

## Review Article

# Bioorthogonal chemistry: implications for pretargeted nuclear (PET/SPECT) imaging and therapy

James C Knight<sup>1,2</sup>, Bart Cornelissen<sup>1,2</sup>

<sup>1</sup>CR-UK/MRC Gray Institute for Radiation Oncology and Biology, University of Oxford, Oxford, OX3 7LJ, United Kingdom; <sup>2</sup>Radiobiology Research Institute, Churchill Hospital, Oxford, OX3 7LJ, United Kingdom

Received September 23, 2013; Accepted December 6, 2013; Epub March 20, 2014; Published March 30, 2014

**Abstract:** Due to their rapid and highly selective nature, bioorthogonal chemistry reactions are attracting a significant amount of recent interest in the radiopharmaceutical community. Over the last few years, reactions of this type have found tremendous utility in the construction of new radiopharmaceuticals and as a method of bioconjugation. Furthermore, reports are beginning to emerge in which these reactions are also being applied *in vivo* to facilitate a novel pretargeting strategy for the imaging and therapy of cancer. The successful implementation of such an approach could lead to dramatic improvements in image quality, therapeutic index, and reduced radiation dose to non-target organs and tissues. This review will focus on the potential of various bioorthogonal chemistry reactions to be used successfully in such an approach.

**Keywords:** Pretargeting, bioorthogonal chemistry, molecular imaging, PET, SPECT, click chemistry

### Directly radiolabelled antibodies in imaging and therapy

The success of molecular imaging and therapy depends on the selective accumulation of the diagnostic probe or therapeutic agent at the site of interest. For imaging studies, this ability is required to effectively contrast the target site against surrounding tissues, organs and biological fluids, whilst in therapy it helps confine the toxic agent to the site(s) of disease and prevent off-target effects. In oncology, a multitude of targets have been identified corresponding to the various known hallmarks of cancer [1]. These include elevated rates of glucose metabolism [2], regions of hypoxia [3], changes in pH [4], and a plethora of cellular and chemical biomarkers [5]. This review will focus on the latter category and the development of novel imaging and therapeutic strategies towards such targets based on bioorthogonal chemistry.

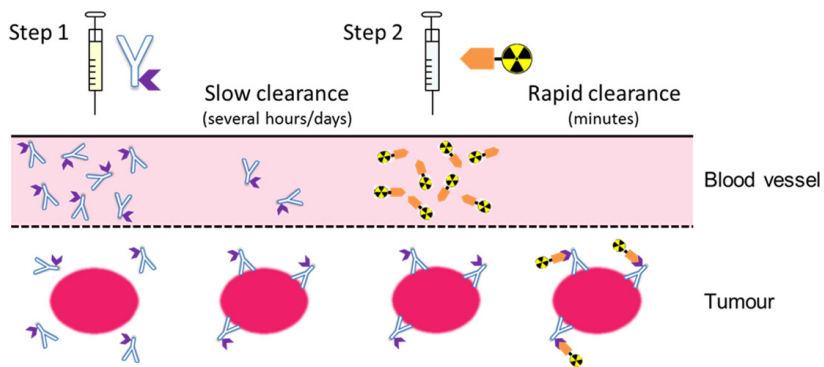
Upon identifying a compelling target, several important considerations are required in order to develop the most effective agent. One of these is the selection of an appropriate targeting vector which will both localize at the site of interest and lead to the specific accumulation

of the desired 'payload'. When developing an agent against a particular biomarker it is important that the targeting vector has (i) high binding affinity for its intended target, (ii) high specificity, (iii) high signal-to-background ratio, (iv) high metabolic stability, and (v) low immunogenicity [6, 7].

Since the beginning of the 20<sup>th</sup> century, antibodies have been considered as suitable vehicles for the delivery of imaging and therapeutic agents, mostly due to their high affinity and specificity [8-15]. Paul Ehrlich first conceived of antibody vectors as 'magic bullets' capable of delivering a payload of toxic agent to antigens associated with certain diseases, thus causing irreversible damage to sites of disease whilst sparing healthy tissue of the toxins deleterious effects [8]. Consequently, a vast array of epitopes (mostly at the cell surface) have been targeted by antibodies for non-invasive imaging and therapeutic applications.

The first examples of radiolabelled antibodies for cancer therapy emerged in the early 1950's [16, 17], although it took roughly two decades before their ability to target human tumour-associated antigens was demonstrated in can-

## Pretargeted nuclear (PET/SPECT) imaging and therapy



**Figure 1.** A simplified representation of a two-step pretargeting approach.

cer patients [18]. It was the development of hybridoma technology in 1975 that enabled the relatively facile generation of monoclonal (murine) antibodies (mAbs) in useful quantities [19] and, as a consequence, the number of studies in this area quickly increased. The use of murine antibodies in these applications was found to be problematic due to the provocation of a human anti-murine antibody response [20]. Therefore, significant efforts have been focused on the production of chimeric, humanized, and human monoclonal antibodies which are much less likely to invoke an immune reaction. Currently, the vast majority of approved mAbs are either chimeric or humanized [21].

Despite their attractive properties, antibodies have a few important shortcomings that have prevented their development into the magic bullets that Ehrlich had envisioned. One of the most critical barriers to achieving high tumour-to-blood (T/B) and tumour-to-muscle (T/M) ratios is the slow rate of clearance of antibodies from the blood and non-target tissues due to their high molecular weight [22, 23]. This necessitates the use of radionuclides with correspondingly long radioactive half-lives (e.g.  $^{89}\text{Zr}$ ;  $t_{1/2} = 78.41 \text{ h}$  [24]). Of course, the radiation dose to the patient increases as a function of exposure time and therefore there is a clear incentive to improve the pharmacokinetic properties of antibody constructs. One method of achieving this has involved a variety of lower molecular weight vectors which retain the essential antigen binding pharmacophore yet exhibit more rapid elimination. In decreasing order of size, these include minibodies, diabodies, single chain variable fragments (scFv), and single variable domain fragments (Fv), etc.

### Pretargeted imaging and therapy: an alternative approach

In the mid-1980s, an alternative strategy referred to as pretargeted radioimmunotherapy (PRIT) was developed to circumvent the issues associated with the prolonged residence times of radiolabelled antibody constructs [25, 26]. In essence, this approach involves the sequential

administration of (i) a macromolecular (usually antibody-based) targeting vector and (ii) a low molecular weight radiolabelled effector species (**Figure 1**). Crucially, the radiolabelled species is administered following a predetermined lag period to allow the antibody sufficient time to accumulate at the target site and for any residual antibody to be cleared from the circulation.

To ensure the two component parts bind strongly upon interaction at the site of interest, each must be suitably modified with complementary reactive species. In some cases, an additional 'chaser' species is administered prior to the radiolabelled effector, thus creating a 'three-step' approach. The purpose of this chaser species is to assist in the removal of any unbound antibody from the circulation. As a result, improved T/B ratios have been achieved [27].

The successful implementation of a pretargeting approach combines the high target specificity and affinity offered by antibody vectors with the superior pharmacokinetic properties of a low molecular weight compound. As a result, pretargeting strategies can lead to an improvement of imaging contrast at earlier time points following administration of the radiolabelled agent. Furthermore, this approach has been shown to decrease the overall radiation burden to non-target organs and tissues [28].

Whilst the concept of pretargeting has been known for several decades, the number of pretargeting systems has been limited to a few distinct classes. This is mostly due to the inherent difficulties of developing chemical reactions which proceed rapidly within living systems without also reacting with the vast array of

chemical entities that are present often in vast molar excesses.

### Conventional pretargeting systems

#### *Bispecific antibodies and radiolabelled haptens*

The original pretargeting concept is based on the use of bispecific antibodies which are capable of binding to both a target antigen and a radiolabelled hapten [11, 29-33]. This approach was made possible when Reardan *et al.* developed monoclonal antibodies which were capable of binding radiometal chelates, specifically  $^{111}\text{In}$  complexes based on the hexadentate chelator ethylenediamine tetraacetic acid (EDTA) [25]. It was later found that an affinity enhancement effect could be achieved by tethering two of these haptens together *via* a short two amino acid linker [34]. This modification led to improved uptake and retention of the radiolabelled divalent hapten at the tumour site whilst still retaining the essential rapid clearance from the circulation and surrounding tissues.

A notable example of this approach involved an anti-CEA  $\times$  anti- $^{111}\text{In}$ -benzyl-EDTA Fab'  $\times$  Fab' bispecific mAb and an  $^{111}\text{In}$ -EDTA derivative ( $^{111}\text{In}$ -EOTUBE) as the radiolabelled effector [35]. A clinical trial involving 14 patients with recurrent or metastatic adenocarcinoma of the colon revealed rapid clearance of the radiolabelled species from normal tissues while affording high T/M ratios [35].

Potential limitations of this approach include the practical complexities and high costs involved in the development of bispecific antibodies. Furthermore, a critical aspect of any pretargeted imaging approach is the affinity between the radiolabelled effector species and the antibody vector. Here, the binding interactions between radiolabelled haptens and bispecific antibodies are entirely non-covalent and binding constants greater than  $\sim 10^{-10}$  M are rarely achieved. In an effort to obtain greater binding constants, alternative systems offering much higher affinities such as the biotin-(strept)avidin interaction have been explored.

#### *Biotin-(strept)avidin systems*

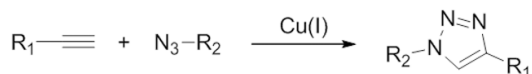
Shortly after the development of bispecific antibodies for pretargeting, Hnatowich *et al.* report-

ed an alternative strategy exploiting the extremely high binding affinity between biotin and (strept)avidin ( $K_d = 4 \times 10^{-14}$  M) [36, 37]. This approach has since been used in various forms which are discussed in depth in several comprehensive reviews [38-42]. The benefits of this approach were clearly demonstrated in a study by Axworthy *et al.* who compared the uptake of a  $^{90}\text{Y}$ -radiolabelled biotin in a tumour pretargeted with a streptavidin-modified mAb against a conventional directly radiolabelled antibody [43]. Promisingly, significantly higher T/B ratios were found using the pretargeting method.

Whilst this system shows clear promise, there are a number of limitations to this approach which require consideration. Perhaps most significant is the immunogenic response that occurs following administration of avidin/streptavidin foreign proteins. Another consideration is the presence of endogenous biotin ( $10^{-7}$ - $10^{-8}$  M) which could interfere with (strept)avidin pretargeting systems by saturating the biotin binding sites, as well as endogenous biotinidase which mediates the hydrolysis of radiolabelled biotin effector species. Lastly, more so than the other conventional pretargeting strategies discussed herein, it is often necessary to administer a chaser species to remove residual antibody from the circulation prior to the administration of the radiolabelled effector [44-49].

