doi:10.2533/chimia.2018.771 Chimia 72 (2018) 771–776

Bioorthogonal Decaging Reactions for Targeted Drug Activation

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Abstract: Bioorthogonal decaging reactions are highly selective transformations which involve the cleavage of a protecting group from a molecule of interest. Decaging reactions can be classified into subgroups depending on the nature of the trigger; they can be photo-, metal- or small molecule-triggered. Due to their highly selective and biocompatible nature, they can be carried out in living systems as they do not interfere with any endogenous processes. This gain-of-function allows controlled activation of proteins and release of fluorophores and drugs *in vivo*. Although there are many examples of fluorophore/protein release, this review focuses on the application of bioorthogonal decaging reactions for targeted drug activation. One strategy for targeted drug delivery is tissue-selective activation of prodrugs and antibody–drug conjugates (ADCs). Bioorthogonal decaging provides a highly selective, controllable method for activating prodrugs and ADCs, reducing toxicity due to the off-target drug release that occurs in endogenous activation strategies. Here we focus on the development of bifunctional linkers that enable studies of bioorthogonal chemistry for activation of ADCs.

Keywords: Antibody–drug conjugates · Bifunctional linkers · Bioorthogonal decaging · Targeted drug delivery

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1. Introduction

A reaction can be classified as bioorthogonal if it can be carried out in living systems without affecting any endogenous processes.[1] For this to be the case, the reaction must be highly selective with no cross reactivity observed with any biomolecules. The reaction should also have a high rate constant under physiological conditions and at the low concentrations (μM–nM) that are required for the reagents to be non-toxic. In reality, most reported bioorthogonal reactions do not fulfil all these criteria and are often limited by either slow reaction rates, cross reactivity with biomolecules, stability to biological conditions or toxicity.

Initial research focussed on bioorthogonal ligation (bond-forming) reactions which led to developments in the labelling and studying of biomolecules. The applications of bioorthogonal chemistry have been greatly expanded by bioorthogonal decaging reactions in which a bond is broken to release a molecule of interest. This

enables gain-of-function studies of compounds which have previously been chemically modified to render them inactive. This strategy can be applied to biomolecules such as proteins, fluorophores and small molecule drugs.[2] In this review we focus on the use of bioorthogonal decaging reactions for targeted drug activation.

Localised, controlled drug release is advantageous as it reduces toxicity due to off-target interactions and therefore allows higher doses to be administered. Prodrugs have the potential to be selectively activated at the target site, resulting in targeted drug release. However, previously they relied on activation by endogenous factors, such as low pH, metabolic enzymes or high thiol concentrations in the target cells.[3] This results in low selectivity and often background release occurs due to degradation of the prodrug elsewhere in the body. Prodrug activation using bioorthogonal decaging offers a greater degree of control and selectivity as, without the external trigger, no drug release should be observed.

One method of selectively targeting drug release is by using antibody–drug conjugates (ADCs). Bioorthogonally cleavable ADCs have been reported, where an antibody is connected to a drug *via* a bioorthogonally cleavable bifunctional linker.[4–9] After administration, the ADC is allowed to accumulate at the desired site before the trigger is used to react with the linker and release the active drug. As the activation mechanism does not rely on intracellular triggers, this also allows non-internalising antibodies to be used.

This review covers the three subclasses of bioorthogonal decaging reactions: photo-, metal- and small molecule-triggered decaging, with representative examples of bifunctional linkers being discussed in each case. Their application to targeted drug activation is discussed, with a particular focus on the development of bifunctional linkers for drug release from ADCs.

2. Near-infrared-triggered Decaging

In a photo-triggered decaging reaction, a protecting group absorbs photons and is transferred to an excited state where it then undergoes a reaction which causes release of the molecule of interest (Fig. 1a). UV-triggered decaging is the most extensively used bioorthogonal decaging reaction. However, UV light (350–365 nm) suffers from issues of cytotoxicity and poor tissue penetration, limiting its use *in vivo*. Blue visible light has also been utilised successfully for the RNA-templated decaging of fluorophores in zebrafish.[10] The self-immolative linker used in this system has been demonstrated in cell culture for the release of drugs[11] and therefore the blue-light mediated reaction should be adaptable to drug release. Using light of a lower energy and longer wavelength (near-IR, 650–900 nm) is appealing as it offers increased tissue penetration and low toxicity in comparison to UV radiation. Therefore, near-IR cleavable linkers offer greater potential for drug activation *in vivo*.

