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An efficient site-selective, dual bioconjugation approach exploiting N-terminal cysteines as minimalistic handles to engineer tailored anti-HER2 affibody conjugates

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Abstract: Site-selective, dual-conjugation approaches for the incorporation of distinct payloads are key for the development of molecularly targeted biomolecules, such as antibody conjugates, endowed with better properties. Combinations of cytotoxic drugs, imaging probes, or pharmacokinetics modulators enabled for improved outcomes in both molecular imaging, and therapeutic settings. We have developed an efficacious dual-bioconjugation strategy to target the N-terminal cysteine of a chemically-synthesized, third-generation anti-HER2 affibody. Such two-step, one-purification approach can be carried out under mild conditions (without chaotropic agents, neutral pH) by means of a slight excess of commercially available *N*-hydroxysuccinimidyl esters and maleimido-functionalized payloads, to generate dual conjugates displaying drugs (DM1/MMAE) or probes (sulfo-Cy5/biotin) in high yields and purity. Remarkably, the double drug conjugate exhibited an exacerbated cytotoxicity against HER2-expressing cell lines as compared to a combination of two monoconjugates, demonstrating a potent synergistic effect. Consistently, affibody-drug conjugates did not decrease the viability of HER2-negative cells, confirming their specificity for the target.

Introduction

Development of methodologies for site-selective functionalization of proteins is an extremely active field that provides an ever-growing arsenal of strategies to design next-generation tools for both fundamental and biomedical research.[1] Dual functionalization approaches that allow to introduce two different types of cargos can tremendously amplify the scope of engineered proteins.[2] Conjugation of distinct moieties onto target proteins can be either carried out at two different sites, or at a single site.[2] The latter case can be particularly advantageous for targeted therapy and imaging applications – such as antibody conjugates – owing to the possibility of selecting an appropriate region for cargo derivatization, thus limiting potential interference phenomena that may disrupt target binding efficacy. Recent examples of antibody conjugates that have been derivatized with two different moieties in a single site include: a cytotoxic drug and a PEG chain as a pharmacokinetic modifier,[3] two different auristatin derivatives (MMAE and MMAF) to overcome drug resistance,[4] or a cyanine 5 derivative and a ¹⁸F-containing moiety as a dual-modality (PET/fluorescence) imaging tool.[5]

Single-site dual conjugation of proteins has been carried out by means of three general approaches: (i) reactive linkers already functionalized with two cargos;[3] (ii) hetero-bifunctional linkers, pre-functionalized with a first cargo, that are reacted in a chemoselective manner to introduce the second cargo subsequently to protein conjugation;[6–10] (iii) hetero-trifunctional linkers.[11,12] Most of these strategies are directed towards the thiol group of solvent-exposed cysteine residues.

Recently, increasing attention has been given to selective modifications involving N-terminal Cys residues (NCys).[13] Thanks to the binucleophilic nature of its 1,2-aminothiol group, NCys have been largely exploited for chemoselective ligations that engage both nitrogen and sulfur atoms. Reported NCys-directed strategies include reaction with aldehydes to yield thiazolidines,[14–18] with nitriles to yield thiazolines,[19–24] with maleimide derivatives to give thiazines,[25] and with *N*-hydroxy succinimide (NHS) acrylates to yield 1,4-thiazepan-5-ones.[26]

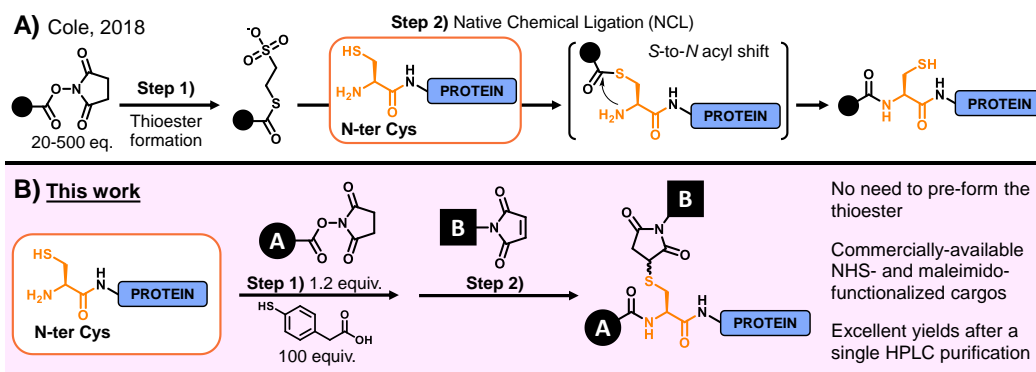
With a focus on NCys-directed dual functionalization, we have been interested by ligation methods that would harness the 1,2-aminothiol group while restoring the thiol functionality, thus enabling for the introduction of a second cargo at the same site. As additional prerequisites for an ideal dual-conjugation strategy, mild reaction conditions and readily available cargos are highly desirable. The Native Chemical Ligation (NCL)[27] developed by Kent is among

