Immunogenic Stapled Proteins

Oxetane Grafts Installed Site-Selectively on Native Disulfides to Enhance Protein Stability and Activity In Vivo

Nuria Martínez-Sáez[+], Shuang Sun[+], Davide Oldrini, Pietro Sormanni, Omar Boutureira, Filippo Carboni, Ismael Compañón, Michael J. Deery, Michele Vendruscolo, Francisco Corzana, Roberto Adamo, and Gonçalo J. L. Bernardes

Abstract: A four-membered oxygen ring (oxetane) can be readily grafted into native peptides and proteins through site-selective bis-alkylation of cysteine residues present as disulfides under mild and biocompatible conditions. The selective installation of the oxetane graft enhances stability and activity, as demonstrated for a range of biologically relevant cyclic peptides, including somatostatin, proteins, and antibodies, such as a Fab arm of the antibody Herceptin and a designed antibody DesAb-Aβ against the human Amyloid-β peptide. Oxetane grafting of the genetically detoxified diphtheria toxin CRM$_{197}$ improves significantly the immunogenicity of this protein in mice, which illustrates the general utility of this strategy to modulate the stability and biological activity of therapeutic proteins containing disulfides in their structures.

The rational modification of the structure of peptides and proteins offers a wide range of opportunities for the modulation of their biological activity.[1] Many efforts have been made to develop strategies that induce such conforma-

tional changes and modulation. Towards this end, macro-cyclization and stapling have emerged as useful tactics to chemically manipulate peptides and proteins, increasing their proteolytic stability, cell permeability, and producing changes in polarity, binding activity, and pharmacokinetic properties.[2] During the last years, different approaches have been developed for the covalent tethering of the side chains of natural or non-canonical amino acids.[2] Considering natural residues, cysteine (Cys) has been the residue of choice for stapling through alkylation,[3] arylation,[4] cycloaddition,[4b] and disulfide forming reactions both at native[5] or engineered[6] Cys residues. More recently, nitrogen arylation has also been shown to be a useful strategy for macrocyclization of lysine residues on peptides.[7] Otherwise, efficient macro-cyclization of linear peptides through the formation of an oxadiazole has also been reported.[8] However, a large number of stapling/macrocyclization/re-bridging strategies consist of the introduction of non-canonical amino acids and their subsequent ligation by ring-closing metathesis,[9] lactam-ization,[10] or cycloaddition reactions.[2c, 4b] Common to many of these strategies is either the requirement for complicated orthogonal protection procedures, sequence engineering, the appendage of bulky/constrained linkers between the two residues, or the use of organic solvents. These conditions have limited, for instance, the application of such methods for the stapling of residues on intact, full-length proteins to impart structural conformational constraints leading to enhanced stability and activity. Thus, there remains a need for simple and robust strategies for stapling native peptides and proteins.

Herein we report a method for site-selective peptide and protein stapling through the bis-alkylation of the sulfhydryl side chain of Cys residues resulting from disulfide reduction, using commercially available 3,3-bis(bromomethyl)oxetane **1** (Figure 1). Oxetanes have become common motifs in drug design due to their ability to modulate parameters including solubility, basicity, lipophilicity, and metabolic stability.[11] While there are examples of the modification of small peptides with oxetanes,[12] their incorporation and modulation of the structure and activity of complex biomolecules[13] remain mostly unexplored.
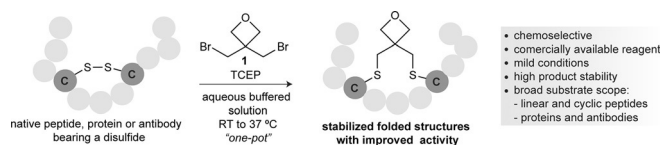
[*] Dr. N. Martínez-Sáez,[+] S. Sun,[+] Dr. P. Sormanni, Dr. O. Boutureira, Prof. M. Vendruscolo, Dr. G. J. L. Bernardes
Department of Chemistry, University of Cambridge
Lensfield Road, CB2 1EW Cambridge (UK)
E-mail: gb453@cam.ac.uk

Dr. G. J. L. Bernardes
Instituto de Medicina Molecular, Faculdade de Medicina
Universidade de Lisboa
Avenida Professor Egas Moniz, 1649-028 Lisboa (Portugal)
E-mail: gbernardes@medicina.ulisboa.pt

D. Oldrini, F. Carboni, Dr. R. Adamo
GSK Vaccines
Via Fiorentina 1, 53100 Siena (Italy)

I. Compañón, Dr. F. Corzana
Departamento de Química, Centro de Investigación en Síntesis
Química, Universidad de La Rioja
26006 Logroño (Spain)

M. J. Deery
Cambridge Centre for Proteomics, Cambridge Systems Biology
Centre, Department of Biochemistry, University of Cambridge
Tennis Court Road, Cambridge CB2 1QR (UK)

[+] These authors contributed equally to this work.