In an early example, near-IR light was used to decage an oestrogen receptor antagonist, hydroxy-cyclofen, in mammalian cells (Fig. 1b).[12] A prodrug consisting of a cyanine fluorophore connected to an alcohol-containing drug was developed and it was shown that upon irradiation with near-IR light, photooxidative C–C bond cleavage occurs. This is followed by hydrolysis to release a free amine which then undergoes intramolecular cyclisation to release the alcohol.

In another example, an alcohol-containing drug, combretastatin A4 (Z-CA4), was converted into a silicon phthalocyanine prodrug (Fig. 1c).[13] Light with a wavelength of 690 nm was shown to result in drug release *via* axial ligand exchange in hypoxic conditions. Near-IR light produces an excited singlet state which then undergoes intersystem crossing to a triplet state. In the presence of reducing agents, such as glutathione, electron transfer occurs to generate a radical anion which then releases the alcohol. Meanwhile, under aerobic conditions toxic reactive oxygen species (ROS) are generated.

2.1 *Bifunctional Linkers*

Schnermann reported an ADC containing a near-IR cyanine-based cleavable linker (Fig. 1d), which connected (Z-CA4), an inhibitor of microtubule polymerization, to monoclonal antibody panitumumab (pan).[4,5] Z-CA4 is significantly less potent than most ADC payloads, therefore was deemed unsuitable for *in vivo* tumour treatment. However, using the fact that the cyanine caging group is intrinsically fluorescent, it was shown that this first generation ADC had good *in vivo* stability, high tumour uptake and that irradiation with near-IR caused a decrease in fluorescence, implying decay of the linker and therefore drug release.[5] In a later publication, the linker was modified in order to improve stability and to shift the absorbance maxima to a longer wavelength. This modified linker was then conjugated to a more po-