#### *Complementary oligonucleotides*

A comparatively more recent approach (also developed by Hnatowich and co-workers) relies on the high affinity interaction between complementary oligomers (such as DNA) [50-59]. Depending largely on the length and the base sequence of the complementary oligomeric chains, this chemical pairing can potentially lead to binding affinities that would rival, or even exceed, that of the biotin-(strept)avidin interaction. This approach can also potentially eliminate some of the inherent limitations of the biotin-(strept)avidin approach. For example, studies in which high doses of single strand DNAs have been repeatedly administered to patients have not revealed any significant immunogenic response or obvious toxicity [60]. Furthermore, unlike the biotin-(strept)avidin approach, the use of complementary oligomers would not be complicated or obstructed by the presence of competing endogenous species. It



**Figure 2.** The Huisgen 1,3-dipolar cycloaddition is an extremely efficient click chemistry reaction, however the requirement of a toxic copper(I) catalyst to enhance the reaction rate has prevented its application in living systems.

is important, however, that oligonucleotides are suitably modified to prevent their rapid degradation by nucleases [61]. The most successful oligomers from a pretargeting perspective have been those based on a morpholino backbone (MORFs). These agents have been used in conjunction with a variety of radionuclides for applications in imaging ( $^{99m}Tc$  [51-54, 58],  $^{111}In$  [55, 56]) and therapy ( $^{90}Y$  [50],  $^{188}Re$  [57]).

#### Using bioorthogonal chemistry for pretargeted imaging of cancer

For a chemical reaction to be described as being truly bioorthogonal, it must result in the rapid formation of a covalent bond (even at low concentrations) whilst remaining completely selective against any other chemical species present within a living system. Given the abundance and variety of reactive functional groups within such a biologically and chemically complex environment, this reduces the number of possible reactions to a small selection [62-73]. In addition, it is important that at least one of the bioorthogonal species is small, and that both reacting components exert minimal toxicity [64].

Click chemistry reactions (as defined by Sharpless *et al.* in 2001 [74]) offer important advantages which give them the potential to translate well into an *in vivo* setting and all of the bioorthogonal reactions discussed in this review fall under this umbrella term.

The archetypal and most prominent click chemistry reaction evolved from work started by Rolf Huisgen in 1963 [75-77], although the development of the click chemistry concept itself did not arise until much later [74]. The Huisgen 1,3-dipolar cycloaddition involves the reaction between azide and alkyne starting materials yielding a triazole species (**Figure 2**). By itself, the reaction requires elevated temperatures or pressures and often results in a mixture of

regioisomers (specifically, 1,4- and 1,5-substituted triazoles). However, in the early 2000s, the laboratories of both Sharpless and Meldal independently discovered that in the presence of a copper(I) catalyst the reaction proceeds at room temperature, at much higher rates ( $\sim 10^6$ -fold), and yields only the 1,4-substituted regioisomer [78, 79]. This click chemistry reaction is particularly attractive as both the alkyne and azide functional groups are easily incorporated into a diverse array of organic species and exhibit high stability under a variety of reaction conditions. As such, the Huisgen 1,3-dipolar cycloaddition has found tremendous utility in the construction of radiopharmaceuticals [80, 81]. In particular, this click chemistry reaction has been used very effectively in the mild preparation of [ $^{18}F$ ]-radiolabelled peptides [82-84].

Despite having many desirable qualities that would render the Huisgen 1,3-dipolar cycloaddition an attractive choice for a pretargeting strategy, the necessity of a copper(I) catalyst presents a major limitation due to its *in vivo* toxicity. As a result, the comparatively recent development of alternative click chemistry reactions which proceed without the presence of a copper(I) catalyst has attracted a significant amount of interest in the molecular imaging community [85]. The copper-free click chemistry reactions are instead usually driven either by the relief of steric strain (strain-promoted azide-alkyne cycloadditions, or SPAAC [63-68, 72]), or by a so-called inverse electron-demand Diels-Alder mechanism [86] (**Table 1**).

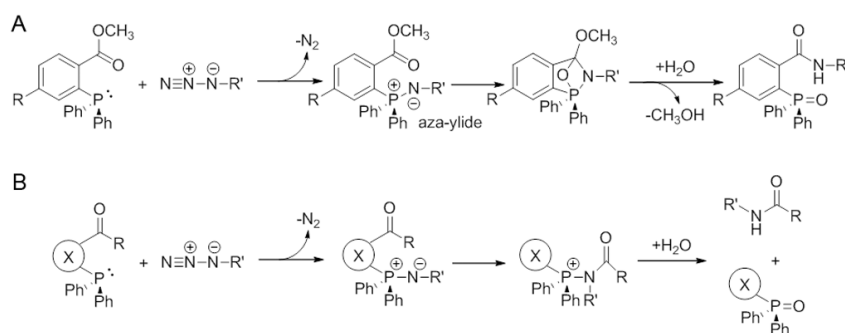
One of the first copper-free bioorthogonal reactions to be evaluated was the Staudinger ligation (**Figure 3**) [87]. This involves the reaction between azide and phosphine functional groups resulting in the formation of an amide bond. There are essentially two forms of this reaction: (i) a non-traceless version in which a phosphine oxide moiety remains attached to the final product, and (ii) a traceless version in which the phosphine oxide group is eliminated. This ligation and its precise mechanism [88] have been discussed in detail in several excellent review articles [89, 90].

The Staudinger ligation is a reliable and highly selective reaction which has been used successfully for the modification of proteins [91], and the engineering of cell surfaces both *in vitro* [92, 93] and in living animals [94]. It has

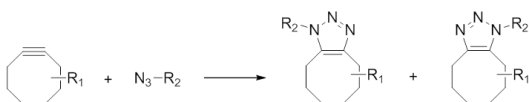
**Table 1.** Rates of reaction for a selection of bioorthogonal reactions

Reaction type	Reacting species	$k$ ( $\times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$ ) <sup>a</sup>	References
Staudinger (non-traceless)	Phosphine + azide	0.83-3.8	[88, 89]
Staudinger (traceless)	Phosphine + azide	0.12-7.70	[89, 90]
Strain-promoted cycloadditions	Cycloalkyne + azide	0.9-4,000	[93, 99-106, 108, 110, 119, 132, 170-174]
	Cycloalkyne + diazo	2.5-13,500	[174]
	Cycloalkyne + nitrene	1,660-58,800	[105, 115, 116, 136]
IEDDA	Cyclopropene + tetrazine	137-137,000	[140]
	Norbornene + tetrazine	41-20,000	[145, 148, 150, 151, 175]
	TCO + tetrazine	3,100-380,000,000	[152, 166, 175-177]

<sup>a</sup>The reported rates of each reaction are dependent on a number of experimental conditions, including the choice of solvent, pH, temperature, etc. For a more precise comparison, refer to the individual references.



**Figure 3.** The non-traceless (A) and traceless (B) versions of the Staudinger ligation have been used for *in vivo* bioorthogonal chemistry reactions.



**Figure 4.** The use of highly strained cyclooctyne derivatives has circumvented the requirement of a toxic copper(I) catalyst and these reactions are therefore more compatible with living organisms compared to the traditional Huisgen 1,3-dipolar cycloaddition.

also been used in combination with fluorophores in *in vitro* studies targeting azide groups present on the surface of live cells [95, 96]. Furthermore, the traceless form of this reaction has also been used to synthesize heterobifunctional linkers to facilitate the construction of radiometal-containing imaging agents [97].

A study conducted by Vugts *et al.* in 2011 explored the possibility of using the Staudinger ligation to facilitate a pretargeted imaging strategy [98]. In this case, an anti-CD44v6 chimeric monoclonal antibody which had been modified with multiple azide functionalities was employed as a targeting vector. A series of phosphine-containing small molecules incorporat-

ing radionuclides for imaging ( $^{67/68}\text{Ga}$ ,  $^{89}\text{Zr}$ ,  $^{123}\text{I}$ , and  $^{177}\text{Lu}$ ) and therapeutic applications ( $^{177}\text{Lu}$ ) were then evaluated as secondary agents. Following the administration of a  $^{67}\text{Ga}$ -DFO-phosphine agent in non-tumour bearing mice, the presence of Staudinger products in the blood pool was monitored, however no evidence

of any ligation was observed. Experiments in serum revealed that the phosphine species is prone to oxidation which renders it unable to undergo reaction with azide groups. Furthermore, the rate of the Staudinger ligation was found to be sub-optimal for *in vivo* bioorthogonal reactions, particularly considering the rapid clearance and elimination of the secondary phosphine agent.

#### Strain-promoted alkyne-azide (SPAAC) and alkyne-nitrene (SPAN) cycloadditions

In another effort to circumvent the use of a toxic Cu(I) catalyst required for traditional Huisgen-type click chemistry reactions, a related class of azide-alkyne [3 + 2] cycloadditions which are promoted by the relief of steric strain have gained prominence. Mostly, these reactions involve an alkyne moiety within a cyclic 8-membered (cyclooctyne [99]) system which causes the bond angles surrounding the two alkyne  $sp$ -hybridised carbon atoms to be severely constrained away from the ideal  $180^\circ$  (**Figure 4**).

A host of cyclooctyne derivatives have been evaluated in terms of their ability to undergo copper-free click chemistry reactions. The purpose of these studies is often focused on enhancing the rate of the cycloaddition reaction by making structural modifications to the cyclooctyne species. For example, it has been found that introducing one or more electron-withdrawing substituents (e.g. fluorine [100]) in close proximity to the alkyne group leads to a substantial increase of rates of reaction. Other modifications, such as one [101] or two [102-104] adjacent aryl rings (e.g. dibenzocyclooctyne, DBCO), and bicyclic systems (e.g. bicyclononynes [105, 106]) have also been found to improve reaction rates yet further. One cyclooctyne derivative 'BARAC' has achieved second order rates of reaction up to  $0.96 \text{ M}^{-1}\text{s}^{-1}$  [104], although a recent study has found evidence that BARAC itself is prone to rearrangement yielding tetracyclic products [107].

Cyclooctyne derivatives can now be purchased from commercial suppliers and this increase in availability has led to the use of this technology in a wide variety of applications [93, 105, 108-123]. Notably, SPAAC reactions have been used in the preparation of fluorine-18 [124-130] and copper-64 [131] radiolabelled compounds for PET imaging applications.

Several studies have now demonstrated the ability of cyclooctyne derivatives to undergo bioorthogonal reactions with azide groups *in vitro* [119, 121, 132], in zebrafish [123], and in live mice [120]. In most of these examples, the azide groups are usually incorporated *via* metabolic glycoengineering.

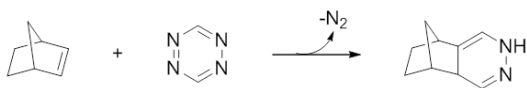
Given their apparent high selectivity and promising rates of reaction in some cases, it is not surprising that SPAAC reactions have been considered as candidates to facilitate a pretargeting approach [130, 133, 134]. Recently, van den Bosch *et al.* reported an in depth study in which the reaction between a series of  $^{177}\text{Lu}$ -containing cyclooctyne derivatives and an azido-functionalised anti-CD20 mAb (Rituximab) was evaluated in non-tumour-bearing mice. In this case, the cyclooctyne species were derived from DIFO and DIBO as both had previously been shown to undergo rapid reactions with benzyl azide [63, 116]. Preliminary *in vitro* studies in phosphate buffered saline and 50% mouse serum indicated sufficient stability

within the expected circulation time of these low molecular weight probes. Mice were initially administered the azide-modified mAb and after a lag time of 5 minutes were subsequently injected with the relevant  $^{177}\text{Lu}$ -labelled cyclooctyne probe. Evidence of bioorthogonal reaction products was monitored in the blood pool and selected tissues. Unfortunately, this approach was unsuccessful as it was evident that the cyclooctyne probes did not have sufficiently high *in vivo* reactivity towards azides, particularly considering the rapid blood clearance of these agents. Furthermore, in some examples, binding to serum proteins was apparent which, whilst slightly lengthening the circulatory residence times, ultimately limited the availability of these secondary agents and therefore reduced the potential for *in vivo* cycloaddition reactions.