the most employed chemoselective reactions that take place at the NCys to form a native amide bond, while liberating the cysteine thiol group once ligation has occurred.[28] It involves a transthioesterification between a thioester and the NCys thiol, to form a transient intermediate that rearranges *via* an *S*-to-*N* acyl shift. This approach requires the preparation of thioester-functionalized cargos, and has been employed to generate site-selective protein mono-conjugates,[29–31] as well as dual conjugates on short peptides,[32,33] *via* subsequent thiol-Michael addition with a maleimide moiety. Recently, Gao and co-workers reported a more sophisticated 2-step dual conjugation strategy that requires a N-terminal “CIS” (Cys-Ile-Ser) tag in the target protein.[34] Reaction with a 2-cyanobenzothiazole-functionalized cargo yields an amidine in which the cysteine thiol group is amenable for further derivatization.[34]

A remarkable improvement in terms of simplicity was reported by Cole and co-workers:[35–37] a straightforward methodology that exploits NHS esters for NCys-specific protein labeling (Scheme 1A). The scope of such approach resonates with the large-scale portfolio of commercially-available NHS-functionalized reagents, including drugs, imaging probes, and so on. Following treatment of a large excess of NHS-cargos (typically 50 to 500 equiv.) with a 100-fold amount of 2-mercaptoethanesulfonic acid (MESNA) to provide the corresponding thioesters, addition of 1 equiv. of NCys-protein yields monoconjugates *via* an NCL reaction.

In this study, we have developed a simple strategy that allows for NCys-selective protein dual conjugation by means of common, commercially-available reagents such as NHS- and maleimido-functionalized drugs and probes (Scheme 1B). Our 2-step process involves direct addition of a slight excess (1.2 equiv.) of the NHS-moiety to the protein solution containing a thiol additive (4-mercaptophenylacetic acid = MPAA); the formation of the corresponding thioester takes place in the reaction mixture with extremely fast kinetics, enabling for subsequent selective NCL-mediated functionalization at the NCys. Following MPAA removal, the second step is carried out *via* thiol-Michael addition on a maleimide, to generate protein dual conjugates in excellent yields. Our synthetic strategy capitalizes on markedly reduced amounts of NHS and maleimide reagents, which despite being readily available in the market, can become extremely expensive when large excesses of sophisticated cargos are used for bioconjugation purposes.

We demonstrated the efficiency of our approach by generating dual-functionalized derivatives of an anti-HER2 affibody[38]



Scheme 1. (A) NCys-specific protein mono conjugation via thioester pre-formation described by Cole and co-workers (Ref. [35–37]). (B) Our proposed strategy for NCys-selective protein dual conjugation using NHS- and maleimido-functionalized cargos.

displaying either cytotoxic drugs or biochemical probes, and validated the resulting conjugates on cells expressing the clinically-relevant HER2 receptor.

Results and discussion

Design and synthesis of NCys-affibody dual conjugates

Affibody (Afb) proteins are engineered affinity ligands based on the three-helical-bundle Z domain, a stabilized version of the B domain of staphylococcal protein A.[39] This small protein scaffold (58 amino acids) was selected according to its excellent biophysical properties (high melting temperature, reversible and rapid folding, extended binding surface, high solubility in aqueous solutions) and for its ease of production in *Escherichia coli*. [40–43] Phage display [44] approaches allowed for the generation of Afb proteins with nanomolar-to-picomolar affinities towards a spectrum of biological targets, [45–47] including human epidermal growth factor receptor 2 (HER2), [48,49] epidermal growth factor receptor (EGFR), [50,51] tumor necrosis factor α (TNF α) [52,53] and amyloid- β (A β) peptide. [54,55] The Afb scaffold is devoid of Cys residues, allowing for site-specific modifications upon introduction of a unique cysteine. Mono-functionalized Afb constructs displaying cytotoxic payloads, radiotracers and optical imaging probes have been built via either C-terminal [56–62] or N-terminal [63,64] cysteine functionalization. In this study, we aimed to implement our NCys-selective dual functionalization strategy to generate unprecedented Afb constructs that display two different cargos at the same site.