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
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Figure 1. Stabilization of folded structures of peptides and proteins through bis-alkylation of Cys residues present in the form of a native disulfide using an oxetane graft.

A requirement for a direct method to graft Cys residues present as disulfides on proteins is the compatibility of the reagent with a reducing agent, such as tris(2-carboxyethyl)-phosphine (TCEP). Importantly, we found that a model pentapeptide bearing two Cys residues reacted with **1** in the presence of TCEP to afford the corresponding stapled cyclic peptide in 75% yield (see the Supporting Information, Figures S2–S5, S34–S39, 48–49 for characterization and discussion of structural features). We then explored the use of **1** to graft two cyclic and biologically relevant peptides: somatostatin **2** and its analogue octreotide **4**, which can be used for imaging and treating neuroendocrine tumors.[14] Peptides **2** and **4** were reacted simultaneously with TCEP and oxetane **1** in a 1:9 mixture of DMF/H$_2$O at 25°C for 12 h. After HPLC purification, the grafted cyclic peptides **3** and **5** were obtained in 76% and 81% yield, respectively (Figure 2a). The affinity of these derivatives to the natural
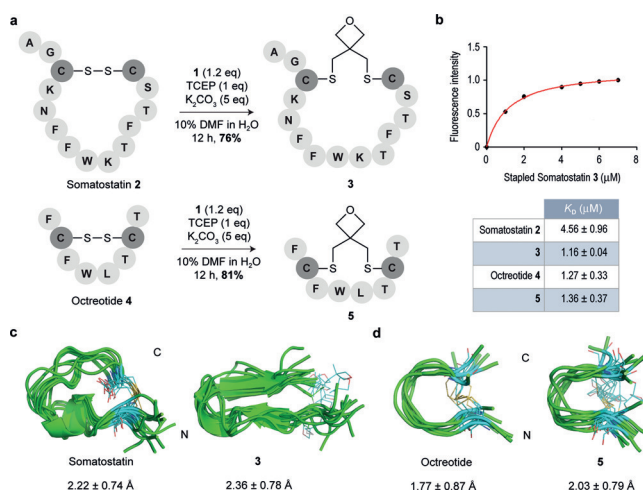

Figure 2. a) Stapling of disulfide-containing cyclic peptides **2** and **4**. b) Binding affinity studies. K_D values were determined by tryptophan fluorescence spectroscopy. c),d) Structural ensembles obtained by 0.5 μs MD simulations. The peptide backbone is in green. Carbon atoms of Cys residues as well as of the oxetane moiety are in cyan. The numbers indicate the root-mean-square deviation (RMSD) for heavy-atom superimposition of the backbone with respect to the average structure.