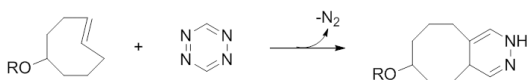
Lee *et al.* have also recently utilised SPAAC-based bioorthogonal chemistry for *in vivo* pretargeting using, in this case, fluorine-18 for PET imaging [134]. Here, rather than use an antibody as the primary targeting agent, the authors employed mesoporous silica nanoparticles (MSNs; 100-150 nm) which were expected to accumulate in tumours *via* the enhanced permeability and retention (EPR) effect. The MSNs were PEGylated and then modified with DBCO (~0.12 mmole of DBCO per gram of product, DBCO-PEG-MSN). These nanoparticles were administered intravenously to mice bearing U87 MG tumour xenografts and, after a lag time of 24 h, the secondary agent ( $^{18}\text{F}$ fluoropentaethylene glycolic azide) was subsequently injected. Promisingly, PET-CT images acquired between 0.25-2 h p.i. revealed accumulation of radioactivity at the tumour site while control experiments performed without prior injection of the DBCO-PEG-MSNs exhibited substantially lower tumour uptake. This observation was supported by data obtained from *ex vivo* biodistribution experiments which showed an improved tumour-to-blood ratio using the pretargeting approach. These experiments provide a good indication that bioorthogonal chemistry reactions based on SPAAC can be used successfully to facilitate *in vivo* pretargeting for imaging and therapeutic applications.

A few studies focused on achieving faster rates of reaction have found that cyclooctyne derivatives also undergo rapid strain-promoted cycloaddition reactions with nitrene species (SPANC)

## Pretargeted nuclear (PET/SPECT) imaging and therapy



**Figure 5.** The norbornene-tetrazine ligation has been used successfully in the radiosynthesis of PET imaging agents based on peptide and antibody constructs. The rate of reaction between these species (and derivatives thereof) appears to be too slow to facilitate a pretargeting strategy.



**Figure 6.** The TCO-tetrazine ligation has been the most successful bioorthogonal chemistry reaction for implementing a pretargeting strategy due to its extremely high rates of reaction.

[135]. The reaction between BARAC and one cyclic nitrene derivative yielded a second-order rate constant of  $47.3 \text{ M}^{-1}\text{s}^{-1}$ , representing a 47-fold increase compared to the equivalent reaction involving benzyl azide [136]. To the best of the authors knowledge this class of reactions has not yet been evaluated in a pretargeting strategy, although it holds clear promise particularly as cyclic nitrenes can be readily conjugated to amine and carboxylic acid functional groups and have already been used successfully to functionalise extracellular targets on live cancer cells [115].

### *Inverse electron-demand Diels-Alder cycloadditions*

In an opposite fashion to the traditional Diels-Alder reaction, inverse electron-demand Diels-Alder (IEDDA) cycloaddition reactions involve the use of an electron-rich dieneophile and an electron-deficient diene [86]. The reaction is formally accepted as a [4 + 2] cycloaddition, however it is still not clear whether it is a truly concerted mechanism. In the early 1990s, Sauer *et al.* demonstrated that the rates of reaction between electron deficient tetrazines and a variety of dienophiles were extremely fast [137], and consequently tetrazines have been commonly employed as dienes in these reactions.

From a bioorthogonal chemistry perspective, the two most prominent dienophiles which have so far been explored are norbornene and *trans*-cyclooctene (TCO) [138], although recent-

ly other species such as cyclopropene [139, 140] and terminal alkenes [141] have also shown promise in this area.

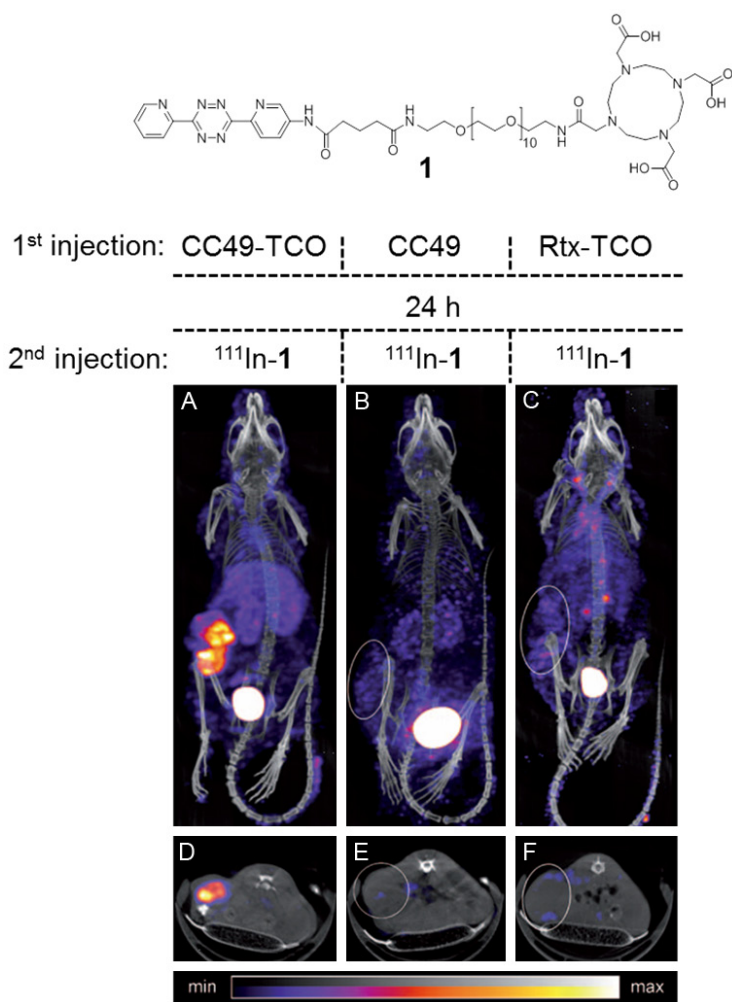
The norbornene-tetrazine ligation was first reported in the 1980s [137, 142] and has seen a resurgence of interest in recent years (Figure 5). Whilst this reaction leads to the formation of multiple isomers, it otherwise meets the criteria of a click chemistry reaction and is often placed in this category. In particular, this ligation has been shown to be very rapid, modular in scope, and high yielding, and has consequently been used very effectively in a variety of applications [143-148], including the preparation of various radiopharmaceutical agents. In 2011, Zeglis *et al.* demonstrated that this ligation can be applied as an effective tool for bioconjugation in their development of radiometallated antibody constructs [149]. Here, the authors synthesized both  $^{64}\text{Cu}$ -NOTA- and  $^{89}\text{Zr}$ -DFO-containing norbornene derivatives which were able to rapidly conjugate to a tetrazine-modified antibody under mild reaction conditions. In a more recent example, Knight *et al.* synthesised an [ $^{18}\text{F}$ ]-containing norbornene prosthetic group ( $^{18}\text{F}$ )-NFB) which was used successfully to radiolabel a bombesin peptide-derivative, also under mild reaction conditions [150].

This reaction has also been used by Devaraj *et al.* in *in vitro* pretargeting experiments involving SKBR3 human breast cancer cells [151]. In this case, norbornene-modified trastuzumab (Herceptin) was used to pretarget Her2/neu growth factor receptors at the cell surface. A near-infrared fluorescent VT68-tetrazine species was then used as a secondary labelling agent. Rapid and highly selective labelling was observed which highlights the utility of this bioorthogonal reaction.

In addition, experiments performed in whole mouse blood have further demonstrated the ability of this reaction to proceed efficiently even in complex biological environments [150]. This reaction has therefore been considered as a candidate to facilitate a pretargeted imaging strategy, however it should be noted that the rate of this reaction ( $\sim 2 \text{ M}^{-1}\text{s}^{-1}$ ) appears to be sub-optimal for *in vivo* pretargeting applications.

The related TCO-tetrazine ligation proceeds via a similar inverse electron-demand Diels-Alder

## Pretargeted nuclear (PET/SPECT) imaging and therapy



**Figure 7.** In the first example of *in vivo* pretargeting using the TCO-tetrazine ligation, Rossin *et al.* used the indium-111 complex of **1** (top) to obtain clearly distinguishable SPECT images of colon cancer xenografts. Tumour-bearing mice were injected with (A) CC49-TCO (100 µg) and after 24 h were subsequently injected with [<sup>111</sup>In]-**1** (25 equiv. to CC49; 3.4 equiv. to TCO); (B) CC49 (100 µg) followed 24 h later with [<sup>111</sup>In]-**1** (identical amount as in (A)); and (C) Rtx-TCO (100 µg) followed 24 h later by [<sup>111</sup>In]-**1** (same amount as in (A)); (D-F) single transverse planar images intersecting the tumours in (A-C). Images acquired at 3 h p.i. Rossin R, Verkerk PR, van den Bosch SM, Vuldere RCM, Verel I, Lub J, Robillard MS, *In Vivo Chemistry for Pretargeted Tumor Imaging in Live Mice*, *Angew Chem Int Ed.* 2010; 122: 3447-3450. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

mechanism (Figure 6), although it is more rapid than the norbornene-tetrazine ligation by several orders of magnitude. Reaction rates of up to  $3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  have been determined [152], making this among the most rapid bioorthogonal copper-free click chemistry reactions. As a result, the TCO-tetrazine ligation was quickly adopted by the radiopharmaceutical community and has been used in the preparation of

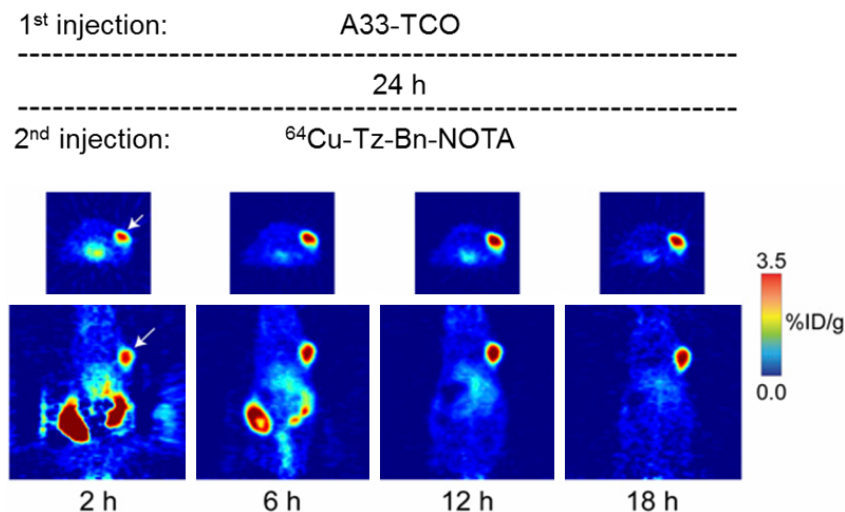
radiolabelled small molecules [153] (including PARP inhibitors [154, 155]), peptides (e.g. cyclic-RGD peptide antagonists of  $\alpha_v\beta_3$  [156, 157], Exendin-4 [158]), and proteins (e.g. VEGF [157]).

