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Trisulfide Modification Impacts the Reduction Step in Antibody–Drug Conjugation Process

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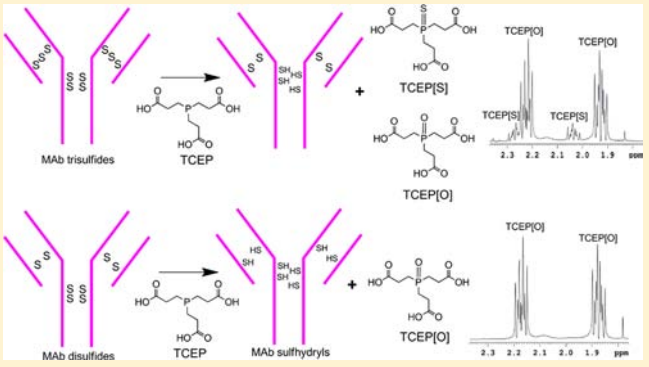
ABSTRACT: Antibody–drug conjugates (ADCs) utilizing cysteine-directed linker chemistry have cytotoxic drugs covalently bound to native heavy–heavy and heavy–light interchain disulfide bonds. The manufacture of these ADCs involves a reduction step followed by a conjugation step. When tris(2-carboxyethyl)phosphine (TCEP) is used as the reductant, the reaction stoichiometry predicts that for each molecule of TCEP added, one interchain disulfide should be reduced, generating two free thiols for drug linkage. In practice, the amount of TCEP required to achieve the desired drug-to-antibody ratio often exceeds the predicted, and is variable for different lots of monoclonal antibody starting material. We have identified the cause of this variability to be inconsistent levels of interchain trisulfide bonds in the monoclonal antibody. We propose that TCEP reacts with each trisulfide bond to form a thiophosphine and a disulfide bond, yielding no net antibody free thiols for conjugation. Antibodies with higher levels of trisulfide bonds require a greater TCEP:antibody molar ratio to achieve the targeted drug-to-antibody ratio.



INTRODUCTION

Antibody–drug conjugates (ADCs) are monoclonal antibodies (mAbs) coupled to potent cytotoxic molecules. These molecules combine the biological specificity of antibodies with the high potency of chemotherapeutic compounds. The result of conjugation is a more targeted and efficacious single-agent therapeutic with lower systemic toxicity.[1,2] Several ADC molecules are currently in clinical development for indications including CD22-positive acute lymphoblastic leukemia and HER2-positive metastatic breast cancer.[3,4] Recently, an ADC targeting CD30-positive relapsed lymphomas was approved as a marketed product in the United States.[5]

Cytotoxic compounds can be coupled to antibodies using lysine- or cysteine-directed linker chemistry.[2] Using cysteine-directed chemistry, cytotoxins can either be linked to native cysteines derived from reduction of interchain disulfide bonds or to specially engineered cysteines.[2,6] When conjugation is to interchain cysteines of IgG1 molecules, the resulting conjugates are composed of mixtures containing predominantly species with 0, 2, 4, 6, or 8 drugs per antibody molecule. The average number of drug molecules conjugated per antibody, the drug-to-antibody ratio (DAR), is an important quality attribute in ADC products.[7] The average DAR reflects the amount of cytotoxin delivered per dose, and therefore, may affect both the safety and efficacy of the ADC.[8]

ADCs with interchain cysteine linkages are generated by reducing a fraction of the total interchain disulfide bonds. The newly available free thiols are then conjugated with the small molecule (which is already part of a larger linker-drug intermediate). As partial reductions are performed under nondenaturing conditions, linkage to cysteines participating in intrachain disulfide bonds is not observed.[9]

The linker–drug containing the thiol reactive moiety (such as a maleimide) is added in excess to ensure conjugation of all available free thiols. Tris(2-carboxyethyl)phosphine (TCEP) is a preferred reductant in these processes due to its favorable reaction kinetics, solution stability prior to reaction, and because it cannot form mixed disulfides with antibody thiols.[10,11] Although not discussed here, other reducing agents, including dithiothreitol (DTT), may also be used in the ADC conjugation process.[6] Stoichiometrically, one molecule of TCEP is expected to reduce one disulfide bond, exposing two free thiols for drug conjugation. Following reduction with one molar equivalent of TCEP (1.0X TCEP:mAb), the expected average DAR value is 2.0. Similarly, in order to achieve a targeted average DAR value of 4.0, a predicted TCEP addition of 2 mol equiv (2.0X TCEP:mAb) would be required. In practice, the reduction step is performed using a predetermined TCEP:antibody (TCEP:mAb) molar ratio.

During the development of multiple cysteine-directed ADC products with specifically targeted average DAR values, the required TCEP:mAb molar ratios deviated from the theoretical

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predictions. The required TCEP:mAb ratio, although reproducible for a given lot of antibody, varied between antibodies as well as between different lots of the same antibody. Although some antibody lots required amounts of TCEP very close to the theoretical predictions, most lots required an increased TCEP:mAb molar ratio to achieve the targeted average DAR value.