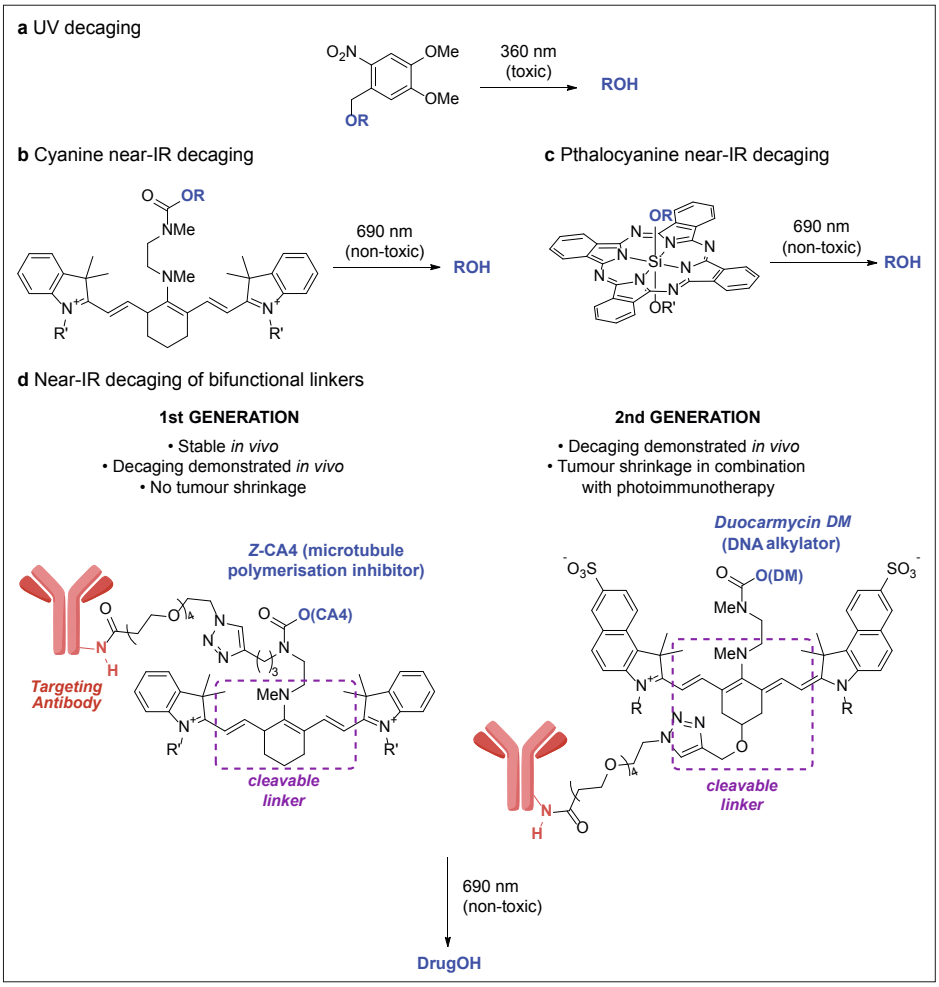

Fig. 1. Photo-triggered drug decaging. a. Example of UV decaging using the most widely reported *o*-nitrobenzyl linker. b. Examples of near-IR decaging from prodrugs *in vitro*. c. near-IR bifunctonal linkers for targeted drug activation.

tent duocarmycin payload to produce a second generation ADC which resulted in a significant decrease in tumour size and increased survival of the mice after a single dose.[6] It has since been shown that a larger therapeutic effect is observed when this near-IR cleavable ADC is used in combination with an antibody–photoabsorber conjugate.[14] This is an antibody connected to a photosensitizer that absorbs near-IR light and generates reactive oxygen species which can kill tumour cells. This combination therapy resulted in greater tumour shrinkage and survival time of the mice and highlights the potential of near-IR light for decaging reactions *in vivo* and next-generation targeted therapeutics. Near-IR cleavable bifunctional linkers have already made fantastic progress, with the second generation ADC showing increased stability and therapeutic index. Important challenges remain however, in developing a site-selective conjugation strategy, determining the depth of biological tissue at which decaging is still possible and the potential of using antibody fragments or derivatives to enable greater tumour penetration.

3. Metal-triggered Decaging

Metal-triggered decaging reactions have been more extensively reported than small molecule-mediated decaging. Palladium-mediated decaging is the most studied method, with the largest number of prodrug examples and *in vivo* applications reported (Fig. 2a). Palladium complexes have been shown to be cell-penetrant and do not form reactive oxygen species in mammalian cells.[15] Furthermore, effective methods to administer the palladium trigger *in vivo* have been reported, for example *via* nanoencapsulated palladium.[16,17] When administered in tumour-bearing mice, these palladium nanoparticles resulted in efficient, targeted activation of a doxorubicin prodrug, which led to the inhibition of tumour growth and increased survival time of the mice.[16]

In 2014 Weiss demonstrated the use of a biocompatible palladium resin to cleave a propargyl group from a 5-fluorouracil prodrug *in vitro*. Fluorescence recovery was performed *in vivo* for the imaging of zebrafish embryos.[18] The same group then reported carbamate prodrugs of gemcitabine which could also be activated using palladium-mediated decaging.[19] The most successful carbamate protecting group tested was *N*-propargyloxycarbonyl (*N*-Proc) which resulted in the greatest amount of free drug release, and therefore cytotoxicity, in cells. In addition, the activation of *N*-Proc-rhodamine in zebrafish embryos was shown by fluorescence to

