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Bioconjugation through Mesitylene Thiol Alkylation

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Abstract

The design and generation of complex multifunctional macromolecular structures by bioconjugation is a hot topic due to increasing interest conjugates with therapeutic applications. In this regard, the development of efficient, selective, and safe conjugation methods is a major objective. In this report, we describe the use of the bis(bromomethyl)benzene scaffold as a linker for bioconjugation with special emphasis in antibody conjugation.

We first performed the monothioalkylation of 1,3,5-tris(bromomethyl)benzene, which rendered the reactive dibromotrimethylbenzyl derivatives to be used in thiol bis-alkylation. Next, we introduced either into a bis(Cys)-containing peptide and also with anti-CD4 and -CD13 monoclonal antibodies, previously subjected to partial reduction of disulphide bonds, into the linker. Mass spectrometry, UV-Vis spectra and SDS-PAGE experiments revealed that this bis-alkylating agent for bioconjugation preserved both antibody integrity and antibody-antigen binding affinity, as assessed by flow cytometry. Taken together, our results show that the mesitylene scaffold is a suitable linker for thiol-based bioconjugation reactions. This linker could be applicable in a near future for the preparation of antibody drug conjugates.

Introduction

Drug discovery efforts are being channelled into finding efficient, selective, and stable pharmaceutical agents with minimum side effects. Due to the biological activity of peptides, proteins and antibodies, therapies based on these biomolecules have become the strategies of choice to treat a wide range of diseases.[1,2] The chemical modification of these biomolecules can enhance their biological properties and strengthen their selectivity.[3–6] The approval of new chemically modified biomolecules by the FDA in recent years[7] underscores the increasing scientific interest and potential of this field.[8]

In nature, chemical modifications occur in a wide variety of amino acid side chains. Thus, the challenge has been to mimic these kinds of natural modification. Regioselectivity in

bioconjugation is achieved through a variety of reactive groups, such as amines, thiols, alcohols and even carboxylic acids.[6] Several methods involve bioconjugation[9] through Lys, Asp/Glu, and Ser/Thr, but the random distribution of these amino acids over the biomolecule structure leads to heterogeneous conjugations and, as a result, a broad number of chemical species. Given this observation, Cys, which is generally found as a disulphide bond and requires reduction of a disulfide bond to generate the active thiol group,[10,11] has been successfully used for regioselective bioconjugation in peptides, proteins and antibodies. Thus, Cys emerges as an amino acid of choice to perform site-specific conjugations and, consequently, to generate homogeneous conjugates with improved stability and therapeutic index. Accordingly, strategies using Cys have been specifically applied for the development of antibody-drug conjugates (ADCs).[12–16]

The most widely used method for Cys conjugation is the well-known thiol group alkylation *via* Michael addition over the double bond of a maleimide ring.[17,18] However, the undesired retro-Michael reaction can lead into the loss of conjugated linker in the plasma.[16,19] In order to avoid this side reaction, alkylating agents such as haloacetyl derivatives or alkyl halides are also used for this purpose.[20,21] Most Cys-conjugation procedures involve the thiol monoalkylation of the residue; however, as mentioned above, in nature Cys residues are often found as disulfide bonds, where they play a key role in the structural conformation of proteins. Although bis-alkylating agents have been widely used for peptide cyclization and labelling,[22–38] only a few examples of bis-alkylation of a previously reduced pair of Cys residues of an antibody using a bis-sulfone linker and bromomaleimides have been described in the literature.[39–43] Thus new chemical entities able to preserve the native structure of the antibodies after bioconjugation are needed.

Hartman and co-workers extended the use of 1,3,5-tris(bromomethyl)benzene (TBMB) as a peptide stapler and also as a linker for carboxylic acids.[44] They described the monoalkylation of TBMB using various kinds of carboxylic acid in basic conditions, yielding functionalized dibromobenzyl derivatives, which allowed simultaneous cyclization and peptide labelling in response to bis-Cys containing peptides. This remarkable simultaneous approach, together with the use of dibromobenzyl derivatives as linkers,[45,46] inspired us to broaden it using more complex bis-Cys-containing molecules such as antibodies.

The chemical stability of the thioether bond to the benzylic scaffold makes the former a suitable linker for a variety of bioconjugation reactions, including those aimed to produce ADCs. To this end, here we describe a novel antibody(Ab)-based bioconjugation strategy (**Figure 1**), which comprises previous mono-functionalization of TBMB using thiol derivatives as potential cargo of the TBMB scaffold, and subsequent conjugation to peptides or Abs. This strategy involves the reduction of a disulfide bond in the antibody in order to release two thiol groups, which finally react with the mono-functionalized dibromobenzyl derivative to yield the desired trimethylbenzyl-mediated conjugate.

