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REVIEW

Toward Realization of Bioorthogonal Chemistry in the Clinic

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Abstract
In the last decade, the use of bioorthogonal chemistry toward medical applications has increased tremendously. Besides being useful for the production of pharmaceuticals, the efficient, nontoxic reactions open possibilities for the development of therapies that rely on in vivo chemistry between two bioorthogonal components. Here we discuss the latest developments in bioorthogonal chemistry, with a focus on their use in living organisms, the translation from model systems to humans, and the challenges encountered during preclinical development. We aim to provide the reader a broad presentation of the current state of the art and demonstrate the numerous possibilities that bioorthogonal reactions have for clinical use, now and in the near future.

Keywords Bioconjugation · Bioorthogonal chemistry · Bioorthogonal reaction · Click chemistry · Click-to-release · In vivo chemistry

1 Introduction

Bioorthogonal chemistry is a rapidly evolving field at the intersection of chemistry and biology. The term was introduced in 2003 by the group of the 2022 Nobel Prize winner, Carolyn Bertozzi, to describe chemical reactions between nonnative components that are so selective that they can occur in living systems without interfering with surrounding biology [1]. Bioorthogonal reactions are a subgroup of the broader category of click reactions, which are reactions that follow a set of stringent criteria, which include modularity, wideness in scope, high yield, stereospecificity, and the generation of inoffensive byproducts [2].

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Bioorthogonal ligation reactions

Bioorthogonal release reactions

Fig. 1 Main classes of bioorthogonal reactions. Bioorthogonal ligation reactions aim to connect two molecular entities, while bioorthogonal release reactions link two entities while expelling a third entity

Table 1 Bioorthogonal ligation reactions discussed in this review and their reactivity

Entry	Reaction type	Reaction	Reactivity (rate constant k_2 in $M^{-1}s^{-1}$)
1	Staudinger ligation	R_1, O, O–R_2, PPh_2 + N_3–R_3 → ($-N_2$, $+H_2O$, $-HO$–R_2) R_1, O, N(H)–R_3, PPh_2=O	~10^{-3} [a]
2	Strain-promoted azide alkyne cycloaddition (SPAAC)	R_1 + N_3–R_2 → R_1, R_2, N, N, N	~10^{-1}-10^{0} [b]
3	Inverse electron-demand Diels-Alder reaction (IEDDA) tetrazine ligation	R_1 + R_2, N, N, N, N, R_3 → ($-N_2$) R_1, R_2, N, NH, R_3	~10^{2}-10^{6} [c]

[a]Measured in acetonitrile/water (19:1) at room temperature [27]

[b]Measured in acetonitrile/water (2:1) at room temperature [99]

[c]Measured in phosphate buffered saline (PBS) (pH 7.4) at 37 °C [26]

The first bioorthogonal reaction (Fig. 1), the Staudinger ligation, was introduced by the Bertozzi group in 2000, and it involves an azide and a phosphine (Table 1, entry 1) [3]. In the first step of the ligation, on the basis of the earlier Staudinger reaction, an azide and a phosphine react to give an unstable phosphoraza-ylide. A proximal acyl group on the phosphine then reacts intramolecularly to give a stable amide ligation product. A total of 2 years after the Staudinger ligation, the copper-catalyzed azide-alkyne cycloaddition (CuAAC) was reported on by the groups of Meldal and Sharpless [4, 5]. The requirement of a toxic copper catalyst rendered this ligation reaction non-bioorthogonal. However, in 2004 the Bertozzi group developed a copper-free variant of this reaction, the strain-promoted azide-alkyne cycloaddition (SPAAC), in which reactivity toward the azide is promoted by the use of cyclic strained alkynes, rather than by a catalyst (Table 1, entry 2) [6]. Following this discovery, various substituted strained alkynes with enhanced reactivities were developed [7–13]. In 2008, the faster tetrazine ligation was reported by Fox et al., which proceeds through the inverse electron-demand Diels–Alder (IEDDA) reaction between a diene (a tetrazine)

and a dienophile (a *trans*-cyclooctene; TCO), with the release of dinitrogen gas (Table 1, entry 3) [14]. An overview of several bioorthogonal ligation reactions is provided in Table 1. These fundamental reactions and more bioorthogonal reactions are described in detail in Chapter 1.

Besides ligation, bioorthogonal reactions leading to intramolecular cleavage were developed (Fig. 1). Generally, these two-step reactions start with a ligation step (commonly referred to as “click”) followed by a cleavage step (referred to as “release”). The first of these “click-to-release” reactions, introduced by our group in 2013, was termed the IEDDA pyridazine elimination and occurs between a tetrazine and a *trans*-cyclooctene (TCO) functionalized on the allylic position with a carbamate linked to a payload [15]. After the initial tetrazine-TCO click step, an intramolecular electronic cascade within the formed dihydropyridazine leads to cleavage of the carbamate and release of an amine-containing payload and CO_2. After 2013, more bioorthogonal cleavage reactions were developed, as described in Chapter 4, of which a selection, including the most promising ones for in vivo applications, are summarized in Table 2.

Another important class of bioorthogonal reactions involves the use of transition metal catalysts [16]. While abiotic metals generally are not well tolerated by living organisms, smart designs have been recently developed that enable the use of these catalysts in vitro and even in vivo [17–20]. Although promising for future clinical applications, a thorough description of bioorthogonal organometallic reactions is beyond the scope of this chapter, and here we limit our analysis to bioorthogonal reactions utilizing organic molecules.

Table 2 Bioorthogonal release reactions discussed in this review and their reactivity

	Ligation reaction step			Release reaction step	
Entry	Reaction type	Ligation reaction	Reactivity (rate constant k_2 in $M^{-1}s^{-1}$)	Reaction type	Release reaction
1	Inverse electron-demand Diels-Alder reaction (IEDDA)		~10^{-1}-10^{2} [a]	Pyridazine elimination	
2			~10^{-5}-10^{-3} [b]	Pyridazine elimination	
3			~10^{-1}-10^{0} [c]	Lactonization	
4	Strain-promoted cycloaddition		~10^{-1}-10^{0} [d]	Cheletropic reaction	
5			~10^{0}-10^{2} [e]	Retro-Diels Alder, hydrolysis	
6	Staudinger ligation		~10^{-3} [f]	Hydrolysis	

[a] Measured in acetonitrile at room temperature [15]

[b] Measured in water/dimethylformamide (1:9) at 37 °C [100]

[c] Measured in PBS/dimethylsulfoxide (DMSO) (1:9) at 37 °C [88]

[d] Measured in DMSO/PBS (1:9) at 37 °C [101]

[e] Measured in water at room temperature [84]

[f] Measured in acetonitrile/water (19:1) at room temperature [27]

1.1 Bioorthogonal Reactions in Drug Development

To date, bioorthogonal chemistry finds applications in many phases of the drug development process.

Owing to their high efficiency and selectivity, bioorthogonal reactions are used for the production of pharmaceuticals. A major application is the ligation of toxins to antibodies to make antibody–drug conjugates (ADCs). Contrary to standard lysine and cysteine conjugations, enzymatic incorporation of bioorthogonal click handles in proteins allows site-selective and quantitative drug coupling, and reproducible ADC preparation. At the moment, a handful of companies have initiated clinical trials with ADCs prepared using bioorthogonal chemistry [21] and, undoubtedly, more will follow.

The benefit of bioorthogonal reactions for the construction of radiotracers and radiotherapeutics has been recognized [22, 23]. Similar to ADC preparation, click reactions can be used for site-specific and reproducible conjugation of radiolabels to form radiopharmaceuticals. But bioorthogonal chemistry has more features that are appealing for radiosynthesis. The production of radiopharmaceuticals is often a race against time owing to the decay of radionuclides. In addition, radiolabeling procedures often require high temperatures or organic solvents, conditions that might not be compatible with the biomolecule to be labeled. To overcome these drawbacks, prosthetic groups can be used, which are radiolabeled in harsh conditions and are subsequently rapidly conjugated to the biomolecule under mild conditions via simple bioorthogonal reactions. Several clinical trials on radiopharmaceuticals radiolabeled with clickable prosthetic groups are ongoing [23].

Another highly promising application of bioorthogonal chemistry is the controlled assembly or disassembly of molecules inside the body of a patient for on-target drug assembly or activation, or off-targeted therapy deactivation. In general, in vivo chemistry approaches can be divided into two categories (Fig. 2):

1. Approaches that employ bioorthogonal chemistry as a tool for in vivo conjugation (Sect. 4.1). For radioimmuno-imaging and -therapy (imaging and therapy using radioactive antibodies) this includes tumor pretargeting, in which a targeted antibody conjugates to a radioactive probe on-target (Sect. 4.1.1). Moreover, off-target removal of antibodies or drugs can be achieved by in vivo conjugation to a clearing agent (Sect. 4.1.2). On-tumor drug assembly belongs to this category of in vivo chemistry, but has not yet been explored in living animals and therefore will not be discussed in this chapter.
2. Approaches relying on click-to-release bioorthogonal reactions (Sect. 4.2). This category includes on-target drug activation (by means of drug release or unmasking of a prodrug) upon click-to-release reaction with an activator molecule (Sect. 4.2.1). Also in this category, in the context of radioimmunoimaging and therapy, is the enhancement of radioactivity clearance by trigger-stimulated separation of a radioactive moiety from an antibody (off-target deactivation) (Sect. 4.2.2).