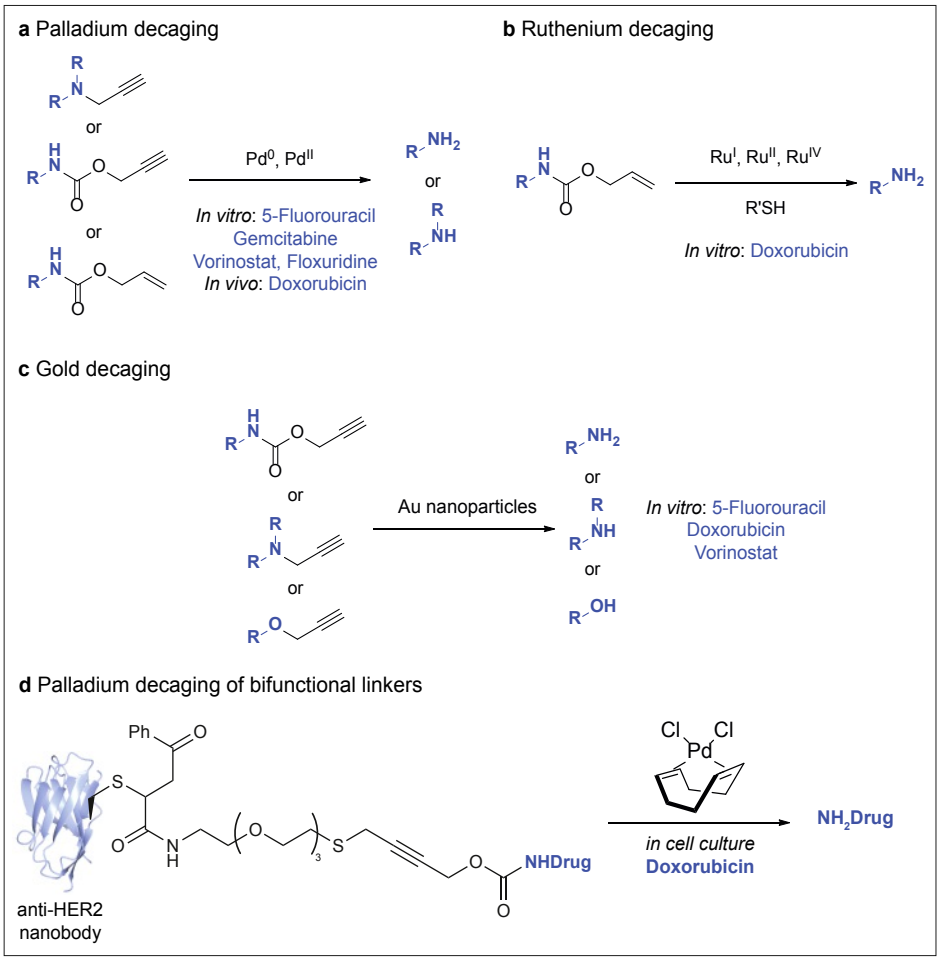

Fig. 2. Metal-triggered decaging. a–c. Examples of decaging from prodrugs. d. Palladium-cleavable bifunctional link

occur locally at the site of the palladium resin. Palladium-catalysed depropargylation of Floxuridine, a cytotoxic anti-cancer drug, under hypoxic conditions in cells has also been reported.[20] Palladium-mediated decaging has also been extended to the release of hydroxamic acid functional groups. Successful activation of vorinostat, a histone deacetylase, was achieved in cells using palladium-catalysed depropargylation to release a phenol which then undergoes 1,6-elimination to release the alcohol of the hydroxamic acid group.[21]

Ruthenium has also been used for prodrug activation (Fig. 2b). Meggers developed organometallic ruthenium catalysts for decaging of *N*-allyloxycarbonyl (*N*-Alloc) protected amines which have high turnover numbers of up to 270 cycles under biological conditions.[22] These catalysts were shown to be highly cell permeable and were active in the presences of thiols at millimolar concentrations. Catalytic activation of doxorubicin was achieved in the cellular cytoplasm of HeLa cells which resulted in apoptosis.

Recently, the first example of gold-mediated decaging was reported (Fig 2c).[23] Gold is known to coordinate preferentially to alkynes, however it also has a high affinity for thiols. The authors proposed that by using solid-supported gold nanoparticles, reaction with large thiol-containing biomolecules could be prevented, allowing deprotection of small molecule drugs in biological systems. The decaging of three alkyne-protected cancer drugs (doxorubicin, 5-fluorouracil and vorinostat) using heterogeneous gold nanoparticles was shown in live cells. This was also the first bioorthogonal decaging reaction to be carried out in the brain of zebrafish embryos. Intracranial activation of a caged rhodamine was achieved, highlighting the potential of gold-mediated decaging reactions *in vivo*.

3.1 *Bifunctional Linkers*

In the area of drug activation, palladium-triggered decaging has mainly been used to cleave monofunctional protecting groups from anticancer prodrugs. To our knowledge, only two examples of bifunctional linkers have been reported. The first of these was developed for target pull-down assays for application in phenotype screening.[24] Kinase inhibitor BIRB796 was conjugated to a reactive capture tag *via* a palladium cleavable bifunctional linker. Once it had bound to its targets in cell lysates, the reactive tag was captured onto immobilised HaloTag and separated from the cell lysates. Palladium complex-

es were then used to cleave the linker and the cellular targets of kinase inhibitors BIRB796 could be identified using mass spectrometry.