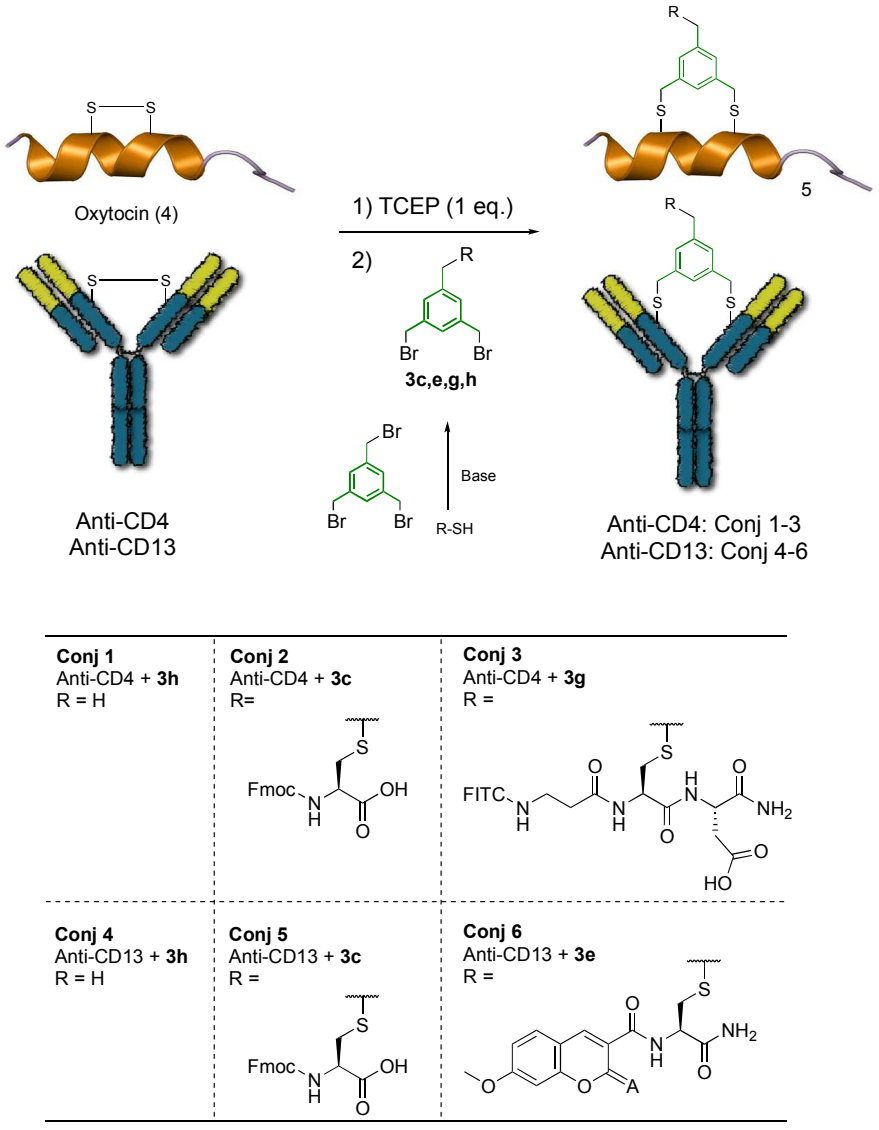


Figure 1. Conjugation of bromobenzyl derivatives to Anti-CD4 and Anti-CD13 antibodies.

The relatively low abundance of Cys in peptides and proteins confers this methodology an advantage since conjugation can be restricted to a particular Cys in the protein. In the case of Abs subjected to this bioconjugation reaction, the stable dibenzylic dithioether can replace the native interchain disulphide bond of the Ab. Given that disulphide bonds are sensitive to agents such as reduced glutathione, which is present in our body,[47] the introduction of the mesytilene scaffold confers additional stability to the peptide, protein or Ab due to the robustness of the thioether bond.[9]

Specifically, we tested the cargo bioconjugation through dibromobenzyl derivatives into a peptide –reduced Oxytocin– and monoclonal antibodies HP2/6 Anti-CD4 and TEA1/8 Anti-CD13. Moreover, we examined whether this bioconjugation strategy affects Ab-antigen binding affinity, a feature that is crucial for Ab function and efficacy.

Results and discussion

The optimization of the thiol monoalkylation of TBMB using various thiol derivatives (phenolic, benzylic or alkylic thiols) was tackled (SI). Afterwards, the bioconjugation step was studied using the dibromobenzyl derivatives previously obtained.

Monoalkylation studies and linker-cargo preparation

As a starting point, a range of conditions (number of equivalents, solvent, reaction time, and temperature) were studied for the monoalkylation of TBMB with phenolic, benzylic or alkylic thiols in the presence of DIEA, to render the corresponding dibromobenzyl derivatives (**SI**). These were further used for bioconjugation. Some thiol derivatives were commercially available (**2a,b,f**) while others were previously synthesized for the purpose of this study (**2c-e,g**) (see **SI**). Thus, in addition to the simple phenyl (**2a**), we used benzyl (**2b**), and Fmoc-Cys-OH (**2f**) as well as some Cys-bearing FITC (**2d**), 7-methoxycoumarin (**2e**) or short peptides such as reduced glutathione (Gln) (**2f**), and a FITC-labelled tripeptide FITC-β-Ala-Cys-Asp-NH_2 (**2g**).

Table 1-SI shows the conditions used for testing the monoalkylation reaction, the conversion ratio detected by HPLC or UPLC in function of the reaction time, the temperature, and the solvent. The monoalkylation reaction was performed using TBMB (1.0–1.5 eq.) and the corresponding thiol (1 eq.) in the presence of diisopropylethylamine (DIEA).

Our results revealed the aliphatic thiols (**2c-g**) as the best choice in terms of reaction conversion, but also because, unlike aromatic thiols, they did not produce an unpleasant smell. Accordingly, monoalkylation of less polar thiols (entries 1-13) gave better results than more polar substrates (entries 14-16). For long reaction times, polyalkylation products appeared in the reaction mixture, thereby hindering the isolation of the desired monoalkylate derivative (see **SI**). Moreover, the average of the reaction conversions corroborated that bimolecular nucleophilic substitution (S_N2) rates were increased in polar solvents (DCM < MeCN < DMF). Although the syntheses of dibromoxylene derivatives were challenging, we obtained the amount of **3c**, **3e** and **3g** required to carry out the bioconjugation experiments. A rigorous control of the reaction conditions (e.g., reaction time, temperature, and solvents) was needed to obtain the monoaddition products and to minimizing the undesired polyalkylation. This is exacerbated due to the high reactivity of the thiol group in response to the halobenzylic compounds. In addition, product isolation was tricky since the overall yields decreased drastically due to decomposition (see **SI**).

Bioconjugation

With the dibromoxylenes derivatives **3c**, **3e** and **3g** in hand, various assays were tested to perform the bioconjugation using a peptide and Abs. As proof of concept, the bioconjugation was assayed with a dithiol-containing model peptide. For this purpose, the reduced form of the natural hormone oxytocin (**4**) was prepared by automatic microwave-assisted solid-phase peptide synthesis (SPPS) (see **SI**). The thiol of Cys protected with the *S*-tetrahydropyran (Thp) group previously developed in our group was introduced into the SPPS.[48] Thp minimizes Cys racemization during coupling and is removed during global deprotection with trifluoroacetic (TFA) cocktail. The conjugation between **4** and the dibromobenzyl derivative **3c** was performed in basic $NaHCO_3$ buffer (pH = 8) at room temperature. The conjugation

reaction took place rapidly and was completed after 15 min, as demonstrated by the absence of **4** (**Figure 2**). These results prompted us to use the dibromobenzyl system for conjugation to Abs.