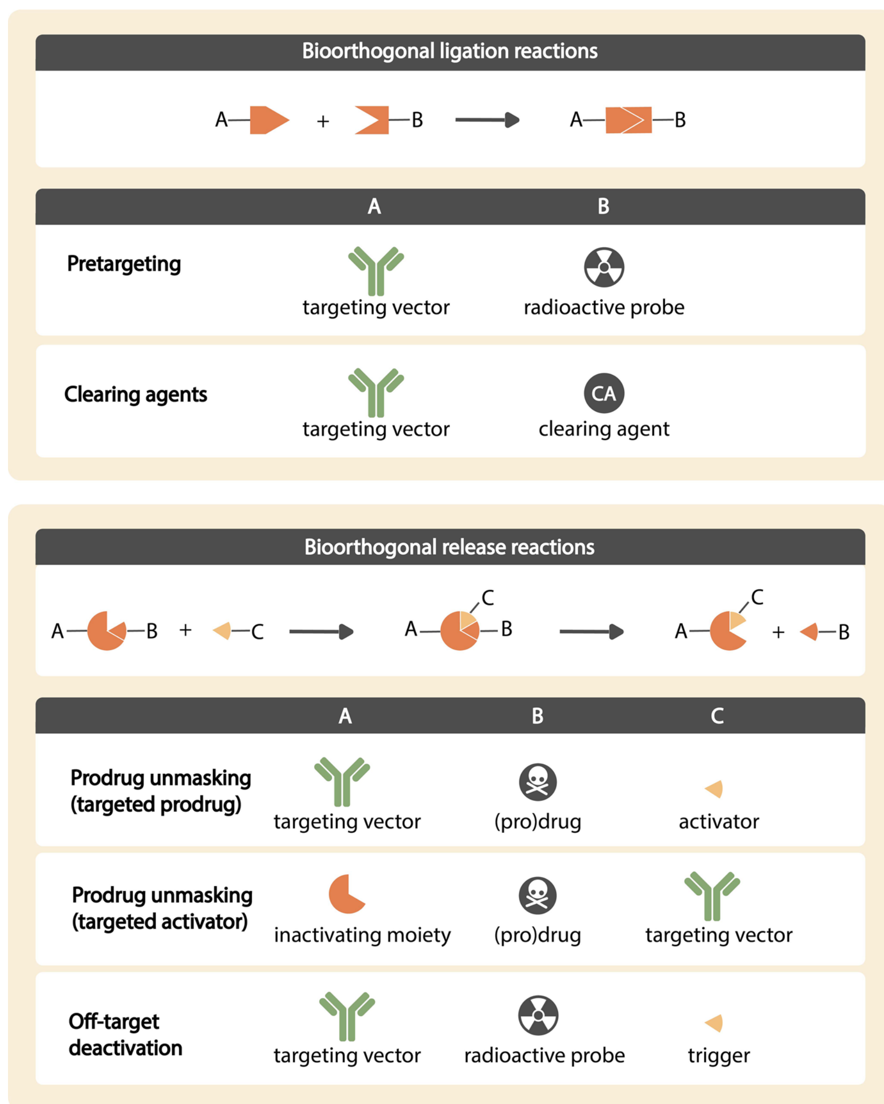


Fig. 2 Overview of the most promising applications of bioorthogonal ligation and release reactions

To date, no in vivo chemistry approach has been approved for clinical use by the Food and Drug Administration (FDA) or European Medicines Agency (EMA); a few trials are ongoing. In the next sections we will describe the most promising applications of bioorthogonal chemistry that are making their way to or through the clinic, and we will discuss the potential challenges in this translation process.

2 Requirements of Bioorthogonal Reactions for In Vivo Chemistry

Performing a reaction on a bench is clearly distinct from eliciting a reaction in a living patient. Reactions used for this purpose need to adhere to more stringent requirements in order to obtain a high reaction yield. Despite the high bar for a click reaction to be considered bioorthogonal, not all bioorthogonal reactions are suited to be used in vivo in medically relevant conditions owing to, among other reasons, pharmacokinetics constraints. The yield of a chemical reaction when performed in vivo is controlled by several factors that should be optimized to obtain maximal conversion, but are in part constrained by the limitations imposed by the living system. These factors will be discussed in this chapter and include: (1) concentration, (2) reaction rate, (3) available reaction time, (4) stability, and (5) bioavailability.

2.1 Concentration of the Reagents

Up to the present moment, life-threatening conditions such as cancer have been the key application areas for in vivo chemistry. In most of these cases, the first of the two bioorthogonal reagents is incorporated in a targeting moiety that binds cancer cells via interaction with tumor-specific antigens. This greatly limits the in vivo concentration of at least one of the two reagents as, in general, the concentration of biological targets is very low (micromolar or lower). In contrast, the second reagent, if it is administered intravenously and is not targeted, is subject to enormous dilution in the body, given the average 3 L of blood plasma and an additional 11 L of extracellular interstitial fluid present in an adult [24]. High reactivity of a bioorthogonal system is therefore crucial to obtain sufficient reaction yields at medically relevant concentrations within the available reaction time.

As the reactions are governed by second order kinetics, one method to increase the in vivo reaction yield is to increase the concentration of one of the two reagents, usually the least toxic one. For instance, in the case of on-tumor prodrug activation, the activator is generally a nontoxic molecule and can therefore be administered in large excess. The activator can also be locally implanted at the tumor site or can be coupled to a nanocarrier to enhance local concentration. Tumor pretargeting for radioimmunoimaging and -therapy cannot make use of these “tricks”, as the first reagent is usually a tagged antibody which must bind the limited amount of tumor receptors, and the second reagent is a radioactive moiety that cannot be administered in large excess as overexposure leads to toxicity. This approach is therefore the most demanding one among those that we will discuss in the next sections.

2.2 Reaction Rate of the Reagents

The bioorthogonal reactions available to date make use of very different reagents with a large variation in substituents influencing electron density and/or strain on the reaction center, thereby providing a wide range of rate constants. Most of these reactions occur via second order kinetics characterized by a second-order rate constant (k_2). The k_2 of the Staudinger ligation is about 10^{-3} M^{-1} s^{-1} [25]. At 1

micromolar (μM) concentration of both reactants, this leads to a reaction half-life of about 32 years, while for most in vivo applications, reagents must react in seconds to maximally days (see Sect. 3.3). The development of SPAAC reactions utilizing dibenzocyclooctyne (DBCO) derivatives and azides strongly improved reactivities, affording rate constants of about 1 M^{-1} s^{-1} (12 days reaction half-life with reagent concentrations of 1 μM) [11]. The real improvement, however, came with the development of the IEDDA reaction, which is the fastest bioorthogonal reaction known to date. The reaction between an electron-deficient 1,2,4,5-tetrazine with a strained *trans*-cyclooctene (TCO) occurs with a second order rate constant up to 10^6 M^{-1} s^{-1} [14, 26]. For reagent concentrations of 1 μM, the reaction half-life for reactions with this rate is merely 1 s. Therefore, the IEDDA reaction is an excellent candidate for in vivo chemistry at submicromolar concentrations or for reagents with limited stability or circulation time.

2.3 Available Reaction Time

To achieve maximum reaction yields in vivo, a reaction must reach full conversion within the available time. The time required for a reaction to be completed is determined by the reaction rate and the concentration of the reagents, as elaborated in preceding Sects. 3.1 and 3.2. Whether this time is available in an in vivo setting depends on the pharmacokinetic properties of both reagents, which dictate their in vivo behavior in terms of absorption, distribution, metabolism, and excretion. As most reagents for in vivo chemistry developed thus far are administered via a systemic injection, clearance from blood and (other) target organs is the major parameter governing the available reaction time. Clearance is strongly dependent on the structure and type of a reagent, and elimination half-lives in blood can vary from seconds (small molecules) to multiple days (full size immunoglobulins (IgGs)). For a treatment in which the two reagents are subsequently administered with an interval, it is crucial that a sufficient amount of the first-administered reagent remains present after the interval. Increasing size, altering hydrophobicity, or using a targeting moiety may be effective strategies for prolongation of the retention time in a target tissue. Alternatively, implantable materials with slow clearance could be used to increase the time that a reagent is present in the target tissue. In optimally designed reagents, the elimination half-lives and administration scheme should be matched with the reaction rate at available concentrations.