Recently, our group reported a bifunctional thioether propargyl carbamate linker for palladium-mediated drug release (Fig 2d).[7] The thioether group was shown to bind to palladium and direct the decaging. After demonstrating palladium-triggered drug release from a PEGylated doxorubicin prodrug in live cells, the application was extended to the development of a palladium-activated ADC. Doxorubicin was connected to a nanobody targeted to the HER2 antigen *via* the bifunctional thioether propargyl carbamate linker. The ADC proved stable and thioether-directed palladium-triggered decaging in cells was achieved using non-toxic concentrations of palladium. Although this is a major advance in the area of metal-triggered decaging for drug release from ADCs, further work is required before this can be extended to *in vivo* applications. For example, the potential off-target toxicity of metal complexes and their fate after administration to animals must be assessed. Nanoencapsulation of catalysts may offer a solution to this problem and should therefore be tested in combination with ADCs.

4. Small Molecule-triggered Decaging

Most small molecule decaging reactions are modified forms of bioorthogonal ligation reactions. For example, the SPAAC (strain-promoted azide-alkyne cycloaddition)[25] has been developed into a decaging reaction by attaching the azide to a self-immolative linker (Fig. 3a).[26] When reacted with *trans*-cyclooctene (TCO), the azide undergoes a 1,3-dipolar cycloaddition to give an unstable 1,2,3-triazoline intermediate which rearranges to an imine *via* alkyl migration. Acid-catalysed hydrolysis of the imine then occurs, causing 1,6-elimination and release of a free amine. The Staudinger ligation[27,28] has also been modified to enable the release of amines[11,29,30] and alcohols[30] *in vitro*. Other decaging reactions have been reported which are not extensions of ligation reactions such as the reaction of *N*-oxides with diboron reagents to release tertiary amines.[31] Most examples so far have focussed on the release of amines with some examples of alcohols[32–35] and recently carboxylic acids.[35]

Trans-cyclooctene and its derivatives have proved to be very promising reagents for bioorthogonal chemistry. Due to high ring strain, the alkene is extremely reactive, which results in fast reaction rates. In particular, the inverse electron-demand Diels-Alder (IEDDA) reaction between *trans*-cyclooctene derivatives and tetrazines[36] has kinetics that are several orders of magnitude higher than other reported bioorthogonal reactions (k_2 = 57.7 $M^{-1}s^{-1}$ in MeCN for the cycloaddition step).[37] The group of Robillard demonstrated that this reaction could be converted into a decaging reaction for the release of amines (Fig. 3b).[37] When applied to lysine decaging in cells, a greater decaging yield was observed in the TCO-carbamate reaction with optimised tetrazines than for the corresponding photodecaging method.[38]