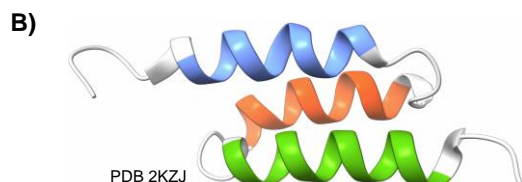
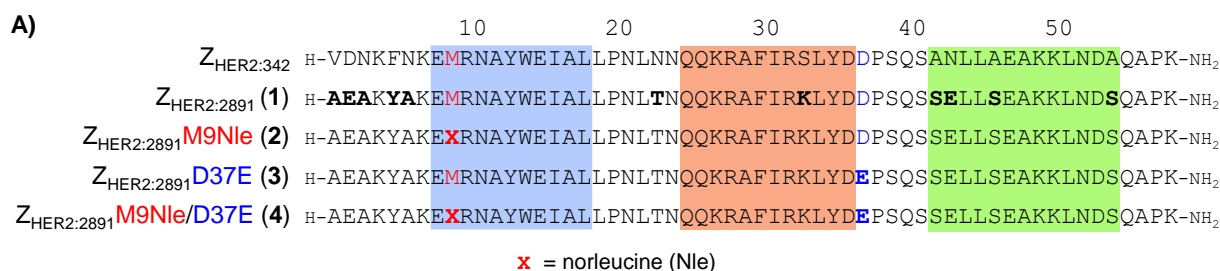
We started from the Z_{HER2:2891} Afb (**1**) sequence, [38] a second-generation anti-HER2 Afb in which mutation of 11 amino acid residues, located in the non-binding surface of the parent Z_{HER2:342} Afb, led to increased hydrophilicity and stability. [38] With the aim to further optimize the chemical stability of Afb **1**, we first generated two new variants *via* solid-phase peptide synthesis: **2** (M9Nle), replacing methionine-9 with isosteric norleucine, and **3** (D37E), replacing aspartate-37 with glutamate (Fig. 1A). In the first case, Met \rightarrow Nle mutation avoids the formation of the methionine sulfoxide by-product arising from oxidation, a problem encountered upon storage of **1** in non-deoxygenated solutions. Remarkably, despite Met9 being located in the interacting surface of Z_{HER2:342} with HER2 (Fig. S16, Supporting Information), Afb **2** exhibited a higher affinity ($K_D = 55$ pM) compared to **1**, and a higher melting temperature ($T_m = 72$ °C) (Fig. 1C). In the second case, the Asp-Pro peptide bond is known to undergo acidolysis, [65] in particular upon standing in TFA-containing HPLC buffers. [66] While not being directly involved in HER2 binding, Asp37 contributes to the stability of Z_{HER2:342} tertiary structure *via* hydrogen bonding with Ser39 (Fig. S17,

Supporting Information). Biophysical assays on Afb **3** showed increased binding affinity (60 pM) compared to **1**, similar to what was observed for Afb **2**; but also had a reduced melting temperature ($T_m = 64$ °C) (Fig. 1C). We then generated the double-mutant Afb **4** (M9Nle/D37E) and we observed that not only was the T_m comparable to that of the original Z_{HER2:2891} Afb **1** (67 vs 69 °C), but Afb **4** affinity for HER2 was additionally increased \approx 3-fold (33 pM) (Fig. 1C), thus validating this third-generation anti-HER2 Afb sequence.

En route to our selective, dual functionalization strategy, we synthesized Afb **5** (Fig. 2), the N-terminal cysteine (NCys)-containing version of Afb **4** (Page S17, Supporting Information).

We first optimized our 2-step dual NCys conjugation protocol to generate bis-conjugate **7a**, derivatized with two different cytotoxic cargos; a maytansinoid drug (DM1) and an auristatin (MMAE) prodrug. In the latter case, the presence of an enzymatically-cleavable Val-Cit motif, in tandem with the self-immolative PAB (*para*-aminobenzyloxycarbonyl) linker, allows the release of the active MMAE form in presence of lysosomal cysteine protease cathepsin B. [67] DM1-SMCC NHS ester and Val-Cit-PAB-MMAE maleimide were chosen for this study because they are the reagents used to synthesize two of the first approved antibody-drug conjugates therapeutics, trastuzumab emtansine (Kadcyla[®]) and brentuximab vedotin (Adcetris[®]).