2.4 Stability of the Reagents

The stability of the reactive handles is another relevant factor when considering the in vivo application of bioorthogonal chemistry and is closely linked to the previous section on available reaction time. Especially for systems with fast reaction kinetics, the stability of individual components may be problematic, as reactivity and stability of a chemical moiety are usually inversely correlated. For example, electron-donating substituents on the phosphines used in the Staudinger ligation significantly speed up reaction kinetics compared with electron-withdrawing groups. However, the extra electron

density makes these more reactive phosphines more sensitive to air oxidation [27]. In IEDDA reactions, the stability of tetrazines in aqueous solutions is limited, especially when strong electron withdrawing groups are introduced on the tetrazine ring to boost reactivity [28]. Furthermore, it was shown that isomerization of TCOs to unreactive *cis*-cyclooctenes (CCOs) may occur in vivo. This transition is thought to result from the interaction of TCOs with circulating copper-containing proteins, such as albumin, and is faster with highly strained (and highly reactive) TCO derivatives [29].

One must consider the time that a bioorthogonal reagent must survive in vivo to exert its effect. In a two-step protocol, for instance, the first reagent needs to be stable in vivo at least until the second one is administered, which can range from a few hours to several days. Therefore, a sufficiently stable reaction handle should be chosen for the (bio)molecule to be injected first, and this may require a trade-off between stability and reactivity. On the contrary, for the second reagent, which is usually excreted within a few minutes, a stability spanning several days becomes superfluous. In this case, reagents with limited stability and higher reactivity may be better suited. As reduced stability decreases the concentration of the reactive molecule, strategies mentioned earlier for enhancing the (local) concentration of a reagent may be used to compensate for its limited stability.

2.5 Bioavailability

Another factor influencing the success of a bioorthogonal reaction in vivo is the bioavailability of the reactive handles. Bioavailability refers to the degree at which a substance is able to access the circulation and reach its target area in the body of a patient unencumbered. This is an important determinant for the in vivo reaction yield as a reagent needs to reach its reaction partner to react with it. Upon entering the patient's body, the reagents may interact with endogenous proteins. Binding of drugs to plasma proteins can be very useful for the prolongation of half-life of drugs [30]. However, binding to plasma proteins can prevent the interaction of a receptor-binding molecule with its target [31]. Similarly, reactive handles can be masked by serum proteins, preventing them from reacting with their chemical partner in vivo. In addition, protein binding can trap reagents within the bloodstream [32], limiting their ability to reach the other reactant in target tissues.

As for the other factors influencing reaction yields in vivo, bioavailability is highly dependent on a drug's chemical structure. Lipophilic structures are more susceptible to nonspecific binding to blood proteins such as albumin [33], and many bioorthogonal reaction handles comprise bulky, lipophilic groups. Lipophilicity can likewise influence target binding and cellular internalization [34]. To maximize in vivo reaction efficiency, optimization of structural properties is essential to ensure high availability of the bioorthogonal reagents at the target site.

3 In Vivo Applications of Bioorthogonal Reactions

Several in vivo applications of bioorthogonal reactions were reported in recent years. Although to date no applications of bioorthogonal chemistry have been clinically approved, ample preclinical studies have been performed, and some of these concepts are already in clinical trials. Those promising in vivo applications will be discussed in the following sections.

3.1 In Vivo Conjugation by Bioorthogonal Ligation Reactions

3.1.1 On-Target Conjugation: Tumor Pretargeting

Radioimmunoimaging and -therapy uses a monoclonal antibody (mAb) to selectively deliver mAb-bound radionuclide to the site of interest (typically cancer cells). Radioimmunotherapy (RIT) of solid tumors is hampered by the low therapeutic index of radiolabeled mAbs and by the relatively high radioresistance of this type of tumors, contrary to that of diffuse malignancies such as lymphomas [35]. Radionuclides emitting beta (e.g., Lu-177 and Y-90) and alpha radiation (e.g., Pb-212, At-211, Ac-225, and Th-227) have a high cell killing potential owing to the DNA damage caused by the emitted radiation when the radioactive decay occurs in close proximity to tumor cells. However, radiolabeled intact IgGs, which typically afford higher tumor uptake and retention in solid tumors compared with antibody fragments [36], exhibit dose-limiting toxicity in radiosensitive bone marrow, caused by the long circulation time in the blood. Furthermore, the use in RIT with highly potent alpha emitters Ac-225 and Th-227, which produce multiple α and β^- emissions, causes additional toxicities. Upon the first radioactive decay, recoil energy causes release of the daughter radionuclide from the circulating radioimmunoconjugate. Accumulation of these secondary emitters in nontarget organs, such as the kidney, produces long term toxicity [37, 38]. Moreover, for radioimmunoimaging, the slow clearance and the localization of radioimmunoconjugates in off-target tissues is problematic, as it leads to strongly reduced image contrast; and the slow clearance prevents the use of radionuclides with short half-lives (e.g., F-18, and Ga-68).

Pretargeting aims to overcome these problems, as in this strategy the tumor-targeting antibody and radioactive probe are administered separately, with an interval in between. After tumor accumulation and clearance of the antibody from circulation, the radioactive probe is administered, which recognizes and binds the antibody and otherwise clears fast from the circulation (Fig. 3).

The main tumor pretargeting approaches developed so far employ antibody-hapten, biotin-avidin/streptavidin, or antisense oligonucleotides interactions for the binding of the probe to the antibody [39, 40]. These "biological" pretargeting approaches, however, showed some disadvantages. Avidin and streptavidin, for instance, are known immunogenic proteins. The production of anti-tumor and anti-hapten bispecific mAbs requires extensive protein engineering. In addition, oligonucleotides suffer from limited stability toward enzymatic degradation. Despite these

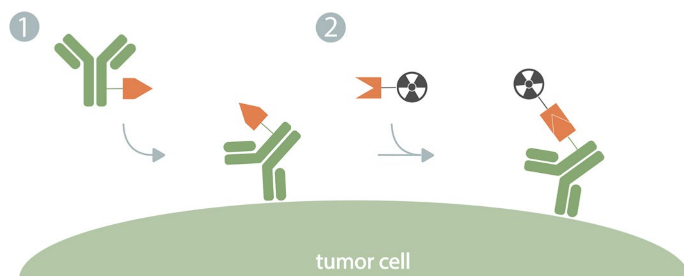


Fig. 3 Schematic representation of a pretargeting approach. In the first step, a targeting vector with a bioorthogonal tag is administered and binds to its target. After an interval, during which the targeting vector clears from circulation, a radioactive probe with a bioorthogonal tag is administered, which ligates to the tumor-bound targeting vector

drawbacks, some of these strategies were tested in a clinical setting, but were later abandoned, as described in other reviews [40, 41].

One new pretargeting approach based on an anti-dodecane tetraacetic acid (DOTA) and anti-tumor (GD2) targeting protein that self-assembles and disassembles (SADA) is currently being tested in a phase I clinical trial (NCT05130255) [42].

Bioorthogonal chemistry offers an attractive alternative to "biological" pretargeting. The tags used for bioorthogonal reactions are small, not prone to immunogenicity, and easy to synthesize and conjugate to tumor targeting vectors. Moreover, the formation of a covalent bond following the chemical reaction between a tagged mAb and a radiolabeled probe prevents the dissociation of the probe from the targeting moiety, thereby reducing the radioactivity washout from tumor.

In 2005, our group started working on new tumor pretargeting strategies involving various bioorthogonal reactions. We developed a pretargeting system on the basis of the Staudinger ligation between an azide functionalized antibody (U36) and a radiolabeled phosphine [43]. While this approach seemed promising in vitro, insufficient reaction rates combined with rapid biological clearance and limited stability of the radiolabeled phosphine probe precluded reaction in vivo. Similarly, when using the SPAAC reaction between azide-conjugated rituximab and radiolabeled cyclooctyne probes for tumor pretargeting, we observed poor in vivo performances in mice owing to low reactivity [44]. Surprisingly, in 2013, Lee et al. showed successful pretargeting in mice using DBCO-conjugated tumor-targeting nanoparticles and a radiolabeled azide as second component [45]. Presumably, the high local concentration of DBCO that was achieved in the tumor via nanoparticle enrichment enabled a sufficient reaction yield despite the low reaction rate of the SPAAC, leading to significant radioactivity uptake in the tumor. Similarly, Au et al. obtained tumor targeting and anti-tumor effects in xenograft and lymphoma murine models with an ^{90}Y-labeled azide dendrimer after a prior injection of DBCO-functionalized anti-CD20 antibody [46].

In 2008, the groups of Fox [14] and Weissleder [47] published the first reports on the IEDDA reaction between a tetrazine and a strained dienophile, respectively TCO and norbornene. The reported IEDDA reactions had ~10,000-fold (for TCO) and

10-fold (for norbornene) higher reactivity than the SPAAC. Shortly after, our group showed that this reaction could be successfully used for pretargeted single-photon emission computed tomography (SPECT) imaging of a mouse model of colon cancer using a TCO-tagged mAb (CC49) and an ^{111}In-labeled electron-deficient tetrazine (Fig. 4) [48]. Noticeably, besides the high reaction yield between the tetrazine and the TCO on-tumor (52–57%), we found good in vivo stability of the TCO in mice (75% intact after 24 h), high tetrazine stability in serum in vitro (94% after 1 h), and rapid tetrazine clearance from blood (9.8 min half-life).

