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A novel family of acid-cleavable linker based on cyclic acetal motifs for the production of antibody-drug conjugates with high potency and selectivity

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Abstract. Cleavable linkers have become the subject of intense study in the field of chemical biology, particularly because of their applications in the construction of antibody-drug conjugates (ADC), where they facilitate lysosomal cleavage and liberation of drugs from their carrier protein. Due to lysosomes' acidic nature, acid-labile motifs have attracted much attention, leading to the development of hydrazone and carbonate linkers among several other entities. Continuing our efforts in designing new moieties, we present here a family of cyclic acetals that exhibit excellent plasma stability and acid lability, notably in lysosomes. Incorporated in ADC, they led to potent constructs with picomolar potency in vitro and similar in vivo efficacy as the commercially available ADC Kadcyla in mouse xenograft models.

Introduction

Cleavable linkers can be defined as chemical groups that can be cleaved in a controlled manner to release at least two distinct molecular entities. In the decade between the late 1980s and 1990s, the development of various cleavable linkers paralleled the emergence and rapid expansion of solid-phase organic synthesis, allowing the smooth traceless release of pure synthetic compounds from their solid support.¹ The resulting diversity of linker structures has been broadly classified into three main domains, depending on the conditions employed for their cleavage: enzymatic, physico-chemical (i.e. photo-irradiation), or chemical, the latter being undoubtedly the most populated and diversified group. Linkers sensitive to acidity/basicity, oxidation/reduction or to nucleophilic species have been thoroughly explored and led to motifs that are now part of the general synthetic chemist toolbox. Interestingly, a second wave of interest in "chemically labile" structures has then emerged with the development of chemical biology in the last two decades, where cleavable linkers are used to connect a payload or a probe to a biomolecule interacting with biological targets.² However, the stringent operating conditions imposed by biological environments (e.g. narrow temperature range, aqueous medium, low concentrations, presence of a myriad of biomolecules) have dictated the development of new types of linkers that can be cleaved under milder conditions, typically those found in various intracellular compartments. Indeed, one of the main applications of cleavable linkers in chemical biology resides in the intracellular delivery of compounds of interest, for example for imaging or therapeutic purposes, which has necessitated the development of chemical moieties with excellent extracellular stability – to avoid premature release and potential off-target delivery of the payload – but rapid and efficient intracellular cleavage. New types of acid-sensitive linkers have been developed for this purpose, to take advantage of the marked differences between the acidity of certain organelles or tissues (e.g. lysosomes or tumor environments, with pH < 5.0), and that of physiological body

fluids, with pH ~7.0 on average. Famous examples of such acid-labile chemical groups are hydrazone,³ *para*-methoxybenzyl,⁴ or dialkyldialkoxysilane derivatives,⁵ which served as basis for the development of various cleavable linkers. With an endocytosis mechanism relying essentially on lysosomal trafficking, it is not surprising to see antibody-drug conjugates (ADC) being equipped with such motifs – Mylotarg and Besponsa both feature a hydrazone linker, while Trodelvy incorporates a carbonate group.^{6,7} However, most of these acid-cleavable linkers also show partial instability in plasma, with half-lives ranging from a few minutes to 2-3 days, leading to risks of early drug cleavage and thus systemic toxicity in vivo.^{8–10}

Reflecting on this issue, our group has actively pursued the development of new acid-cleavable linkers in recent years. Following a semi-serendipitous strategy, we synthesized FRET probes, in which a TAMRA fluorophore was linked to a BHQ-2 quencher via known acid-sensitive moieties such as cyclic orthoester motifs or linear acetals (Figure 1, first and second generation, respectively). Incubating these probes in different media, we saw that some were highly stable in human plasma but readily hydrolyzed under acidic conditions, as determined by monitoring the fluorescence intensity of TAMRA.^{11,12} Building on these results, we designed a new series of compounds, this time bearing cyclic acetal units of different ring sizes and substitution patterns, with the idea of developing acid-cleavable amine-to-thiol cross-linkers that could be used in ADC (Figure 1, third generation).^{13,14} However, these new linkers were found to be extremely stable under acidic conditions, even at pH < 1.0. Intrigued by these unexpected results, we became interested in combining motifs from the second and third generation linkers to see the influence of methoxyphenyl groups on the stability of cyclic acetals, in the hope of developing a fourth generation of acetal-based linkers with an improved stability/cleavage balance.

