinones with H_2O_2 .

studies to support the prodrug strategy for disease targeting (Table 6).

Based on the promising properties of TZ prodrugs and in extension of the aryl boronic acids previously described (Table 4), Clausen and co-workers proposed a thiazolidinone-based MTX prodrug for site-selective delivery.¹⁰⁰ The group reported the synthesis of 57, carrying the TZ group at the MTX- γ -carboxylic acid (Table 6). Prodrug 57 was selectively activated at pathophysiological concentrations of H_2O_2 and displayed good physicochemical and pharmacokinetic properties. In a murine CIA model, prodrug 57 exhibited comparable efficacy to MTX, but showed reduced toxicity, based on the average body weight of the treated animals.

Quinones. Quinones represent a quite complex class of intermediates that can cause toxicity. Their reactivity comes in part from the fact that they are Michael acceptors, and consequently cellular damage can occur through the alkylation of DNA or cellular proteins. Moreover, they are highly redox active molecules, causing formation of ROS with oxidation of lipids, proteins, and DNA as a consequence.¹⁰⁵ Merino and co-workers have published several research papers in which they present, among others, DNA-modifying agents 58 and 59, containing an oxidizable hydroquinone leaving group. They reported how a general hydroquinone analogue leads to the formation of benzoquinone (Hq^{ox}) in the presence of the hydroxyl radical, singlet oxygen or hydrogen peroxide (Table 6). Furthermore, *in vitro* studies and structural characterization demonstrated that Hq^{ox} was added to 2'-deoxyguanosine at the N2–N3 positions forming an annulated adduct. Additional studies helped to identify the structural requirements for oxidizable hydroquinone DNA modifying agents. New lead compounds with cytotoxic effects against the acute myeloid leukemia cell line (AML) as well as a good therapeutic index between the AML model cell line and non-cancerous human CD34+ blood stem/progenitor cells (UCB) were reported.¹⁰² Even more interestingly, compound 60 (Table 6) sensitized dabrafenib-resistant melanoma cells (A375, SK-MEL-24, and WM-115) to the B-Raf protein kinase inhibitor.¹⁰⁶

Photodynamic prodrugs

A different approach to ROS-mediated drug release with spatio-temporal control are the singlet oxygen-responsive photodynamic prodrugs. In contrast to the previously described prodrugs, photodynamic prodrugs are not activated by endogenous ROS but by $^1\text{O}_2$ produced by a covalently linked photosensitizer (PS). Selective irradiation with visible or near infrared (NIR) light triggers the PS to convert ground state molecular oxygen to $^1\text{O}_2$ which in turn can cleave a suitable linker (Fig. 3).

The concept of photodynamic therapy (PDT) is a well-established approach in the treatment of cancer.^{107–109} In classic PDT, a non-toxic PS is administered and then locally activated at the tumour site with visible-NIR light to produce singlet oxygen. The therapeutic effect relies entirely on the reactive nature of singlet oxygen to inflict tissue damage and in turn necrosis and/or apoptosis. Compared to conventional chemotherapy, PDT offers greater selectivity with less toxicity to healthy tissue while at the same time circumventing issues of drug resistance. However, because of the short lifetime ($t_{1/2} < 0.04 \mu\text{s}$) and thus limited diffusion distance ($< 0.02 \mu\text{m}$) of singlet oxygen in biological systems,¹¹⁰ the effectiveness of PDT is closely linked to the localization of the PS. The poor distribution within the tumour microenvironment due to insufficient vasculature is therefore a concern for PDT, as it is for chemotherapy. To tackle this challenge, photodynamic prodrugs have been developed to increase efficacy by combining PDT with release of a drug for additional bystander toxicity.

Photosensitizers

Photosensitizers are highly conjugated structures that can be excited to a triplet state by irradiation at an appropriate wavelength (Chart 1). For effective deep tissue penetration, activation of the PS should ideally be performed using light with wavelengths $> 650 \text{ nm}$. Higher energy light, such as UV light, suffers from poor tissue penetration and is damaging to healthy cells.^{111–113}

Vinyl heteroatom-based linkers

Inspired by the early work on $^1\text{O}_2$ -responsive systems by Breslow and co-workers,¹¹⁴ Jiang and Dolphin reported the first example of a photodynamic prodrug in 2008.¹¹⁵ In a proof-of-

Fig. 3 Schematic illustration of the photodynamic prodrug. A photosensitizer–drug (PS–drug) construct is irradiated with suitable light to promote the conversion of native molecular oxygen (triplet state) into singlet oxygen, which is capable of cleaving a $^1\text{O}_2$ -sensitive linker to release the drug. The therapeutic effect arises from a combination of drug release and $^1\text{O}_2$ -mediated tissue damage.