Afb **5** was solubilized under anaerobic conditions at 1 mM concentration in phosphate buffer (final pH = 6.6), in the presence of 100 mM MPAA and 50 mM *tris*-carboxyethylphosphine (TCEP) to maintain a reductive environment and avoid disulfide formation via oxidation. Then, 1.2 equiv. of DM1-SMCC-NHS were added, and the reaction mixture was incubated at 37 °C overnight (Fig. 2). As shown by HPLC analysis in Fig. 2, the initial step proceeded cleanly towards the formation of mono-adduct **6a** without the need to pre-form DM1-SMCC-thioester. This is due to the extremely fast kinetics of *in-situ* MPAA thioester generation upon addition of DM1-SMCC-NHS to the reaction mixture, accounting for complete conversion in < 5 sec (Fig. S8, Supporting Information), thus preventing non-selective reactions of the NHS ester with the protein amino groups. Upon completion of step 1, the reaction medium was diluted with AcOH, and ice-cold Et₂O was added to induce Afb precipitation, allowing the efficient removal of both MPAA and residual DM1-SMCC-thioester via washing. The resulting pellet was re-solubilized at 1 mM in phosphate buffer (pH = 7.7), and reaction step 2 was carried out by adding 1.0 equiv. of maleimide (*i.e.* MMAE-PAB-Cit-Val-maleimide), which was completely converted to the desired dual-functionalized Afb **7a** within 1 h at room temperature (Fig. 2). A single final HPLC purification yielded pure **7a** in 40% yield. In order to exemplify the scope of the methodology with very diverse cargos, a similar protocol was used to generate bis-conjugate **7b** using biotin-PEG₄-NHS and



C)

Sequence	k _a (1/M·s) ^[a]	k _d (1/s) ^[a]	K _D (pM) ^[a]	T _m (°C) ^[b]
1	9.7·10 ⁶	8.7·10 ⁻⁴	90	69
2	6.9·10 ⁶	3.7·10 ⁻⁴	55	72
3	6.0·10 ⁶	3.6·10 ⁻⁴	60	64
4	6.0·10 ⁶	2.0·10 ⁻⁴	33	68

Figure 1. (A) Sequences of anti-HER2 affibody (Afb) molecules Z_{HER2:342} (Ref. [68]), Z_{HER2:2891} (1), Z_{HER2:2891}M9Nle (2), Z_{HER2:2891}D37E (3), Z_{HER2:2891}M9Nle/D37E (4). X stands for a norleucinyl (Nle) residue (B) Solution structure of Z_{HER2:342} (PDB 2KZJ, ref. [69]) with its three helices highlighted in blue (helix 1), orange (helix 2), and green (helix 3). (C) Table summarizing biophysical analyses carried out on sequences 1–4. [a] association rate constant (k_a), dissociation rate constant (k_d) measured on HER2 ectodomain *via* surface plasmon resonance (Page S16, Supporting Information); affinity values for Z_{HER2:2891} (1) in agreement with the literature (76 pM, Ref. [70]) ; [b] melting temperature (T_m) measured via circular dichroism (Page S13, Supporting Information), in agreement with the literature (67 °C, Ref. [69]).

