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Comparative Analysis of Monoclonal Antibody *N*-Glycosylation using Stable Isotope Labelling and UPLC-Fluorescence-MS

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A twoplex method using $^{12}C_6$ and $^{13}C_6$ stable isotope analogues (Δmass = 6 Da) of 2-aminobenzoic acid (2-AA) is described for quantitative analysis of *N*-glycans present on monoclonal antibodies and other glycoproteins using ultra performance liquid chromatography with sequential fluorescence and accurate mass tandem quadrupole time of flight (QToF) mass spectrometric detection.

N-glycans present at the asparagine 297 residue in the C_H2 domain of the Fc region of IgG play a crucial role in modulating the structural stability and functional activity relationship of the antibody.[1, 2] Quantitative structural characterisation of *N*-glycans present on antibody therapeutics, such as monoclonal antibodies (mAbs) and Fc fusion proteins, is required under the regulatory guidelines. Characterisation of the oligosaccharides present is normally performed using liquid phase separation techniques with optical detection following enzymatic liberation of the *N*-glycans from the antibody and derivatisation with a fluorophore to increase detection sensitivity.[3, 4] Despite recent advances in separation chemistries for oligosaccharides,[5, 6] peak area based quantitation remains a subjective process due to integration issues for distorted peaks containing poorly or partially resolved oligosaccharides, or due to the presence of multiple glycans within a single chromatographic peak.[7] Such effects can result in either under reporting of a particular *N*-glycan or create difficulties in the alignment of data for subsequent statistical evaluation.

Relative or absolute quantitation, for the elucidation of differential expression of proteins, using stable isotope labelling technology has become widely used in proteomics.[8] Application of stable isotope methods for quantitative glycomics has also attracted attention with published reports focusing upon heavy isotope incorporation during permethylation of oligosaccharides prior to MALDI-MS analysis.[9-11] A limitation to this approach is that isotope incorporation and the associated light-heavy mass difference varies with the number of methylation sites available on the oligosaccharide. This limitation has been overcome through the labelling of oligosaccharide samples for comparison with either $^{13}CH_3I$ or $^{12}CH_2DI$, respectively, thereby introducing a mass difference of 2.922 mDa per methylation site, however high resolution FT-ICR-MS was required to distinguish between the resulting isotopic pairs.[12, 13] Stable isotope incorporation using reductive amination has also been reported prior to MALDI-MS analysis using $^{13}C_6$ or deuterated analogues of commonly used reagents for high sensitivity fluorescence detection.[14-21] Specifically designed hydrophobic hydrazide reactive reagents have been reported that facilitate MS based quantitation based upon $^{13}C_6$ incorporation and also increase the hydrophobicity of the glycoconjugate, thereby, increasing its ionisation efficiency.[22, 23] Metabolic generation of isotopically enriched aminosugars and their products resulting from ^{15}N glutamine feeding of mammalian cells, in an analogous manner to heavy lysine or arginine incorporation through SILAC, has also been described.[24, 25] MS/MS isobaric tagging methods were also reported for quantitative glycomics, however concerns exist regarding the attainment of a balance during fragmentation to ensure complete reporter ion release while avoiding over fragmentation of the oligosaccharide being characterised.[26-29] A limitation of many of the cited methods is their use with either direct infusion ESI-MS or MALDI-MS, thereby limiting the possibility to gain quantitative information for positional or linkage oligosaccharide isomers present in the *N*-glycans pools under study due to the lack of an analytical separation.

Here, the use of $^{12}C_6$- and $^{13}C_6$ 2-aminobenzoic acid (2-AA) differential labelling of *N*-glycans followed by ultra performance hydrophilic interaction liquid chromatography with online fluorescence and tandem mass spectrometry using a quadrupole time of flight instrument (UPLC-Flr-QToF-MS/MS) is described for the comparative analysis of the glycosylation present of different batches of a commercial chimeric IgG1 mAb. Initial evaluations focused on the quantitative performance of the $^{12/13}C_6$ 2-AA labelling using *N*-glycan standards (oligomannose and bi-antennary *N*-glycans with and without core fucose, antennary galactose and sialic acid residues) to assess linearity and accuracy of the quantitative response. The molar ratios of the light and heavy labelled *N*-glycan