Results and discussion

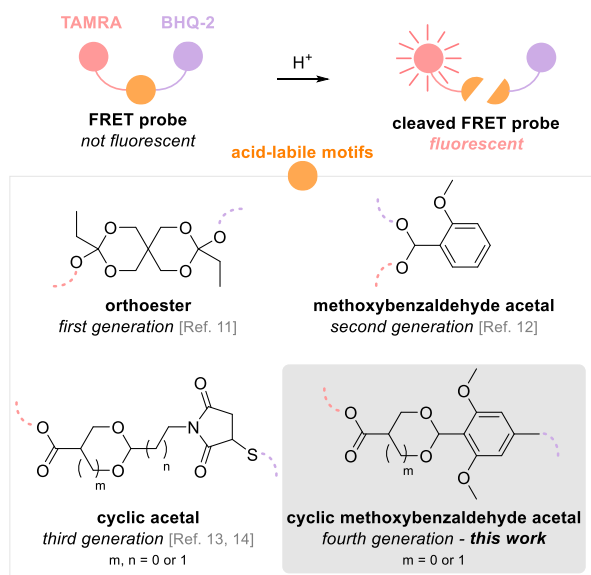


Figure 2 Design of FRET probes incorporating acid-cleavable linkers. TAMRA fluorescence is directly related to probe hydrolysis, separating the fluorophore from its quencher, and serves as an indirect way to evaluate the stability of our linkers.

For this purpose, we elected 4-bromo-2,6-dimethoxybenzaldehyde **1** as the carbonyl source for the construction of 5- and 6-membered cyclic acetals (Figure 2). We first engaged it in a Sonogashira coupling with ethynyltrimethylsilane, effectively delivering terminal alkyne **2** after a subsequent desilylation step. Reacting the latter with either a 1,2- or a 1,3-diol in the presence of a catalytic amount of *p*-toluenesulfonic acid in refluxing toluene gave the expected 5- and 6-membered acetal rings, isolated as mixtures of *cis-trans* isomers that could be readily separated

at this stage (see ESI). Saponification of the ester group afforded carboxylic acids **3** and **4** with excellent yields, bearing now two orthogonal functional sites – an alkyne and a carboxylate – that could be employed to build the FRET probes. In this regard, an azide-containing black-hole quencher 2 (BHQ-2-azide) was first coupled to **3** and **4** via copper-catalyzed azide-alkyne cycloaddition (CuAAC), before a BEP-mediated coupling with a TAMRA-amine derivative led to the final adducts **5** and **6**, isolated with a good overall yield (23%-35% over six steps).

The acid lability of these novel cyclic acetals was then assessed in various media by measuring the evolution of TAMRA fluorescence intensity at 580 nm over time, as previously discussed (Figure 2). Our two probes **5** and **6** were thus incubated in human plasma and in various buffers at different pH for 15 h, and the fluorescence values obtained were normalized to that of an equimolar solution of TAMRA and BHQ in the same buffer and at the same concentration. While our third generation probes had shown extreme stability even at pH < 1.0, acetals **5** and **6** were found to be readily hydrolyzed at pH ≤ 4.0 whilst being stable at neutral pH and in human plasma, highlighting the strong influence of the 2,6-dimethoxyphenyl group on the linker cleavability in acidic media. Contrary to what we had previously observed for other cyclic acetal probes, the rates of acidolysis were very similar between the 5- and 6-membered rings **5** and **6**, suggesting that stabilization of the transient oxonium ion by the strong electron-donating 2,6-dimethoxyphenyl group overcomes other stereoelectronic aspects, such as ring size and conformation.^{15,16}