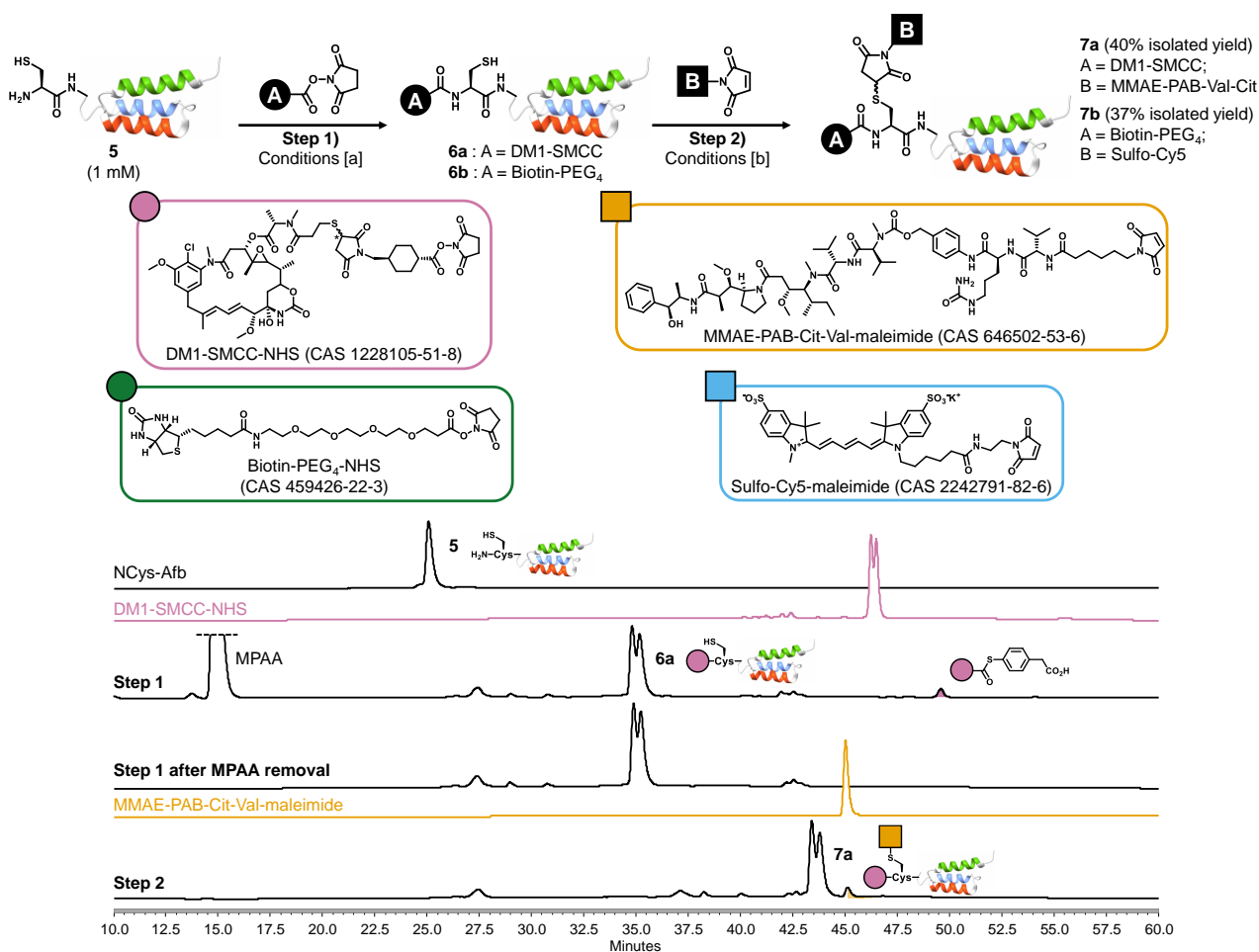


Figure 2. NCys-selective dual functionalization on anti-HER2 Afb 5 to generate dual conjugates 7a (DM1/MMAE) and 7b (biotin/sulfo-Cy5). Conditions [a]: 100 mM 4-mercaptophenylacetic acid (MPAA), 50 mM TCEP-HCl, 200 mM phosphate buffer (pH = 6.6), 37 °C (for 6a, 40% volume in NMP was used to solubilize DM1-SMCC-NHS). Conditions [b]: 200 mM phosphate buffer (pH = 7.7), RT (for 7a, 40% volume in NMP was used to solubilize MMAE-PAB-Cit-Val-maleimide). RP-HPLC chromatograms ($\lambda = 214$ nm) showing the two-step synthesis of 7a (See pages S19-S20 for detailed procedure, Supporting Information). DM1-SMCC-NHS and DM1-SMCC-containing affibodies show two closely eluted peaks due to the presence of two diastereoisomers.

sulfo-cyanine5-maleimide (37% isolated yield) (Page S25, Supporting Information).

Biological evaluation on HER2-expressing cells

intracellular C-terminus (Fig. 3A); western blot analysis confirmed cell transfection, and the absence of endogenous HER2 receptor on non-transfected cells (Fig. 3B, and Fig S18, Supporting Information). Next, dual affibody conjugate **7b**, displaying a biotin tag and a sulfo-cyanine 5 (Cy5) fluorophore was used to carry out flow cytometry assays on both HEK 297 cells, and transfected ones, referred to as HER2(-) and HER2(+), respectively.

Upon incubation with **7b** (50 nM), only transfected cells (Fig. 3C, right panel) induced increased signals relative to both GFP (488 nm laser, 530/30 nm band pass filter) and Cy5 (633 nm laser, 670/30 nm band pass filter), in sharp contrast with HEK 293 (Fig. 3C, left panel). In figure 3D, Cy5 fluorescence readouts from the above experiment were plotted against concentrations of **7b** added to HER2(+) (filled circles) and HER2(-) (hollow circles); indicating a dose-response behavior only for the positive cell line. Flow cytometry analysis also

With our dual affibody conjugates **7a** and **7b** in hand, we next sought to validate their functionality through cellular assays.

First, HEK 293 cells were transfected to express the HER2 receptor fused with a green fluorescent protein (GFP) on its

indicated that about 40% of the cells have been transfected with HER2-GFP, thus conveniently allowing to run the negative control experiment concomitantly: population Q3 in Fig. 3C right panel corresponds to non-transfected cells that do not bind the anti HER2 Afb, and Q2 to transfected ones that do. Absence of signals in the Q1 region confirms that non-transfected cells do not bind **7b**.

In addition, we performed ELISA assays to probe the binding to HER2 by exploiting the biotin moiety displayed on **7b** (Fig. 3E). For this, cells were seeded in multi-well plates and serial dilutions of **7b** were added. Next, addition of streptavidin-HRP (horseradish peroxidase) conjugate followed by TMB (3,3',5,5'-tetramethylbenzidine) substrate provided absorbance values that were fitted in a curve, from which we extrapolated an $EC_{50} = 1.1 \pm 0.4$ nM.[71] Having validated the binding properties of affibody construct **7a**, we next sought to undertake functionality studies.