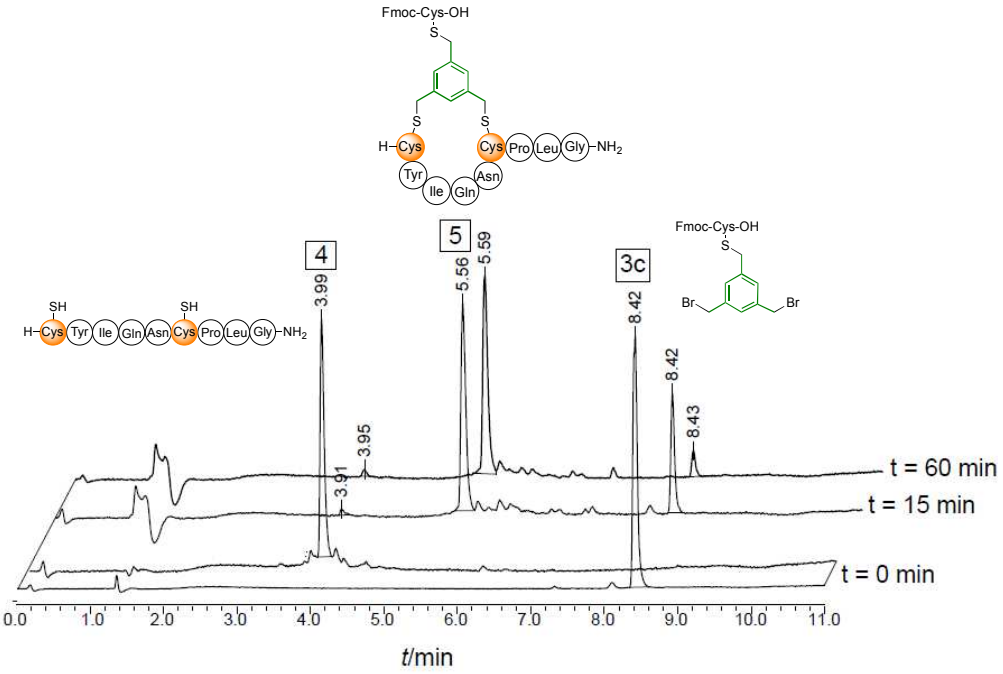


Figure 2. HPLC chromatograms of the starting materials **3c** and reduced Oxytocin (**4**), and the bioconjugation product **5**.

The monoclonal antibodies (mAbs) used for bioconjugation were the anti-CD4 HP2/6 and the anti-CD13 TEA1/8. Both CD4 and CD13 are targets for therapeutic intervention.[49–51] Indeed, a humanized anti-CD4 mAb, Zanolimumab, has shown promising results in the treatment of reumathoid arthritis, psoriasis, Sezary syndrome and against T cell lymphomas.[49,52]

To perform the corresponding bioconjugation with the dibromoxylene system, a previous disulfide reduction step of the mAbs was needed to release reactive thiol groups. To study the behavior of bromobenzyl derivatives in response to two or more reactive thiols, an anti-CD4 solution was treated with a range of amounts of TCEP (1 eq., 3 eq. and an excess >100 eq. for conjugates **2a, 2b** and **2c** respectively). After disulfide reduction, the dibromobenzyl derivative **3c** was added to each mAb solution (for experimental details see **SI**). Then the conjugates (**Conj2a-c**) were purified, and characterized by SDS-PAGE (see **SI**). Using SDS-PAGE in non-reducing conditions, the entire mAb band (≈150 kDa) was observed with the naked antibody and with the conjugate analyzed which was reduced using equimolar amount of TCEP and conjugated with the dibromobenzyl derivative **3c** (**Conj2a**). However, when the amount of TCEP was higher than that of **3c** (more than 1 eq.) new bands appeared in the electrophoresis gel (see **SI**). The SDS-PAGE under reduced conditions showed that the reduction step affected the integrity of the conjugate. As expected**,** the more equivalents of TCEP used, the greater the degree of Ab reduction. The SDS-PAGE analysis indicated that with 1 and 3 eq. of TCEP (**Conj2a and 2b**), the bands corresponded to the light and heavy chains (25 and 50 kDa, respectively). In contrast, when using excess of TCEP (**Conj2c**), more than one band corresponding to the light chain was observed, thereby indicating that these conditions affected the structure of the conjugate. Due to the low molecular weight of **3c**

[(459.6 g/mol) corresponding to $C_{27}H_{25}NO_4S^{2+}$] in the SDS-PAGE analysis, the differences between the conjugate and the naked antibody were minimal, thus hindering the analysis of the results by this technique.

Having determined the optimal conditions to reduce disulfide bonds without affecting the integrity of the Ab, we next prepared a set of conjugates. To this end, the dibromobenzyl derivatives **3c**, **3e** and **3g** and also the commercially available *m*-dibromoxylene (**3h**) were conjugated to the previously disulfide-reduced Anti-CD4 and Anti-CD13 Abs by adding the dibromobenzyl compounds (from 1 eq. to an excess) dissolved in DMSO (<10%) and stirring the mixture at 4ºC for 30 min (**Figure 1).**

The conjugates obtained were characterized by emission spectroscopy, SDS-PAGE, HPLC, and mass spectrometry (see **SI**). One of the most encouraging results was the preparation of conjugate **3**, where the introduction of a labelled tripeptide (**3g**) into the Ab was observed in the emission spectrum of this compound (**Figure 3**). The emission, which was recorded at approximately 475 nm, indicated the presence of a FITC moiety in **3**. Unfortunately, the emission spectrum for the 7-methoxycumarin conjugates (conjugates **6**) failed to show clear conjugation due to the small UV variation at approximately 330 nm (see **SI**). This finding suggests that the degradation of the dibromobenzyl compound decreased the conjugation ratio.

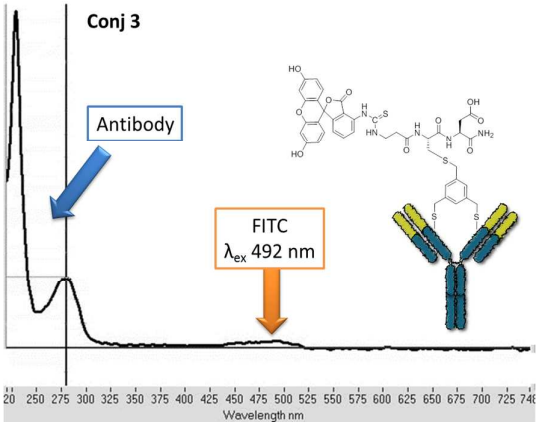


Figure 3. Emission spectrum (from 220 to 750 nm) for **Conjugate 3** (Compound **3g** conjugated to Anti-CD4 Ab).

The analysis by SDS-PAGE and mass spectrometry revealed that the integrity of the Ab was maintained during the bioconjugation protocol. The analysis by SDS-PAGE in non-reducing conditions (**SI**) showed that the antibodies were not completely reduced using 1 eq. of TCEP. Moreover, the appearance of a faint band of approximately 100 kDa in some conjugates indicated that some molecules conjugated to the mAb, linking its two heavy chains (HC) (50+50 kDa). Since not all the heavy chains were linked, more equivalents of dibromobenzyl derivatives were needed to achieve complete bioconjugation.