Having observed a good balance between plasma stability and acid lability for our two probes, we next compared their

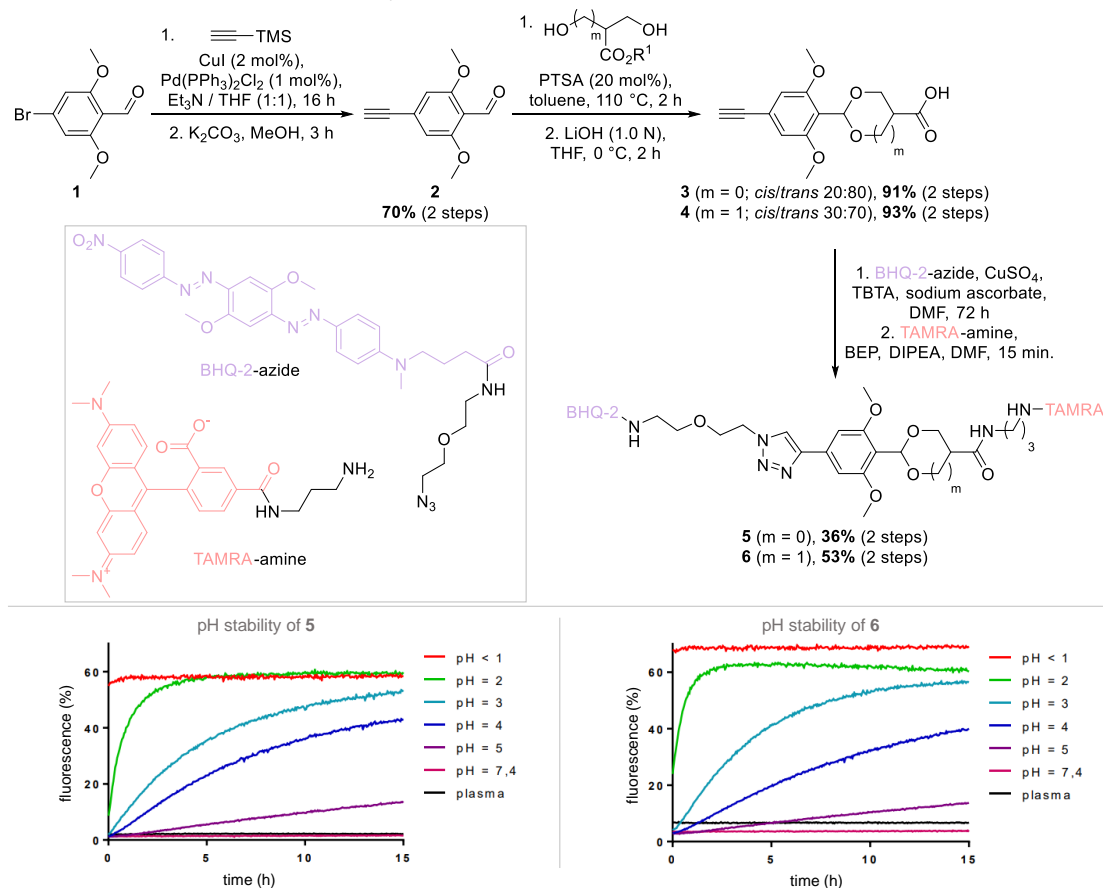


Figure 1 Synthesis of FRET probes **5** and **6** incorporating a five-membered (*m* = 0) and a six-membered (*m* = 1) ring acetal, respectively (top panel). Stability study of FRET probes **5** and **6** at different pH and in human plasma – the measured fluorescence is a direct indication of acetal hydrolysis and, by extension, of linker cleavage (bottom panel).