There are now several reports in which the TCO-tetrazine ligation has been used to facilitate an *in vitro* pretargeting strategy [132, 159-166]. In each of these examples, it is the TCO species which is coupled to the targeting vector, and a fluorescently-tagged tetrazine species is employed as the secondary agent. Epitopes at the cell surface [132, 159, 161, 163, 165] and in intracellular regions [160-164] have been successfully targeted using this strategy.

In their landmark study, Rossin *et al.* were first to demonstrate the utility of bioorthogonal chemistry as a pretargeting strategy in live mice bearing colon cancer xenografts [167]. In this example, the antigen TAG72 was selected as a model target due to its limited degree of internalisation (which would otherwise render it inaccessible) and its resistance to shedding. TAG72 also has clinical relevance as it is overexpressed in a variety of cancer cell lines. As a targeting vector, an anti-TAG72 mAb (CC49) was functionalised with an average of 7.4 TCO species using conventional NHS-based conjugation chemistry. A complementary dipyrindyl tetrazine derivative was selected based on its high reactivity with TCO and good

stability. This tetrazine species was modified with a DOTA chelating agent to facilitate radiolabelling with the SPECT radionuclide <sup>111</sup>In (<sup>111</sup>In-**1**; Figure 7). Preliminary *in vitro* experiments conducted in PBS revealed that the bioorthogonal reaction between CC49-TCO and <sup>111</sup>In-**1** proceeded with a second order rate constant of  $13,090 \pm 80 \text{ M}^{-1} \text{ s}^{-1}$ . In their *in vivo* experiments, a lag time of 1 day was used between





**Figure 8.** Zeglis *et al.* successfully employed the TCO-tetrazine ligation for *in vivo* pretargeted PET imaging of SW1222 tumour xenografts (white arrow; transverse [top] and coronal [bottom] images) in mice. A33-TCO (100 µg) was administered *via* tail vein injection and after a lag time of 24 h were then administered <sup>64</sup>Cu-Tz-Bn-NOTA (10.2-12.0 MBq [275-325 µCi], 1.2-1.4 µg, for 2.5-2.8 Tz-to-A33 ratio). Reprinted by permission of SNMMI from: Zeglis BM, Sevak KK, Reiner T, et al. A Pretargeted PET Imaging Strategy Based on Bioorthogonal Diels-Alder Click Chemistry. *J Nucl Med.* 2013; 54(8): 1389-1396. **Figure 4.**

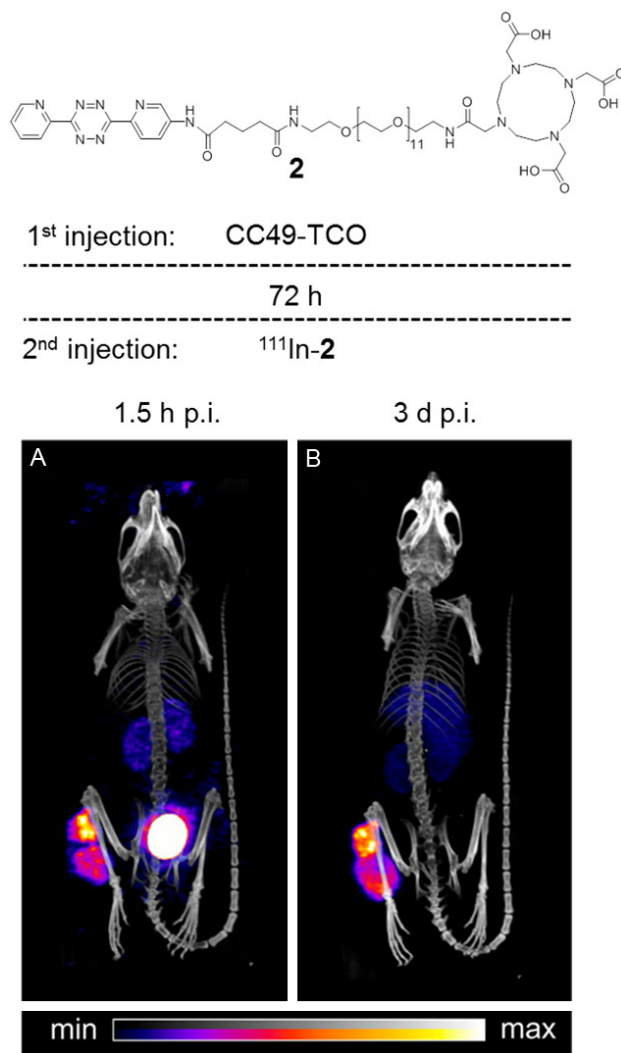
the administration of the TCO-mAb construct and the radiolabelled tetrazine derivative. Promisingly, a rapid reaction between the two complementary bioorthogonal species occurred *in vivo* and SPECT/CT images acquired at 3 h p.i. revealed high contrast images of the tumour xenografts, yielding a T/M ratio of 13:1 (**Figure 7**). Importantly, only low levels of radioactivity were observed in the blood and non-target tissues (including the liver) at 24 h p.i. which were attributed to small amounts of TCO-CC49 which remained circulating in the blood pool.

Shortly after, Devaraj *et al.* reported a study which was focused on further elucidating the parameters linked to the *in vivo* behaviour of the TCO-tetrazine ligation [168]. In this case, the secondary agents were based on dextran scaffolds (10 kDa and 40 kDa) modified with multiple tetrazine species. In non-tumour-bearing mice, the longer residence times of these high molecular weight constructs proved beneficial in achieving higher *in vivo* labelling efficiencies in the blood pool. This study also included pretargeting experiments aimed at imaging the A33 glycoprotein in LS174T tumour-bearing mice. The A33 glycoprotein was selected as a suitable target due to its high persistence at the cell surface and practical half-life

(>2 days). A33 is also overexpressed in >95% of all human colorectal cancers, including the LS174T cell line, and therefore represents a target with high clinical relevance. The targeting vector in this case was an anti-A33 mAb modified with ~3 TCO moieties as well as a near-infrared VT680 fluorophore to enable the acquisition of complementary optical imaging data. For the acquisition of high contrast PET images, an [<sup>18</sup>F]-containing 10 kDa dextran construct ([<sup>18</sup>F]PMT10) containing multiple tetrazine species was administered as the secondary agent. In

a similar manner to the previous study, a lag time of 24 h was used between the primary and secondary agent, and PET/CT images were acquired at 3 h p.i. of the radiolabelled probe. While T/M or T/B ratios were not reported, the A33 tumours were clearly contrasted against the surrounding tissues and had substantially higher uptake on comparison to control tumours which did not express the A33 antigen.

In another example, Zeglis *et al.* used the TCO-tetrazine ligation to successfully implement a pretargeted imaging strategy in mice bearing SW122 colorectal tumour xenografts (**Figure 8**) [28]. This group also used an anti-A33 mAb (this time modified with approximately 5 TCO moieties per mAb) and importantly found no significant detrimental effect on the overall immunoreactivity. Here, the secondary agent comprised of a tetrazine group modified with a NOTA chelator for labelling with the PET radio-metal copper-64. In common with the two previous studies, a lag time of 24 h was used and, in this case, PET images were acquired at various time points between 2-18 h. This group compared their findings against the directly labelled construct, <sup>64</sup>Cu-NOTA-A33, as well as the comparable <sup>89</sup>Zr-labelled antibody (in which



**Figure 9.** Rossin *et al.* significantly improved the *in vivo* stability and reactivity of the TCO-tetrazine ligation by increasing the steric hindrance of the TCO tag. An LS174T tumour-bearing mouse was administered CC49-TCO (100 µg) and after a lag-time of 72 h was then administered the <sup>111</sup>In-labelled tetrazine secondary agent (**2**). SPECT/CT images were acquired 1.5 h (A) and 3 days (B) after injection of the tetrazine species. Adapted with permission from Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J, and Robillard MS, Highly Reactive trans-Cyclooctene Tags with Improved Stability for Diels-Alder Chemistry in Living Systems. *Bioconjugate Chem.* 2013; 24(7): 1210-1217. Copyright 2013 American Chemical Society.

a DFO chelating agent was used). It was found that whilst the directly labelled antibody constructs resulted in higher overall tumour uptake at 12 h and 24 h p.i., the pretargeting approach yielded greater T/M ratios. Furthermore, it was also found that as a result of the faster clearance rate of the radiolabelled secondary agent, the overall radiation burden to off-target tissues and organs was substantially reduced.

A major concern regarding the application of TCO in a pretargeting strategy has been its sub-optimal *in vivo* stability. Rossin *et al.* recently addressed this issue and were able to determine that protein-bound copper likely deactivates TCO by promoting its conversion to the comparatively unreactive *cis*-cyclooctene isomer (CCO) [169]. By shortening the distance between the TCO moiety and the lysine residue to which it binds, an increase in steric hindrance on the TCO tag was found to obstruct this deactivation. As a result, the *in vivo* stability of TCO in mice was greatly improved. In addition, the authors also reported significantly improved rates of reaction for TCO derivatives substituted in axial rather than equatorial positions with bulky linking groups (~10-fold higher). *In vivo* pretargeting experiments were performed (**Figure 9**) using a very similar experimental design compared to their previous study [167], however in this instance the more stable TCO-derivative allowed a longer three-day lag period prior to the addition of the <sup>111</sup>In-labelled tetrazine secondary agent (<sup>111</sup>In-2; **Figure 9**). The extended lag period resulted in reduced background signal in the circulation and, promisingly, a similar degree of tumour uptake which substantiates the improved *in vivo* stability of this TCO species. Furthermore, after 3 days the radioactivity in the tumour was unchanged which indicates that the bioorthogonal reaction product is extremely stable *in vivo*.

### Summary and future perspectives

Pretargeting is a more complex approach compared with the use of directly radiolabelled imaging or therapeutic agents and it will require careful optimisation in order to be successfully translated into a clinical setting. Ultimately, the incentive to undertake this costly endeavour will come from the demonstrable proof that this approach is superior to the use of directly radiolabelled macromolecules. To date, a small number of clinical trials involving conventional pretargeting strategies have shown such promise, despite suffering from a few inherent shortcomings.

Bioorthogonal chemistry has the potential to circumvent many of the limitations of its predecessors and has shown significant promise in a

few pioneering preclinical studies. From a selection of potential candidates, only one bioorthogonal chemistry reaction has been used successfully to enable pretargeting of specific cancer biomarkers, namely the TCO-tetrazine ligation. However, as bioorthogonal reactions continue to be refined through innovative chemical design, this number is likely to increase, yielding chemical pairings with improved bioavailability, bioorthogonality, metabolic stability, and rates of reaction.

