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**Herein we describe the development of a novel azobenzene-containing glutathione-cleavable linker for use in antibody–drug conjugates (ADCs). This linker demonstrated efficient payload release under elevated glutathione levels while maintaining stability in human plasma. An anti-HER2 ADC incorporating this linker exhibited potent *in vitro* cytotoxicity and selective activity towards HER2-positive cell lines.**

Antibody–drug conjugates (ADCs) are a rapidly growing class of targeted therapeutics. They combine the selectivity of monoclonal antibodies for cancer cells with the antitumor activity of cytotoxic payloads. With 13 FDA-approved therapies on the market and over 100 candidates advancing through clinical trials, ADCs display significant therapeutic potential as cancer therapies.<sup>1–3</sup>

The desired therapeutic effect of an ADC depends on three key components: the antibody, the cytotoxic payload, and the linker that connects the two. Linker chemistry determines the mechanism of cytotoxin release, influencing the stability and metabolic fate of the ADC. To minimize off-target toxicity, high linker stability is required in circulation.<sup>4</sup>

Cleavable linker motifs enable selective payload release.<sup>5–7</sup> One example are reducible disulfides, cleaved in the presence of glutathione (GSH). GSH, a tripeptide synthesised in the cytosol of the cell and distributed across different cellular organelles, is an intracellular reducing agent and a potent antioxidant, essential for several cellular processes including thiol-disulfide exchange and cellular signalling.<sup>8</sup> Typically, the cytosol of a healthy cell contains 1–11 mM of GSH, but its levels are several folds higher in a tumorous cell, to help restore intracellular redox homeostasis.<sup>9</sup>

Since unmodified disulfides within linkers are highly reactive and susceptible to nucleophilic attack in circulation,<sup>9,10</sup> the introduction of steric bulk has been used to prevent premature linker cleavage and payload release (Fig. 1a). This was achieved

## Towards a glutathione-cleavable azobenzene linker for antibody–drug conjugates

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by installing aromatic rings and methyl groups adjacent to the disulfide, or by using sterically hindered cytotoxic payloads.<sup>11–14</sup> As an alternative strategy, direct disulfide conjugation to engineered cysteine residues on the antibody have been utilised to sterically protect the disulfide from solvent accessibility.<sup>12</sup>

To expand the toolbox of GSH-cleavable ADC linkers beyond reducible disulfides, we describe the development of a novel azobenzene-containing linker (Fig. 1b). A tetra-*ortho*-methoxy-substituted motif was selected to establish the proof-of-concept due to its ability to undergo reductive cleavage of the azo bond under reducing conditions (similarly to other azobenzene analogues), resulting in the formation of aromatic anilines.<sup>15–19</sup> Such motifs are primed for self-immolation and subsequent cytotoxic payload release, similarly to Val-Cit linkers often used in FDA-approved ADC therapies.<sup>20</sup> While azobenzenes have been utilized as self-immolative triggers in ADCs,<sup>21</sup> to the best of our knowledge there is no reported use with GSH as the trigger.

Synthesis of the linker began with oxidation of ethyl 4-aminobenzoate to generate the nitroso adduct **1a** (Fig. 2). This was followed by Mills coupling with 4-aminobenzyl alcohol to afford the unsubstituted azobenzene **1b**. To avoid oxidation of the hydroxyl handle in the next step, **1b** was protected to generate **2a**. Subsequently, methoxy substituents were introduced into the azobenzene's *ortho* positions *via* a palladium-catalysed C–H oxidation developed by Müller-Deku *et al.*<sup>22</sup> TBS deprotection followed to afford tetra-*ortho*-methoxy azobenzene **2b**. A fluorescent 7-amino-4-methyl coumarin (AMC) payload was installed onto the azobenzene, forming a model linker-payload conjugate whose payload release and stability could be monitored by fluorimetry. AMC-isocyanate was first generated *in situ* then reacted with **2b** in the presence of dibutyltin dilaurate catalyst to afford tetra-*ortho*-methoxy azobenzene-AMC conjugate **2c** in two steps.

To investigate payload release, fluorimetry experiments were conducted. **2c** (50  $\mu$ M) was incubated in PBS (pH 7.4) in the presence and absence of GSH at 37 °C, over 24 hours (Fig. 3a and Fig. S8). Low levels of GSH (6 eq., 300  $\mu$ M) mimicked the concentration found in circulation, while elevated levels (200 eq., 10 mM)