Prior to the mass spectrometry analysis, the *N*-glycosides from the Ab were removed by treating the conjugates with PNGase F[53] in PBS for 17 h to improve the mass spectra resolution.[54,55] This methodology was used to accurately determine the number of molecules attached to the Ab. The mass analysis showed that most of the conjugates maintained their

integrity after the conjugation step. The analysis of the molecules attached into the Abs was done by comparing the mass spectra of naked Abs with that of the respective conjugate **(**see **SI).**

Regarding anti-CD4 mAb and its conjugates, due to the complexity of the mass spectra, the analysis showed unreliable information about the number of molecules covalently attached to the immunoglobulin; however, a mass increment was observed for the deglycosylated conjugates (see **SI**). In contrast, the mass spectra for the anti-CD13 mAb presented four main peaks from approximately 144 to 148 kDa (see **SI**), thus simplifying the analysis of the conjugate. The clearest evidence of conjugation was shown by conjugates **3** and **4** for the anti-CD4 and -CD13 mAbs, respectively (see **SI**).

Antibody-conjugate cell binding analysis

To determine whether the conjugation of the linkers to the anti-CD4 HP2/6 and anti-CD13 TEA1/8 mAbs had any effect on the binding to their corresponding epitopes, an antibody binding analysis was performed using cell lines that constitutively express CD4 (Jurkat cell line) or CD13 (U937 cell line). For both cell lines, increasing concentrations (0.1–10 μg/ml) of the naked mAbs (anti-CD4 HP2/6 and anti-CD13 TEA1/8) and their corresponding conjugates (1-3 for anti-CD4 and 4-6 for anti-CD13) were used. As shown in **Figure 4A and B**, the naked Abs showed a higher mean fluorescence intensity (MFI) and binding affinity compared to those of the conjugates.

Regarding anti-CD4 mAb conjugates, **2a** and **2b** had similar binding affinities to the anti-CD4 mAb (AC_{50} 0.30 and 0.36 μg/ml compared to 0.38 μg/ml, respectively), and only a slightly lower maximum MFI value (71 and 89%, respectively) than that of the native anti-CD4 mAb. This result suggests that **conjugates 2a** and **2b** are fully functional and would coat most CD4 molecules on the cell surface. **Conjugates 1** and **3** had an AC_{50} of 0.49, 0.31 μg/ml, respectively, but they achieved a significantly smaller maximum MFI compared to that obtained with the naked anti-CD4 mAb. This result is consistent with diminished binding of the secondary FITC antibody, as a result of epitope masking caused by the linkers. Finally, the lack of binding of **conjugate 2c** could be attributed to the abrogation of Ab activity caused by the extensive reduction required for the synthesis of this molecule (**Figure 4A**).

On the other hand, all the anti-CD13 conjugates were functional, although they showed less binding affinity than the naked Ab but preserved the ability to coat all the CD13 molecules present on the cell surface (**Figure 4B**).

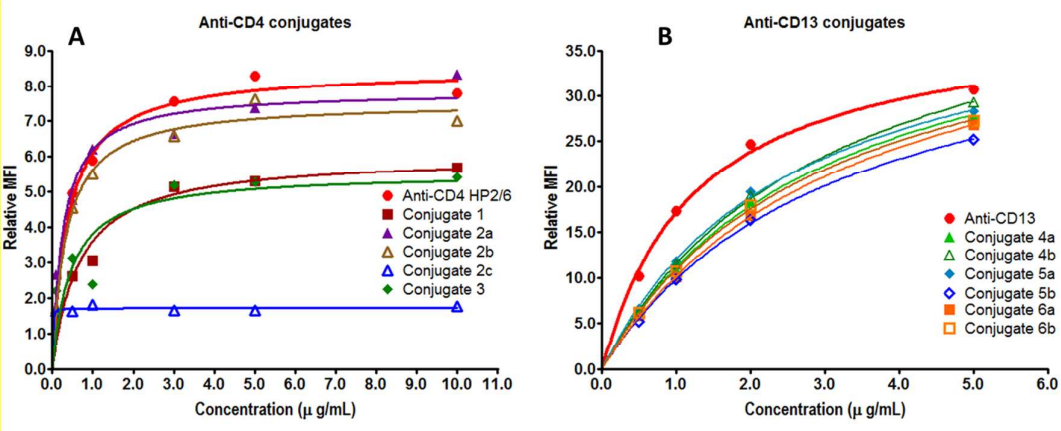
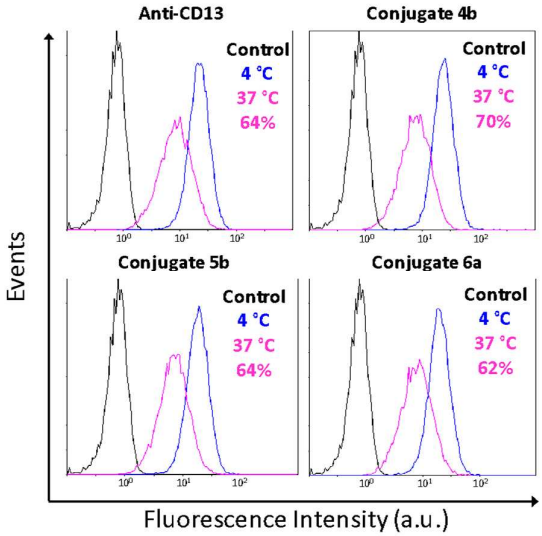


Figure 4. Cell binding analysis of antibody-conjugates by flow cytometry. **A**) The binding of naked anti-CD4 HP2/6 mAb and the anti-CD4 **conjugates** indicated to CD4-expressing Jurkat cells was assessed **B**) The binding of naked anti-CD13 TEA1/8 mAb and the anti-CD13 **conjugates** indicated to CD13-expressing U937 cells was assessed. After incubation with the indicated concentrations of mAb or the **conjugates 1-6**, cells were washed and incubated with FITC-labeled goat anti-mouse IgG antibodies, as described in Materials and Methods. Cytometry analysis was performed, and the mean fluorescence intensity (MFI) ratio was calculated using the MFI value or an irrelevant monoclonal antibody (basal MFI value) as reference.

Some proteins on the cell surface have the ability to internalize upon activation by their natural ligands or by agonistic Abs. The ability of some Abs to induce internalization inside the cell this way makes them suitable candidates for ADC design, since the endocytosis of the conjugates is essential to achieve the release of the drug into the target cell, thus resulting in higher therapeutic efficiency. We have evidence that the TEA1/8 anti-CD13 mAb induces efficient CD13 internalization (Perez-Chacon, Sanchez-Madrid and Zapata, unpublished results). Therefore, we examined the internalization efficiency of our anti-CD13 conjugates. The naked anti-CD13 mAb induced 64% internalization of the CD13 present on the cell surface (**Figure 5**). The percentage of endocytosis induced by the three anti-CD13 conjugates studied (70, 64 and 62% for **4b**, **5b** and **6a**, respectively) was very similar to that shown by the naked Ab. These results demonstrate that this type of bioconjugation does not affect the capacity of the Ab to internalize and is therefore suitable for the development of ADCs.