Trisulfides were identified as a potential source of the TCEP:mAb ratio variability observed during the manufacture of ADCs. Protein trisulfide bonds are post-translational modifications in which an extra sulfur atom is bound between two cysteines involved in disulfide bonding.[12] Although trisulfides had been previously reported in smaller recombinant proteins such as human growth hormone,[13,14] a truncated IL-6,[15] and a Cu,Zn-superoxide dismutase,[16] they were only recently reported in mAbs.[12,17] To date, trisulfides have only been reported within interchain bonds, predominantly between the heavy and light chains.[12]

Here we demonstrate how trisulfides impact ADC production and we use NMR to confirm the reaction mechanism for the elimination of interchain trisulfides. This work is broadly applicable to other ADCs that employ interchain cysteine chemistry with TCEP as the reductant.

EXPERIMENTAL PROCEDURES

Materials. IgG1 monoclonal antibodies mAb1, mAb2, and mAb3 were produced in Chinese hamster ovary (CHO) cells and were purified and formulated at Genentech (South San Francisco, CA). Data from several lots of mAb1 are discussed below. Each lot will be identified using the following nomenclature: lot 1 is mAb1−1, lot 2 is mAb1−2, and lot 3 is mAb1−3.

Antibody−Drug Conjugation. Unconjugated mAbs (>5 g/L) were adjusted to pH 7.5. Following determination of the protein concentration, the pH-adjusted samples were reduced using a predetermined TCEP:mAb ratio (from a 10 mM stock solution of TCEP in water) for 90 min at room temperature. In the following experiments, a range from 0.1X to 2.2X molar excess TCEP:mAb ratios was used. The samples were immediately conjugated using an excess of the desired maleimide-containing linker−drug. Unreacted linker−drug was quenched with an excess N-acetyl cysteine (NAC):linker−drug ratio (from a 10 mM stock solution of NAC in water) and the pH was adjusted to match formulation conditions. If required, the conjugated sample was buffer exchanged into a formulation buffer containing excipients for increased stability.

Measuring DAR. The method described here was based on a previously published protocol.[7] Neat samples of conjugated antibody (approximately 50 μg injections) were analyzed by hydrophobic interaction chromatography (HIC) on a Tosoh Bioscience Butyl-NPR column (4.6 mm × 3.5 cm, 2.5 μm particle) equilibrated with 100% solvent A (1.5 M ammonium sulfate, 25 mM sodium phosphate, pH 6.95) using an Agilent 1200 HPLC system. The column flow rate was 0.8 mL/minute and the temperature was maintained at 24 °C. The DAR species of the conjugated antibodies were resolved with a linear gradient from 0 to 100% solvent B (75% of 25 mM sodium phosphate, 25% isopropyl alcohol, pH 6.95) over 12 min. The chromatogram was developed with a multistep gradient as follows: 1−100% solvent B over 12 min, back to 0% solvent B in 0.1 min, and equilibration with 0% solvent B for 5.9 min. Absorbance was monitored at 280 nm and data were analyzed using Chemstation (Agilent).

The resulting chromatogram provides a quantitative understanding of the ADC drug load and drug distribution (data not provided). Each peak in the chromatogram corresponds to a specific drug species between 0 and 8 drugs per antibody. The peaks are manually integrated and assigned a DAR value according to the number of conjugated drug molecules. The average DAR is calculated by multiplying the percent peak area for each peak by its assigned DAR value. The resulting values are then summed and the total is divided by 100.

Measuring Trisulfides. Nonreduced Lys-C peptide map analysis, in conjunction with high-resolution liquid chromatography-tandem mass spectrometry, was used to detect trisulfides between the heavy and light chains of IgG1 monoclonal antibodies (conjugated and unconjugated). Previous work showed no trisulfides between the heavy−heavy interchain disulfide bonds (data not provided) or in any intrachain disulfide bonds.[12] To samples containing 1 mg of antibody, 8 M guanidine (Pierce), and 100 mM N-ethylmaleimide (Pierce) were added to final concentrations of 6 M and 4 mM, respectively. After a two hour incubation at 37 °C, the denatured and capped antibody samples were diluted from 20 mg/mL to approximately 0.5 mg/mL with buffer containing 100 mM sodium phosphate and 2 mM EDTA at pH 7.0. Endoproteinase Lys-C (sequencing grade, Roche) was added to sample aliquots at a 1:10 (w:w) ratio. Digestion was allowed to proceed for 16 h at 37 °C, and then TFA was added to bring the samples to 0.3% TFA by volume.