in vitro stability with that of the model probe **7** containing the enzyme-cleavable valine-citrulline (ValCit) motif, the most commonly encountered linker in ADC.¹⁷ A rapid evaluation was conducted in five different tumor cell lines, each incubated with a 1.0 μM solution of probe for 1.5 h at 37 $^{\circ}\text{C}$ before analysis by fluorescence-activated cell sorting (see ESI). This study indicated a better cleavage performance from our two probes **5** and **6** compared with the ValCit control **7**, and showed that the highest levels of mean fluorescence intensity (MFI) were obtained with SKBR-3 cancer cells (human, breast – HER2-positive). We thus selected this cell line as our model in a more comprehensive study of the intracellular cleavage mechanism of **5** and **6**. SKBR-3 cells were thus incubated with four different probes – **5**, **6**, the model ValCit **7** and our second-generation probe **8**, which served as an acetal benchmark – under similar conditions (1.25 μmol , 5 μM , 37 $^{\circ}\text{C}$, 90 min) before their nuclei were stained with Hoechst 33258 (Figure 3). Both cyclic acetals **5** and **6** led to the detection of TAMRA fluorescence, as did ValCit probe **7**, albeit in this case with lower intensity. This was in stark contrast with the results obtained with linear acetal probe **8**, for which no fluorescence was detected, confirming our previous observation on the intracellular stability of these constructs and demonstrating again the strong influence of stereoelectronic effects on the hydrolytic sensitivity of acetals.¹² To validate that the vesicle-like distribution of TAMRA fluorescence was due to a cleavage in acidic organelles, subsequent incubation of SKBR-3 cells with 2 μM of LysoSensorTM Green DND-189 was also conducted. To our delight, red and green fluorescence signals were shown to be colocalized as indicated by an orange-yellow fluorescence signal in merged pictures, reinforcing our hypothesis of a lysosomal cleavage. While both probes **5** and **6** led to higher fluorescence values than ValCit probe **7** by flow cytometry analysis, even after only 30 min of incubation, it was interesting to notice that **6** gave

systematically a 10-fold increase in fluorescence intensity compared with its 5-membered ring homologue **5**, a behavior that had not been seen during our stability studies in buffers (Figure 3). On the contrary, linear acetal probe **8** led to barely detectable fluorescence, confirming the microscopy results and its intracellular stability.

In order to confirm that these sharp differences were not caused by differential cellular uptake, we also made calibration curves of all four probes **5-8** in a 9:1 (v/v) mixture of DMEM/DMSO and used free TAMRA as internal standard. These allowed us to determine the amount of probes left in cell-culture medium after a 90-min incubation, which was found to be almost identical for probes **5**, **6** and **7**, ranging from 34% to 39%, but much lower for linear acetal **8** (< 5%). Overall, these results show that the observed discrepancies in fluorescence levels cannot be explained by variable cellular uptake, but rather by a difference in stability in the intracellular medium.

Having demonstrated that cyclic acetal motifs **5** and **6** were stable in plasma but superior to the classical ValCit linker in terms of cellular cleavage, we decided to take this new family of linkers a step further and considered their use in the production of ADC (Figure 4). We initially planned to develop a traceless approach, selecting anticancer drugs with 1,2 or 1,3-diol motifs to use these directly to construct the acetal ring. We opted for the broadly used anticancer drug gemcitabine, a therapeutic nucleoside analogue based on a 2-deoxy-2,2-difluororibose sugar that had previously shown to be amenable to acetal derivatization and incorporation in drug-delivery systems.^{18,19} However much to our dismay our numerous attempts in acetal formation with aldehydes **1** and **2** were systematically met with failure. We thus rerouted our efforts toward another cytotoxic drug classically employed for the production of ADC, monomethyl auristatin F (MMAF). Although lacking a 1,2- or 1,3-diol unit, which prompted us to abandon a traceless approach, we were still confident in