	Control	Anti-CD13		Conjugate 4b		Conjugate 5b		Conjugate 6a	
		4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC	4 ºC	37 ºC
MFI	0.72	21.2	8.18	23.0	7.41	17.6	6.77	18.9	7.54

$MFI_{control}$-MFI Ab	0	20.49	7.47	22.29	6.70	16.89	6.06	18.19	6.83
% Endocytosis	**0**	**64**		**70**		**64**		**62**	

Figure 5. Endocytosis of anti-CD13 and its conjugates in U937 cells. The average results are expressed using the MFI determined by flow cytometry. U937 cells were saturated with anti-CD13 and its conjugates under at 4 ºC and 37ºC, at 3 h the incubated cells were processed for analysis. Table shows the MFI for conjugates **4b**, **5b** and **6a** at 4ºC and 37ºC and the resulting percentage of internalization.

Next, we studied whether any of the linkers in our anti-CD13 conjugates impaired cell viability. For these experiments, U937 cells were incubated with increasing concentrations of the Ab or its conjugates (0.05–10 μg/mL) for 72 h at 37ºC. Cell viability was then measured by a luminescence assay. **Figure 6** shows the percentage of cell viability *vs.* the log [Ab] for **conjugates 4-6**. As a positive control, we used an ADC based on the anti-CD13 mAb (Dominguez and Cuevas, unpublished results).

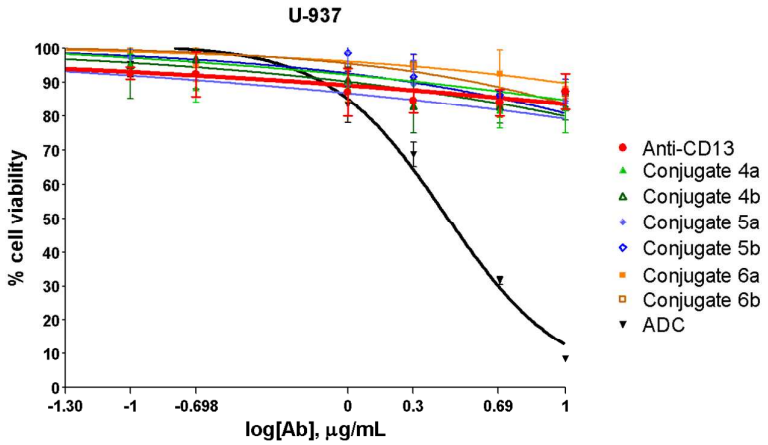


Figure 6. U-937 cell viability over 72 h.

As expected, the anti-CD13 conjugates did not have any cytotoxic effect on U937 cells while the anti-CD13-based ADC used as control efficiently killed these leukemic cells. This result indicates that neither the mesitylened antibodies nor any byproduct from them that might be produced by the target cell (i.e degradation products) had any significant effect of cell viability, thus supporting the use of this conjugation strategy not only for ADC generation but also for antibody conjugation of non-toxic bioactive molecules. In summary, this new conjugation method based on mesitylene thiol alkylation provides a new tool for antibody conjugation. In this regard, this approach preserves the structure and function of the Ab, thus making it a suitable technique for the development of ADCs.

Conclusions

Here we describe a bioconjugation strategy through thiols from partially reduced antibodies, using thioether-like mesitylene scaffolds (**3c,e,g** and **h**) as non-cleavable linkers. The strong nucleophilicity of the thiol group as the potential cargo was found to be the main drawback regarding the thiol monoalkylation of the TBMB core, as reflected by the observation of polyalkylation products in most of the conditions tested. In this regard, an exhaustive control of the reaction conditions is needed to avoid undesired thiol polyalkylation or final product degradation. Reaction time, temperature and solvent play a key role in the S_N2 of the thiol group into halobenzylic carbons. The successful conjugation of the previously prepared linker-cargo (**3c**, **3e** and **3g**) to the antibodies (Anti-CD4 or Anti-CD13) was carried

out at 4°C for 30 min using the biomolecules in their reduced form. The conjugates obtained were isolated using a PD-10 desalting column. The conjugates were characterized via UV, HPLC, SDS-PAGE and Mass spectrometry. Moreover, in order to determine whether these conjugates are biologically affected, antibody/conjugate-antigen binding affinity experiments were carried out. During the conjugation process, it was assumed that harsh reaction conditions, such as the complete reduction of the antibody, would directly affect its properties as immunoglobulin misplacement impairs binding affinity. However, most of the conjugates maintained their capacity to bind to CD4 (**Conjugates 2a,b**) or CD13 (**Conjugates 4-6**), showing behaviour similar to that of the the naked antibodies. This observation indicates that this kind of conjugation strategy does not disrupt antibody activity, mesitylene-like compounds being suitable linkers for thiol bioconjugation and more specifically for ADCs.

Materials and methods

Products and commercial sources. Commercial products and solvents were used as received without further purification. More information at SI.

Antibody production is detailed at the SI.

Solid-phase peptide synthesis (SPPS). Manual SPPS was performed on a 10-mL polypropylene syringe with a porous disc following standard protocols (See SI). The Automatic SPPS was carried out in a CEM Liberty Blue™ Microwave Peptide Synthesizer. The coupling reactions were performed with Fmoc-L-amino acids (0.2 M), *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) (0.5 M) and *N,N*-diisopropylethylamine (DIEA) (2 M) in DMF. First of all, the coupling reaction was heated at 75ºC (170 W) for 20 s and then at 90ºC (50W) for 3 min. The Fmoc group was removed by treatments with piperidine/*N,N*-dimethylformamide (DMF) (20:80, *v/v*) first for 30 s at 75ºC (155 W) and then for 90 s at 90ºC (30 W).

Analysis and characterization. Instrument technical details are described at the SI. *HPLC*: HPLC reversed-phase column Xbridge™ BEH130 C_{18} 3.5 μm (4.6×100 mm) (Waters). Acquity UPLC BEH C_{18} Column, 130 Å, 1.7 μm, 2.1 mm×100 mm) (Waters). For HPLC and UPLC, linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 1 mL/min over 8 min and 0.61 mL/min over 2 min respectively.

HPLC-ESMS: A HPLC-ESMS reversed-phase column SunFire™ C_{18} 3.5 μm (2.1×100 mm) (Waters).

ESI-MS (Electro spray ionization mass analysis): BioSuite pPhenyl 1000RPC 2.0×75 mm; 10 μm column (Waters). Micromass LCT-Premier mass spectrometer (Waters), equipped with an Acquity UPLC Binary Sol MGR chromatograph with an Acquity UPLC Autosampler MO (Waters). Linear gradients from 95:5 to 20:80 of H_2O/MeCN were run for 60 min using MeCN UPLC quality (+1% formic acid) into MilliQ water (+1% formic acid) at a flow rate of 100 μL/min.