The digested samples (50 μg injections) were analyzed by RP-HPLC on a Phenomenex Jupiter C18 column (2 × 250 mm, 5 μm particle) equilibrated with 100% solvent A (0.1% TFA in water) using an Agilent 1200 HPLC system. The chromatography method was optimized for the elution of peptides containing disulfide and trisulfide bonds between the heavy and light chains of IgG1 antibodies. The column flow rate was 0.25 mL/minute and the temperature was maintained at 45 °C. The intact trisulfide and disulfide linked peptides of interest were resolved with a linear gradient from 0 to 15% solvent B (0.1% TFA in acetonitrile) over 75 min. The chromatogram was developed with a multistep gradient as follows: 0−15% solvent B over 75 min, 15−95% solvent B over 5 min, cleaning with 95% solvent B for 5 min, back to 0% solvent B in 0.1 min, and equilibration with 0% solvent B for 14.9 min. Absorbance was monitored at 214 nm and column flow was directed into the source of an LTQ Orbitrap XL mass spectrometer.

Relative trisulfide concentrations were determined by manual integration of extracted ion chromatograms using Xcalibur software. The trisulfide peak area was divided by the sum of the trisulfide and disulfide peak areas to obtain the percent heavy−light chain trisulfides for each sample, similar to a previously published protocol.[12] This approach was also used to determine trisulfide levels in conjugated samples. As the trisulfide level percentage reflects the ratio of remaining intact trisulfide and disulfide bonds, drug-linked peptides were not accounted for in conjugated sample calculations.

Reducing and Reoxidizing Antibodies. Samples were adjusted to pH 7.5 and were subsequently fully reduced by adding DTT at a 20X molar equivalent to mAb. The samples were incubated for 16 h at room temperature. This was followed by a buffer exchange into Tris-HCl pH 7.5 buffer. To regenerate the disulfide bonds, samples were incubated for 2 h

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with dehydroascorbic acid (Sigma-Aldrich) at 6X to 10X molar equivalents to the mAb. Following reoxidation, samples were buffer exchanged into the appropriate formulation buffer.

Artificially Inducing Trisulfide Formation. A hydrogen sulfide incubation was performed based on a previously reported method.[12] Antibody samples were buffer exchanged by gel filtration using PD-10 columns (GE Healthcare) into 50 mM Tris buffer, pH 7.8 to a concentration of 7.5 mg/mL. The samples were treated with either a 125 mM hydrogen sulfide water solution (Ricca Chemical) to a final concentration of 2.5 mM or an equivalent volume of purified water (method control sample) and were incubated for 24 h at room temperature. After incubation, the samples were buffer exchanged using PD-10 columns into a formulation buffer containing excipients for increased stability. To generate a third sample with an intermediate level of trisulfides, aliquots of the control and hydrogen sulfide treated samples were mixed at a 1:1 ratio.

Identifying Reduction Products using NMR. Oxidized TCEP (TCEP[O]) and thiophosphine (TCEP[S]) standards for NMR testing were generated using the following protocol. A solution of allyl trisulfide (Santa Cruz Biotechnology) containing 0.08 mmol in 2.5 mL of acetonitrile-d_3 (Cambridge Isotope Laboratories) was sparged with nitrogen and was heated to 75 °C.[18] A TCEP solution containing 0.08 mmol in 2.5 mL of deuterium oxide was added, by single drops, to the heated allyl trisulfide solution. The resulting mixture was stirred overnight. The mixture was washed twice with hexanes to remove excess allyl trisulfide, and was then evaporated under reduced pressure to yield a white residue containing TCEP[O] and TCEP[S] in a 64:36 ratio, respectively, as confirmed by NMR.

To evaluate the formation of TCEP[O] and TCEP[S] species from antibody reduction, unconjugated samples (approximately 200 mg) were first buffer exchanged by gel filtration using PD-10 columns (GE Healthcare) into 100 mM ammonium bicarbonate, pH 8.0. The samples were concentrated to approximately 20 mg/mL for the reduction step using 100 kD MWCO centrifugal filters (Amicon). Antibody concentrations were tested using the Nanodrop prior to sample reduction. TCEP was added to the concentrated samples from a 10 mM stock solution to achieve a molar ratio of 1 mol of TCEP per mol of antibody (1X TCEP:mAb). The solution was incubated at 20 °C for 90 min. After the reduction, 3 volumes of chilled methanol were used to precipitate the protein. The samples were held at −20 °C for 30 min and were subsequently centrifuged for 30 min to pellet the protein. The supernatant was collected and vacuum-dried. The samples were reconstituted in deuterium oxide (approximately 300 μL) for NMR analysis.

^{1}H and ^{1}H−^{13}C heteronuclear multiple-quantum correlation (HMQC) NMR spectra were acquired on a Bruker 600 MHz spectrometer with a 5 mm gradient-equipped TCI cryoprobe. ^{1}H spectra were acquired using a 1D pulse sequence coupled with an excitation sculpting pulse sequence designed to minimize the resonance signal from water in solution. The experiment comprised 64 scans with an acquisition time of 3.41 s, a block size of 64 K data points, and a spectral width of 16 ppm, with an offset of 4.7 ppm. ^{1}H−^{13}C HMQC NMR spectra were acquired using a phase sensitive heteronuclear zero and double quantum coherence HMQC pulse sequence with gradient pulses for selection. The spectral width was set to 6 ppm in the direct (F2) dimension and 90 ppm in the indirect dimension (F1), and the center of the spectrum was set to 2 ppm in the F2 dimension, and 45 ppm in the F1 dimension. The acquisition data size was set to 2 K data points in the F2 dimension and 512 data points in the F1 dimension. Sixty-four scans were collected per t1 increment, with an acquisition time of 0.28 s. The final processed size was 2 K data points × 2 K data points.