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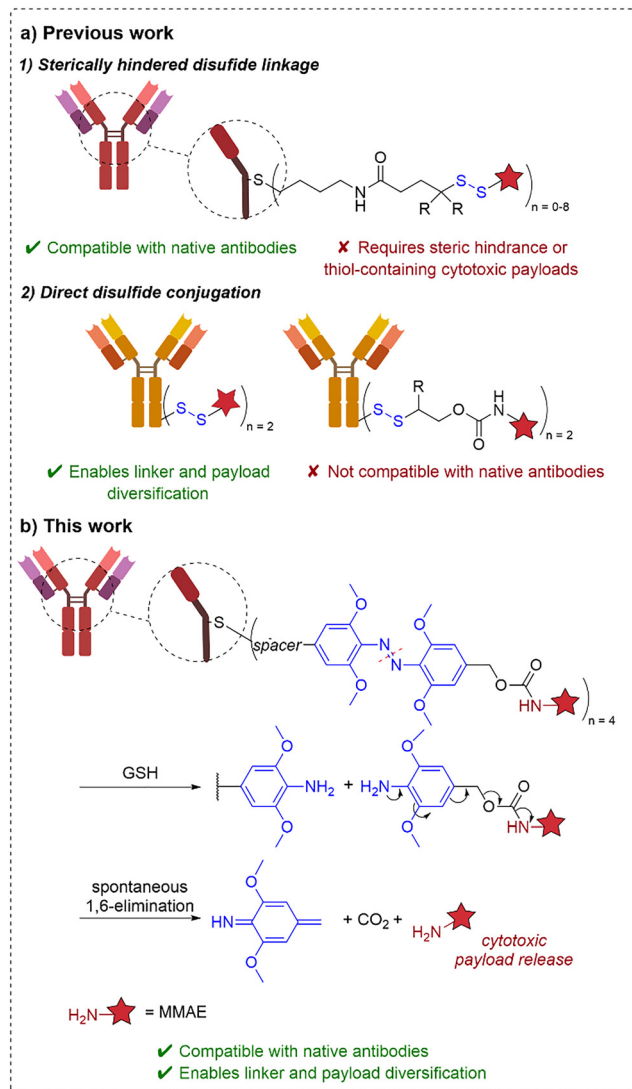


Fig. 1 GSH-cleavable linkers for ADCs. (a) Previous work utilising steric bulk and direct disulfide conjugation to enable stability of ADC constructs. (b) This work: azobenzene-containing GSH-cleavable linker.

mimicked those found in the tumour microenvironment.<sup>10</sup> Azobenzene-AMC conjugate **2c** displayed excellent stability in the absence of and at low levels of GSH, while azo bond cleavage and AMC payload release were observed at elevated GSH levels.

To evaluate the stability of the novel linker in the conditions of our cell-based assay, **2c** (50  $\mu\text{M}$ ) was incubated in DMEM, McCoy's and RPMI cell growth media in the presence (200 eq., 10 mM) and absence of GSH at 37 °C, over 4 days (Fig. 3b, c and Fig. S12). Excellent stability of **2c** was observed in each cell growth media in the absence of GSH, while GSH-mediated release of AMC was observed upon GSH addition.

The plasma stability of **2c** was further investigated. **2c** (50  $\mu\text{M}$ ) was incubated in human plasma at 37 °C, over 10 days (Fig. 3d and Tables S3, S4, Fig. S13–S15). Compound **2c** demonstrated therapeutically relevant stability in human plasma, with no significant release of AMC observed over 10 days.

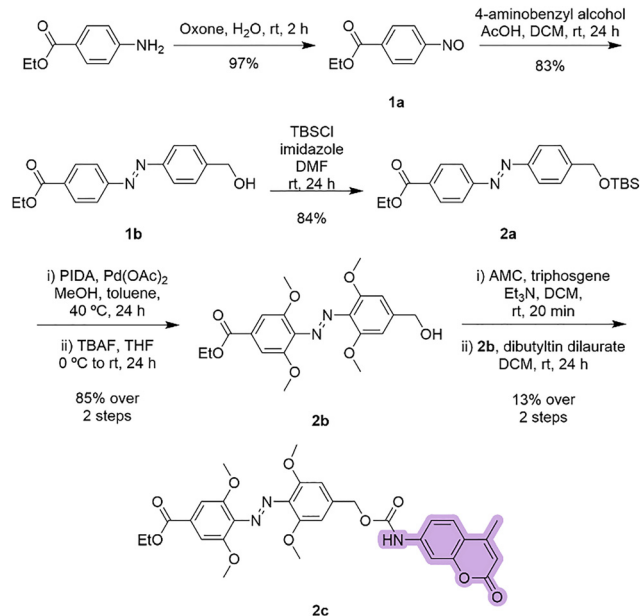


Fig. 2 Synthetic route towards azobenzene-AMC conjugate **2c**, with AMC highlighted in purple.