It is also worth noting that many of the most compelling biomarkers of cancer exist within the intracellular (and, indeed, intranuclear) compartments of tumour cells. This presents a significant challenge to the imaging community as these targets are much less accessible, particularly to the types of macromolecular targeting vectors that are involved in a pretargeting strategy. Therefore, research efforts should also be focussed on enhancing the cell permeability of these constructs. So far, only a handful of publications have addressed this.

In summary, the continual improvement of bioorthogonal chemistry reactions over recent years has facilitated an alternative pretargeting strategy which is demonstrating much promise for both imaging and therapy of cancer. This approach offers key advantages over more conventional strategies which will undoubtedly increase its potential for translation into a clinical setting.

### Acknowledgements

The authors would like to thank Cancer Research UK for supporting this work.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Bart Cornelissen, CR-UK/MRC Gray Institute for Radiation Oncology and Biology, University of Oxford, Radiobiology Research Institute, Churchill Hospital, Oxford, OX3 7LJ, United Kingdom. Tel: +44 (0)1865 857126; E-mail: bart.cornelissen@oncology.ox.ac.uk

### References

[1] Hanahan D and Weinberg Robert A. Hallmarks of Cancer: The Next Generation. *Cell* 2011; 144: 646-674.

- [2] Gambhir SS, Czernin J, Schwimmer J, Silverman DHS, Coleman RE and Phelps ME. A Tabulated Summary of the FDG PET Literature. *J Nucl Med* 2001; 42: 1S-93S.
- [3] Vavere AL and Lewis JS. Cu-ATSM: A radiopharmaceutical for the PET imaging of hypoxia. *Dalton Trans* 2007; 4893-4902.
- [4] Warburg O. On the Origin of Cancer Cells. *Science* 1956; 123: 309-314.
- [5] Knight JC and Wuest FR. Nuclear (PET/SPECT) and optical imaging probes targeting the CXCR4 chemokine receptor. *Med Chem Comm* 2012; 3: 1039-1053.
- [6] Chen K and Chen X. Design and Development of Molecular Imaging Probes. *Curr Top Med Chem* 2010; 10: 1227-1236.
- [7] Chen K and Conti PS. Target-specific delivery of peptide-based probes for PET imaging. *Adv Drug Deliv Rev* 2010; 62: 1005-1022.
- [8] Strebhardt K and Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer* 2008; 8: 473-480.
- [9] Wu AM and Olafsen T. Antibodies for molecular imaging of cancer. *Cancer J* 2008; 14: 191-197.
- [10] Knowles SM and Wu AM. Advances in Immuno-Positron Emission Tomography: Antibodies for Molecular Imaging in Oncology. *J Clin Oncol* 2012; 30: 3884-3892.
- [11] Sharkey RM, Rossi EA, Chang CH and Goldenberg DM. Improved Cancer Therapy and Molecular Imaging with Multivalent, Multispecific Antibodies. *Cancer Biother Radiopharm* 2010; 25: 1-12.
- [12] Boerman OC and Oyen WJG. Immuno-PET of Cancer: A Revival of Antibody Imaging. *J Nucl Med* 2011; 52: 1171-1172.
- [13] Olafsen T and Wu AM. Antibody Vectors for Imaging. *Semin Nucl Med* 2010; 40: 167-181.
- [14] Wu AM. Antibodies and Antimatter: The Resurgence of Immuno-PET. *J Nucl Med* 2009; 50: 2-5.
- [15] Goldenberg DM. Targeted Therapy of Cancer with Radiolabeled Antibodies. *J Nucl Med* 2002; 43: 693-713.
- [16] Pressman D and Korngold L. The in vivo localization of anti-Wagner-osteogenic-sarcoma antibodies. *Cancer* 1953; 6: 619-623.
- [17] Wissler RW, Barker PA, Flax MH, La Via MF and Talmage DW. A Study of the Preparation, Localization, and Effects of Antitumor Antibodies Labeled with I131. *Cancer Res* 1956; 16: 761-773.
- [18] Goldenberg DM, DeLand F, Kim E, Bennett S, Primus FJ, van Nagell JR, Estes N, DeSimone P and Rayburn P. Use of Radio-Labeled Antibodies to Carcinoembryonic Antigen for the Detection and Localization of Diverse Cancers by External Photoscanning. *New Engl J Med* 1978; 298: 1384-1388.

## Pretargeted nuclear (PET/SPECT) imaging and therapy

- [19] Kohler G and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256: 495-497.
- [20] Tjandra JJ, Ramadi L and McKenzie IFC. Development of human anti-murine antibody (HAMA) response in patients. *Immunol Cell Biol* 1990; 68: 367-376.
- [21] Chames P, Van Regenmortel M, Weiss E and Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 2009; 157: 220-233.
- [22] Rosebrough SF, Grossman ZD, McAfee JG, Kudryk BJ, Subramanian G, Ritter-Hrncirik CA, Witanowski LS, Tillapaugh-Fay G, Urrutia E and Zapf-Longo C. Thrombus Imaging with Indium-111 and Iodine-131-Labeled Fibrin-Specific Monoclonal Antibody and Its F(ab')<sub>2</sub> and Fab Fragments. *J Nucl Med* 1988; 29: 1212-1222.
- [23] Murray JL, Rosenblum MG, Lamki L, Glenn HJ, Krizan Z, Hersh EM, Plager CE, Bartholomew RM, Unger MW and Carlo DJ. Clinical Parameters Related to Optimal Tumor Localization of Indium-111-Labeled Mouse Antimelanoma Monoclonal Antibody ZME-018. *J Nucl Med* 1987; 28: 25-33.
- [24] Deri MA, Zeglis BM, Francesconi LC and Lewis JS. PET imaging with 89Zr: From radiochemistry to the clinic. *Nucl Med Biol* 2013; 40: 3-14.
- [25] Reardan DT, Meares CF, Goodwin DA, McTigue M, David GS, Stone MR, Leung JP, Bartholomew RM and Frincke JM. Antibodies against metal chelates. *Nature* 1985; 316: 265-268.
- [26] Goodwin DA, Mears CF, McTigue M and David GS. Monoclonal antibody hapten radiopharmaceutical delivery. *Nucl Med Commun* 1986; 7: 569-580.
- [27] Goodwin DA, Meares CF, McCall MJ, McTigue M and Chaovapong W. Pre-Targeted Immunoscintigraphy of Murine Tumors with Indium-111-Labeled Bifunctional Haptens. *J Nucl Med* 1988; 29: 226-234.
- [28] Zeglis BM, Sevak KK, Reiner T, Mohindra P, Carlin SD, Zanzonico P, Weissleder R and Lewis JS. A Pretargeted PET Imaging Strategy Based on Bioorthogonal Diels-Alder Click Chemistry. *J Nucl Med* 2013; 54: 1389-1396.
- [29] Goldenberg DM, Rossi EA, Sharkey RM, McBride WJ and Chang CH. Multifunctional Antibodies by the Dock-and-Lock Method for Improved Cancer Imaging and Therapy by Pretargeting. *J Nucl Med* 2008; 49: 158-163.
- [30] Goldenberg DM, Chatal JF, Barbet J, Boerman O and Sharkey RM. Cancer imaging and therapy with bispecific antibody pretargeting. *Update Cancer Ther* 2007; 2: 19-31.
- [31] Sharkey RM, Karacay H and Goldenberg DM. Improving the treatment of non-Hodgkin lymphoma with antibody-targeted radionuclides. *Cancer* 2010; 116: 1134-1145.
- [32] Sharkey RM, Rossi EA, McBride WJ, Chang CH and Goldenberg DM. Recombinant Bispecific Monoclonal Antibodies Prepared by the Dock-and-Lock Strategy for Pretargeted Radioimmunotherapy. *Semin Nucl Med* 2010; 40: 190-203.
- [33] Goldenberg DM, Chang CH, Rossi EA, McBride WJ and Sharkey RM. Pretargeted Molecular Imaging and Radioimmunotherapy. *Theranostics* 2012; 2: 523-540.
- [34] Le Doussal JM, Martin M, Gautherot E, De-laage M and Barbet J. In Vitro and In Vivo Targeting of Radiolabeled Monovalent and Divalent Haptens with Dual Specificity Monoclonal Antibody Conjugates: Enhanced Divalent Hapten Affinity for Cell-Bound Antibody Conjugate. *J Nucl Med* 1989; 30: 1358-1366.
- [35] Stickney DR, Anderson LD, Slater JB, Ahlem CN, Kirk GA, Schweighardt SA and Frincke JM. Bifunctional Antibody: A Binary Radiopharmaceutical Delivery System for Imaging Colorectal Carcinoma. *Cancer Res* 1991; 51: 6650-6655.
- [36] Hnatowich DJ, Virzi F and Rusckowski M. Investigations of Avidin and Biotin for Imaging Applications. *J Nucl Med* 1987; 28: 1294-1302.
- [37] Green NM. Avidin and streptavidin. *Methods Enzymol* 1990; 184: 51-67.
- [38] Boerman OC, van Schaijk FG, Oyen WJG and Corstens FHM. Pretargeted Radioimmunotherapy of Cancer: Progress Step by Step. *J Nucl Med* 2003; 44: 400-411.
- [39] Liu G and Hnatowich DJ. A Semiempirical Model of Tumor Pretargeting. *Bioconjugate Chem* 2008; 19: 2095-2104.
- [40] Sharkey R, Chang CH, Rossi E, McBride W and Goldenberg D. Pretargeting: taking an alternate route for localizing radionuclides. *Tumor Biol* 2012; 33: 591-600.
- [41] Sharkey RM and Goldenberg DM. Advances in Radioimmunotherapy in the Age of Molecular Engineering and Pretargeting. *Cancer Invest* 2006; 24: 82-97.
- [42] Goldenberg DM, Sharkey RM, Paganelli G, Barbet J and Chatal JF. Antibody Pretargeting Advances Cancer Radioimmunodetection and Radioimmunotherapy. *J Clin Oncol* 2006; 24: 823-834.
- [43] Axworthy DB, Fritzberg AR, Hylarides MD, Mallett RW, Theodore LJ, Gustavson LM, Su FM, Beaumier PL and Reno JM. Preclinical Evaluation of An Anti-Tumor Monoclonal Antibody/Streptavidin Conjugate for Pretargeted 90Y Radioimmunotherapy in A Mouse Xenograft Model. *J Immunother* 1994; 16: 158.
- [44] Mohsin H, Jia F, Bryan JN, Sivaguru G, Cutler CS, Ketrang AR, Miller WH, Simón J, Frank RK, Theodore LJ, Axworthy DB, Jurisson SS and Lewis MR. Comparison of Pretargeted and Conventional CC49 Radioimmunotherapy Us-