^{31}P NMR and ^{1}H−^{31}P heteronuclear multiple-bond correlation (HMBC) NMR spectra were acquired on a Bruker 500 MHz spectrometer with a 5 mm gradient-equipped BBI probe. ^{31}P NMR spectra were acquired using a 1D pulse sequence with power gated decoupling, and a 30° flip back angle. The experiment comprised 16 K scans, an acquisition time of 0.40 s, a block size of 64 K data points, and a spectral width of 400 ppm, with the offset at −50 ppm. ^{1}H−^{31}P HMBC NMR spectra were acquired using a heteronuclear zero and double quantum coherence HMBC pulse sequence with gradient pulses for selection. The spectral width was set to 8 ppm in the direct (F2) dimension and 300 ppm in the indirect dimension (F1), and the center of the spectrum was set to 4 ppm in the F2 dimension and 150 ppm in the F1 dimension. The acquisition data size was set to 1 K data points in the F2 dimension and 512 data points in the F1 dimension. Four scans were collected per t1 increment, with an acquisition time of 0.13 s. The final processed size was 1 K data points × 1 K data points.

All NMR spectra were acquired at 300 K, and were acquired and processed using Bruker software *TOPSPIN 2.1*. For 1D integration values, integration regions were manually baseline corrected if necessary.

RESULTS AND DISCUSSION

Trisulfide Impact on Conjugation. Three independently produced lots of a single antibody, with consistent product quality profiles as measured by SEC, iCIEF, and binding (data not provided) performed quite differently during conjugation. When reducing and conjugating lots mAb1−1 and mAb1−2 with identical molar ratios of TCEP to antibody, mAb1−1 always yielded lower average DAR values than mAb1−2. At each TCEP:mAb ratio, both lots mAb1−1 and mAb1−2 always had lower average DAR values than mAb1−3. The average DAR values of mAb1−1 and mAb1−2 differed by about 0.2 units (moles drug per mole of antibody). The average DAR values of mAb1−3 nearly matched the theoretical values (dashed line in Figure 1) based on the reduction reaction stoichiometry and the TCEP:mAb ratios used (Figure 1).

One possible explanation for the observed variation in TCEP required for these three lots, based on recent publication of a novel post-translational modification of antibodies, was the presence and level of trisulfide bonds.[12] Lot mAb1−1 contained 7.4% trisulfide bonds, mAb1−2 contained 3.0% trisulfide bonds, and mAb1−3 had almost no detectable trisulfides with 0.4%. For any targeted average DAR value, the levels of trisulfides correlated directly with the required TCEP:mAb ratio. To obtain an average DAR of 3.5 (horizontal dotted line in Figure 1), mAb1−3 required approximately 1.75X TCEP:mAb (the theoretical stoichiometry), mAb1−2 required approximately 1.9X TCEP:mAb, and mAb1−1 required about a 2.0X TCEP:mAb ratio. Samples with higher trisulfide content required an increased TCEP:mAb ratio to achieve the same average DAR value. Based on these data, interchain trisulfide bonds appeared to cause the different linear responses of these lots to TCEP treatment, as well as caused the deviation in required TCEP of lots mAb1−1 and mAb1−2 from the

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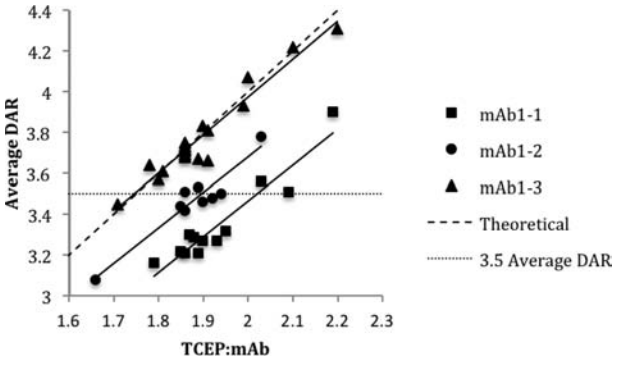

Figure 1. Each point represents a separate reduction and then conjugation experiment. mAb1−1 (7.4% trisulfides) has n = 12 points, mAb1−2 (3.0% trisulfides) has n = 9 points, and mAb1−3 (0.4% trisulfides) has n = 15 points. The lines are best fit curves for each lot. The stoichiometrically predicted average DAR values for each TCEP:mAb ratio is also shown (dashed line). The dotted line at 3.5 average DAR represents the targeted average DAR value for this ADC. Where the lines cross 3.5 average DAR reflects the required TCEP:mAb ratio to achieve the targeted DAR value for a particular mAb lot.

theoretical reduction stoichiometry. After conjugation at any of the TCEP:mAb ratios shown, interchain trisulfide bonds in the ADCs were undetectable by nonreduced Lys-C peptide map analysis (data not provided).