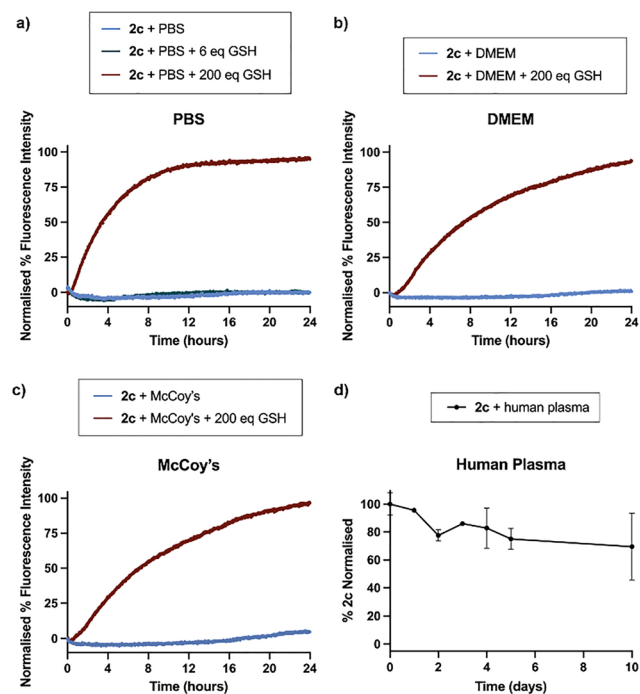


Fig. 3 Fluorometric analysis of AMC release from azobenzene-AMC conjugate **2c** with/without GSH at 37 °C over 24 hours in PBS (a), DMEM (b) and McCoy's (c). (d) Stability of **2c** in human plasma at 37 °C over 10 days.

Once the stability and AMC release of azobenzene-AMC conjugate **2c** was confirmed, synthesis of the corresponding azobenzene-cytotoxin was carried out (Fig. 4a). First, the ester handle of azobenzene **2b** was hydrolysed to enable amide coupling with an azide-functionalised polyethylene glycol (PEG) amine, affording the desired intermediate **3a**. Subsequently,



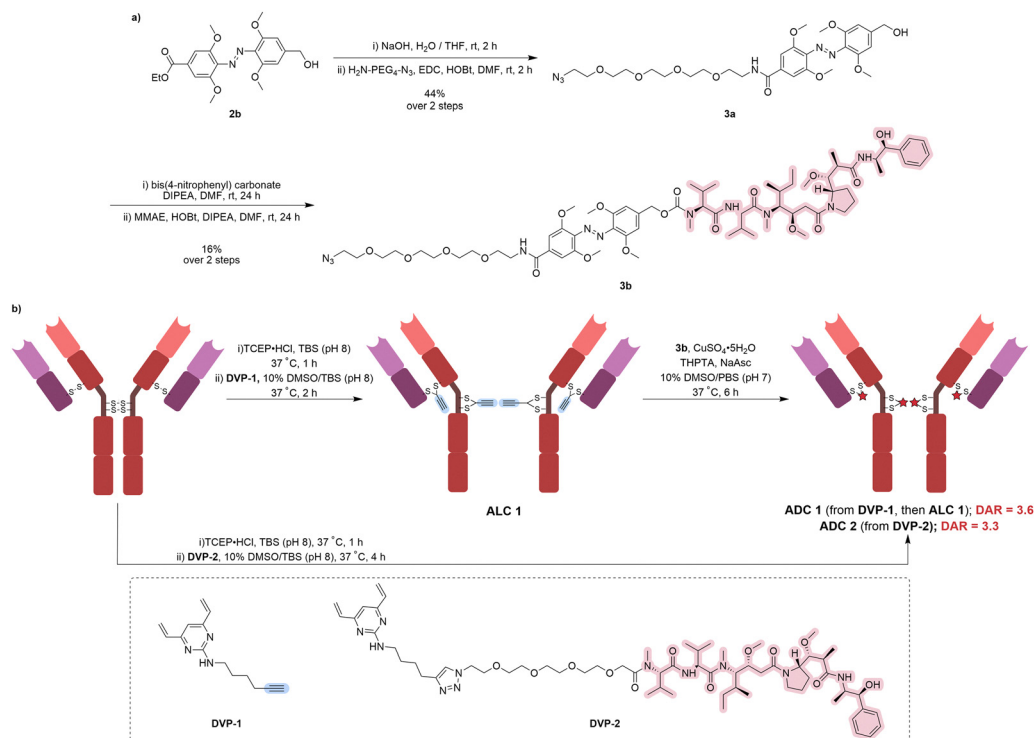


Fig. 4 (a) Synthetic route towards azobenzene-MMAE conjugate **3b**. (b) Bioconjugation conditions towards **ADC 1** and **ADC 2**, with alkyne handles and MMAE highlighted in blue and pink, respectively.

the benzylic alcohol was activated using bis(4-nitrophenyl) carbonate and coupled with monomethyl auristatin E (MMAE) to afford azobenzene-MMAE conjugate **3b** in two steps.