These first findings demonstrated the potential application of the TCO-tetrazine IEDDA reaction for tumor pretargeting in the clinic and led to the development of other tetrazine-TCO-based pretargeting systems. The groups of Lewis and Zeglis developed a TCO-conjugated humanized A33 antibody, which targets colorectal cancers [49]. A ^{64}Cu-labeled sarcophagine-tetrazine probe (Tz-SarAr) was then developed (Fig. 5), which showed sustained circulation in blood (~ 16 min half-life) and rapid elimination via the kidneys [50]. Besides imaging, the Lewis and Zeglis groups performed pretargeted tumor therapy using the same antibody and a therapeutic ^{177}Lu-labeled DOTA-PEG$_7$-tetrazine probe [51, 52]. Successful imaging [53] and therapy [54, 55] of pancreatic tumors was achieved using the carbohydrate antigen 19.9 (CA19.9) targeting antibody 5B1. Despite the internalizing and shedding nature of this target, pretargeting with high tumor-to-tissue ratios was accomplished

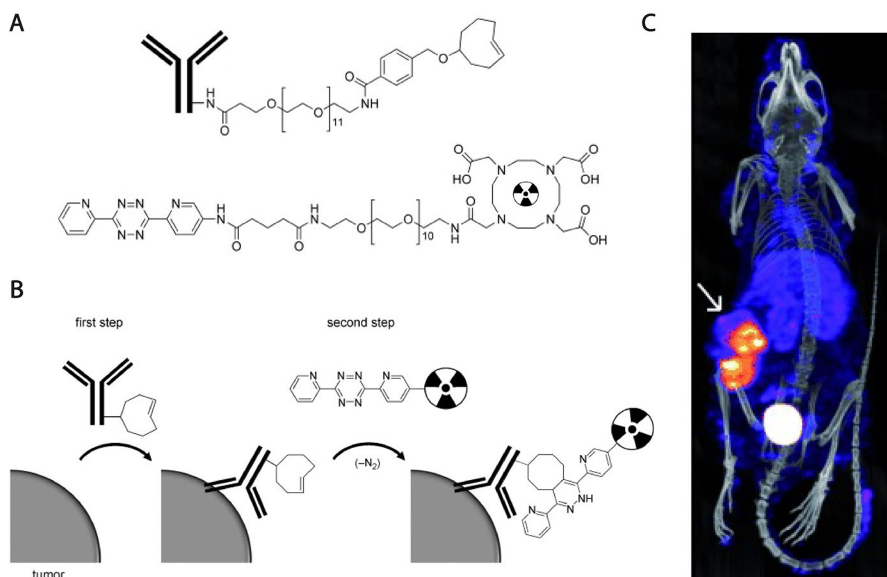


Fig. 4 First TCO-tetrazine pretargeting system as presented by Rossin et al. [48]. **A** Chemical structures of TCO-conjugated CC49 antibody (top) and radiolabeled tetrazine-DOTA probe (bottom). **B** Schematic representation of the two steps in an IEDDA-based pretargeting system. **C** SPECT/computed tomography (CT) image of a mouse administered with CC49-TCO, followed 1 day later by ^{111}In-labeled tetrazine. The tumor is indicated by a white arrow [48]. Reprinted and adapted with permission from Rossin, R., et al., "In vivo chemistry for pretargeted tumor imaging in live." *Angew Chem Int Ed Engl*, 2010. 49(19): p. 3375–8. Copyright 2010 John Wiley and Sons

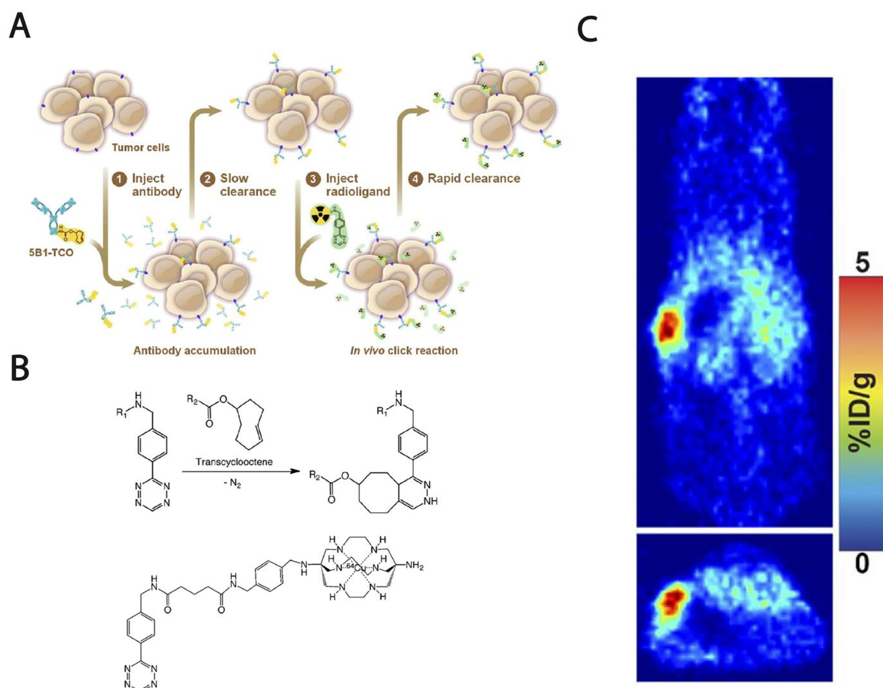


Fig. 5 Pretargeting components used in the first clinical trial of bioorthogonal-chemistry-based pretargeting. **A** Schematic representation of 5B1-TCO and tetrazine-based pretargeting system [53]. **B** Tetrazine ligation reaction between the tetrazine probe and antibody conjugated TCO (top), and chemical structure of [^{64}Cu]Cu-Tz-SarAr (bottom) [50]. Reprinted with permission from Zeglis, B.M., et al., "Optimization of a pretargeted strategy for the PET imaging of colorectal carcinoma via the modulation of radioligand pharmacokinetics." *Mol Pharm*, 2015. 12(10): p. 3575–87. Copyright 2015 American Chemical Society. **C** Positron emission tomography (PET) image of tumor-bearing mouse administered with 5B1-TCO followed by [^{64}Cu]Cu-NOTA-PEG$_7$-Tz 72 h later [53]. Reprinted with permission from Houghton, J.L., et al., "Pretargeted immuno-PET of pancreatic cancer: overcoming circulating antigen and internalized antibody to reduce radiation doses". *J Nucl Med*, 2016. 57(3): p. 453–9. Copyright 2016 Society of Nuclear Medicine and Molecular Imaging

after an interval of 72 h, thus showing that optimal dosing and timing interval of injections are critical factors in determining the success of a pretargeting strategy.

The importance of rational design of tetrazine probes for pretargeting was underlined by Herth and colleagues, who demonstrated that bispyridyl tetrazines with more hydrophilic substituents were the more reactive ones, which correlated with increased in vivo tetrazine/TCO ligation efficiency [26]. These insights were used for the development of tetrazine-ligation-based pretargeting with radiohalogen tetrazine probes. While ^{18}F is the most used radionuclide for clinical PET imaging, pretargeting using radiofluorinated tetrazines was limited by their challenging production, especially for the more reactive mono- or bis-(hetero)aryl substituted tetrazines. The group of Herth overcame these problems by the development of a procedure for direct fluorination of these highly reactive tetrazines [56–59], and demonstrated their use in pretargeting procedures [60]. The group also developed

methods for direct labeling of tetrazines with ^{11}C, another frequently used PET isotope [61, 62]. Besides possibilities for tumor imaging, the ability of some of these ^{18}F- and ^{11}C-labeled tetrazines to cross the blood–brain barrier opens possibilities for pretargeted brain imaging [63].