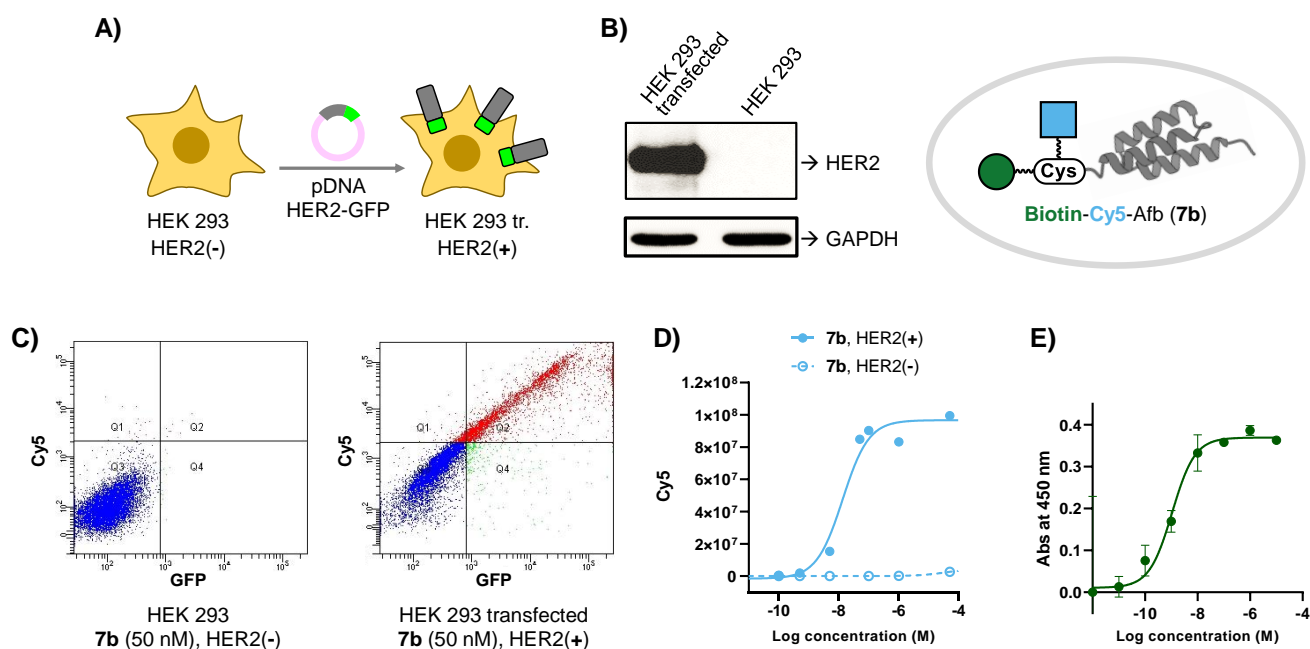


Figure 3. (A) Cellular models employed for the biological characterization of affibody conjugates, abbreviated as HER2(-) and HER2(+). HEK 293 cells were transfected with a plasmid containing the sequence of HER2 gene fused to a green fluorescent protein (GFP) to obtain HER2(+) cells. (B) Western blot analysis for HER2 expression in HEK 293 cells and transfected ones using an anti-HER2 antibody. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression was used as a loading control and was detected with an anti-GAPDH antibody. (C) Flow cytometry analysis carried out on HER2(-) (left panel) and HER2(+) cells (right panel) with Afb conjugate **7b**. (D) Increasing concentrations of **7b** induced an increase of double-positive cells (Q2, see Fig. 3C and 3D), *i.e.* cells expressing HER2 (GFP signal) and labelled with affibody (Cy5 signal). (E) Binding of **7b** has been confirmed by ELISA assays based on the streptavidin-biotin system. HER2(-) and HER2(+) cells have been incubated with **7b** and absorbance readouts detected in HER2(-) cells were subtracted from those obtained for HER2(+) cells.

Antibody-drug conjugates (ADCs) displaying a dual cytotoxic cargo system are promising tools for anticancer therapy.[4,72–74] They hold the potential to address intratumor heterogeneity and multi-drug resistance through concomitant delivery of drugs endowed with complementary or synergistic activities. Seminal studies in tumor challenge models reported improved outcomes when ADCs loaded with both MMAE and MMAF auristatin derivatives were systematically compared with analogs displaying only one drug.[4,74]

Various formats of anti-HER2 affibody conjugates displaying either MMAE or DM1 cytotoxic drugs have been reported,[57–61,64,75–77] and to the best of our knowledge, no affibody conjugate displaying different drugs has been reported so far. Thus, we sought

to exploit our dual DM1-MMAE-Afb conjugate **7a** to perform *in vitro* viability assays with our HER2(+) and HER2(-) cellular models (Fig. 3A).