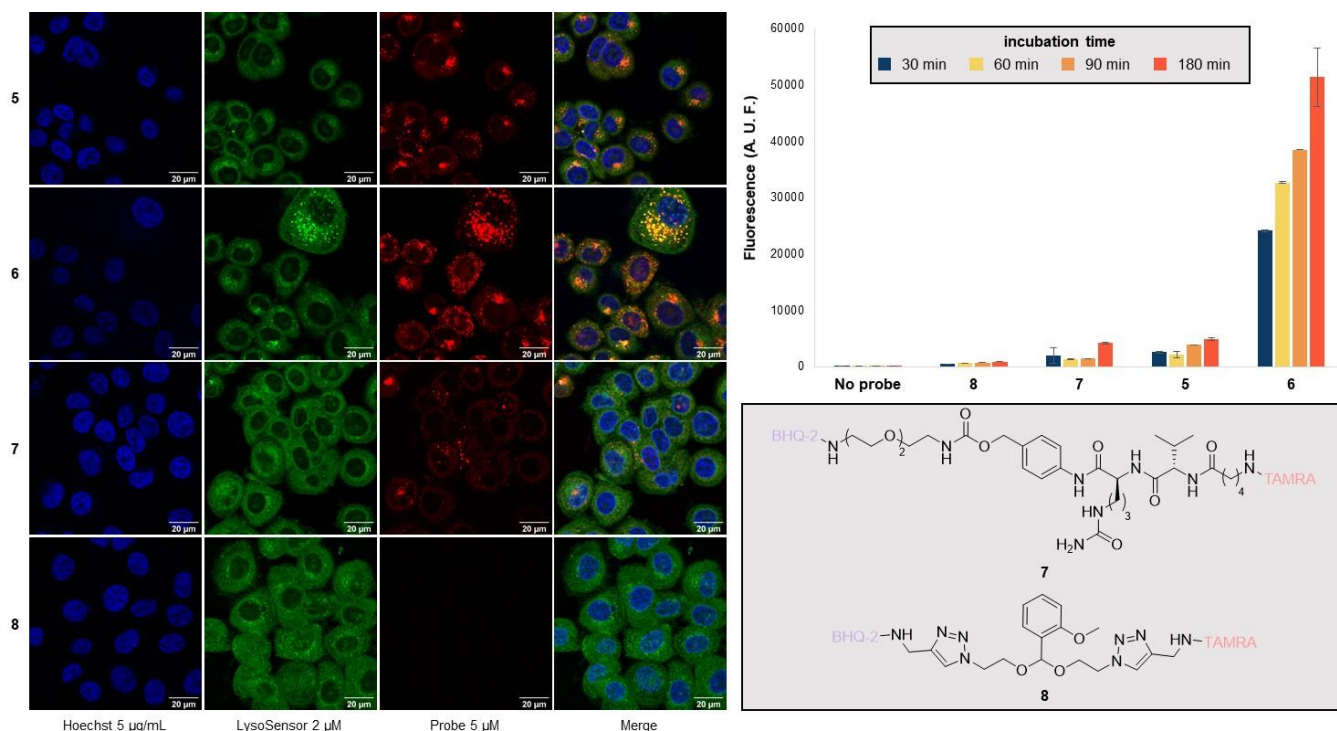


Figure 3 Microscopy and cytometry analyses. Left-hand panel: distribution and colocalisation studies of probes **5-8** in live SKBR-3 cells by fluorescence microscopy (blue channel, λ_{ex} = 359 nm: Hoechst 33258 fluorescence; green channel, λ_{ex} = 488 nm: LysoSensorTM Green DND-189 fluorescence; red channel, λ_{ex} = 555 nm: TAMRA fluorescence); 30 000 live cells were incubated with 5.0 μM of probe for 90 min followed by Hoechst 33258 (5 $\mu\text{g/mL}$, 30 min) to stain nuclei and LysoSensorTM Green DND-189 (2 μM , 30 min) to stain acidic organelles before they were analysed by confocal microscopy (scale bar = 20 μm). Right-hand panel: flow cytometry analysis (10 000 cells per assay) of SKBR-3 cells incubated with 1.0 μM probes **5-8** for different time (N = 4).