Conjugation Effects from Reduced Trisulfide Levels. Others have previously shown that trisulfides can be removed under mildly reductive conditions.[12,19,20] Samples of mAb1−1 and mAb1−2 were fully reduced under native conditions, and were subsequently reoxidized, thereby removing all of the trisulfide bonds. These lots were then conjugated using a 2X TCEP:mAb ratio, resulting in the expected average DAR of 4.0 and an average DAR/TCEP value around 2.0 (Table 1). An

Table 1. Average DAR/TCEP Results from Native and Reduced/Reoxidized Sample Preparations Conjugated Using a 2X TCEP:mAb Ratio[a]

sample	sample treatment	% trisulfides	average DAR/TCEP
mAb1−1	none	7.4	1.73
	reduced/reoxidized	ND[b]	2.02
mAb1−2	none	3.0	1.85
	reduced/reoxidized	ND[b]	2.00

[a]The average DAR/TCEP value for: the native mAb1-1 is the mean of n = 12 average DAR/TCEP points, the reduced/reoxidized mAb1-1 is the mean of n = 4 points, the native mAb1-2 is the mean of n = 9 points, and the reduced/reoxidized mAb1-2 is the mean of n = 4 points. [b]ND indicates that trisulfide bonds were not detected.

average DAR/TCEP value reflects the efficiency of the reduction and conjugation process. Theoretically, for a 2X TCEP:mAb ratio with a resultant average DAR value of 4.0, the average DAR/TCEP value would be 2.0. In samples containing trisulfides, however, the average DAR/TCEP values were lower than expected for a 2X TCEP:mAb addition.

When mAb1−1 and mAb1−2 samples containing trisulfides (not pretreated with reduction/reoxidation) were reduced using 2X TCEP:mAb ratio and then conjugated, the substoichiometric average DAR/TCEP values of 1.73 and 1.85, respectively, were obtained (Table 1). Indeed, the sample with the highest trisulfide level (lot mAb1−1) had the lowest average DAR/TCEP value, meaning that fewer mAb free thiols were generated for the given TCEP addition.

Conjugation Effects from Induced Trisulfide Formation. Earlier work showed that antibody trisulfide levels could be artificially increased by incubating samples with hydrogen sulfide.[12,21] Using this method, trisulfide levels were increased in lot mAb1−3 (from a starting level of 0.4% trisulfide bonds). Postincubation, four samples were conjugated using a 2X TCEP:mAb ratio: a control, an untreated sample, a 50/50 co-mix of the untreated and treated samples, and the hydrogen sulfide treated sample. The control sample was the same material as the untreated sample, but underwent no additional sample preparation prior to trisulfide testing and conjugation. The control sample ensured that the processing used for the other three samples did not affect either the trisulfide or conjugation results.

Hydrogen sulfide incubation increased levels of trisulfides, resulting in decreased average DAR/TCEP values following conjugation (Table 2). The control and untreated samples, with

Table 2. Samples Conjugated Using a 2X TCEP:mAb Ratio with an Expected Average DAR/TCEP Value of 2.0[a]

sample	% trisulfides	average DAR/TCEP
control	0.4	1.97
untreated	0.5	1.92
50/50 co-mix	2.3	1.85
treated	5.3	1.73

[a]mAb1-3 was incubated with hydrogen sulfide to artificially increase trisulfide levels. Data shown represents a single conjugation experiment for each sample.

the lowest levels of trisulfides, had the highest average DAR/TCEP values. These average DAR/TCEP values nearly matched the theoretical value of 2.0. The 50/50 co-mix sample, with an intermediate level of trisulfides at 2.3%, had an intermediate average DAR/TCEP value of 1.85. Finally, the hydrogen sulfide treated sample with 5.3% trisulfides had the lowest average DAR/TCEP value at 1.73. As stated earlier, increased trisulfide levels result in lower average DAR values that directly result in lower average DAR/TCEP values for a fixed TCEP:mAb reduction.

Preferential Reduction of Trisulfide Bonds by TCEP. All conjugated samples tested to date have undetectable levels of trisulfides by nonreduced Lys-C peptide mass spectrometry. These samples were all conjugated, however, following reductions using a TCEP:mAb ratio far in excess of the level of antibody trisulfides. A TCEP:mAb ratio titration was performed using mAb2, with a basal level of 7.2% trisulfides, to better understand trisulfide elimination at low TCEP additions (Figure 2). The reduction ratios started below the molar level of trisulfide bonds present in the starting material, and increased to levels more typical of current mAb conjugations.