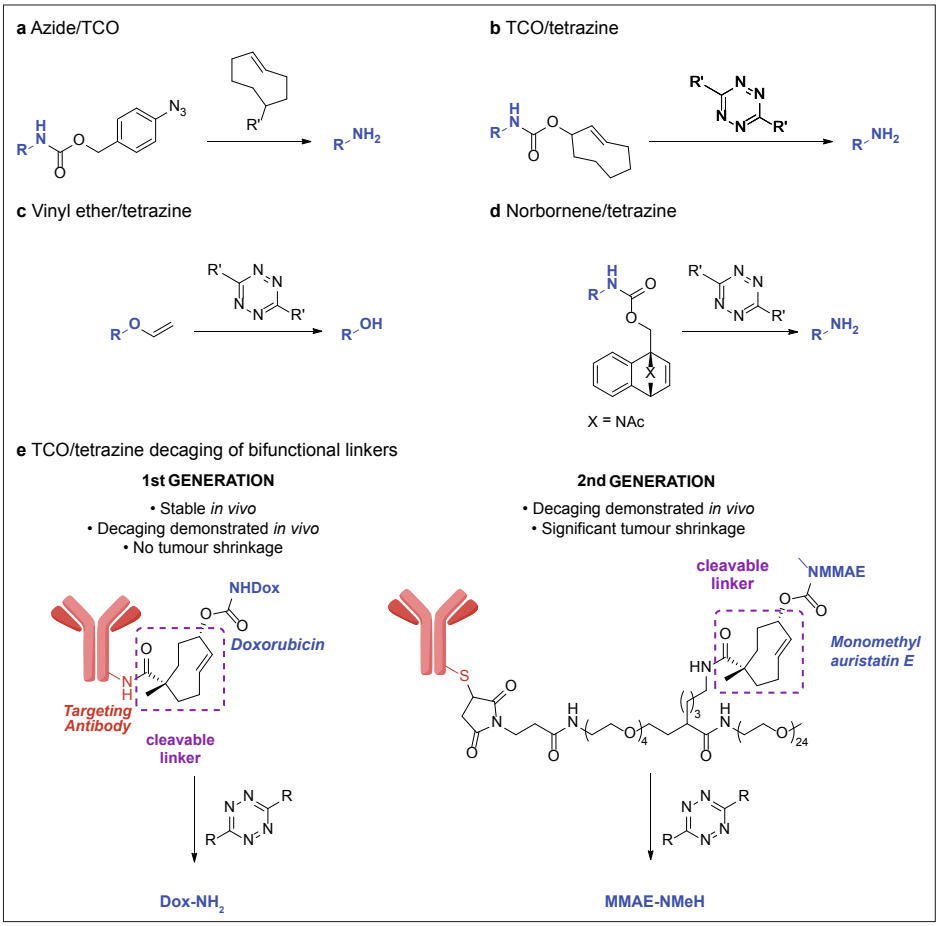

Fig. 3. Small molecule drug decaging. a-d Selected examples of small molecule decaging of prodrugs. e. Bifunctional TCO linkers for tetrazine-triggered drug release from ADCs.

Other examples of tetrazine-mediated IEDDA decaging have been reported, for example, the vinyl ether-tetrazine reaction for the release of alcohols.[32–34] Our group applied this to the decaging of a vinyl ether-duocarmycin prodrug in live cells (Fig. 3c).[32] This reaction is considerably slower than the TCO-tetrazine reaction (k_2 = 5.37 x 10^{-4} $M^{-1}s^{-1}$ in 10% H_2O/DMF for the cycloaddition step), however, it has expanded the scope of functional groups that can be used in bioorthogonal chemistry. The vinyl ether handle has also been connected to a self-immolative linker and applied to the tetrazine-triggered release of doxorubicin *via* remote functional group activation followed by 1,6-elimination.[34] Tetrazines have also been used for the cleavage of a benzonorbornadiene protecting group from amines (Fig. 3d). This reaction is also significantly slower than the TCO-carbamate reaction (k_2 = 0.028 $M^{-1}s^{-1}$ in 10% H_2O/DMSO for the cycloaddition step). However, it offers other advantages, such as the simple synthesis of the benzonorbornadiene probes and the high stability of the probe under physiological conditions.[39]

Recently, 3-isocyanopropyl protecting groups have been cleaved by reaction with tetrazine, resulting in the release of drugs containing amines (doxorubicin and mitomycin C), alcohols (SN-38) and thiols (mercaptopurine) *in vitro*.[40] The cyano group undergoes a [4+1] cycloaddition with the tetrazine to generate a pyrazole-imine intermediate which is then hydrolysed to a 3-oxypropyl species. β-elimination then occurs to release the molecule of interest and acrolein, a highly toxic compound which may be a limitation of this reaction. Advantages include fast kinetics, near-quantitative yields and the synthetic ease of the protecting group. Tetrazine-modified beads were then implanted in zebrafish embryos and the release of mex-

iletine (a voltage-gated sodium channel blocker) was successfully shown to cause a decrease in heart rate.