standards were mixed in theoretical ratios of 1:1, 2:1, 5:1 and 10:1, Supplementary Fig. 1. Quantitation was performed by determining the ratio of the sum of the extracted mass chromatogram peak areas generated using the combination of the $^{12}C_6$ isotopes relative to the $^{13}C_6$ isotopic data for the $[M-2H]^{2-}$ pseudomolecular ion. Evaluation of the experimental data revealed close agreement of the experimental ratios with the theoretically mixed values. Translation of the data into linearity plots resulted in correlation coefficients for fitted least squares regression trend lines of $R^2 > 0.99$ for all glycan standards tested, Supplementary Fig. 1. Quantitative precision of the $^{12/13}C_6$ 2-AA labelling strategy was next established using IgG *N*-glycans released from polyclonal IgG that was extracted from healthy human control serum using Protein A affinity chromatography. Normalisation was performed based upon the total amount of IgG taken for each deglycosylation (500 µg). Six technical replicate pairs of the IgG *N*-glycans were prepared with an expected light heavy ratio of 1:1. Each technical replicate was analysed using hydrophilic interaction UPLC-fluorescence-MS, the mean and standard deviation of the six technical replicates was determined, resulting data is depicted in Fig. 1. Annotation of the *N*-glycans present in each chromatographic peak was based upon the oligosaccharide composition as derived from the m/z value and MS/MS spectral annotation for structural confirmation where required. Excellent precision was established for the six replicate analyses of the light heavy pairs with the expected 1:1 light heavy ratio obtained and an average percentage error of ~5% across all *N*-glycans annotated, (range 2-9%), demonstrating excellent precision for the described twoplex stable isotope labelling quantitative glycomics approach.

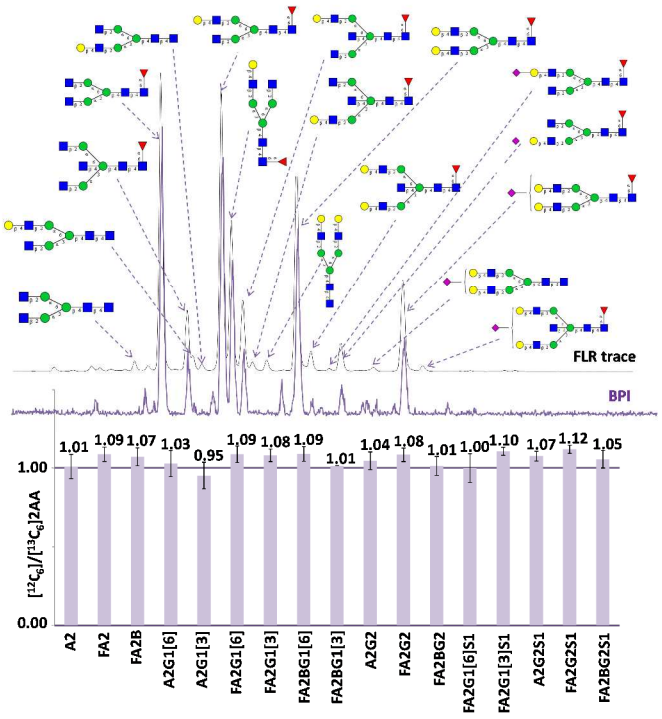

Fig.1: Annotated fluorescence and base peak intensity (BPI) chromatograms for *N*-glycans released from polyclonal human serum IgG. The bar chart underneath the chromatograms depicts the mean light to heavy relative ratio as experimentally determined using UPLC-fluorescence-MS for the six technical replicate pairs analysed, y-error bars are also included at ± the standard deviation of the six technical replicates.

The developed twoplex $^{12/13}C_6$ 2-AA labelling hydrophilic interaction UPLC-fluorescence-MS approach was then applied to investigate the consistency of the *N*-glycans present on two different commercial lots of a chimeric IgG1 mAb. To ensure validity of the generated data, the experimental design used focused upon triplicate analysis of technical replicates of lot 1 *versus* lot 1, lot 2 *versus* lot 2 and finally, lot 1 *versus* lot 2, with each comparison normalised based upon the amount of mAb taken for deglycosylation (500 µg). Fig. 2 (A) depicts the annotated fluorescence and base peak intensity (BPI) chromatograms for the *N*-glycans present on the chimeric IgG1 mAb.