With conjugate **3b** in hand, MMAE release was monitored. **3b** (100  $\mu$ M) was incubated in PBS (pH 7.4) in the presence of GSH (200 eq., 20 mM) at 37 °C, over 72 hours. MMAE release was detected by both LC-MS and HPLC within 1 hour, with > 50% release over 72 hours (Table S2 and Fig. S9–S11).

To evaluate biological activity, linker-cytotoxin conjugate **3b** was incorporated into trastuzumab, an anti-HER2 antibody (Fig. 4b). Following a synthetic procedure developed by the Spring group,<sup>23</sup> ADC synthesis commenced with a 1-hour reduction of the four interchain disulfides of trastuzumab with tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Subsequent bioconjugation was achieved upon the addition of **DVP-1** and a 2-hour incubation at 37 °C to afford antibody-linker conjugate **ALC 1**. Copper-catalysed azide-alkyne cycloaddition (CuAAC) was performed with the addition of azide-functionalised azobenzene-MMAE conjugate **3b**, to afford GSH-cleavable azobenzene-containing **ADC 1**. A non-cleavable variant of the ADC (**ADC 2**) was also synthesised, to serve as a control for *in vitro* cytotoxicity studies.<sup>24</sup> High conversion to the desired ADCs was observed by protein LC-MS (Fig. S2–S4), SDS-PAGE (Fig. S5) and hydrophobic interaction chromatography (Fig. S6), while size-exclusion chromatography showed low aggregation (Fig. S7).

**ADC 1** and **ADC 2** were subsequently tested for their *in vitro* cytotoxicity against HER2-positive (SKBR3 and BT474; HER2+) and HER2-negative (MCF7 and MDA-MB-468; HER2-) breast cancer cell lines (Fig. 5). Pleasingly, GSH-cleavable **ADC 1**

demonstrated potent dose-dependent cell killing effect against HER2+ cell lines. The activity of GSH-cleavable **ADC 1** was comparable to that of the non-cleavable positive control, **ADC 2**, suggesting that linker conjugation has not interfered with antigen binding and internalisation. Furthermore, the GSH-cleavable ADC showed slightly higher cytotoxicity than the non-cleavable ADC, with a 2.2-fold increase in potency in BT474 cells and a 1.4-fold increase in SKBR3 cells (Table S5). In HER2- cell lines, GSH-cleavable **ADC 1** displayed cell killing effect only at high ADC concentration. This may be due to the diffusion of GSH into the extracellular space causing GSH-mediated payload release extracellularly.<sup>25</sup> Nonetheless, from a clinical perspective, this activity at high ADC concentration in HER2- cells lacks relevance due to the potent activity in HER2+ cells, yielding selectivity between the cell lines.

In summary, we have developed a novel azobenzene-containing cleavable linker that exhibited strong potential for ADC applications. The linker enables efficient GSH-mediated payload release while maintaining high stability in both human plasma and cell-based conditions. Using this platform, a GSH-cleavable anti-HER2 ADC (**ADC 1**) was successfully generated, which showed selective cytotoxicity toward HER2-positive cell lines and demonstrated an accessible therapeutic window compared to a relevant positive control.

M. K. – conceptualisation, investigation, visualization, data analysis, writing original draft. R. P., M. P., K. K., and T. W. – investigation and writing – review and editing. F. J. P. A. and D. R. S. – supervision and writing – review and editing.

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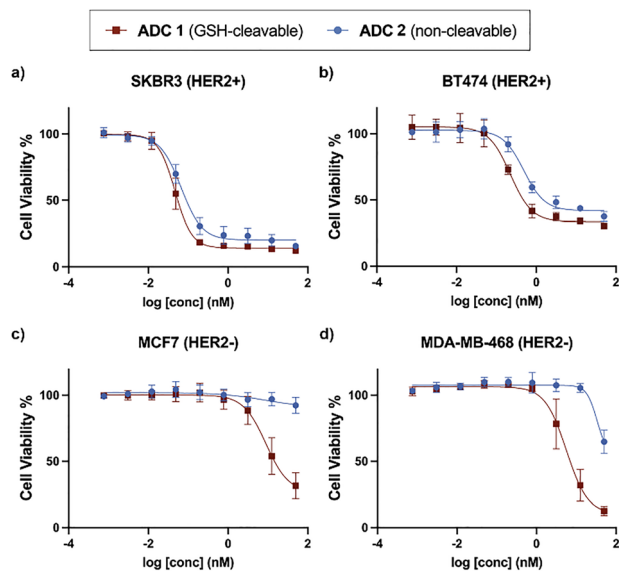


Fig. 5 *In vitro* cytotoxicity of GSH-cleavable **ADC 1** and non-cleavable **ADC 2** in HER2-positive (SKBR3 (a), BT474 (b)) and HER2-negative (MCF7 (c), MDA-MB-468 (d)) breast cancer cell lines.

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## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5cc05824d>.

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