The low TCEP:mAb ratios (0.1X, 0.25X, and 0.5X) did not eliminate all of the initially present trisulfide bonds. At a molar TCEP ratio of approximately 0.75X TCEP:mAb, the trisulfide level dropped to about 0.1%. At a 2X TCEP:mAb ratio, trisulfides were undetectable in the final antibody conjugate, thus confirming what had been seen in all conjugated samples previously tested (Figure 2). TCEP molar excess currently used in typical ADC processing (>1.75X) should completely remove trisulfides at these levels. The data in Figure 2 also suggests that

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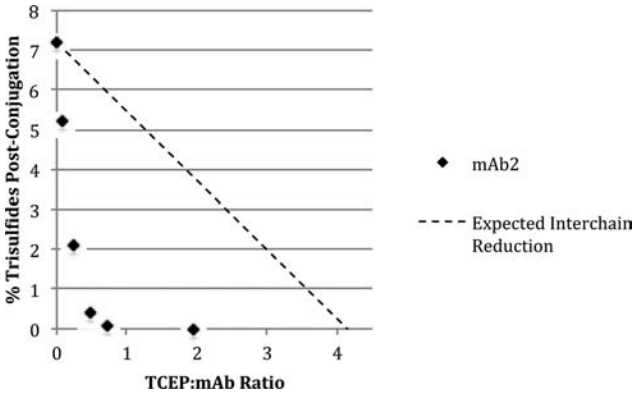

Figure 2. Trisulfide levels in an ADC produced from mAb2 (containing 7.2%) were tested after reducing the antibody with varying TCEP:mAb ratios and then conjugating with an excess of the desired maleimide-containing linker-drug. Trisulfide levels in the conjugated samples were determined using nonreduced Lys-C peptide mass spectrometry. The dashed line represents the expected reduction behavior of all interchain bonds if trisulfides and disulfides were reduced equivalently.

TCEP elimination of trisulfides occurs preferentially to disulfide reduction, which magnifies the impact of trisulfides on the process stoichiometry. If trisulfide reduction were not favored over disulfide reduction, meaning that trisulfides reacted in a linear manner with TCEP addition, levels of trisulfides would not be expected to drop to zero until all eight interchain bonds had reacted with TCEP (approximately 4X TCEP:mAb; see dashed line in Figure 2).

Previous data has shown that bonds between the heavy and light chains of antibodies are more susceptible to reduction than the bonds between the two heavy chains due to greater solvent exposure.[22] The greater accessibility of this bond may explain why trisulfides have been detected at significant levels between the heavy and light chains in the IgG1 mAbs tested, and not within other, more protected, inter- or intrachain bonds (data not provided). The preferential reduction of the heavy−light bond also helps to explain the reduction behavior of trisulfides. The additional sulfur atom may serve to enhance the reducibility of a bond that is already preferentially reduced over other interchain bonds.

Understanding the Trisulfide-TCEP Reaction Mechanism by NMR. Reduction of a disulfide bond by TCEP produces an oxidized TCEP (TCEP[O]) and two antibody free thiols, which are available for subsequent drug conjugation.[10] In contrast, previous work has suggested that reactions between reductive phosphines and trisulfide bonds produce a thiophosphine and a disulfide bond via a nucleophilic attack on the central sulfur atom.[18] NMR was used to confirm that the previously elucidated reaction mechanism is applicable to reduction of antibody trisulfides by TCEP. The presence of the thiophosphine, TCEP[S], in samples containing antibody trisulfides, and its absence in samples without trisulfides, was used to confirm that trisulfides consume TCEP during antibody reduction without producing any mAb free thiols for conjugation. The consumption of TCEP without a corresponding increase in free thiols alters the stoichiometry for the conjugation process, directly leading to the lower average DAR values observed during the conjugation of mAbs containing trisulfides.

NMR testing of TCEP[S] and TCEP[O] standards showed unique proton and phosphorus chemical shifts for each of the species. To ensure that the TCEP[S] signal was not overwhelmed by the TCEP[O] signal in the NMR spectra, the TCEP[S]:TCEP[O] ratio was maximized by using a low TCEP:mAb ratio. Based on the preferential reduction of trisulfides (Figure 2), a 1X TCEP:mAb ratio was selected to enable full elimination of trisulfides while simultaneously minimizing disulfide reduction. Samples of mAb1−3 (0.4% trisulfides) and mAb3 (6.5% trisulfides) were reduced using 1X TCEP:mAb and were then conjugated. Retesting of the conjugated samples showed that trisulfide levels were undetectable (data not provided). Additional samples of mAb1−3 and mAb3 were reduced with 1X TCEP:mAb and were processed to collect TCEP[S] and TCEP[O] as described in the Experimental Procedures.