A variation on the antibody-based pretargeting systems was presented by Valliant and colleagues in 2016 [64]. Instead of using a mAb-TCO for tumor targeting, this group used a TCO-conjugated bisphosphonate (BP-TCO) agent for the targeting of sites with active bone metabolism in the context of PET imaging for bone cancer and metastases [64]. In contrast to antibodies, the low molecular-weight BP does not suffer from lengthy blood circulation time and therefore, in principle, it does not require a two-step pretargeting approach. Despite this, this system was used for the first translation of IEDDA pretargeting into larger animals, and Maitz et al. combined TCO-BP with [^{64}Cu]Cu-Tz-SarAr for PET imaging of canine patients with osteodestructive lesions (Fig. 6) [65]. This study paved the way for the first human clinical trials with tetrazine ligation-based pretargeting. Currently, the mAb-based pretargeting system using hu5B1-TCO and [^{64}Cu]Cu-Tz-SarAr (Fig. 5) is being

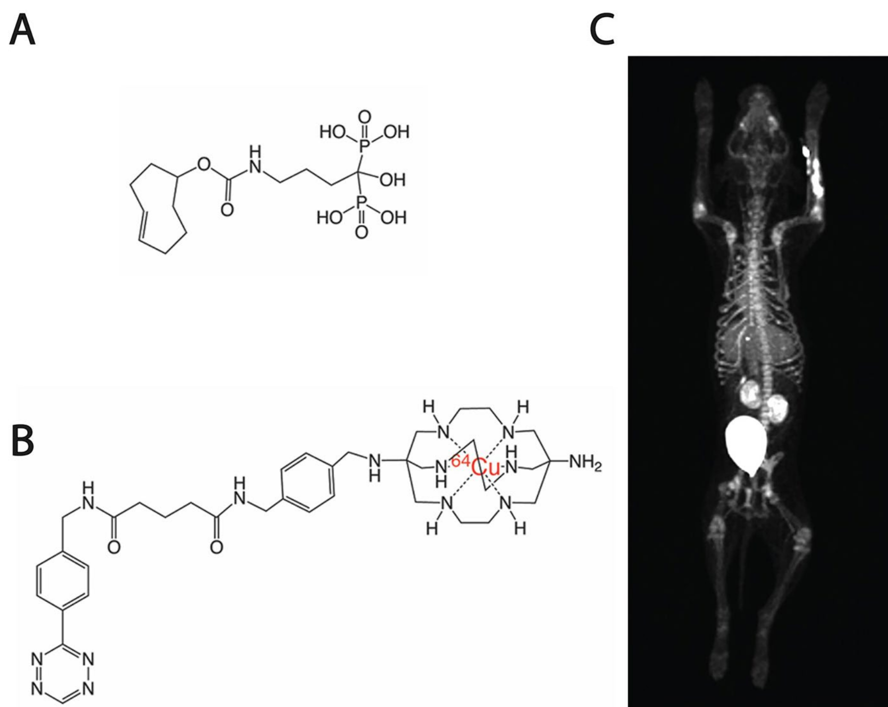


Fig. 6 First clinical trial and demonstration of bioorthogonal chemistry in large animals. **A** Chemical structure of TCO-conjugated bisphosphonate (BP-TCO). **B** Chemical structure of [^{64}Cu]Cu-SarAr-Tz probe. **C** Pretargeted PET image of a dog with an osteodestructive lesion in left ulna administered with TCO-BP and [^{64}Cu]Cu-SarAr-Tz [65]. Reprinted with permission from Maitz, C.A., et al., "Pretargeted PET of osteodestructive lesions in dogs." *Mol Pharm*, 2022. 19(9): p. 3153–3162. Copyright 2022 American Chemical Society

evaluated in patients with pancreatic cancer in a phase I clinical trial at the Memorial Sloan Kettering Cancer Center (NCT05737615).

3.1.2 Off-Target Conjugation: Clearing Agent Conjugation

To maximize tumor-to-blood ratios, pretargeting procedures require complete clearance of tagged antibodies from circulation prior to probe injection. In humans, antibodies can circulate for several weeks, which requires a lengthy interval and an unrealistic stability of the tumor-bound antibody and bioorthogonal reaction handle. To overcome these challenges, clearing agents can be used to enhance clearance of antibodies from circulation (Fig. 7). Our group improved the effectivity of the tetrazine ligation-based pretargeting approach with TCO-tagged CC49 antibody by combination with a tetrazine-containing clearing agent [66]: either albumin coated with galactose and tetrazine moieties or polystyrene beads coated with tetrazine moieties. Both designs resulted in rapid reaction with circulating antibody and its hepatobiliary clearance. Subsequent administration of ^{177}Lu-labeled tetrazine probe led to a boost of the tumor-to-blood ratio from 2 (without clearing agent) to 80 and 250, with one and two doses of clearing agent, respectively. Dosimetry calculations indicated that this approach could afford an order of magnitude higher tumor dose in humans compared with non-pretargeted radioimmunotherapy [66]. A similar strategy was developed for the earlier mentioned TCO-tagged 5B1 antibody [67]. Here, a tetrazine-decorated dextran masking agent was made that reacts and thereby inactivates circulating antibody-bound TCO. The large size of the masking agent prevents extravasation and inactivation of A33-TCO in the tumor. Tumor-to-blood ratios were improved, while the interval between the antibody and radiolabeled probe could be reduced [67].

In addition, outside pretargeting in vivo conjugation of clearing agents can be of use, as in vivo ligation to a radiolabeled antibody can reduce radiation exposure in blood. While initial designs were based on clearing agent binding via biological

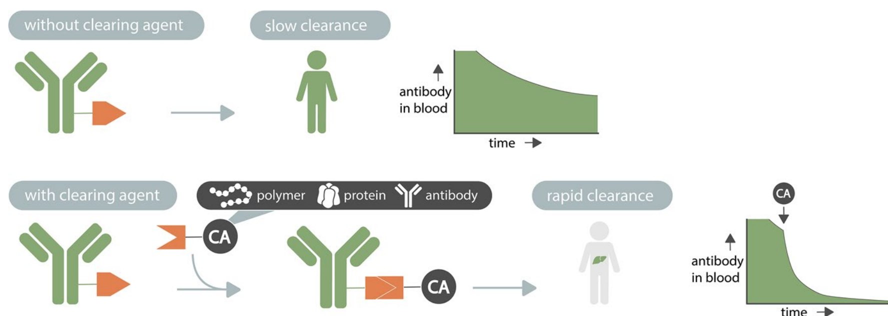


Fig. 7 In vivo bioorthogonal ligation of a clearing agent to tagged antibodies for pretargeting. Without clearing agent, antibodies undergo slow clearance from blood. In vivo conjugation of a clearing agent directs circulating antibodies toward rapid excretion (i.e., via the liver) and/or inactivates them. Instead of antibodies, this strategy can likewise be used on other bioorthogonally-tagged drug molecules

interactions, the usefulness of bioorthogonal chemistry for this purpose was recently demonstrated in the context of high contrast brain imaging [68].

Another application of bioorthogonal ligation reactions is their use for neutralization and enhanced clearance of molecular drugs upon iatrogenic accidents. This was illustrated by the group of Wagner using the anti-coagulant drug warfarin, which was modified to carry an azide without altering its coagulant activity (Fig. 8) [69]. Via an in vivo SPAAC with a bicyclononyne-polyethylene glycol (BCN-PEG)-based clearing agent, the drug could be inactivated, and at the same time its clearance was enhanced, thereby almost completely restoring coagulant properties of the blood of mice [69].

3.2 In Vivo Cleavage by Bioorthogonal Release Reactions

3.2.1 On-Target Activation: Prodrug Unmasking and Drug Release from ADCs

Cleavage reactions are the latest addition to the toolbox of bioorthogonal reactions and are the basis of the major application of bioorthogonal chemistry in vivo, i.e., on-target activation of prodrugs (Fig. 9). While conversion of an inactive prodrug to an active drug in tumors by means of enzymes is an established approach [70], it is not universally applicable (see below).

In 2013, our group reported the first example of a cleavage reaction based on the IEDDA cycloaddition between TCO and tetrazine, termed the IEDDA pyridazine elimination, demonstrating chemically triggered release of doxorubicin (Dox) from a TCO-Dox prodrug in cell culture [15]. Subsequently, we applied this "click-to-release" reaction for on-tumor activation of click-cleavable antibody–drug conjugates (ADCs) [15, 71].

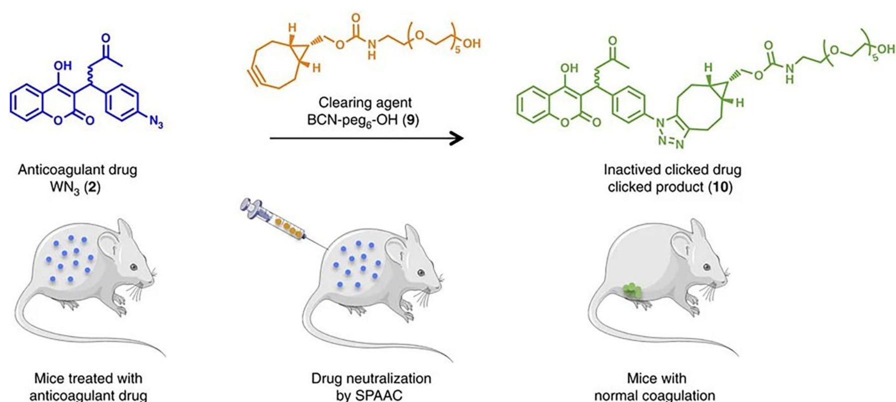


Fig. 8 Neutralization and clearance enhancement of a warfarin drug by in vivo conjugation to a clearing agent. Azido-warfarin (WN_3) reacts in vivo with a BCN-PEG$_6$-OH clearing agent to give an inactivated drug [69]. Reprinted with permission from Ursuegui, S., et al., "An in vivo strategy to counteract post-administration anticoagulant activity of azido-Warfarin." *Nat Commun*, 2017. 8: p. 15,242. Copyright 2017 Springer Nature, reproduction under CC BY 4.0