somatostatin receptor 2 (SSTR2) was experimentally determined by tryptophan fluorescence spectroscopy (Supporting Information, Figure S1). While octreotide **4** and surrogate peptide **5** showed a similar affinity against this receptor, grafted somatostatin **3** displayed improved binding properties, showing a 4-fold enhancement in K_D value (Figure 2b). The improvement of binding activity is a considerable advantage of the incorporation of the oxetane graft when compared, for instance, with the recently reported methylene thioacetal that led to a decrease in binding affinity to SSTR2.[3b] Interestingly, 0.5 μs MD simulations performed on these derivatives in explicit water and using ff14SB amber force-field[15] suggested that octreotide **4** and its stapled derivative **5** presented a similar conformational behavior in solution (Figure 2d; Supporting Information, Figure S8),[16]
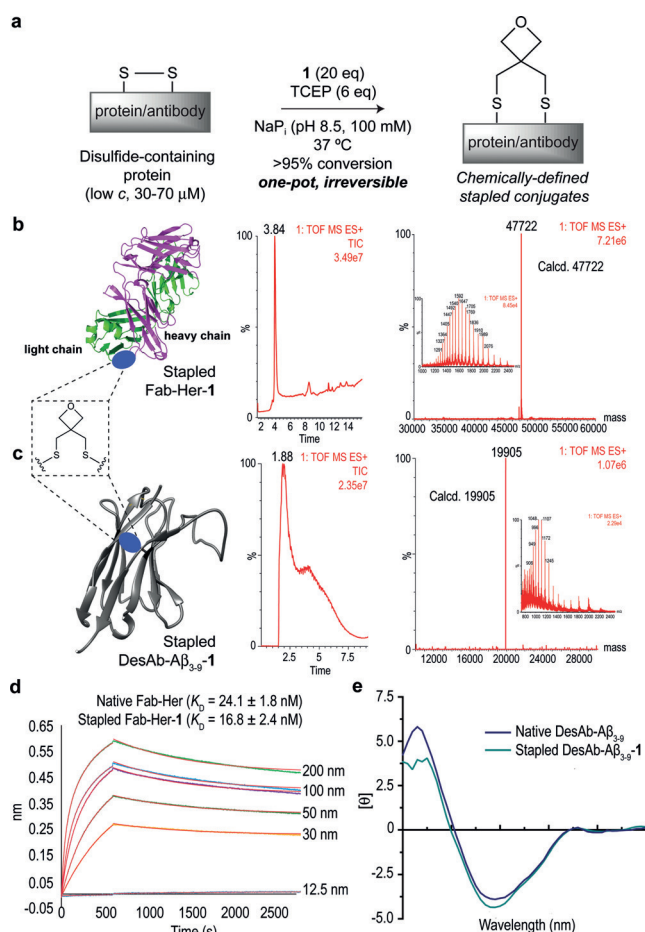
displaying an equilibrium between antiparallel β-sheet structures and conformations in which the C-terminal residues form a 3$_{10}$ helix-like fold, as reported in DMSO solution. In contrast, stapled somatostatin **3** was more rigid and displayed a more defined conformation in solution than **2** (Figure 2c; Supporting Information, Figures S6 and S7).[17] In fact, **3** showed a closely related β-sheet arrangement in solution stabilized by a typical hydrogen bond network, which is apparently ideal for a more efficient binding to the receptor. Finally, analysis of the stability of **3** and **5** both in human plasma as well in the presence glutathione (GSH) showed that the oxetane grafted peptides remain intact under these conditions (Supporting Information, Figures S13–S18).

To initially test the potential of using this one-pot, site-selective bis-alkylation oxetane stapling method directly on proteins, we chose thioredoxin (Trx) as a model protein that features a naturally occurring, solvent-exposed disulfide bond. We could reduce and staple the disulfide bond in a straightforward manner through selective bis-alkylation with **1** in the presence of TCEP and 10% DMF in sodium phosphate buffer at pH 8.5. Complete conversion was achieved after 24 h at 37°C, as confirmed by HPLC-MS analysis (Supporting Information, Figures S19 and S20). Furthermore, analysis of the CD spectra of the native and stapled Trx-**1** (Supporting Information, Figure S22) indicated that both molecules present very similar conformational preferences in solution. Although this is supported by MD simulations performed on both proteins in explicit water, the calculations indicate a small increase in flexibility for the peptide backbone of stapled Trx-**1** (Supporting Information, Figure S11). This result may be explain attending to the greater S−S distance in Thrx-**1** when compared to the native Trx (4.18 and 2.04 Å, respectively). Finally, we confirmed the suppression of Trx redox activity[18] through the selective and covalent disulfide stapling (Supporting Information, Figure S21).

Next, we demonstrated the utility of the oxetane graft to build stapled antibodies. First, the exposed disulfide bond tethering the heavy and light chains of a Fab fragment of Herceptin (Fab-Her), an antibody currently used to treat Her2+ breast cancer patients,[19] was readily stapled using **1** under aqueous buffered conditions in the presence of TCEP at pH 8.5 and at 37°C (Figures 3 a,b; Supporting Information, Figures S23 and S24). The oxetane stapled Fab-Her-**1**, unlike the disulfide native antibody, was stable under reducing conditions and in human plasma (Supporting Information, Figures S25 and S26). This stability is a key aspect of antibody therapeutics design as thiol-exchange reactions in plasma lowers efficacy and adds side-toxicity.[20] Importantly, a relatively small but significant increase in binding affinity to the Her2 receptor, as determined by bio-layer interferometry (BLI) experiments (Figure 3 d; Supporting Information, Figure S27), was observed for Fab-Her-**1** when compared with the native antibody. Next, we extended our stapling strategy to the antibody DesAb-Aβ$_{3-9}$, which was designed to target the region 3–9 of human Amyloid-β (Aβ42) peptide, the aggregation of which is a hallmark of Alzheimer's disease.[21] This antibody features a challenging, hindered intra-domain disulfide typical of VH domains. Of note, complete conversion into the oxetane grafted antibody DesAb-Aβ$_{3-9}$-**1** was