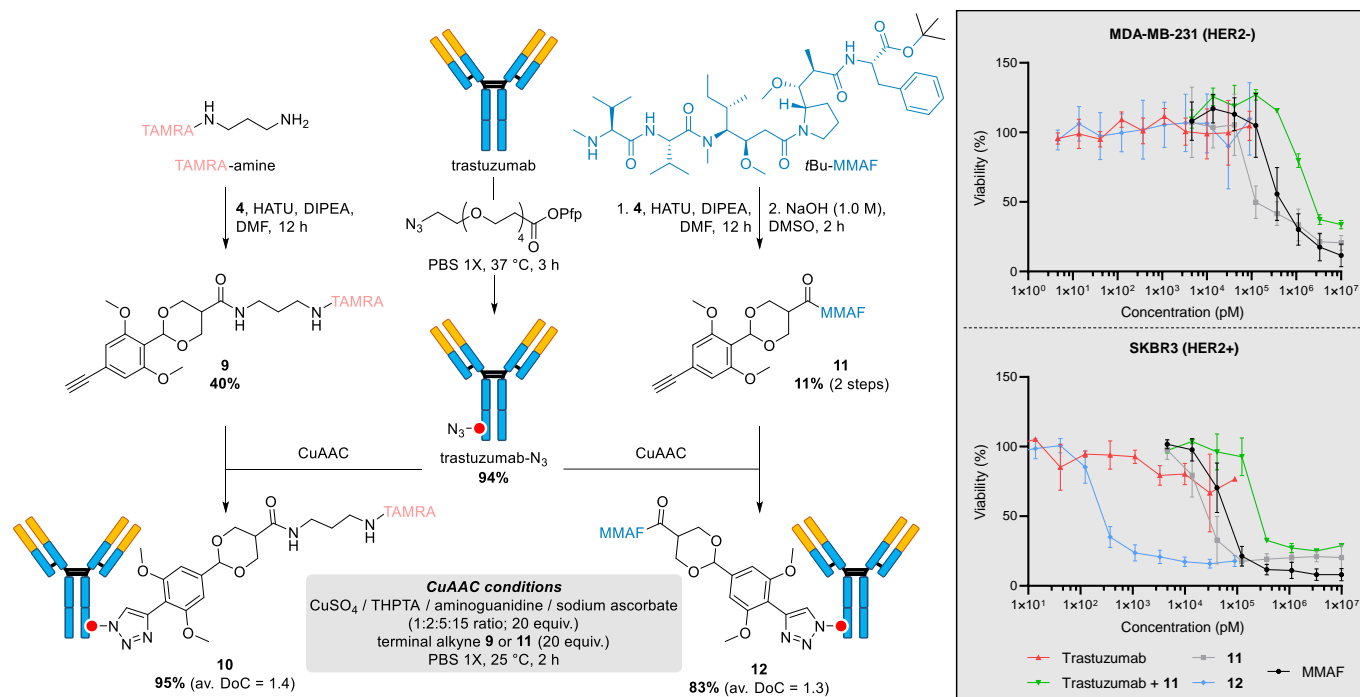


Figure 4 Synthesis of trastuzumab conjugates via CuAAC. Fluorescent immunoconjugate **10** was obtained from the previously described TAMRA-amine in two steps. ADC **12** was synthesized in three steps from commercially available tBu-MMAF; its cytotoxicity was evaluated on two cell lines – MDA-MB-231 (HER2-) and SKBR-3 (HER2+) – which were incubated with varying concentrations of **12** for 96 h before cell viability was determined by MTS assay. Data correspond to N = 6, except for trastuzumab + **11** (green line), N = 3.

the potential success of this second option, MMAF being known to tolerate *N*-terminal modifications,²⁰ as demonstrated by the recent approval of Blenrep for the treatment of multiple myeloma.²¹ Our first task was thus to equip MMAF with a cyclic acetal motif bearing an orthogonal reactive group for subsequent conjugation to an antibody. Previously synthesized intermediates **3** and **4** seemed to be ideal candidates for that purpose, possessing a carboxylic acid for MMAF attachment and a terminal alkyne for CuAAC-mediated conjugation to an antibody functionalized with azide groups.