TCEP[O] and TCEP[S] were initially identified by 1D NMR (Figure 3). In a typical ^{1}H NMR spectrum, the peak

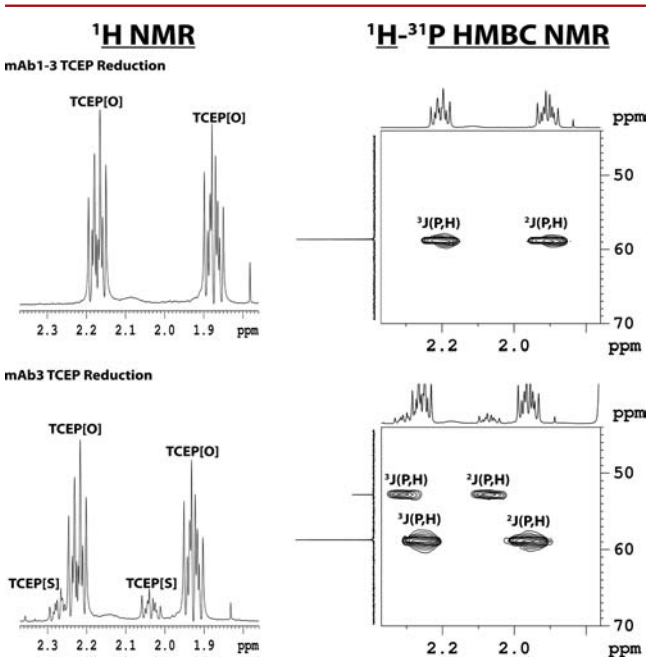

Figure 3. NMR spectra showing the detection of TCEP[O] and TCEP[S] species upon reduction of mAb1−3 (0.4% trisulfides) and mAb3 (6.5% trisulfides) at 1X TCEP:mAb. ^{1}H spectra are on the left and ^{1}H−^{31}P HMBC spectra are on the right. In mAb1−3, with low basal trisulfides, mostly TCEP[O] was detected. In mAb3, with a higher basal level of trisulfides, TCEP[O] and TCEP[S] were detected.

resonances of the alkyl protons in the TCEP species fell within the region of δ_H = 1.8−2.7 ppm. Although the chemical shifts of these protons were sensitive to changes in solution pH and ion strength, the relative chemical shifts of the protons in each species were consistent (TCEP[S] was shifted downfield from TCEP[O]).

An HMBC experiment further confirmed the chemical structure of the TCEP species (Figure 3). This experiment showed multiple bond correlations of protons to the phosphorus motifs in TCEP[O] and TCEP[S]. The TCEP[O] phosphorus peak (δ_P = 56.41 ppm) had correlations in the proton dimension at δ_H = 2.20 ppm (^{2}J) and δ_H = 2.66 ppm (^{3}J). The TCEP[S] phosphorus peak (δ_P = 52.96 ppm) had correlations in the proton dimension at δ_H = 2.30 ppm (^{2}J) and δ_H = 2.71 ppm (^{3}J). An HMQC experiment provided an

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additional check to confirm the identity of the peaks (data not provided).

As expected, mAb3, with a high level of trisulfides, was found to yield both TCEP[S] and TCEP[O] as reaction products. By contrast, following reduction of mAb1−3, which had a very low starting trisulfide level, the primary NMR signals detected were consistent with TCEP[O]. The 1D NMR results were integrated and the peak area ratios of TCEP[S] to TCEP[O] were then compared to the calculated molar ratio of trisulfides to disulfides (Table 3). The trisulfide to disulfide ratio was assumed to be a good predictive surrogate for comparison with the TCEP[S] to TCEP[O] ratio as only trisulfides could form TCEP[S].

Table 3. Theoretical vs. Experimental Results For Determining the Trisulfide/Disulfide Ratio[a]

sample	% trisulfide	theoretical mol trisulfide/ mol disulfide	experimental TCEP[S]/ TCEP[O]
mAb1−3	0.4	0.01	0.02
mAb3	6.5	0.15	0.17

[a]mAb1-3 (0.4% trisulfides) and mAb3 (6.5% trisulfides) were reduced and processed for the NMR analysis used to identify the TCEP[S] produced from trisulfide elimination and TCEP[O] from disulfide reduction. ^{1}H NMR peaks were integrated and the TCEP[S]/ TCEP[O] peak area ratios were compared to the calculated ratio of mole trisulfides/mol disulfides (determined from the amount of TCEP added and the initial level of sample trisulfide bonds).

For mAb1−3, the trisulfide to disulfide molar ratio was calculated to be 0.01 based on a basal trisulfide level of 0.4% and a 1X TCEP:mAb addition. This predicted value was very similar to the TCEP[S] to TCEP[O] ratio that was experimentally determined. The same held true for mAb3. The theoretical trisulfide to disulfide molar ratio, predicted based on a basal trisulfide level of 6.5% and a 1X TCEP:mAb addition, was 0.15. The experimental ratio, derived from the peak areas of the TCEP[S] and TCEP[O] peaks, was similar at 0.17. These results confirmed that trisulfides were eliminated using TCEP, and that this reaction formed a thiophosphine as previously described. Based on this trisulfide TCEP reduction mechanism, the average DAR from a conjugation with a given amount of TCEP decreases in accordance with the TCEP consumed due to trisulfide elimination. Variability in mAb trisulfide levels directly accounts for the variability in the required TCEP:mAb ratios observed during ADC production.