Figure 3. a) Representation of disulfide stapling of native antibody sequences using an oxetane graft. b),c) Total ion chromatogram, combined ion series and deconvoluted mass spectrum reconstructed from the ion series using the MaxEnt algorithm. b) Fab-Her-**1** (pdb ID Fab-Her: 1N8Z) and c) DesAb-Aβ$_{3-9}$-**1** (3D model of DesAb-Aβ$_{3-9}$ generated using ABodyBuilder, see main text). d) BLI and fit curves obtained for Fab-Her-**1**, together with the derived K_D constants for Fab-Her and Fab-Her-**1**. e) CD spectra of DesAb-Aβ$_{3-9}$ and DesAb-Aβ$_{3-9}$-**1**.

achieved using our method (Figure 3c; Supporting Information, Figures S28 and S29). Owing to the fact that the disulfide is deeply buried, an excess of TCEP (40 equiv) and longer reaction times were required (Supporting Information). Unlike the reduced antibody that readily reacts with thiol-specific Elman's reagent, the stapled DesAb-Aβ$_{3-9}$-**1** did not react suggesting complete consumption of the reduced Cys during stapling (Supporting Information). Finally, analysis of secondary structural content by CD showed no significant differences between the original and stapled antibodies (Figure 3e). MD simulations performed on a 3D model of DesAb-Aβ$_{3-9}$, previously generated using ABodyBuilder,[22] and on the stapled derivative DesAb-Aβ$_{3-9}$-**1**, suggest that, although the 3D structure is maintained upon the chemical modification, the oxetane motif provokes a slightly increase in the degree of flexibility (Supporting Information, Figure S31). Collectively, these data demonstrate the suitability of the oxetane motif to staple solvent accessible disulfide bonds on proteins with minimal secondary structure alterations.

To demonstrate the practical application of our method to therapeutic proteins, we investigated the effects of the selective introduction of the oxetane staple into the genetically detoxified diphtheria toxin CRM$_{197}$, which features four Cys residues in the form of two disulfides. Recently, it has been shown that antibodies against CRM$_{197}$ neutralized diphtheria toxin in HIV infected young individuals.[23] Furthermore, CRM$_{197}$ is a clinically used carrier in many glycoconjugate vaccines.[24] Previous structural studies showed that only the disulfide C186-C201 connecting the fragment A (C domain) and B (T/R domain) of CRM$_{197}$ is selectively reduced in the presence of the highly hindered C461–C471 disulfide upon treatment with dithiothreitol.[24b] Addition of a slight excess of TCEP to CRM$_{197}$ under aqueous buffered conditions at pH 8.5 and 37 °C, followed by an excess of **1**, led to the introduction of one oxetane graft (Figure 4 a,b and the Supporting Information, Figure S32 for mass spectrometry analysis), presumably at C186–C201 according to our previous findings.[25] The impact of the installation of the oxetane moiety into CRM$_{197}$ on its structure and thermal stability was studied by CD and differential scanning calorimetry (DSC) analysis, respectively, and compared with the native protein (Figure 4 c–e). We found that both the far and near UV CD spectra of CRM$_{197}$-**1** were nearly identical to those of CRM$_{197}$, which indicates that the 3D structure is preserved upon the chemical stapling. The DSC curves also corroborate this finding. Although CRM$_{197}$-**1** exhibited a broader DSC peak when compared to the sharp change in
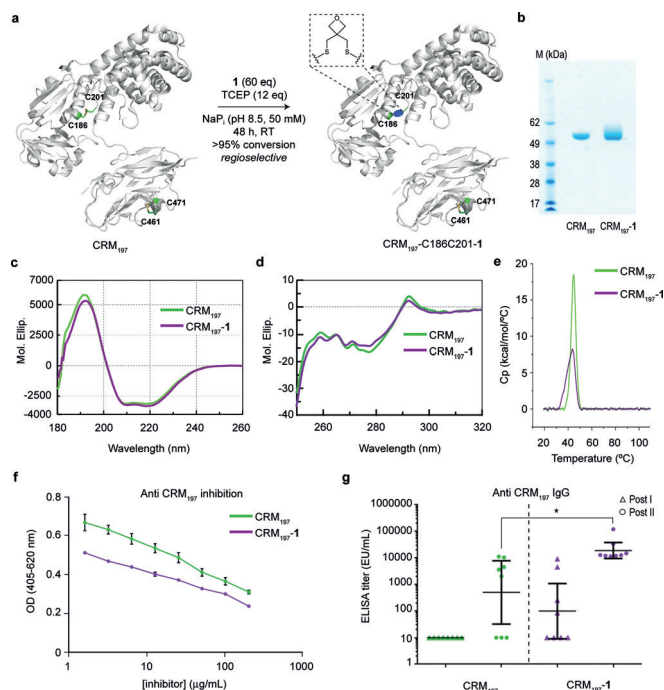