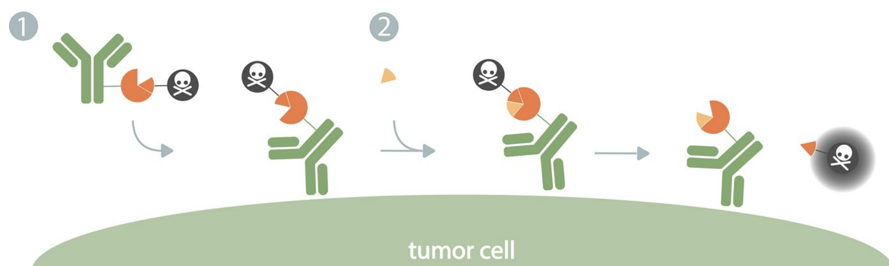


Fig. 9 Schematic representation of prodrug unmasking using a bioorthogonal reaction. In the first step, a targeted prodrug is administered and accumulates in its target tissue. In the second step, an activator molecule is administered that reacts with the prodrug, and subsequently releases the drug in an active form. In addition, prodrug unmasking systems exist in which a targeted activator is administered prior to a non-targeted prodrug

ADCs are potent biopharmaceuticals that combine the tumor targeting of receptor-specific mAbs with the cell killing capabilities of highly potent toxins (the warhead). Nowadays, the field of ADC development is booming, with over 100 new compounds in clinical trials [72]. Current ADCs rely on internalization into tumor cells for warhead release through protease-based cleavage, among others, leading to cell killing. However, the number of tumor-specific receptors with fast internalization and recycling, suitable for ADC applications, is limited [73]. Moreover, intracellular warhead release preferentially kills tumor cells that have relatively high receptor expression, leaving pockets of receptor-negative cells beyond the reach of the drug, free to continue proliferating [74]. On the contrary, click-cleavable ADCs are designed to target tumor-specific extracellular and stromal receptors and markers, thus expanding the scope of current ADCs. Such ADCs burst-release the warhead in the extracellular space upon reaction with a chemical activator, which is administered in a second step after ADC localization, thus giving complete control of the release. In addition, the released cell-permeable drug can diffuse through the tumor microenvironment and enters both receptor positive and receptor negative cells, thus maximizing the “bystander effect”.

In our initial ADC design, Dox was conjugated to the anti-TAG72 mAb CC49 via a TCO linker using a carbamate bond [15]. In mice bearing LS174T xenografts, CC49-TCO-Dox showed the expected sustained blood circulation and high tumor uptake [71]. As previously observed in vitro with the Dox-TCO prodrug [15], we showed that electron donating substituents on the tetrazine increased efficiency of the elimination reaction, but reduced the reactivity toward the TCO linker [71]. As a result, to enable sufficient click reaction in the tumor, a tetrazine with sustained circulation in the blood was required [75]. Finally, owing to the long circulation of the full-size IgG, a clearing agent was used to remove CC49-TCO-Dox from blood and nontarget tissues before activator administration to prevent systemic drug release. In our second ADC design, we switched to a faster clearing PEGylated CC49 diabody carrying the potent anti-tubulin monomethyl auristatin E (MMAE) warhead linked via TCO and combined this with a low molecular weight tetrazine activator with sufficient blood circulation (half-life 12 min in mice) (Fig. 10). The optimized

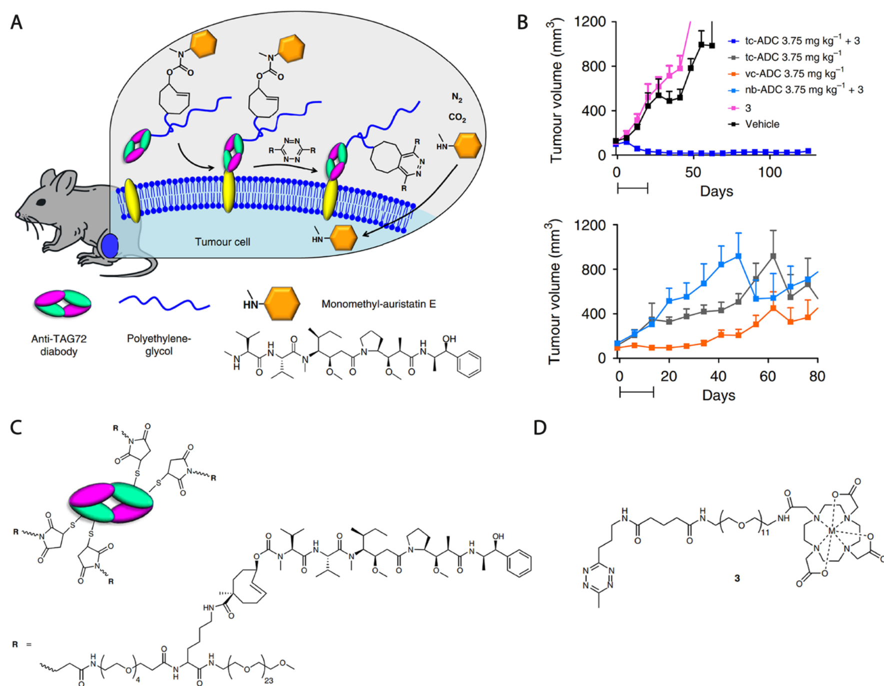


Fig. 10 Bioorthogonally activated prodrug system with optimized ADC and activator. **A** Schematic representation of IEDDA-based ADC prodrug activation. **B** Therapeutic efficacy of ADC (tc-ADC) alone or in combination with activator (3), and comparison with ADCs with an enzymatically cleavable linker (vc-ADC) and non-tumor binding antibody (nb-ADC), as evaluated in OVCAR-3 ovarian carcinoma mouse models. **C** Chemical structures of anti-TAG72 diabody-TCO ADC and tetrazine activator [75]. Reprinted and adapted with permission from Rossin, R., et al., "Chemically triggered drug release from an antibody–drug conjugate leads to potent antitumour activity in mice." *Nat Commun*, 2018. 9(1): p. 1484. Copyright 2018 Springer Nature, reproduction under CC BY 4.0

ADC/activator combination yielded 90% drug release in PBS after 1 h. A 1000-fold enhancement of cytotoxicity was observed in cell culture after tetrazine-triggered release of the drug from the ADC. In mice, a 2-day interval was chosen between the administration of the ADC and the activator, enabled by a near-complete clearance of the ADC at this time without the need for a clearing agent. The 2-day interval matched well with the in vivo stability of the TCO, having a deactivation half-life of 5.5 days [75]. This protocol afforded pronounced delay of tumor growth in a TAG72-positive colon cancer model (LS174T) and complete tumor remission in an ovarian cancer model (OVCAR-3). Importantly, an analogous ADC with a protease cleavable linker instead of TCO was not effective in these models, demonstrating the added benefit of click-cleavable linkers for non-internalizing ADCs. A further improved version of this ADC/activator combination (Tagworks' program TGW101) yielding complete drug release in minutes with low doses of highly reactive tetrazine activator is currently in investigational new drug (IND)-enabling studies in preparation for a phase I trial in patients with advanced solid tumors.

The click-to-release approach described above was adopted by Mejia-Oneto and Royzen for a different on-tumor prodrug activation application. Their initial work centered on a tetrazine-loaded alginate hydrogel that was deposited peri-tumorally in mice bearing soft tissue sarcoma xenografts. Following systemic administration of Dox-TCO prodrug [15], the hydrogel captured the prodrug locally at the tumor site, after which free Dox was released via the click-to-release mechanism (Fig. 11) [76]. This system was later optimized by switching to a tetrazine-modified hyaluronate hydrogel and by adding a hydrophilic moiety on the TCO ring of the prodrug for enhanced solubility. The resulting prodrug had an in vitro cytotoxicity that was 82-fold lower than that of free Dox, and the solubility in PBS was increased by four orders of magnitude [77]. The maximum tolerated dose (MTD) of the TCO-masked Dox was enhanced 10- to 20-fold compared with free Dox in rodents, and off-target Dox exposure was reduced significantly compared with free Dox. Moreover, pharmacokinetic studies in rodents demonstrated largely sustained activity of the tetrazine-modified hydrogel (at least 5 days), gradually decreasing over time [78]. The promising preclinical results led to translation of this polymeric activator/prodrug system to the clinic. The phase I trial involved 40 patients with advanced or metastatic solid tumors that, after the intratumoral injection of the biopolymer, received