Deglycosylation: Antibody deglycosylations were performed by treating the corresponding antibody-conjugate (1–20 μg) with PNGase F (1 μL, 500 000 U/mL) in PBS at pH = 7.4 and incubation at 37ºC for 17 h. After this time, the samples were dialyzed with a Slide-A-Lyzer™ MINI Dialysis Device, 10K MWCO over $NH_4(C_2H_3O_2)$ (50 mM) and then analyzed by mass spectrometry.

Flow cytometry analysis: Flow cytometry analysis was performed in a FACSCanto II cytometer, and data were analyzed with the FACSDiva software (both from BD Biosciences). For data acquisition, a 488 nm excitation laser was used, and 10 000 events were collected for each sample.

NMR spectra: Spectra were recorded on a Varian Mercury-400 (^{1}H 400 MHz, ^{13}C 100 MHz). ^{1}H is reported as follows: chemical shift (δ ppm), [integration, multiplicity (s = singlet,

d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J* in Hz)]. Data for ^{13}C NMR are reported in terms of chemical shift. NMR spectra were referenced by tetramethylsilane (TMS), and the signal for the deuterated solvent that was used in each particular case was taken as an internal reference.

Concentration: The concentration of antibody conjugates was determined using a NanoDrop ND-1000 (Thermo-Fischer) spectrophotometer, analyzing 1 μL of sample at λ = 280 nm.

Gel Electrophoresis: The conjugates were characterized on 4–20% or 10% polyacrylamide precast Mini-PROTEAN® TGX™ Gels (SDS−PAGE). Previously, the samples (5 μL, 1 mg/mL approx.) were treated with reducing loading buffer (5 μL) and heated at 95°C for 5 min, and after that the gel was run at 150 V. The reduced antibody analysis gives two distinct bands corresponding to light and heavy chains. PageRuler™ Unstained Protein Ladder from Bio-Rad (10-250 kDa) was used as standard.

Purification. The automatic purification was performed on CombiFlash® Rf TELEDYNE ISCO using a pre-packed Redisep® Rf Gold 50 g C_{18} column from Teledyne Technology Company. Solid samples were loaded into C_{18} silica and eluted into the column by linear gradients of MeCN (+0.036% TFA) in H_2O (+0.045% TFA), which were run at a flow rate of 40 mL/min. UV detection was at λ = 220 nm. *Conjugate purification*: The antibody conjugates were purified by dialysis when the solution contained more than 1 mL of buffer and by Shephadex™ columns when below this value. The solution of protein to purify was introduced into a Spectra/Por® Dialysis Membrane MWCO: 12-14,000 and dialyzed for 3 days over 2 L of the desired buffer in which we want the purified protein, in that time, the buffer was changed twice a day. The following pre-packed Shephadex™ G-25-containing columns from GE Healthcare were used for antibody purification and/or antibody buffer exchange: PD-10 Desalting Columns (1–2.5 mL of sample), PD MiniTrap™ (0.1–0.5 mL). For antibody conjugate purification, the convenient pre-packed Shephadex™ G-25 column was equilibrated with the indicated buffer or water. Then the conjugate crude (0.1–1 mL) was loaded into the column, and, by means of gravity, the sample was eluted through the column with the corresponding buffer (1–3.5 mL). The fractions were then collected into Eppendorf tubes (0.25–0.5 mL), and the concentrations of the fractions were determined by UV analysis using Nanodrop. When needed, the proteins were concentrated for 15 min at 5ºC and 13,000 rpm using a Vivaspin 500 with a 50 kDa MWCO from GE Healthcare Europe GmbH (Freiburg, Germany).

Thiol monoalkylation. The indicated amount (0.02–0.6 mmol) of the corresponding thiol (2a-g) was added to a solution of 1,3,5-tris(bromomethyl)benzene (1, 0.02–0.6 mmol) in the indicated solvent. Then DIEA (0.02–1.7 mmol) was added to the reaction, and the mixture was left to react until the starting thiol disappeared. See SI for all reaction conditions. The monoalkylated products 3c, 3e and 3g were automatically purified on a pre-packed Redisep Rf Gold C18 column, obtaining compounds 3c (45 mg, 63%), 3e (19.2 mg, 31%) and 3g (1.1 mg, 6%) respectively.

*Fmoc-Cys(DBMB)-OH (**3c**)*: UPLC (H_2O/MeCN from 50:50 to 0:100 over 2 min): t_R: 1.5 min. m/z calculated for $C_{27}H_{25}Br_2NO_4S$ = 619.3; found = 619.9 $[M+H]^+$. ^1H NMR (400 MHz, Acetone-d_6) δ 7.94 – 7.81 (m, 2H), 7.79 – 7.69 (m, 2H), 7.51 (s, 1H), 7.46 – 7.36 (m, 4H), 7.36 – 7.26 (m, 2H), 6.85 (t, *J* = 8.0 Hz, 1H), 4.66 (s, 2H), 4.61 (s, 2H), 4.55 – 4.48 (m, 1H), 4.45 – 4.31 (m, 2H), 4.30 – 4.23 (m, 1H), 3.85 (d, *J* = 15.4 Hz, 2H), 3.05 – 2.97 (m, 1H), 2.90 – 2.83 (m, 1H).

*7-methoxycumarin-Cys-(DBMB)-NH_2 (**3e**)*: UPLC (H_2O/MeCN from 70:30 to 0:100 over 2 min): t_R: 1.6 min. m/z calculated for $C_{23}H_{22}Br_2N_2O_5S$ = 598.3; found = 598.9 $[M+H]^+$.

*FITC-βAla-Cys(DBMB)-Asp-NH_2 (**3g**)*: UPLC (H_2O/MeCN from 80:20 to 0:100 over 2 min): t_R: 1.3 min. m/z calculated for $C_{40}H_{37}Br_2N_5O_{10}S_2$ = 971.69; found = 972.3 corresponding to $[M+H]^+$.

Oxytocin conjugation. Fmoc-Cys(DBMB)-OH in H_2O/MeCN was added to a solution of reduced Oxytocin prepared by automatic SPPS at the indicated concentration in the indicated buffer at pH = 8. The reactions were completed at 1 h, as determined by HPLC analysis (see SI). HPLC (H_2O/MeCN from 95:5 to 0:100 over 8 min): *Oxytocin* (**4**): t_R =3.9 min, *m/z* calculated for $C_{43}H_{68}N_{12}O_{12}S_2$ = 1009.21; found = 1010.4 corresponding to $[M+H]^+$. *Fmoc-Cys(Mesitylene-Oxytocin)-OH conjugate (**5**)*: t_R: 5.59 min. *m/z* calculated for $C_{70}H_{91}N_{13}O_{16}S_3$ = 1466.75; found = 1468.0 corresponding to $[M+H]^+$.