4.1 *Bifunctional Linkers*

So far, the TCO-tetrazine IEDDA reaction is the only small molecule decaging reaction to be applied *in vivo*. Mejía-Oneto demonstrates a method for controlling drug release from a Dox-TCO carbamate prodrug by installing a tetrazine-modified hydrogel at the site of the tumour in mice.[41] In an alternative strategy, Robillard developed an ADC containing a bifunctional TCO linker that enabled targeted drug release in mice (Fig. 1e).[8] The TCO-carbamate prodrug was attached to an antibody *via* functionalisation of the 5-position of TCO with an activated succinimidyl ester. When reacted with a tetrazine ~65% decaging was observed after 2 min in PBS, and ~25% in mouse serum. Isomerisation of the *trans* alkene in the TCO-carbamate prodrug occurs in biological systems due to the presence of copper ions. This limits the utility of the hydrogel approach as deactivation of the prodrug may occur before it can reach the tetrazine-modified hydrogel. However, the ADC displayed good stability *in vivo* with a half-life almost identical to the free antibody, due to steric hindrance preventing this deactivating isomerisation from occurring.[42] This highlights the fact that the ADC affects the stability and reactivity of the caging group. Importantly, when applied to a tumour xenograft in mice, the ADC showed excellent tumour uptake and reaction occurred with a radiolabelled tetrazine. It was shown that good levels of free doxorubicin were retained in the tumour, however it was not shown that this resulted in tumour shrinkage or increased survival of the mice. Although this was a huge breakthrough as the first example of ADC activation using click-chemistry, there are still several factors which limit its widespread application, including the challenging 11-step synthesis (which requires a specialised UV-irradiation under flow).[8]

Recently Robillard reported the first example of therapy using bioorthogonal decaging in tumour-bearing mice.[9] An ADC consisting of a cytotoxic payload, monomethyl auristatin E (MMAE), conjugated to the cysteine residues in a diabody *via* a bifunctional TCO linker was developed for targeting non-internalising receptors. Near-quantitative drug release was achieved by the use of new tetrazine derivatives. It was found that installing a DOTA chelate on the tetrazine reduced the rate of clearance and therefore increased the decaging yield. It also enabled direct imaging of the tetrazine trigger which was used to show that a good distribution throughout the tumour occurred. The ADC showed selective uptake in the tumour and fast blood clearance. Importantly, a potent therapeutic effect was observed in two mouse xenograft models. In the same models, an analogous ADC bearing the valine-citrulline linker that is cleaved intracellularly by proteases failed to control tumour growth. This work expands the scope of ADCs to non-internalising receptors and therefore, the range of solid tumours that can be treated using ADC therapy. It also highlights the potential of small molecule-triggered decaging reactions for therapeutics. Further work is required to make this strategy more widely applicable by increasing the number of targets, antibodies and payloads which can be used.

5. Future Perspectives

The ability of bioorthogonally cleavable bifunctional linkers to allow spatiotemporally controlled release of biologically active compounds has the potential to make them some of the most powerful tools in chemical biology. They have shown promise in challenging applications such as the targeted delivery of toxic compounds to specific cells both *in vitro* and *in vivo*. As prodrug activation from bioorthogonally cleavable linkers occurs *via* an external trigger, they could also offer important insights into the effects of extracellular delivery.

Compared with the number of decaging reactions that have been reported, there are few examples of bioorthogonally cleavable bifunctional linkers. This is likely due to the fact that many of the reported decaging reactions suffer from limitations, mainly slow reaction rates, and efforts are focussed on improving and expanding the repertoire of decaging reactions before applying these reactions *in vivo*. However, as it has been shown that the bifunctional linker can alter the reactivity and stability of the probe compared to the monofunctional linker, there is a need for more emphasis on bifunctional linkers in the area of bioorthogonal decaging.

With a few exceptions, most reported decaging reactions have been applied solely to the release of cancer drugs. As well as the improvement of the decaging reactions, there is potential for widening the scope of payloads and targets which can be used. Recently, tetrazine-triggered decaging of a TCO-protected epitope was shown to control T-cell proliferation *in vivo*.[43] Following this example, future applications should extend to the delivery of more diverse payloads, for example hormones, neurotransmitters, radionuclides or immunotherapeutics. This will enable the study of the effects of these payloads upon specific cell types or tissues in complex biological systems.

The use of bioorthogonal cleavage reactions has already shown potential for targeted drug delivery. The number of reported bioorthogonal decaging reactions and *in vivo* applications is constantly expanding and we anticipate exciting developments in this area in the future.

Acknowledgements

We thank the EPSRC (PhD studentships to S.D. and B.J.S.) and FCT Portugal (iFCT to G.J.L.B.) for funding. G.J.L.B. is a Royal Society University Research Fellow and holds an ERC Starting Grant (TagIt).

Received: July 9, 2018

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