To coherently evaluate the interest of introducing a dual cytotoxic payload system, we synthesized DM1-Afb **8** (Page S29, Supporting Information) and MMAE-Afb **9** (Page S30, Supporting Information) mono-conjugates as controls.

As a preliminary step, we performed viability assays on both HER2(+) and HER2(-) cells using unconjugated DM1 and MMAE drugs; the latter in its active form, devoid of the Val-Cit-PAB moiety (see Table S1, page S28, Supporting Information). This allowed us to define a reference maximal effect to subsequently evaluate the efficacy of our constructs (Fig. 4A). Co-incubation with equimolar

amounts of free drugs exerted comparable effects on both HER2(+) and HER2(-), confirming their expected non-selective cytotoxicity, regardless of HER2 expression (Fig. 4A). A clear dose-response was observed, with no significant increase of cell death by increasing the drug concentration from 50 nM to 1 μ M, highlighting a cell

subpopulation (\approx 40 %) resistant to the MMAE/DM1 combination. Consistently, minimum values of viability at 1 μ M were taken as maximal effect for the next experiments. Under the same experimental conditions, we incubated HER2(+) and HER2(-) cells with increasing concentrations of dual conjugate **7a**.

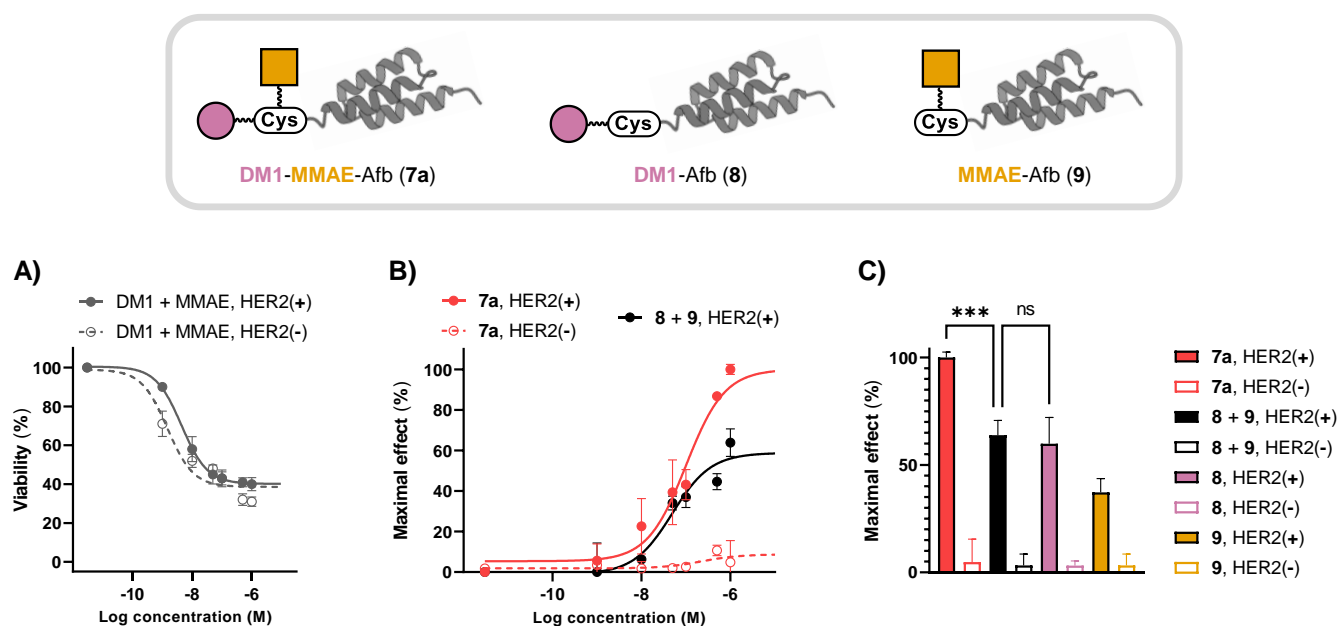


Figure 4. Afb conjugates **7a**, **8** and **9** and free DM1 and MMAE drugs (see Table S1, page S28, Supporting Information) were probed for their ability to inhibit cell proliferation of HER2(+) and HER2(-) models (Fig. 3A). (A) Determination of cell viability in HER2(+) and HER2(-) HEK 293 cells by Alamar Blue assay after treatment with admixed DM1 and MMAE in their free-form. Results expressed in percentage of total viability. (B) Activity induced by dual conjugate **7a** versus admixed mono conjugates **8** and **9**. Results expressed in percentage of the maximal effect, using the percentage of total viability at 1 μ M concentrations displayed in graph 4A as reference (see also Fig. S19-S20, Supporting Information). (C) Activity induced by drug conjugates, administered either alone (**7a**, **8**, **9**) or combined (**8 + 9**) at 1 μ M concentrations. Statistical significance was assessed using one-way ANOVA analysis with post-hoc Tukey's HSD test (***p \leq 0.001).