Test1: Oxytocin (0.17 mg/mL) in $NaHCO_3$ buffer (120 µL), Fmoc-Cys(DBMB)-OH (20 µL of 1 mg/mL solution).

Test2: Oxytocin (2 mg/mL) in Borate buffer (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) (5 mL), Fmoc-Cys(DBMB)-OH in H_2O/MeCN (1 mL of 6.1 mg/mL solution). The conjugated compound was found stable after 72 h in borate buffer.

Antibody conjugation. *General procedure:* A solution of anti-CD4 or anti-CD13 antibody in borate buffer at pH = 8 (25 mM H_3BO_3, 25 mM NaOH, 25 mM NaCl and 1 mM EDTA) was treated with TCEP at 37ºC for 2 h. The reduced antibody was then treated with a solution of the dibromobenzyl derivative solved in DMSO. The mixture was left to react for 30 min at 4ºC. The crude was then purified using PD Minitrap G-25 column and PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) as eluent for antibody storage. The samples collected (approximately 200 µL) were analyzed by UV nanodrop in order to determine their concentration. The samples were analyzed by SDS-PAGE in reducing and non-reducing conditions and by mass spectrometry. For mass analysis, an aliquot of the antibody solution in PBS (18 µL) was treated with PNGase F 500 000 U/mL (1 µL) at 37ºC for 17 h in order to perform conjugate deglycosylation in order to improve the mass analysis spectra.

Anti-CD4 ESI-MS: 149 947, 152 755 (100%) and 155 135. Deglycosylated anti-CD4: 147 142, 148 328 and 149 725 (100%).

*α,α'-Dibromo-m-xylene (**3h**) conjugated to anti-CD4* (**Conjugate 1**): After reduction of 0.5 mL of anti-CD4 (1.6 mg/mL, 10.7 µM) with TCEP (1.5 µg, 5.3 nmol, 10.6 µM). The reduced antibody was treated with 5.0 µL of 1,3-dibromobenzene (0.4 mg, 1.5 µmol, 1.5 mM) solution in DMSO for 30 min at 4ºC. After purification, five samples of approximately 150 µL comprising between 0.1 and 1.0 mg/mL were collected. Deglycosylated Conjugate 1 ESI-MS: 148 451, 149 735, 150 625, 151 251(100%), 152 572.

*Fmoc-Cys(DBMB)-OH (**3c**) conjugated to anti-CD4:*

Conjugate 2a: 0.5 mL of anti-CD4 (0.88 mg/mL, 5.9 µM) was reduced with TCEP (1.7 µg, 5.9 nmol, 5.9 µM). The reduced antibody was treated with 4.0 µL of Fmoc-Cys(DBMB)-OH (**3c**) (1.0 mg, 1.6 µmol, 1.6 mM) solution in DMSO for 30 min at 4ºC. After purification, six samples of approximately 150 µL comprising between 0.1 and 1.1 mg/mL were collected. Deglycosylated Conjugate 2a ESI-MS: 146 800, 149 103 (100%), 149 714, 151 282, 156 586.

Conjugate 2b: 0.5 mL of anti-CD4 (0.7 mg/mL, 4.7 µM) was reduced with TCEP (4.3 µg, 0.015 µmol, 0.015 mM) in borate buffer. Then, 0.5 mL of the reduced antibody (0.67 mg/mL, 4.5 µM) was treated with 2 µL solution of Fmoc-Cys(DBMB)-OH (4 µg, 6.5 nmol, 6.5 µM) solution in DMSO. After purification, four samples of approximately 200 µL comprising between 0.2 and 0.6 mg/mL were collected. Deglycosylated Conjugate 2b ESI-MS: 146 604, 149 693, 151 159, 152 633(100%), 154 230, 155 662, 157 315.

Conjugate 2c: 0.4 mL of anti-CD4 (0.69 mg/mL, 4.6 µM) was reduced with TCEP (2.8 mg, 9.8 µmol, 10 mM) in borate buffer. The reduced antibody was then treated with 1 µL of a solution of Fmoc-Cys(DBMB)-OH (52 mg, 84 µmol, 84 mM) solution in DMSO. After the purification, six samples of approximately 150 µL comprising between 0.1 and 0.5 mg/mL were collected. Deglycosylated Conjugate 2c ESI-MS: 148 425, 149 772, 151 153(100%), 152 647, 154 160.

FITC-β-Ala-Cys(BBMB)-Asp-NH_2 (3g) conjugated to anti-CD4 (**Conjugate 3**)*:* 0.5 mL of anti-CD4 antibody (1.5 mg/mL, 9.7 µM) was reduced with TCEP (1.4 µg, 4.9 nmol, 9.7 µM)

in borate buffer. The reduced antibody was then treated with 10 µL of a solution of FITC-bAla-Cys-Asp-NH_2 (1.2 mg, 1.2 µmol, 1.0 mM) solution in DMSO. After the purification, six samples of approximately 100 µL comprising between 0.2 and 1.0 mg/mL were collected. Deglycosylated Conjugate 3 ESI-MS: 146 786(100%), 149 100, 149 672, 150 605(98%), 151 239, 152 070.

Deglycosylated anti-CD13 ESI-MS: 145 552, 146 997, 147 159(100%), 148 604, 148 776.

*α,α′-Dibromo-m-xylene (**3h**) conjugated to anti-CD13*

Conjugate 4a: 0.5 mL of anti-CD13 (0.84 mg/mL, 5.6 µM) was reduced with TCEP (0.96 µg, 3.4 nmol, 6.7 µM) in borate buffer. Then, 0.25 mL of the reduced antibody was treated with 0.8 µL of a solution of α,α′-dibromo-*m*-xylene (**3h**) (1.1 mg, 4.1 µmol, 3.8 mM) solution in DMSO. After the purification, three samples of approximately 250 µL comprising between 0.10 and 0.34 mg/mL were collected. Deglycosylated Conjugate 4a ESI-MS: 145 552, 145 659, 146 997, 147 160(100%), 147 262, 148 604, 148 764.

*α,α′-Dibromo-m-xylene (**3h**) conjugated to anti-CD13*

Conjugate 4b: 0.25 mL of the reduced antibody (0.84 mg/mL) was treated with 1.6 µL of a solution of α,α′-dibromo-*m*-xylene (**3h**) (1.1 mg, 4.1 µmol, 3.8 mM) solution in DMSO. After the purification, three samples of approximately 250 µL comprising between 0.10 and 0.39 mg/mL were collected. Deglycosylated Conjugate 4b ESI-MS: 145 552, 145 657, 146 996, 147 099, 147 159(100%), 147 207, 147 260, 147 315, 148 600, 148 764.