Figure 4. Enhancing the immunogenicity of a protein carrier through disulfide oxetane stapling. a) The functional stapling of CRM$_{197}$ (pdb ID CRM$_{197}$: 4AE0) with **1**. b) SDS-page of native and stapled CRM$_{197}$-**1**. c) Far UV CD spectrum. d) Near UV CD spectrum. e) DCS analysis. f) Competition of anti-CRM$_{197}$ serum binding to the protein with CRM$_{197}$ and its stapled form as inhibitors. g) Anti-CRM$_{197}$ IgG levels of CRM$_{197}$ and CRM$_{197}$-**1** after first and second boost immunizations in mice, 2 weeks apart.

heat capacity, in the range of 40–55°C for CRM$_{197}$, both proteins present an identical transition midpoint (T_m) of 46°C.

To evaluate the biological effects resulting from the introduction of the oxetane staple into CRM$_{197}$, we first assayed the capacity of competing with the binding of anti-CRM$_{197}$ serum to the proteins. We found that the stapled CRM$_{197}$-**1** induces an inhibition that was slightly lower compared to the unmodified protein (Figure 4f). In contrast, a much better inhibition of the binding to a commercial anti-diphtheria toxoid human recombinant monoclonal antibody was observed for CRM$_{197}$-**1** compared to the unmodified protein (Supporting Information, Figure S33a). To ascertain that protein epitopes were not impaired by the chemical modification of the disulfide bond, groups of 8 BALB/c mice were immunized with both unmodified CRM$_{197}$ and the stapled CRM$_{197}$-**1** (Figure 4g). Remarkably, these in vivo experiments demonstrated that CRM$_{197}$-**1** induced a statistically significant higher level of anti-protein antibodies respect to the unmodified protein. The antibodies generated by CRM$_{197}$-**1** had a threefold higher avidity for the protein antigen compared to the anti-CRM$_{197}$ serum (avidity index = 0.8 ± 0.4 M for CRM$_{197}$-**1** vs. 0.3 ± 0.1 M for CRM$_{197}$), as determined by ELISA using thiocyanate elution (Supporting Information, Figure S33b). These data, together with 200 ns MD simulations performed on both proteins (Supporting Information, Figure S12), suggest that the oxetane bridging of the disulfide bond does not cause relevant structural modifications on the protein but results in improved immunogenic activity in vivo, most likely through chemical stabilization of the antigen against proteases and/or other degradation factors.

In summary, we have presented an efficient method for oxetane stapling of Cys residues present as native disulfides on peptides and proteins under mild and biocompatible aqueous conditions. The four-membered oxetane ring has an ideal distance to enable direct stapling of native disulfides on several protein scaffolds, including antibodies. This approach is however dependent on solvent accessibility of the disulfide within the protein of interest. Furthermore, and unlike current protocols, this method does not require prior sequence engineering neither purification after the disulfide reduction step. The selective installation of the oxetane motif enables stabilization of folded structures and results in disulfide-grafted products with enhanced bioactivity that are stable under biological conditions. We demonstrate the value of oxetane graft installation on protein through the regioselective disulfide stapling of the protein carrier CRM$_{197}$ that showed a significant increase in its immunogenicity in vivo. Because many therapeutic proteins feature Cys residues in the form of disulfide bonds, we anticipate that their direct modulation through oxetane grafting can, in principle, be used as a general strategy to enhance their in vivo stability and to fine-tune their structure for optimal pharmacokinetics and activity.

Conflict of interest

N.M.S., S.S., O.B., F.Corzana, and G.J.L.B. are listed as inventors on a pending patent application related to the technology described in this work. F.Carboni, D.O., and R.A. are employees of GSK companies.

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