CONCLUSION

The presence of trisulfide bonds impacts the reduction reaction stoichiometry for ADCs produced using cysteine-directed chemistry. The demonstrated effect of artificially introducing or removing trisulfides in mAbs prior to reduction confirms the inverse correlation between trisulfide levels and the resultant average DAR values for a given amount of reductant. The impact of trisulfides on antibody reduction was confirmed mechanistically using NMR; TCEP reacts with a trisulfide bond to form one molecule of thiophosphine and one disulfide bond (Figure 4a). Conversely, when TCEP reduces disulfide bonds, the result is two antibody free thiols available for conjugation (Figure 4b). In effect, trisulfide bonds consume an equivalent of TCEP without producing any mAb free thiols for conjugation.

As a result of trisulfide elimination, the average DAR value is below the theoretical value predicted based solely on the TCEP:mAb ratio used for reduction. Trisulfide bonds are completely removed using low molar excesses of TCEP, and, therefore, no trisulfide bonds are found in conjugates produced using current conjugation practices (>1.75X TCEP).

Average DAR is an important quality attribute for antibody−drug conjugates. As a result, a primary goal during the process development of ADCs is to achieve a consistent average DAR value. Improved process controls to achieve a specific average DAR may be required to mitigate the potential impact of variable levels of interchain trisulfide bonds. These could potentially include: removing trisulfides under mildly reductive conditions during the purification process;[19] controlling trisulfide levels during the cell culture process by monitoring cysteine feeding or by stripping the cell culture fluid with a gas such as nitrogen or argon;[19−21] empirically determining the TCEP required on a lot-specific basis using small-scale conjugations; or calculating the TCEP required on a lot-specific basis using quantitative measurement of trisulfide levels

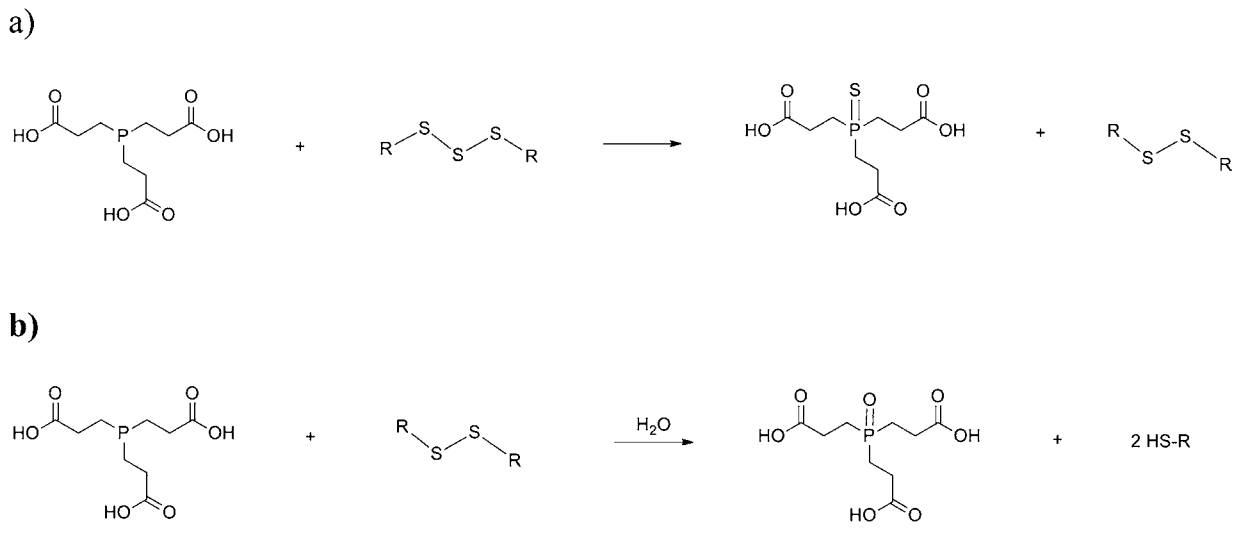

Figure 4. (a) Trisulfide reduction with TCEP yields the thiophosphine and a disulfide bond; (b) disulfide reduction with TCEP yields the phosphine oxide and two free thiols. Both thiophosphine and phosphine oxide derivatives of TCEP are distinguishable and quantifiable by NMR.

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in the starting mAb material. Evaluations of these potential strategies are underway.

The level of trisulfides in the antibody starting material is a major source of variability in the relationship between the excess of TCEP used for reduction and the resultant DAR. There are, however, multiple additional factors that influence the amount of TCEP required including the purity of the TCEP starting material, the amount of air oxidation that occurs during reduction, and the efficiency of the conjugation reaction. Our lab is continuing to investigate these other factors in order to improve predictions of final DAR results based on trisulfide levels for a given amount of TCEP.

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Notes

The authors declare no competing financial interest.

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