In parallel, to evaluate if the combination of DM1 and MMAE in dual conjugate **7a** resulted in a synergistic effect, DM1-Afb **8** and MMAE-Afb **9** were administered at concentrations designed to match the molar amounts of the individual drugs in **7a** (Fig. 4B). For HER2(+) cells, the activity of dual conjugate **7a** appeared to be related (at 1 μ M) to the activity exerted by equimolar concentrations of unconjugated drugs (see also Fig. S20, Supporting Information). In contrast, **7a** activities on HER2(-) cells were close to baseline values (Fig. 4B, red curves). Remarkably, dose-response curves involving HER2(+) cells suggested a synergistic effect between the two drugs when conjugated to a single Afb (**7a**), compared to HER2(+) cells treated with a combination of **8** and **9** (Fig. 4B, red versus black continuous curves). The extent of maximal effect for the complete battery of conjugates at 1 μ M concentration towards positive and negative cell lines confirmed the selectivity of all affibody-drug conjugates (Fig. 4C).

In our model, DM1-Afb **8** displayed increased activity compared to MMAE-Afb **9**; while co-incubation with **8** and **9** did not significantly enhance the ability to selectively kill HER2(+) cells with respect to **9** alone (Fig 4C). Strikingly, activity of dual conjugate **7a** was not only superior to that of singularly-administrated conjugates **8** and **9**, but also when the two mono-functionalized conjugates were combined together, revealing a synergistic effect for the selective killing of HER2-expressing cells.

Conclusions

Overall, we reported a simple synthetic strategy to utilise NCys for single-site double bioconjugation that enables the rapid derivatization of proteins with distinct payloads by means of reduced molar amounts of commercially-available reagents. Our two-step, one purification approach can be carried out under mild conditions and reactions proceeded cleanly, enabling the isolation of target conjugates with high isolated yields.

We have applied this methodology to an anti-HER2 affibody protein scaffold, that we optimized to increase chemical stability by means of two point mutations, affording a third-generation affinity ligand that displayed \approx 3-fold increased affinity for its target, while maintaining comparable thermal stability.

Biological characterization of our small set of affibody conjugates on cellular models, consisting of HEK 293 cells and the same cell line transfected with the HER2 ectodomain fused to a green fluorescent protein, allowed us to validate the functionality of both sulfo-cyanine 5 and biotin moieties displayed by conjugate **7b** for fluorescent staining and binding studies. Cell viability assays confirmed the specificity of all affibody drug conjugates, revealing a synergistic effect for dual affibody **7a**, displaying simultaneously the maytansinoid (DM1) drug, and the auristatin-derived (MMAE) prodrug.

In conclusion, the present study provided a facile but powerful synthetic strategy that will be a valuable addition to the increasing arsenal of single-site, dual modification toolkit to engineer proteins as next-generation conjugates for fundamental and biomedical research.

Author Contributions

V.A. and F.L. conceived the project; V.A. and C.P. coordinated the project. S.M.-L. supervised the cellular biology experiments; S.B. supervised the SPR experiments; F.K. performed the synthesis and purification of affibodies **1-4**, their analyses by circular dichroism, and performed preliminary exploration of dual conjugation; C.P. optimized the dual conjugation protocols and performed the dual conjugations described in the manuscript; A.N. performed all cellular biology experiments; C.P. wrote the manuscript and prepared figures with inputs from all authors; V.A., S.M.-L. and F.L. reviewed and edited the manuscript.

Conflicts of interest

S.B. and F.L. are employees of Laboratoires Servier. The remaining authors declare no competing interests.

Data and materials availability

Integrative experimental details are available in the Supporting Information file.

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Abbreviations

Afb: affibody

Cy5: cyanine 5

DM1: *N*²-deacetyl-*N*²-(3-mercapto-1-oxopropyl)-maytansine

EGFR: epidermal growth factor receptor

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

HEK: human embryonic kidney

HER2: human epidermal growth factor receptor 2

MESNa: 2-mercaptoethanesulfonic acid

MMAE: monomethyl auristatin E

MMAF: monomethyl auristatin F

MPAA: 4-mercaptophenylacetic acid

NCL: native chemical ligation

NCys: N-terminal cysteine

NHS: *N*-hydroxysuccinimide

PAB: *para*-aminobenzyloxycarbonyl

PEG: polyethylene glycol

PET: positron emission tomography

SMCC: succinimidyl-*trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate

TNF α : tumor necrosis factor α

Notes and references

‡ These authors contributed equally to this work.

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