*Fmoc-Cys(DBMB)-OH (**3c**) conjugated to anti-CD13*

Conjugate 5a: 0.5 mL of anti-CD13 (0.90 mg/mL, 6.0 µM) was reduced with TCEP (1.0 µg, 3.6 nmol, 7.2 µM) in borate buffer. Then, 0.25 mL of the reduced antibody (0.9 mg/mL) was treated with 1.8 µL of a solution of Fmoc-Cys(DBMB)-OH (1.6 mg, 2.6 µmol, 1.6 mM) solution in DMSO. After the purification, three samples of approximately 250 µL comprising between 0.10 and 0.34 mg/mL were collected. Deglycosylated Conjugate 5a ESI-MS: 145 552(100%), 146 996, 147 159, 148 604, 148 765.

Conjugate 5b: 0.25 mL of the previous reduced antibody (0.9 mg/mL) was treated with 3.7 µL of a solution of Fmoc-Cys(DBMB)-OH (1.6 mg, 2.6 µmol, 1.6 mM) solution in DMSO. After purification, two samples of approximately 250 µL (0.17 and 0.32 mg/mL) were collected. Deglycosylated Conjugate 5b ESI-MS: 145 561(100%), 145 992, 147 007, 147 166, 147 239, 147 329, 148 611, 148 788.

*7-MeO-cumarin-Cys(DBMB)-NH_2 (**3e**) conjugated to anti-CD13:*

Conjugate 6a: 0.5 mL of anti-CD13 (0.98 mg/mL, 6.5 µM) was reduced with TCEP (1.1 µg, 4.0 nmol, 7.8 µM) in borate buffer. Then, 0.25 mL of the reduced antibody was treated with 4.0 µL of a solution of 7-MeO-cumarin-Cys(DBMB)-NH_2 (0.8 mg, 1.3 µmol, 1.6 mM) solution in DMSO. After the purification, three samples of approximately 250 µL comprising between 0.06 and 0.36 mg/mL were collected. Conjugate 6a ESI-MS: 145 550(100%), 146 995, 147 156, 147 216, 148 601, 148 763.

Conjugate 6b: 0.25 mL of the previously reduced antibody (0.98 mg/mL) was treated with 4.0 µL of a solution of 7-MeO-cumarin-Cys(DBMB)-NH_2 (0.4 mg, 0.67 µmol, 1.7 mM) in DMSO (0.4 mL). After the purification, two samples of approximately 250 µL (0.13 and 0.22 mg/mL) were collected. Conjugate 6b ESI-MS: 145 554(100%), 146 999, 147 162.

Binding affinity experiments. Jurkat (5 x 10^5) and U937 (5 x 10^5) cells were first incubated with 50 μg/ml of γ-globulin for 10 min and then with the anti-CD4 HP2/6 and anti-CD13 TEA1/8 monoclonal antibodies (mAbs), respectively, or their corresponding conjugates, at the indicated concentrations for 1 h on ice. Cells were then washed with PBS twice and incubated with 10 μg/ml of FITC-labeled goat anti-mouse IgG antibodies (BD Biosciences) for 30 min on ice. Finally, they were washed twice and resuspended in PBS for their analysis by flow cytometry using a FACSCanto II cytometer.

Internalization experiments. For these analyses, the maximum levels of expression of CD13 on the surface of U937 (5 x 10^5) cells detected by the naked anti-CD13 mAb or the anti-CD13 conjugates (4b, 5b and 6a) was determined by incubating cells with saturating concentrations of mAb (10 μg/ml) at 4ºC for 3 h, followed by incubation with FITC-labeled goat anti-mouse antibody (t=0 h). To determine endocytosis, the anti-CD13 mAb and the anti-CD13 conjugates were incubated at 4ºC for 30 min as before, to allow binding of the antibodies to the CD13 on the cell surface, and then incubated at 37ºC for 3 h to allow internalization to proceed (t=3h). The CD13/anti-CD13 conjugate complexes remaining on the cell surface were then stained by incubation with FITC-labeled goat anti-mouse antibody. Next, the cells were washed twice, resuspended in PBS and analyzed by flow cytometry using a FACSCanto II cytometer.

Cell viability experiments. U937 (2 x 10^4) cells were incubated in triplicate wells without any further addition (control), with the naked anti-CD13 mAb, with the different anti-CD13 conjugates, or with a cytotoxic anti-CD13-based ADC provided by PharmaMar, at the indicated concentrations (0.05–10 μg/mL) for 72 h at 37ºC. Cell viability was assessed by a luminescence assay (CellTiter-Glo®, Promega Biotech, Madrid, Spain). Analysis was performed considering the control condition as 100% of viability.

ASSOCIATED CONTENT

Supporting information

Experimental procedures and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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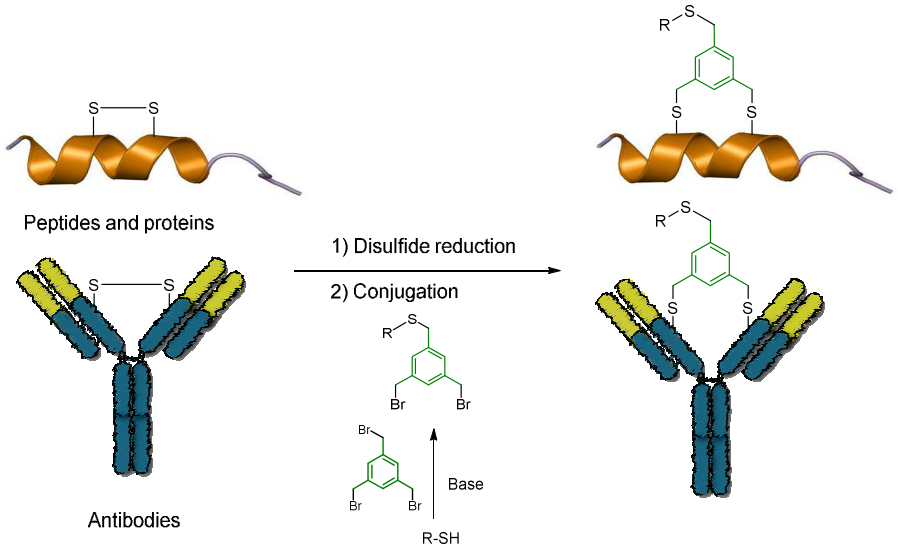
Peptides and proteins
Antibodies
1) Disulfide reduction
2) Conjugation
Base
R-SH