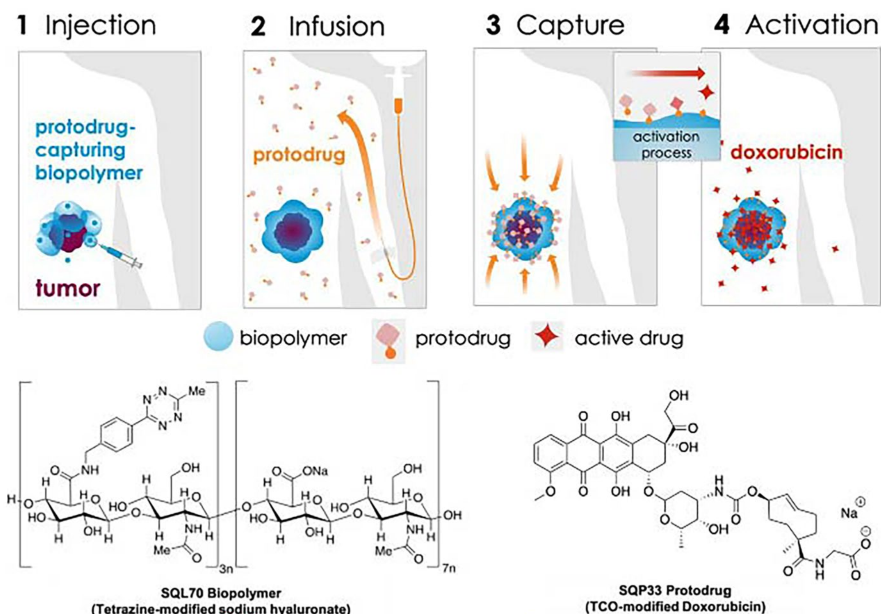


Fig. 11 Hydrogel-based prodrug activation system tested in clinical trials. Top: schematic representation of TCO-modified Doxorubicin prodrug (SQP33) activation by tetrazine-modified hydrogel (SQL70). Bottom: chemical structures of SQL70 biopolymer (left) and SQP33 prodrug (right) [77]. Reprinted with permission from Srinivasan, S., et al., "SQ3370 activates cytotoxic drug via click chemistry at tumor and elicits sustained responses in injected and non-injected lesions." *Adv Ther (Weinh)*, 2021. 4(3). 13(12): p. 4004–4015. Copyright 2021 John Wiley and Sons

five consecutive daily intravenous infusions of prodrug, with no Dox-related adverse effects [79, 80]. This Shasqi program is currently in a phase II clinical trial in patients with advanced solid tumors (NCT04106492).

This approach was tested with prodrugs containing paclitaxel (PTX), etoposide (ETP), and gemcitabine (GCB), but these payloads had a lower degree of deactivation by the TCO mask than Dox. In addition, the plasma stability of the ETP prodrug turned out to be lower than that of the Dox prodrug [78]. Recently, McFarland et al. reported an extension of this approach in which a tumor targeted activator is administered systemically, instead of an intratumoral administration. [81] Targeting of the activator to tumorous tissue is performed via linkage of the activator to a HER2-targeting Fab fragment. Antitumor effects in several mouse models were observed after repeated prodrug administration [81].

More strategies for drug delivery and/or activation in tumors using bioorthogonal chemistry were developed and tested in animal models. Gao and colleagues developed a masked tetrazine pro-activator which, upon systemic administration and enzymatic conversion, self-assembles into a tetrazine-functionalized hydrogel inside tumor cells [82]. Subsequently, a systemically administered cell-permeable TCO-Dox prodrug is injected and activated specifically inside tumor cells [82]. This approach was also tested with TCO-prodrugs of monomethyl auristatin F (MMAF), trichothecene mycotoxin (Mytoxin A) and PTX, but relatively low activation rates were seen in vitro, most likely owing to steric effects during the tautomerization step of the IEDDA reaction [83].

Another creative approach making use of an enzymatically cleavable pro-activator was presented by the group of Taran [84]. In this approach, a drug loaded micelle obtained by self-assembly of iminosydnone-based amphiphiles is injected and accumulates in tumors via EPR, followed by internalization. A cyclooctyne-bearing pro-activator is then administered in a second step and is unmasked selectively in the tumor microenvironment by tumor-associated β-glucuronidase. The resulting lipophilic cyclooctyne then enters the tumor cells and disassembles the micelle via strain-promoted iminosydnone-cycloalkyne cycloaddition (SPICC) release reaction [85, 86]. Proof of concept of this double targeted delivery mechanism was achieved in tumor-bearing mice injected with micelles filled with a fluorogenic dye [84].

Recently, Adhikari et al. designed an antibody-radiodrug conjugate in which the payload, a pyrrolobenzodiazepine (PBD) dimer prodrug, can be followed by nuclear imaging aiming to monitor tissue distribution and to optimize the timing of prodrug activation in a clinical setting [87]. Linkage of the PBD-dimer to a radioactive DOTA-chelate enabled detection of the prodrug, while keeping the prodrug inactive. While the PBD-dimer was linked to the antibody via an enzymatically cleavable dipeptide linker, full activation of the prodrug could only be obtained after activation by cell-permeable tetrazine, triggering release of the DOTA-chelate via a TCO-caged self-immolative *para*-aminobenzyl linker. The researchers found a relatively low tetrazine-triggered release yield and a nonspecific accumulation of the payload, both of which require optimization before the approach becomes clinically relevant. However, first proof of the theragnostic principle was demonstrated in mice [87].

The group of Wang developed chemically controlled release of the gasotransmitter carbon monoxide (CO). The reaction releases CO following an intramolecular

Diels–Alder reaction of an activated alkyne with a cyclopentadienone prodrug [88]. While their initial design comprised a single molecule that released CO upon an intramolecular Diels–Alder reaction [89, 90], more control over the release could be obtained with a bimolecular approach [88]. In this approach both prodrug and activator were targeted to the mitochondria, enhancing the concentration of the reagents in this organelle. The low reactivity of the alkyne-cyclopentadienone system prevents activation in circulation, but enables reaction and CO release upon enrichment in targeted areas. Successful CO release resulted in anti-inflammatory responses in vitro and in vivo [88]. The same group also demonstrated enrichment-triggered release of Dox from a targeted tetrazine prodrug upon reaction with targeted alkyne activators and intramolecular lactonization within the clicked intermediate. Enrichment-dependent cytotoxicity was observed in vitro, but the prodrug system was not evaluated in vivo [88].

3.2.2 Off-Target Deactivation

The previously discussed limitations imparted by the long circulation of radiolabeled mAbs for imaging and RIT can be overcome by using a tumor pretargeting approach for non- or slowly internalizing receptors (discussed in Sect. 4.1.1) or, in the case of efficiently internalizing receptors, by decoupling the radioactivity from the slow-clearing mAb at a moment when sufficient radiolabeled mAb has localized inside the tumor cells.

A first attempt to cleave a radiometal chelate from a circulating targeting vector was performed by Mukai and colleagues [91, 92]. To this end, a urokinase-cleavable linker was installed between a chelator and a gastrin-releasing peptide receptor-targeting bombesin analogue tetramer [92] or HER2-targeting trastuzumab. [91] After radiolabeled conjugate administration and tumor uptake, repeated intravenous urokinase injections were used to trigger radioactivity clearance from the blood. While this approach improved ratios in mice, repeated injections and high amounts of a thrombolytic drug, such as urokinase, may have serious side effects that must be addressed. Therefore, more optimization is necessary before this approach can be translated to the clinic.

To solve the problem of the long circulation of radioactivity, we applied the bioorthogonal cleavage reaction originally designed for ADC applications to RIT, developing click-cleavable radioimmunoconjugates (Fig. 12).

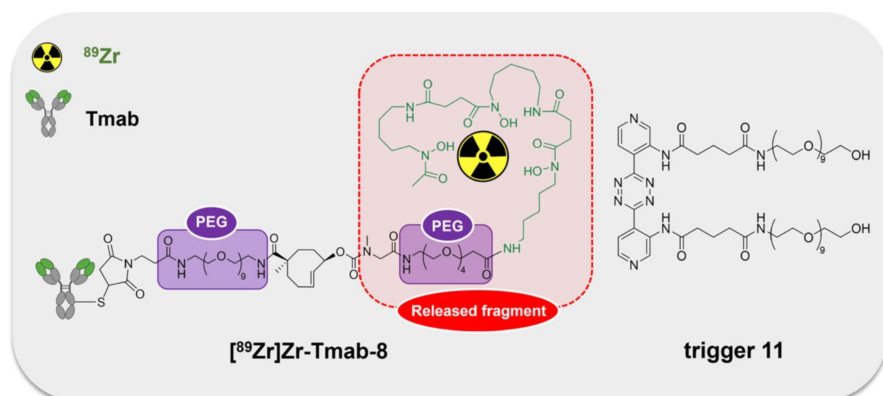
In this approach, the mAb carrying a radiometal at the end of a cleavable TCO linker is administered to a subject in a first step. After tumor accumulation and sufficient internalization in tumor cells, a non-cell-permeable tetrazine trigger is administered in a second step. The trigger reacts with the TCO linker on the mAb in circulation, but not the tumor cell-internalized fraction, releasing a small-molecule radioactive fragment that clears rapidly from the blood and other extracellular non-target tissue via the kidneys, boosting the tumor-to-blood ratios. Therefore, contrary to ADCs, click-cleavable RIT employs mAbs targeting internalizing receptors (e.g., HER2-binding trastuzumab). We refer to this new strategy as off-target deactivation, contrary to on-target activation as used in click-cleavable ADCs.