At this stage, we decided to focus solely on the six-membered ring acetal **4**, 1,3-dioxane motifs having previously displayed superior cleavage than 1,3-dioxolanes in flow cytometry analyses.

Before engaging with MMAF, we started to develop our conjugation procedure with a model TAMRA payload, which was more easily accessible and safer to handle. The previously described TAMRA-amine was therefore derivatized to compound **9** in a single step under classical conditions for amide bond formation (Figure 4). In parallel, we selected trastuzumab – a therapeutic antibody used under the brand name Herceptin for the treatment of HER2-positive breast cancer – as our model carrier protein. Trastuzumab was easily functionalized with azide groups via lysine conjugation with a reactive pentafluorophenyl (Pfp) ester, setting the stage for the final conjugation step by CuAAC. After extensive investigations, it was found that the best conditions for this transformation were those developed by Marelli and coworkers.²² Using a catalytic system composed of CuSO₄, THPTA, aminoguanidine, and sodium ascorbate in PBS at 25 °C, trastuzumab-N₃ delivered 95% of pure fluorescent conjugate **10** with an average degree of conjugation of 1.4. Importantly, this antibody conjugate was shown to be stable in mouse plasma, with no sign of degradation over 48 h (see ESI), confirming the results observed with FRET probe **6**. With these optimized CuAAC

conditions in hand, we next focused on the construction of our desired ADC. Commercially available tBu-MMAF was reacted with **4** under the same conditions as previously described, delivering the expected amide product that was engaged in a subsequent C-terminal ester hydrolysis, an essential step for improving the aqueous solubility of our MMAF derivative. Trastuzumab-N₃ conjugation was performed following our previously optimized method, giving 83% of pure ADC **12** with an average drug-to-antibody ratio of 1.3 (see ESI for details).

In vitro cytotoxicity of our newly synthesized ADC **12** was then assayed on both HER2-positive SKBR-3 and HER2-negative MDA-MB-231 cancer cell lines (Figure 4, grey box; blue line). Naked trastuzumab (red line), free MMAF (black line), acetal-bearing MMAF **11** (grey line), and a non-covalently linked mixture of trastuzumab and **11** (green line) were used as controls – of important note is the fact that the naked trastuzumab had also been exposed prior to its testing to the same CuAAC catalytic mixture as trastuzumab-N₃ in order to eliminate any potential cytotoxicity bias that residual amounts of copper might cause. Cells were incubated with increasing concentrations in tested compounds for 96 h and cell viability was determined by MTT assay. As expected, our ADC proved to be essentially nontoxic on MDA-MB-231 cells – in contrast to the free drugs MMAF and MMAF-acetal **11** – but highly potent on SKBR-3 cells, thus confirming both the targeting effect of trastuzumab and the stability of our linker in the extracellular medium. In addition, we were pleased to observe a marked 100-fold increase in cytotoxicity for our ADC **12** on SKBR-3 cells compared with both free drugs – IC₅₀ (**12**) = 224 pM; IC₅₀ (MMAF) = 55 nM; IC₅₀ (**11**) = 22 nM – perfectly consistent with the known properties of MMAF, whose C-terminally charged carboxylate group makes it less membrane-permeable. Covalent attachment of **11** to trastuzumab appears to be essential for effective cytotoxicity, as shown by the stark differences between **12**

and the equimolar mixture of its two separate components, trastuzumab and **11**. The observed IC₅₀ value of our MMAF-ADC on targeted cells is also coherent with previously reported data in the literature for related ADC, which fluctuate between 8 nM and 25 pM depending on the targeted antigen and the MMAF payload structure (see ESI).^{23–28}