## Pretargeted nuclear (PET/SPECT) imaging and therapy

- ing  $^{149}\text{Pm}$ ,  $^{166}\text{Ho}$ , and  $^{177}\text{Lu}$ . *Bioconjugate Chem* 2011; 22: 2444-2452.
- [45] Pagel JM, Kenoyer AL, Bäck T, Hamlin DK, Wilbur DS, Fisher DR, Park SI, Frayo S, Axtman A, Orgun N, Orozco J, Shenoi J, Lin Y, Gopal AK, Green DJ, Appelbaum FR and Press OW. Anti-CD45 pretargeted radioimmunotherapy using bismuth-213: high rates of complete remission and long-term survival in a mouse myeloid leukemia xenograft model. *Blood* 2011; 118: 703-711.
- [46] Pagel JM, Matthews DC, Kenoyer A, Hamlin DK, Wilbur DS, Fisher DR, Gopal AK, Lin Y, Saganic L, Appelbaum FR and Press OW. Pretargeted Radioimmunotherapy Using Anti-CD45 Monoclonal Antibodies to Deliver Radiation to Murine Hematolymphoid Tissues and Human Myeloid Leukemia. *Cancer Res* 2009; 69: 185-192.
- [47] Pagel JM, Orgun N, Hamlin DK, Wilbur DS, Gooley TA, Gopal AK, Park SI, Green DJ, Lin Y and Press OW. A comparative analysis of conventional and pretargeted radioimmunotherapy of B-cell lymphomas by targeting CD20, CD22, and HLA-DR singly and in combinations. *Blood* 2009; 113: 4903-4913.
- [48] Shen S, Forero A, LoBuglio AF, Breitz H, Khazaeli MB, Fisher DR, Wang W and Meredith RF. Patient-Specific Dosimetry of Pretargeted Radioimmunotherapy Using CC49 Fusion Protein in Patients with Gastrointestinal Malignancies. *J Nucl Med* 2005; 46: 642-651.
- [49] Förster GJ, Santos EB, Smith-Jones PM, Zanzonico P and Larson SM. Pretargeted Radioimmunotherapy with a Single-Chain Antibody/Streptavidin Construct and Radiolabeled DOTA-Biotin: Strategies for Reduction of the Renal Dose. *J Nucl Med* 2006; 47: 140-149.
- [50] Liu G, Dou S, Liu Y, Wang Y, Rusckowski M and Hnatowich DJ.  $^{90}\text{Y}$  labeled Phosphorodiamidate Morpholino Oligomer for Pretargeting Radiotherapy. *Bioconjugate Chem* 2011; 22: 2539-2545.
- [51] Liu G, Dou S, Cheng D, Leif J, Rusckowski M, Streeter PR, Shultz LD, Hnatowich DJ and Greiner DL. Human Islet Cell MORF/cMORF Pretargeting in a Xenogeneic Murine Transplant Model. *Mol Pharm* 2011; 8: 767-773.
- [52] He J, Wang Y, Dou S, Liu X, Zhang S, Liu G and Hnatowich D. Affinity Enhancement Pretargeting: Synthesis and Testing of a  $^{99\text{m}}\text{Tc}$ -Labeled Bivalent MORF. *Mol Pharm* 2010; 7: 1118-1124.
- [53] Liu G, Liu C, Zhang S, He J, Liu N, Gupta S, Rusckowski M and Hnatowich DJ. Investigations of  $^{99\text{m}}\text{Tc}$  Morpholino Pretargeting in Mice. *Nucl Med Commun* 2003; 24: 697-705.
- [54] He J, Liu G, Gupta S, Zhang Y, Rusckowski M and Hnatowich DJ. Amplification Targeting: A Modified Pretargeting Approach with Potential for Signal Amplification-Proof of a Concept. *J Nucl Med* 2004; 45: 1087-1095.
- [55] Liu G, Cheng D, Dou S, Chen X, Liang M, Pretorius PH, Rusckowski M and Hnatowich D. Replacing  $^{99\text{m}}\text{Tc}$  with  $^{111}\text{In}$  Improves MORF/cMORF Pretargeting by Reducing Intestinal Accumulation. *Mol Imaging Biol* 2009; 11: 303-307.
- [56] Liu G, Dou S, Rusckowski M and Hnatowich DJ. An experimental and theoretical evaluation of the influence of pretargeting antibody on the tumor accumulation of effector. *Mol Cancer Ther* 2008; 7: 1025-1032.
- [57] Liu G, Dou S, Baker S, Akalin A, Cheng D, Chen L, Rusckowski M and Hnatowich DJ. A preclinical  $^{188}\text{Re}$  tumor therapeutic investigation using MORF/cMORF pretargeting and an antiTAG-72 antibody CC49. *Cancer Biol Ther* 2010; 10: 767-774.
- [58] Wang Y, Chang F, Zhang Y, Liu N, Liu G, Gupta S, Rusckowski M and Hnatowich DJ. Pretargeting with Amplification Using Polymeric Peptide Nucleic Acid. *Bioconjugate Chem* 2001; 12: 807-816.
- [59] He J, Rusckowski M, Wang Y, Dou S, Liu X, Zhang S, Liu G and Hnatowich D. Optical Pretargeting of Tumor with Fluorescent MORF Oligomers. *Mol Imaging Biol* 2007; 9: 17-23.
- [60] Crooke ST. Advances in Understanding the Pharmacological Properties of Antisense Oligonucleotides. In: August JT, editors. *Advances in Pharmacology*. Academic Press; 1997. pp: 1-49.
- [61] Goodchild J. Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties. *Bioconjugate Chem* 1990; 1: 165-187.
- [62] Boyce M and Bertozzi CR. Bringing chemistry to life. *Nat Meth* 2011; 8: 638-642.
- [63] Debets MF, van Berkel SS, Dommerholt J, Dirks AJ, Rutjes FPJT and van Delft FL. Bioconjugation with Strained Alkenes and Alkynes. *Acc Chem Res* 2011; 44: 805-815.
- [64] Sletten EM and Bertozzi CR. From Mechanism to Mouse: A Tale of Two Bioorthogonal Reactions. *Acc Chem Res* 2011; 44: 666-676.
- [65] Becer CR, Hoogenboom R and Schubert US. Click Chemistry beyond Metal-Catalyzed Cycloaddition. *Angew Chem Int Ed* 2009; 48: 4900-4908.
- [66] Jewett JC and Bertozzi CR. Cu-free click cycloaddition reactions in chemical biology. *Chem Soc Rev* 2010; 39: 1272-1279.
- [67] Baskin JM and Bertozzi CR. Bioorthogonal Click Chemistry: Covalent Labeling in Living Systems. *QSAR Comb Sci* 2007; 26: 1211-1219.
- [68] Sletten EM and Bertozzi CR. Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of

## Pretargeted nuclear (PET/SPECT) imaging and therapy

- Functionality. *Angew Chem Int Ed* 2009; 48: 6974-6998.
- [69] van Berkel SS and van Delft FL. Metal-free bioconjugation reactions. *Drug Discov Today Technol* 2013; 10: e45-e51.
- [70] Devaraj NK. Tetrazine Bioorthogonal Reactions through the Development of New Synthetic Tools. *Synlett* 2012; 23: 2147-2152.
- [71] Best MD. Click Chemistry and Bioorthogonal Reactions: Unprecedented Selectivity in the Labeling of Biological Molecules. *Biochem* 2009; 48: 6571-6584.
- [72] Baskin JM and Bertozzi CR. Copper-free click chemistry: Bioorthogonal reagents for tagging azides. *Aldrichimica Acta* 2010; 43: 15-23.
- [73] Debets MF, van Hest JCM and Rutjes FPJT. Bioorthogonal labelling of biomolecules: new functional handles and ligation methods. *Org Biomol Chem* 2013; 11: 6439-6455.
- [74] Kolb HC, Finn MG and Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew Chem Int Ed* 2001; 40: 2004-2021.
- [75] Huisgen R. 1,3-Dipolar Cycloadditions. Past and Future. *Angew Chem Int Ed* 1963; 2: 565-598.
- [76] Meldal M and Tornøe CW. Cu-Catalyzed Azide-Alkyne Cycloaddition. *Chem Rev* 2008; 108: 2952-3015.
- [77] Huisgen R. Kinetics and Mechanism of 1,3-Dipolar Cycloadditions. *Angew Chem Int Ed* 1963; 2: 633-645.
- [78] Rostovtsev VV, Green LG, Fokin VV and Sharpless KB. A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angew Chem Int Ed* 2002; 41: 2596-2599.
- [79] Tornøe CW, Christensen C and Meldal M. Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J Org Chem* 2002; 67: 3057-3064.
- [80] Nwe K and Brechbiel MW. Growing Applications of "Click Chemistry" for Bioconjugation in Contemporary Biomedical Research. *Cancer Biother Radiopharm* 2009; 24: 289-302.
- [81] Wängler C, Schirrmacher R, Bartenstein P and Wängler B. Click-chemistry reactions in radiopharmaceutical chemistry: fast & easy introduction of radiolabels into biomolecules for in vivo imaging. *Curr Med Chem* 2010; 17: 1092-1116.
- [82] Marik J and Sutcliffe JL. Click for PET: rapid preparation of [<sup>18</sup>F]fluoropeptides using CuI catalyzed 1,3-dipolar cycloaddition. *Tetrahedron Lett* 2006; 47: 6681-6684.
- [83] Hausner SH, Marik J, Gagnon MKJ and Sutcliffe JL. In vivo positron emission tomography (PET) imaging with an  $\alpha\beta 6$  specific peptide radiolabeled using 18F-"click" chemistry: Evaluation and comparison with the corresponding 4-[<sup>18</sup>F]fluorobenzoyl- and 2-[<sup>18</sup>F] fluoropropionyl-peptides. *J Med Chem* 2008; 51: 5901-5904.
- [84] Li ZB, Wu Z, Chen K, Chin FT and Chen X. Click Chemistry for 18F-Labeling of RGD Peptides and microPET Imaging of Tumor Integrin  $\alpha\beta 3$  Expression. *Bioconjugate Chem* 2007; 18: 1987-1994.
- [85] Carroll L, Evans HL, Aboagye EO and Spivey AC. Bioorthogonal chemistry for pre-targeted molecular imaging - progress and prospects. *Org Biomol Chem* 2013; 11: 5772-5781.
- [86] Knall AC and Slugovc C. Inverse electron demand Diels-Alder (IEDDA)-initiated conjugation: a (high) potential click chemistry scheme. *Chem Soc Rev* 2013; 42: 5131-5142.
- [87] Staudinger H and Meyer J. Ueber neue organische Phosphorverbindungen II. Phosphazine. *Helv Chim Acta* 1919; 2: 619-635.
- [88] Lin FL, Hoyt HM, van Halbeek H, Bergman RG and Bertozzi CR. Mechanistic Investigation of the Staudinger Ligation. *J Am Chem Soc* 2005; 127: 2686-2695.
- [89] van Berkel SS, van Eldijk MB and van Hest JCM. Staudinger Ligation as a Method for Bioconjugation. *Angew Chem Int Ed* 2011; 50: 8806-8827.
- [90] Schilling CI, Jung N, Biskup M, Schepers U and Bräse S. Bioconjugation via azide-Staudinger ligation: an overview. *Chem Soc Rev* 2011; 40: 4840-4871.
- [91] Kiick KL, Saxon E, Tirrell DA and Bertozzi CR. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc Natl Acad Sci U S A* 2002; 99: 19-24.
- [92] Saxon E and Bertozzi CR. Cell Surface Engineering by a Modified Staudinger Reaction. *Science* 2000; 287: 2007-2010.
- [93] Agard NJ, Baskin JM, Prescher JA, Lo A and Bertozzi CR. A Comparative Study of Bioorthogonal Reactions with Azides. *ACS Chem Biol* 2006; 1: 644-648.
- [94] Prescher JA, Dube DH and Bertozzi CR. Chemical remodelling of cell surfaces in living animals. *Nature* 2004; 430: 873-877.
- [95] Chang PV, Prescher JA, Hangauer MJ and Bertozzi CR. Imaging Cell Surface Glycans with Bioorthogonal Chemical Reporters. *J Am Chem Soc* 2007; 129: 8400-8401.
- [96] Hangauer MJ and Bertozzi CR. A FRET-Based Fluorogenic Phosphine for Live-Cell Imaging with the Staudinger Ligation. *Angew Chem Int Ed* 2008; 47: 2394-2397.
- [97] Heldt JM, Kerzendorf O, Mamat C, Starke F, Pietzsch HJ and Steinbach J. Synthesis of Short and Versatile Heterobifunctional Linkers