Fig. 12 Schematic representation of an off-target deactivation approach. In the first step, a radioactive antibody is administered. After an interval, a trigger is administered, which leads to release of a radioactive fragment from the antibody after a bioorthogonal release reaction

We recently demonstrated the advantage of the off-target deactivation approach with a click-cleavable trastuzumab derivative for improved PET imaging (Fig. 13) [93]. Trastuzumab was conjugated with $[^{89}Zr]$Zr-deferoxamine (DFO) via a cleavable TCO linker which, upon reaction with a tetrazine, releases a fast-clearing $[^{89}Zr]$Zr-DFO fragment. As the tetrazine trigger is non-cell-permeable, sufficient internalization of the antibody in tumor cells prevents radioactivity release from the tumor. In addition, owing to the impermeable nature of the radiometal-chelate fragment, radioactivity remains trapped in the tumor cells, even after any intracellular reaction or degradation of the conjugate. In tumor-bearing mice injected with ^{89}Zr-labeled trastuzumab followed by a tetrazine only 6 h later, this approach produced a more than twofold radioactivity reduction in blood, and significant increase of the tumor-to-blood ratio [93]. While these initial results support the use of click-cleavable radioimmunoconjugates for PET imaging of tumors early after mAb administration (hours instead of days [93]), decreasing the patient dose and improving logistics, the key application of this technology is RIT. Removing beta- and alpha-emitting radionuclides from the circulation on demand will spare the bone marrow and the kidney, while leaving the tumor radiation largely intact, enabling increased tumor radiation doses and potentially making solid tumor RIT more successful.

Very recently, the Weissleder group used this approach for improved trastuzumab-based imaging, now with the C_2-symmetric TCO (C_2TCO) linker, with releasable groups at each of the two allylic positions, giving fast and complete omnidirectional cleavage of one of the two groups (Fig. 14) [94, 95]. As a result, reaction with aminoethyl-substituted tetrazine released two different DOTA fragments, depending on click orientation. Proof-of-principle studies in mice injected with the trastuzumab construct and tetrazine after a 4 h interval showed 34–43% clearance of radioactivity from the circulation in 2 h and yielded an enhanced tumor-to-background ratio in PET images (~twofold). The same C_2TCO linker was also used for multiplex in vivo imaging [96]. Rather than a radioactive DOTA, for this application, cell-targeting antibodies were functionalized with a fluorophore, enabling the repeated staining and destaining of immune cells in live mice with dorsal and cranial window chambers [96].

Bioorthogonal off-target deactivation for RIT was also tested with a Staudinger ligation-triggered elimination reaction, in which the aza-ylide ligation intermediate undergoes intramolecular cyclization, leading to 1,6-elimination [97]. In this



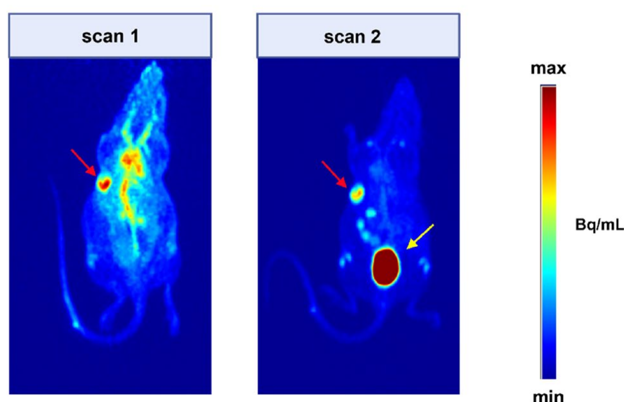


Fig. 13 Bioorthogonal off-target deactivation for enhanced imaging contrast using ^{89}Zr-labeled Trastuzumab. Top: structures of ^{89}Zr-labeled Trastuzumab construct comprising a cleavable TCO linker and a tetrazine-based trigger. Bottom: PET images of a tumor-bearing mouse before (scan 1) and after administration of the trigger (scan 2). The tumor and bladder are indicated with red and yellow arrows, respectively. [93]. Reprinted with permission from Vlastara, M., et al., "Click-to-release: cleavable radioimmunoimaging with [(89)Zr]Zr-DFO-*trans*-cyclooctene-trastuzumab increases tumor-to-blood ratio." *Theranostics*, 2023. 13(12): p. 4004–4015. Copyright 2023 Ivyspring

approach, a trastuzumab derivative containing a cleavable phosphine linker labeled with beta-emitting ^{131}I was administered to mice, followed by reaction with a subsequently administered *N*-glycosyl azide trigger in the circulation. This improved the tumor-to-blood ratio, but owing to the low reaction kinetics of the Staudinger ligation, the effect was modest, and multiple trigger injections were needed to observe an effect [97].

In another variant of this approach, described by Zhang et al., the off-target deactivation system relies on a bioorthogonal cleavage reaction between a dihydropyridine (DHP) linker and nitric oxide (NO). [98]. ^{131}I was linked to nanoparticles via the cleavable DHP linker, which, upon reaction with NO from subsequently injected

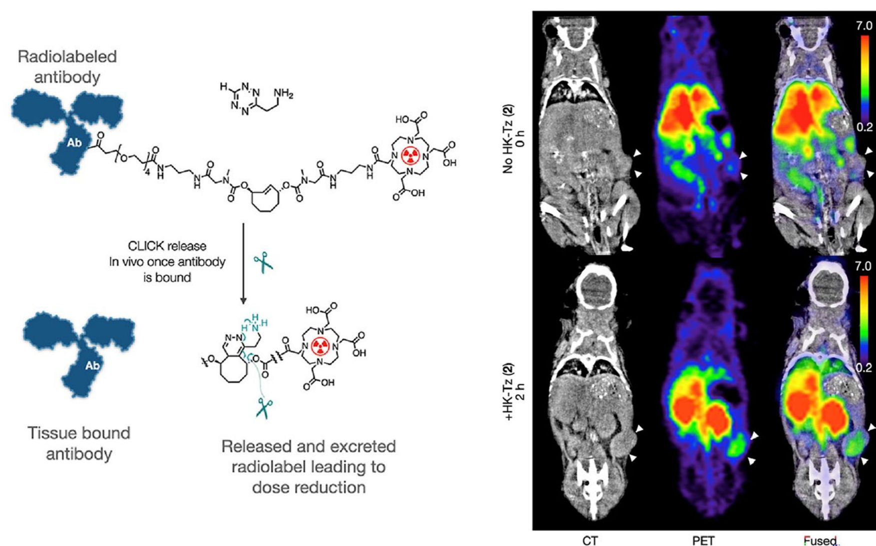


Fig. 14 Off-target deactivation approach with ^{64}Cu-labeled trastuzumab. Left: structures of cleavable C_2TCO linker connecting trastuzumab to ^{64}Cu-labeled DOTA, and HK-Tz trigger. Right: PET and CT images of mice injected with ^{64}Cu-labeled trastuzumab followed by HK-Tz 4 h later, leading to an enhanced tumor-to-background ratio after 2 h. Tumors are denoted by white arrows [94]. Reprinted with permission from Quintana, J.M., et al., *Bioconjug Chem*, 2024 35(10):1543–1552. Copyright 2024 American Chemical Society

glyceryl trinitrate (GTN), was released and cleared. The clearance rates from virtually all organs were enhanced by GTN treatment [98]. This system was designed to “turn off” radioactivity from radioactive antibodies so that in a future application, multiple different radiotracers can be screened rapidly, one after another, in order to determine the cancer phenotype in a patient.

4 Conclusions

Bioorthogonal chemistry has opened numerous new possibilities for the development of innovative pharmaceutical strategies. The high modularity of the available bioorthogonal reactions is clearly illustrated by the versatility of the approaches for diagnostic and therapeutic purposes that are currently being explored in (pre)clinical research.

Despite the fact that each clinical application has its own set of unique requirements, the only two bioorthogonal approaches evaluated in clinical trials to date rely on the IEDDA tetrazine-TCO reaction. In fact, among the established bioorthogonal reactions, the IEDDA reaction is the most suited candidate for in vivo chemistry, owing to the high reactivity, selectivity, and stability of the groups involved in this reaction. However, as we showed in this review, other reactions have produced promising results in small animal models. Therefore, it is quite possible that another chemical reaction will make it to clinical trials in the near future.

In general, clinical applications of bioorthogonal chemistry rely on the administration of two or more components. From a drug development point of view, this may add increased complexity and potentially raise costs. However, when the right applications are pursued, we expect that the patient benefit will far outweigh these hurdles.

Author Contribution K.d.R., R.R., and M.S.R. wrote the main manuscript.

Data Availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest K.d.R., R.R., and M.S.R. are employees of Tagworks Pharmaceuticals.

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