Chart 1 Overview of the photosensitizers used for photodynamic prodrugs: verteporfin (VP), *meso*-tetraphenylporphyrin (TPP), core-modified porphyrin (CMP), silicon phthalocyanine (Pc), phthalocyanine-based IRDyeTM 700DX (IRD), and pro-photosensitizer iodo-substituted 5(6)-carboxyfluorescein diacetate (CF).

principle study, they demonstrated light-induced drug release from a PS-drug complex. The responsive linker was based on the direct incorporation of the carbonyl group of ibuprofen or naproxen into a vinyl diether moiety further conjugated to a PS to obtain **61** and **62** (Table 7). Upon irradiation, the electron-rich olefin underwent a [2 + 2] cycloaddition with $^1\text{O}_2$ to afford a dioxetane intermediate, which decomposed spontaneously to release the NSAID methyl esters (Scheme 4). Prodrug **61** with verteporfin (VP) or **62** with *meso*-tetraphenylporphyrin (TPP) was activated using light with >90% drug release within 10 minutes. The involvement of $^1\text{O}_2$ was demonstrated by addition of the $^1\text{O}_2$ -scavenger 1,4-diazabicyclo[2,2,2]octane (DABCO), which reduced the reaction rate by an order of magnitude. The use of heteroatom-substituted olefin linkers is limited to drugs containing an ester or an amide. Attachment to other functional groups will lead to release of a formylated drug, *e.g.* drug-O-CHO, which requires additional hydrolysis to release the drug. Furthermore, robust and facile synthetic methodologies of such vinyl di(thio)ethers are scarce.^{116–120} For less electron-rich olefin linkers such as vinyl mono-ethers, a competing ene-reaction is a significant concern.^{115,121}

Acrylate linkers

You and co-workers developed a more synthetically accessible aminoacrylate (AA) linker for alcohol containing mole-

cules.^{121,122} Coined “photo-unclick chemistry”, the activation mechanism is identical to the one shown in Scheme 4. In a series of publications,^{122–130} You and co-workers described photodynamic prodrugs containing the aminoacrylate linker and the tubulin polymerization inhibitor combretastatin A-4 (CA4). In combination with the pro-PS iodo-substituted 5(6)-carboxyfluorescein diacetate (CF), the cascade, dual-activation photodynamic prodrug **63** was developed to facilitate easier handling under ambient light (Table 7).¹²³ CF is first activated by intracellular esterases to become photoresponsive, whereupon irradiation releases the drug. Incubation for 24 h in breast cancer cells (MCF-7) followed by irradiation with visible light ($\lambda_{\text{max}} = 540 \text{ nm}$) for 30 min resulted in 99% compound release. Addition of a $^1\text{O}_2$ -quencher (DABCO or β -carotene) effectively delayed the cleavage of the aminoacrylate linker, whereas the superoxide radical quencher 1,4-benzoquinone had no effect. In the dark, the prodrug showed 10 times reduced toxicity compared to irradiated cells and a significant bystander toxicity was demonstrated using partially illuminated wells. In another example, the PS was exchanged for the far-red light responsive core-modified porphyrin (CMP) for enhanced deep tissue activation (Table 7).¹²⁴ The CMP-AA-CA4 prodrug **64** showed >80% drug release after 10 min of irradiation ($\lambda_{\text{max}} = 690 \text{ nm}$) and a 6-fold increased efficacy compared to non-irradiated MCF-7 cells. The photodynamic prodrug showed significant *in vivo* tumour suppression in a mouse

Table 7 Photodynamic prodrugs 61–67

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
61, 62	Ibuprofen and naproxen	Proof of principle	—	—	D. Dolphin	115
63, 64	CA4	Cancer	Cytotoxicity in breast (MCF-7) and murine colon (colon-26) cancer cells	Efficacy and PK in mice (murine colon-26 tumours)	Y. You	122, 123, 131
65	CA4	Cancer	Cytotoxicity in breast (MCF-7) and murine colon (murine colon-26) cancer cells	Efficacy and PK in mice (murine colon-26 tumours)	Y. You	125, 126
66	CA4	Cancer	Cytotoxicity in breast (MCF-7) and murine colon (murine colon-26) cancer cells	Efficacy and PK in mice (murine colon-26 tumours)	Y. You	129
67	CA4	Cancer	Cytotoxicity in rat bladder cancer cells (AY-27)	—	Y. You	132

homograft model (murine colon-26 cells) upon irradiation of tumours as compared to non-irradiated mice and controls. In their second generation of aminoacrylate-based photodynamic prodrugs, You and co-workers incorporated the poten-

tial for optical imaging by switching the PS to silicon phthalocyanine (Pc) – a dual $^1\text{O}_2$ generator and fluorescent reporter.¹²⁶ The Pc-based construct 65 allowed for the conjugation of two molecules of CA4 and showed a larger difference