- for Conjugation of Bioactive Molecules with (Radio-)Labels. *Synlett* 2013; 24: 0432-0436.
- [98] Vugts DJ, Vervoort A, Stigter-van Walsum M, Visser GWM, Robillard MS, Versteegen RM, Vuldiers RCM, Herscheid JDM and van Dongen GAMS. Synthesis of Phosphine and Antibody-Azide Probes for in Vivo Staudinger Ligation in a Pretargeted Imaging and Therapy Approach. *Bioconjugate Chem* 2011; 22: 2072-2081.
- [99] Stockmann H, Neves AA, Stairs S, Ireland-Zecchini H, Brindle KM and Leeper FJ. Development and evaluation of new cyclooctynes for cell surface glycan imaging in cancer cells. *Chem Sci* 2011; 2: 932-936.
- [100] Codelli JA, Baskin JM, Agard NJ and Bertozzi CR. Second-Generation Difluorinated Cyclooctynes for Copper-Free Click Chemistry. *J Am Chem Soc* 2008; 130: 11486-11493.
- [101] Varga BR, Kállay M, Hegyi K, Béni S and Kele P. A Non-Fluorinated Monobenzocyclooctyne for Rapid Copper-Free Click Reactions. *Chem Eur J* 2012; 18: 822-828.
- [102] Mbua NE, Guo J, Wolfert MA, Steet R and Boons GJ. Strain-Promoted Alkyne-Azide Cycloadditions (SPAAC) Reveal New Features of Glycoconjugate Biosynthesis. *Chembiochem* 2011; 12: 1912-1921.
- [103] Ning X, Guo J, Wolfert MA and Boons GJ. Visualizing Metabolically Labeled Glycoconjugates of Living Cells by Copper-Free and Fast Huisgen Cycloadditions. *Angew Chem Int Ed* 2008; 47: 2253-2255.
- [104] Jewett JC, Sletten EM and Bertozzi CR. Rapid Cu-Free Click Chemistry with Readily Synthesized Biarylazacyclooctynones. *J Am Chem Soc* 2010; 132: 3688-3690.
- [105] Dommerholt J, Schmidt S, Temming R, Hendriks LJA, Rutjes FPJT, van Hest JCM, Lefebvre DJ, Friedl P and van Delft FL. Readily Accessible Bicyclononynes for Bioorthogonal Labeling and Three-Dimensional Imaging of Living Cells. *Angew Chem Int Ed* 2010; 49: 9422-9425.
- [106] Gordon CG, Mackey JL, Jewett JC, Sletten EM, Houk KN and Bertozzi CR. Reactivity of Biarylazacyclooctynones in Copper-Free Click Chemistry. *J Am Chem Soc* 2012; 134: 9199-9208.
- [107] Chigrinova M, McKay CS, Beaulieu LPB, Udachin KA, Beauchemin AM and Pezacki JP. Rearrangements and addition reactions of biarylazacyclooctynones and the implications to copper-free click chemistry. *Org Biomol Chem* 2013; 11: 3436-3441.
- [108] Agard NJ, Prescher JA and Bertozzi CR. A Strain-Promoted [3 + 2] Azide-Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *J Am Chem Soc* 2004; 126: 15046-15047.
- [109] Bernardin A, Cazet AI, Guyon L, Delannoy P, Vignet Fo, Bonnaffé D and Texier I. Copper-Free Click Chemistry for Highly Luminescent Quantum Dot Conjugates: Application to in Vivo Metabolic Imaging. *Bioconjugate Chem* 2010; 21: 583-588.
- [110] Debets MF, van Berkel SS, Schoffelen S, Rutjes FPJT, van Hest JCM and van Delft FL. Azadibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3 + 2) cycloaddition. *Chem Commun* 2010; 46: 97-99.
- [111] van der Linden WA, Li N, Hoogendoorn S, Ruben M, Verdoes M, Guo J, Boons GJ, van der Marel GA, Florea BI and Overkleeft HS. Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: A comparative study. *Bioorg Med Chem* 2012; 20: 662-666.
- [112] Reddington SC, Tippmann EM and Dafydd Jones D. Residue choice defines efficiency and influence of bioorthogonal protein modification via genetically encoded strain promoted Click chemistry. *Chem Commun* 2012; 48: 8419-8421.
- [113] van Hest JCM and van Delft FL. Protein Modification by Strain-Promoted Alkyne-Azide Cycloaddition. *Chembiochem* 2011; 12: 1309-1312.
- [114] Bostic HE, Smith MD, Poloukhine AA, Popik VV and Best MD. Membrane labeling and immobilization via copper-free click chemistry. *Chem Commun* 2012; 48: 1431-1433.
- [115] McKay CS, Blake JA, Cheng J, Danielson DC and Pezacki JP. Strain-promoted cycloadditions of cyclic nitrones with cyclooctynes for labeling human cancer cells. *Chem Commun* 2011; 47: 10040-10042.
- [116] Ning X, Temming RP, Dommerholt J, Guo J, Ania DB, Debets MF, Wolfert MA, Boons GJ and van Delft FL. Protein Modification by Strain-Promoted Alkyne-Nitrone Cycloaddition. *Angew Chem Int Ed* 2010; 49: 3065-3068.
- [117] Temming RP, Eggermont L, van Eldijk MB, van Hest JCM and van Delft FL. N-terminal dual protein functionalization by strain-promoted alkyne-nitrone cycloaddition. *Org Biomol Chem* 2013; 11: 2772-2779.
- [118] Colombo M, Sommaruga S, Mazzucchelli S, Polito L, Verderio P, Galeffi P, Corsi F, Tortora P and Prospero D. Site-Specific Conjugation of ScFvs Antibodies to Nanoparticles by Bioorthogonal Strain-Promoted Alkyne-Nitrone Cycloaddition. *Angew Chem Int Ed* 2012; 51: 496-499.
- [119] Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA and Bertozzi CR. Copper-free click chemistry for dynamic in vivo imaging. *Proc Natl Acad Sci U S A* 2007; 104: 16793-16797.
- [120] Chang PV, Prescher JA, Sletten EM, Baskin JM, Miller IA, Agard NJ, Lo A and Bertozzi CR. Cop-

- per-free click chemistry in living animals. *Proc Natl Acad Sci U S A* 2010; 107: 1821-1826.
- [121] Koo H, Lee S, Na JH, Kim SH, Hahn SK, Choi K, Kwon IC, Jeong SY and Kim K. Bioorthogonal Copper-Free Click Chemistry In Vivo for Tumor-Targeted Delivery of Nanoparticles. *Angew Chem Int Ed* 2012; 51: 11836-11840.
- [122] Dehnert KW, Baskin JM, Laughlin ST, Beahm BJ, Naidu NN, Amacher SL and Bertozzi CR. Imaging the Sialome during Zebrafish Development with Copper-Free Click Chemistry. *Chem-biochem* 2012; 13: 353-357.
- [123] Laughlin ST, Baskin JM, Amacher SL and Bertozzi CR. In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish. *Science* 2008; 320: 664-667.
- [124] Bouvet V, Wuest M and Wuest F. Copper-free click chemistry with the short-lived positron emitter fluorine-18. *Org Biomol Chem* 2011; 9: 7393-7399.
- [125] Campbell-Verduyn LS, Mirfeizi L, Schoonen AK, Dierckx RA, Elsinga PH and Feringa BL. Strain-Promoted Copper-Free "Click" Chemistry for <sup>18</sup>F Radiolabeling of Bombesin. *Angew Chem Int Ed* 2011; 50: 11117-11120.
- [126] Sachin K, Jadhav VH, Kim EM, Kim HL, Lee SB, Jeong HJ, Lim ST, Sohn MH and Kim DW. F-18 Labeling Protocol of Peptides Based on Chemically Orthogonal Strain-Promoted Cycloaddition under Physiologically Friendly Reaction Conditions. *Bioconjugate Chem* 2012; 23: 1680-1686.
- [127] Hausner SH, Carpenter RD, Bauer N and Sutcliffe JL. Evaluation of an integrin  $\alpha\beta 6$ -specific peptide labeled with [<sup>18</sup>F]fluorine by copper-free, strain-promoted click chemistry. *Nucl Med Biol* 2013; 40: 233-239.
- [128] Carpenter RD, Hausner SH and Sutcliffe JL. Copper-Free Click for PET: Rapid 1,3-Dipolar Cycloadditions with a Fluorine-18 Cyclooctyne. *ACS Med Chem Lett* 2011; 2: 885-889.
- [129] Arumugam S, Chin J, Schirrmacher R, Popik VV and Kostikov AP. [<sup>18</sup>F]Azadibenzocyclooctyne ([<sup>18</sup>F]ADIBO): A biocompatible radioactive labeling synthon for peptides using catalyst free [3 + 2] cycloaddition. *Bioorg Med Chem Lett* 2011; 21: 6987-6991.
- [130] Evans HL, Slade RL, Carroll L, Smith G, Nguyen QD, Iddon L, Kamaly N, Stockmann H, Leeper FJ, Aboagye EO and Spivey AC. Copper-free click-a promising tool for pre-targeted PET imaging. *Chem Commun* 2012; 48: 991-993.
- [131] Chen K, Wang X, Lin WY, Shen CKF, Yap LP, Hughes LD and Conti PS. Strain-Promoted Catalyst-Free Click Chemistry for Rapid Construction of <sup>64</sup>Cu-Labeled PET Imaging Probes. *ACS Med Chem Lett* 2012; 3: 1019-1023.
- [132] Karver MR, Weissleder R and Hilderbrand SA. Bioorthogonal Reaction Pairs Enable Simultaneous, Selective, Multi-Target Imaging. *Angew Chem Int Ed* 2012; 51: 920-922.
- [133] van den Bosch SM, Rossin R, Renart Verkerk P, ten Hoeve W, Janssen HM, Lub J and Robillard MS. Evaluation of strained alkynes for Cu-free click reaction in live mice. *Nucl Med Biol* 2013; 40: 415-423.
- [134] Lee SB, Kim HL, Jeong HJ, Lim ST, Sohn MH and Kim DW. Mesoporous Silica Nanoparticle Pretargeting for PET Imaging Based on a Rapid Bioorthogonal Reaction in a Living Body. *Angew Chem Int Ed* 2013; 52: 10549-10552.
- [135] McKay CS, Moran J and Pezacki JP. Nitrones as dipoles for rapid strain-promoted 1,3-dipolar cycloadditions with cyclooctynes. *Chem Commun* 2010; 46: 931-933.
- [136] McKay CS, Chigrinova M, Blake JA and Pezacki JP. Kinetics studies of rapid strain-promoted [3 + 2]-cycloadditions of nitrones with biaryl-azacyclooctynone. *Org Biomol Chem* 2012; 10: 3066-3070.
- [137] Thalhammer F, Wallfaher U and Sauer J. Reaktivität einfacher offenkettiger und cyclischer dienophile bei Diels-Alder-reaktionen mit inversem elektronenbedarf. *Tetrahedron Lett* 1990; 31: 6851-6854.
- [138] Selvaraj R and Fox JM. trans-Cyclooctene - a stable, voracious dienophile for bioorthogonal labeling. *Curr Opin Chem Biol* 2013; 17: 753-60.
- [139] Sauer J, Bäuerlein P, Ebenbeck W, Gousetis C, Sichert H, Troll T, Utz F and Wallfaher U. [4 + 2] Cycloadditions of 1,2,4,5-Tetrazines and Cyclopropenes - Synthesis of 3,4-Diazanocaradienes and Tetracyclic Aliphatic Azo Compounds. *Eur J Org Chem* 2001; 2001: 2629-2638.
- [140] Yang J, Šečková J, Cole CM and Devaraj NK. Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions. *Angew Chem Int Ed* 2012; 51: 7476-7479.
- [141] Niederwieser A, Späte AK, Nguyen LD, Jüngst C, Reutter W and Wittmann V. Two-Color Glycan Labeling of Live Cells by a Combination of Diels-Alder and Click Chemistry. *Angew Chem Int Ed* 2013; 52: 4265-4268.
- [142] Boger DL. Diels-Alder reactions of heterocyclic aza dienes. Scope and applications. *Chem Rev* 1986; 86: 781-793.
- [143] Alge DL, Azagarsamy MA, Donohue DF and Anseth KS. Synthetically Tractable Click Hydrogels for Three-Dimensional Cell Culture Formed Using Tetrazine-Norbornene Chemistry. *Biomacromol* 2013; 14: 949-953.
- [144] Plass T, Milles S, Koehler C, Szymański J, Mueller R, Wießler M, Schultz C and Lemke EA. Amino Acids for Diels-Alder Reactions in Living Cells. *Angew Chem Int Ed* 2012; 51: 4166-4170.
- [145] Lang K, Davis L, Torres-Kolbus J, Chou C, Deiters A and Chin JW. Genetically encoded nor-



- bornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nat Chem* 2012; 4: 298-304.
- [146] Han HS, Devaraj NK, Lee J, Hilderbrand SA, Weissleder R and Bawendi MG. Development of a Bioorthogonal and Highly Efficient Conjugation Method for Quantum Dots Using Tetrazine–Norbornene Cycloaddition. *J Am Chem Soc* 2010; 132: 7838-7839.
- [147] Hansell CF, Espeel P, Stamenović MM, Barker IA, Dove AP, Du Prez FE and O'Reilly RK. Additive-Free Clicking for Polymer Functionalization and Coupling by Tetrazine–Norbornene Chemistry. *J Am Chem Soc* 2011; 133: 13828-13831.
- [148] Schoch J, Wiessler M and Jäschke A. Post-Synthetic Modification of DNA by Inverse-Electron-Demand Diels-Alder Reaction. *J Am Chem Soc* 2010; 132: 8846-8847.
- [149] Zeglis BM, Mohindra P, Weissmann GI, Divilov V, Hilderbrand SA, Weissleder R and Lewis JS. Modular Strategy for the Construction of Radiometalated Antibodies for Positron Emission Tomography Based on Inverse Electron Demand Diels-Alder Click Chemistry. *Bioconjugate Chem* 2011; 22: 2048-2059.
- [150] Knight JC, Richter S, Wuest M, Way JD and Wuest F. Synthesis and evaluation of an 18F-labelled norbornene derivative for copper-free click chemistry reactions. *Org Biomol Chem* 2013; 11: 3817-3825.
- [151] Devaraj NK, Weissleder R and Hilderbrand SA. Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging. *Bioconjugate Chem* 2008; 19: 2297-2299.
- [152] Schoch J, Staudt M, Samanta A, Wiessler M and Jäschke A. Site-Specific One-Pot Dual Labeling of DNA by Orthogonal Cycloaddition Chemistry. *Bioconjugate Chem* 2012; 23: 1382-1386.
- [153] Li Z, Cai H, Hassink M, Blackman ML, Brown RCD, Conti PS and Fox JM. Tetrazine-trans-cyclooctene ligation for the rapid construction of 18F labeled probes. *Chem Commun* 2010; 46: 8043-8045.
- [154] Keliher EJ, Reiner T, Turetsky A, Hilderbrand SA and Weissleder R. High-Yielding, Two-Step 18F Labeling Strategy for 18F-PARP1 Inhibitors. *ChemMedChem* 2011; 6: 424-427.
- [155] Reiner T, Lacy J, Keliher EJ, Yang KS, Ullal A, Kohler RH, Vinegoni C and Weissleder R. Imaging Therapeutic PARP Inhibition In Vivo through Bioorthogonally Developed Companion Imaging Agents. *Neoplasia* 2012; 14: 169-177.
- [156] Selvaraj R, Liu S, Hassink M, Huang CW, Yap LP, Park R, Fox JM, Li Z and Conti PS. Tetrazine-trans-cyclooctene ligation for the rapid construction of integrin  $\alpha\beta 3$  targeted PET tracer based on a cyclic RGD peptide. *Bioorg Med Chem Lett* 2011; 21: 5011-5014.
- [157] Liu S, Hassink M, Selvaraj R, Yap LP, Park R, Wang H, Chen X, Fox JM, Li Z and Conti PS. Efficient 18F Labeling of Cysteine-Containing Peptides and Proteins Using Tetrazine–Trans-Cyclooctene Ligation. *Mol Imaging* 2013; 12: 121-128.
- [158] Wu Z, Liu S, Hassink M, Nair I, Park R, Li L, Todorov I, Fox JM, Li Z, Shively JE, Conti PS and Kandeel F. Development and Evaluation of 18F-TTCO-Cys40-Exendin-4: A PET Probe for Imaging Transplanted Islets. *J Nucl Med* 2013; 54: 244-251.
- [159] Devaraj NK, Upadhyay R, Haun JB, Hilderbrand SA and Weissleder R. Fast and Sensitive Pretargeted Labeling of Cancer Cells through a Tetrazine/trans-Cyclooctene Cycloaddition. *Angew Chem Int Ed* 2009; 48: 7013-7016.
- [160] Yang KS, Budin G, Reiner T, Vinegoni C and Weissleder R. Bioorthogonal Imaging of Aurora Kinase A in Live Cells. *Angew Chem Int Ed* 2012; 51: 6598-6603.
- [161] Carlson JCT, Meimetis LG, Hilderbrand SA and Weissleder R. BODIPY-Tetrazine Derivatives as Superbright Bioorthogonal Turn-on Probes. *Angew Chem Int Ed* 2013; 52: 6917-6920.
- [162] Budin G, Yang KS, Reiner T and Weissleder R. Bioorthogonal Probes for Polo-like Kinase 1 Imaging and Quantification. *Angew Chem Int Ed* 2011; 50: 9378-9381.
- [163] Liu DS, Tangpeerachaikul A, Selvaraj R, Taylor MT, Fox JM and Ting AY. Diels-Alder Cycloaddition for Fluorophore Targeting to Specific Proteins inside Living Cells. *J Am Chem Soc* 2011; 134: 792-795.
- [164] Reiner T, Earley S, Turetsky A and Weissleder R. Bioorthogonal Small-Molecule Ligands for PARP1 Imaging in Living Cells. *Chembiochem* 2010; 11: 2374-2377.
- [165] Devaraj NK, Hilderbrand S, Upadhyay R, Mazitschek R and Weissleder R. Bioorthogonal Turn-On Probes for Imaging Small Molecules inside Living Cells. *Angew Chem Int Ed* 2010; 49: 2869-2872.
- [166] Karver MR, Weissleder R and Hilderbrand SA. Synthesis and Evaluation of a Series of 1,2,4,5-Tetrazines for Bioorthogonal Conjugation. *Bioconjugate Chem* 2011; 22: 2263-2270.
- [167] Rossin R, Renart Verkerk P, van den Bosch SM, Vulderson RCM, Verel I, Lub J and Robillard MS. In Vivo Chemistry for Pretargeted Tumor Imaging in Live Mice. *Angew Chem* 2010; 122: 3447-3450.
- [168] Devaraj NK, Thurber GM, Keliher EJ, Marinelli B and Weissleder R. Reactive polymer enables efficient in vivo bioorthogonal chemistry. *Proc Natl Acad Sci U S A* 2012; 109: 4762-4767.
- [169] Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J and Robillard

## Pretargeted nuclear (PET/SPECT) imaging and therapy

- MS. Highly Reactive trans-Cyclooctene Tags with Improved Stability for Diels-Alder Chemistry in Living Systems. *Bioconjugate Chem* 2013; 24: 1210-1217.
- [170] Poloukhine AA, Mbua NE, Wolfert MA, Boons GJ and Popik VV. Selective Labeling of Living Cells by a Photo-Triggered Click Reaction. *J Am Chem Soc* 2009; 131: 15769-15776.
- [171] Sletten EM and Bertozzi CR. A Hydrophilic Azacyclooctyne for Cu-Free Click Chemistry. *Org Lett* 2008; 10: 3097-3099.
- [172] de Almeida G, Sletten EM, Nakamura H, Palaniappan KK and Bertozzi CR. Thiacycloalkynes for Copper-Free Click Chemistry. *Angew Chem Int Ed* 2012; 51: 2443-2447.
- [173] Kii I, Shiraishi A, Hiramatsu T, Matsushita T, Uekusa H, Yoshida S, Yamamoto M, Kudo A, Hagiwara M and Hosoya T. Strain-promoted double-click reaction for chemical modification of azido-biomolecules. *Org Biomol Chem* 2010; 8: 4051-4055.
- [174] McGrath NA and Raines RT. Diazo compounds as highly tunable reactants in 1,3-dipolar cycloaddition reactions with cycloalkynes. *Chem Sci* 2012; 3: 3237-3240.
- [175] Lang K, Davis L, Wallace S, Mahesh M, Cox DJ, Blackman ML, Fox JM and Chin JW. Genetic Encoding of Bicyclononynes and trans-Cyclooctenes for Site-Specific Protein Labeling in Vitro and in Live Mammalian Cells via Rapid Fluorogenic Diels-Alder Reactions. *J Am Chem Soc* 2012; 134: 10317-10320.
- [176] Blackman ML, Royzen M and Fox JM. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels-Alder Reactivity. *J Am Chem Soc* 2008; 130: 13518-13519.
- [177] Taylor MT, Blackman ML, Dmitrenko O and Fox JM. Design and Synthesis of Highly Reactive Dienophiles for the Tetrazine-trans-Cyclooctene Ligation. *J Am Chem Soc* 2011; 133: 9646-9649.