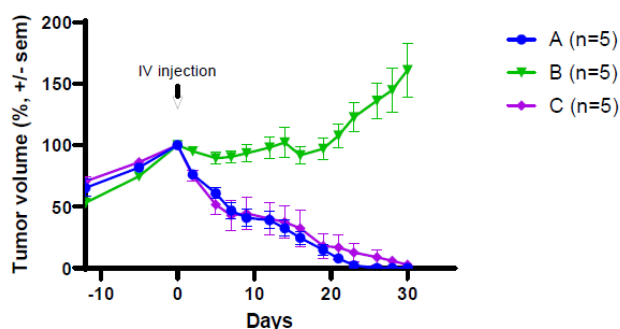


Figure 5 In vivo assays. NOD-SCID mice with heterotopic SKBR-3 tumor xenografts were injected intravenously with either ADC 12 (1.12 mg.kg⁻¹; group A), PBS 1X (vehicle; group B) or Kadcylla (1.12 mg.kg⁻¹; group C) and tumor volume was measured every 2–3 days for 30 days.

Having succeeded in the development of a potent antibody-drug conjugate with excellent targeting properties, we finally evaluated its cytotoxic activity in vivo (Figure 5). A cohort of 15 six-week-old immunodeficient NOD-SCID mice were thus first implanted heterotopically with SKBR-3 tumor xenografts and bred for 21 days. They were then split into three groups of five mice each, which received a single intravenous injection of the tested substances: group A was injected with ADC 12 (1.12 mg.kg⁻¹), group B received the vehicle (PBS 1X, negative control) and group C the marketed ADC Kadcylla (1.12 mg.kg⁻¹, positive control). Pleasingly, following a steady decline in volume over time, complete tumor regression was observed in group-A mice after 23 days post-injection, a remarkable activity at such low dose, comparable with that of the positive control Kadcylla (group C). This brings the final proof that our new family of acetal-based linkers possess an excellent stability/cleavability balance and that they can be used for in vivo applications.

Conclusions

We reported the synthesis of two new families of cyclic acetals derived from 2,6-dimethoxybenzaldehyde that could be used as acid-cleavable linkers. We showed that these structures were stable at neutral pH and in mouse and human plasma but readily cleaved at acidic pH ≤ 5.0. Interestingly, they even outcompeted the classical ValCitPAB linker in terms of intracellular lability as demonstrated by microscopy and cytometry experiments, which suggest a cleavage in acidic cellular compartments. When installed as the linker connecting the drug MMAF to the antibody trastuzumab, the newly synthesized antibody-drug conjugate was shown to be extremely potent in targeted cells, with an IC₅₀ = 224 pM in HER2-positive SKBR-3 cells, but non-toxic in HER2-negative MDA-MB-231 cells. This excellent potency was further demonstrated in in vivo xenograft assays, where full tumor regression was observed in NOD-SCID mice after 23 days following a single injection. Altogether, these results indicate that this new class of acid-labile linker are excellent candidates for payload-release applications where lysosomal trafficking is expected.

Author Contributions

TR and LT took care of investigation, methodology, validation, formal analysis, visualization and data curation. ET took care of investigation (preliminary results and original synthesis of probes). SE took care of formal analysis (mass spectrometry), data curation and visualization. FT took care of investigation and formal analysis (first in vitro experiments). OHA and SC took care of supervision and validation (mass spectrometry analyses). FD took care of data curation, formal analysis, investigation, methodology, resources, validation and visualization (in vivo experiments). AW took care of funding acquisition, resources, supervision and reviewing & editing of the final manuscript. GC took care of conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, visualization, writing of the original draft and reviewing & editing of the final manuscript.

Conflicts of interest

There are no conflicts to declare.

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Supporting Information Available

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.xxxx> and contains the following: experimental materials, instrumentation, and procedures, characterization of all new compounds, details of stability studies and cellular uptake of the different probes evaluated, native mass spectra of all new conjugates, copies of ¹H and ¹³C NMR spectra.

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