Scheme 4 General mechanism for the singlet oxygen mediated cleavage of olefin linkers. Irradiation of the photosensitizers (PS) promotes the production of singlet oxygen, which reacts with electron-rich olefins in a [2 + 2] cycloaddition. The resulting dioxetane decomposes spontaneously to release two carbonyl compounds. The resulting species can either be part of the drug as an ester (R = alkyl) or be a formyl group (R = H), which requires additional hydrolysis to release the free drug.

between dark- and photo-toxicity in breast cancer cells (MCF-7) than the CMP-based prodrug 63 (Table 7). Increased anti-tumour activity was also observed in tumour-bearing mice; however, administration was performed in poly(ethylene glycol)-poly(D,L-lactide) (PEG-PLA) micelles due to the high lipophilicity of the construct. Optical imaging was also performed *in vivo* and showed high prodrug accumulation in tumours. The therapeutic relevance of the multifunctional prodrug was further improved by the addition of tumour-targeting properties in the form of folic acid (FA) replacing one CA4 molecule, to obtain 66 (Table 7).¹²⁹

Incorporation of a PEG-spacer further increased aqueous solubility and resulted in more specific uptake. *In vivo*, the targeted prodrug demonstrated tumour suppression in mice over an impressive 75-day period. Indeed, mice treated with a non-targeting prodrug suffered more skin damage than tumour damage, confirming the benefit of targeting. Optical imaging verified the accumulation in tumour tissue, peaking around seven hours after injection. Similar Pc-based constructs with the anticancer agent paclitaxel (PTX) were also synthesized showing the feasibility of using the linker with secondary alcohols and not only phenolics.^{127,128,130}

Recently, You and co-workers developed the mitochondria-targeting prodrug 67 based on an intermolecular prodrug/PS system.¹³² The prodrug was assembled from CA4, an AA linker, and the targeting ligand Rhodamine B (RhB). A PDT effect was aided by addition of hexyl-5-aminolevulinate (HAL), which is converted to the PS protoporphyrin IX (PpIX) inside the mitochondria (Table 7).^{133–135} In rat bladder cancer cells (AY-27), the prodrug localized to mitochondria and showed increased cytotoxicity upon irradiation compared to PDT alone.

Oxathio- and thioketal linkers

Thioketals (TK) are robust protecting groups for carbonyl groups that can be unmasked under oxidative conditions.^{139–141} This has inspired researcher to explore their potential as ROS-responsive linkers in drug delivery systems^{142–144} and later as photodynamic prodrugs. One advantage of thioketals is their compatibility with amines, alcohols, and carbonyl functional groups.¹⁴⁵ Lamb and Barbas investigated a variety of oxathiolanes and dithiolanes as linkers in sensors and prodrugs (Table 8).¹³⁶ Aryl 1,3-oxathiolane and 1,3-dithiolane were found to be the most promising for selective activation by ¹O₂ (although several hours of irradiation were needed) while exhibiting tremendous stability towards other ROS including H₂O₂, O₂^{•−}, and OH[•]. The concept oxathiolane-doxorubicin prodrug 68 was cleanly activated *in vitro* in the presence of different PSs. A proposed mechanism for the cleavage of (oxa)thioketals by ¹O₂ is given in Scheme 5. Based on a thioketal linker, Zhang and co-workers created the gemcitabine photodynamic prodrug 69 for image-guided treatment of cancer (Table 8).¹³⁷ Incorporation of a carbonate group in the linker effectively trapped the intermediate thiol in an intramolecular cyclization to release the drug and an oxathiolanone. The prodrug performed well *in vitro* against cervical cancer cells (HeLa) demonstrating a clear bystander effect. Due to the lipophilicity of the construct, *in vivo* studies were carried out by either subcutaneous or intravenous injection in PEG-PLA micelles. In a H22-bearing mouse model, the prodrug effectively suppressed tumour growth with significantly better results than both gemcitabine and PDT controls for both routes of

Table 8 Photodynamic prodrugs 68–70

Cpd.	Drug/active species	Indication	<i>In vitro</i> efficacy	<i>In vivo</i> studies	Group	Ref.
68	Doxorubicin	Cancer	—	—	C. F. Barbas	136
69	Gemcitabine	Cancer	Cytotoxicity in cervical cancer cells (HeLa)	Efficacy and PK in mice (murine hepatic H22 tumours)	X. Z. Zhang	137
70	mbc94-analogue	Cancer	Cytotoxicity in mouse brain cancer cells (CB ₂ -mid DBT) and cell viability in healthy human embryonic kidney cells (HEK-293)	—	M. Bai	138

Scheme 5 Proposed mechanism for the $^1\text{O}_2$ -mediated cleavage of thioketals ($\text{X} = \text{S}$) and oxathioketals ($\text{X} = \text{O}$).^{148,149}

administration. No significant body weight loss or gross organ changes were observed, indicating good systemic tolerability.

Bai and co-workers also exploited the $^1\text{O}_2$ -sensitivity of the thioetal linker to develop the tumour targeting photodynamic prodrug 70.¹³⁸ Using a heterobifunctional thioetal linker,¹⁴⁵ the hydrophilic photosensitizer IR700DX was linked to the cannabinoid drug mbc94, which also served as a targeting ligand against type-2 cannabinoid receptor (CB_2R) overexpressing cancer cells (Table 8). In mouse brain tumour cells (CB_2 -mid DBT), prodrug 70 showed significantly improved therapeutic potential when compared to a non-cleavable cannabinoid-PS construct, which had previously been reported for classic PDT.^{146,147} Notably, healthy human embryonic kidney cells (HEK-293) not expressing CB_2R were mainly unaffected at therapeutically relevant doses.

With PDT already approved for cancer treatment, the use of photodynamic prodrugs seems to be an easy improvement of this approach. Clear evidence of increased bystander effects has been demonstrated *in vitro* with increased efficacy *in vivo* and should in principle help to improve the shortcomings of PDT. With that said, only a handful of PS-linker-drug combinations have been tested so far and many of the photodynamic prodrugs discussed in this review suffer from low aqueous solubility. Further studies into new constructs are therefore highly recommended, possibly drawing on inspiration from adjacent fields.

Conclusions and future perspectives

As evident from the many contributions and ingenious designs disclosed since Cohen's seminal paper in 2010,³⁵ the research into ROS-activated prodrugs is thriving. The approaches, drugs, and ROS-responsive promoieties are becoming more diverse and the biological investigations accompanying new reports more sophisticated. Nonetheless, there is a way to go before these types of systems can be brought all the way to the clinic. Some of the obstacles and areas that warrant further investigation are: 1) improving QIC_{50} values (a comparative toxicity ratio between the prodrug and drug). Although the activity of the prodrugs is attenuated compared to the parent drugs, it is rare that there is no background activity. This is almost inevitable, since these designs rely on a labile functionality – sensitive to ROS but oftentimes also to background hydrolysis. However, to truly harness the potential of site-selective release of the active ingredient, this parameter has to be controlled. 2) Better

understanding of the actual mechanism of action *in vivo*. Despite the fact that most newer studies include data from a relevant animal model, the focus is often on proving the activity of the prodrugs. The mechanism of activation is understood to be due to reactive oxygen species, but this is not often investigated and thus not supported by critical experiments. 3) These two issues raises a third point: is the pathophysiological concentration of ROS sufficient to ensure a targeted delivery of the payload to diseased tissue? Several studies have shown that ROS levels are elevated at sites of inflammation (in effect H_2O_2 concentration, since this is the only ROS long-lived enough for reliable measurements to be made). This is underscored by the large number of studies, where the prodrugs are artificially activated through *e.g.* the addition of exogenous H_2O_2 or endogenous chemically induced H_2O_2 generation – presumably because the endogenous ROS levels are insufficient to demonstrate the concept. Furthermore, ROS measurements are cumbersome and the variation in concentration significant, so better solutions for correlating efficacy of prodrugs with reliable ROS measurements would benefit the field greatly. 4) What is the systemic exposure of the parent drug following prodrug activation? Release can be as desired (at the site of elevated ROS concentrations) or form elsewhere (hydrolysis during systemic circulation of the prodrug or through metabolism in the liver, *e.g.* deboronation or thioether oxidation) – but regardless, limiting the systemic exposure of the parent drug is paramount to a beneficial effect from the more complex prodrug strategy.

Investigations that address the four points above are pivotal to successful preclinical development of these designs and it will behove research in this area to pay attention to these factors as early as possible. Nevertheless, amazing strides are being made within prodrugs and their clinical development and approval, a trend that surely will also benefit ROS activation strategies in the future.

Conflicts of interest

Three of the authors (JPC, NST, and MHC) are inventors of patents filed by DTU regarding MTX prodrugs. MHC is a co-founder of ROS Therapeutics, a company with a mission to develop better treatment for rheumatoid arthritis.

Acknowledgements

We are grateful for financial support from the Independent Research Fund Denmark (grant no. 7017-00026), the Novo

Nordisk Foundation (Novo Scholarship to NST) and the Technical University of Denmark (PoC funding and PhD scholarships for JPC and NST).

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