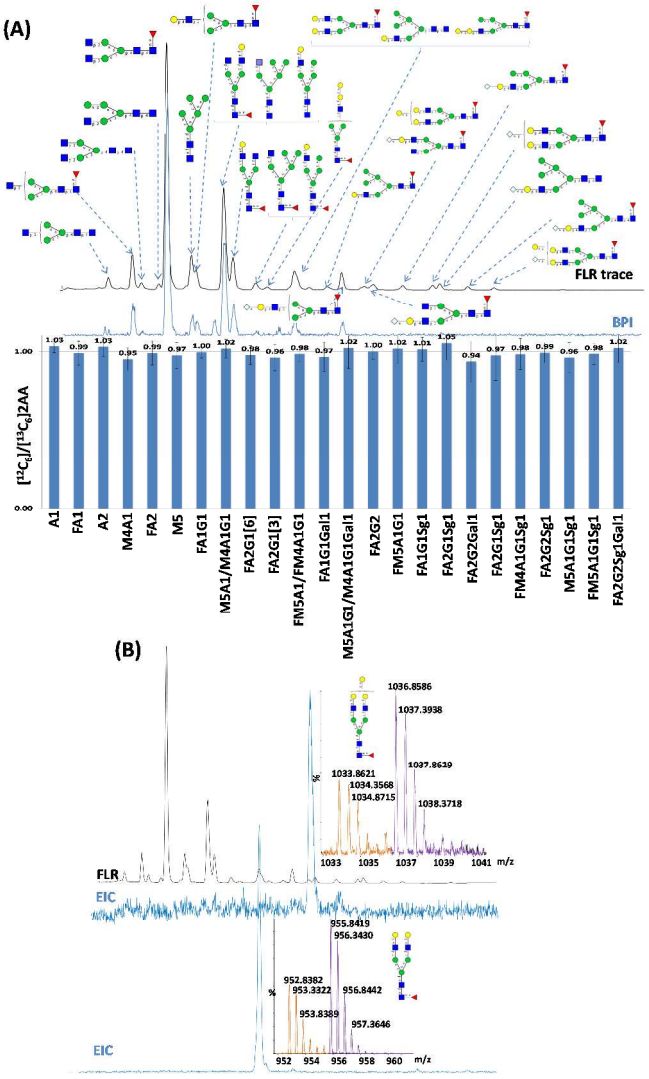

Fig.2: (A) Annotated fluorescence and BPI chromatograms for the *N*-glycans released from the commercial chimeric IgG1 mAb analysed. The bar chart underneath represents the mean ± standard deviation of the light to heavy ratios calculated for a triplicate analysis of the oligosaccharides (Lot 2 *versus* Lot 2). The 'Sg' in the glycan names underneath the bar chart represents the *N*-glycolyl neuraminic acid form of sialic acid. (B) Example extracted ion chromatograms and associated spectra demonstrating differences in the relative amounts of a core fucosylated bi-antennary glycan (lower trace) and a core fucosylated bi-antennary glycan containing a galactose α1-3 linked galactose epitope (upper trace) in the two commercial lots of the mAb analysed.

Twenty seven *N*-glycan structures were annotated including high mannose, hybrid and complex bi-antennary glycans with variable degrees of core fucosylation, galactosylation, sialylation and galactose α1-3 linked galactose epitopes. Relative quantitation data was inferred from the $^{12/13}C_6$ light to heavy ratios for each oligosaccharide present, resulting data is displayed in Table 1 which lists the mean light heavy ratio for each *N*-glycan, the standard deviation of the triplicate measurement is displayed in parenthesis.

Table 1: Relative light to heavy ratios determined for the analysis of the individual lots of mAb analysed as indicated in the table header.

GLYCAN STRUCTURE	RATIO ($^{12}C_6$)Lot 1:($^{13}C_6$)Lot 1	RATIO ($^{12}C_6$)Lot 2:($^{13}C_6$)Lot 2	RATIO ($^{12}C_6$)Lot 1:($^{13}C_6$)Lot 2
A1	0.96 (0.02)	1.03 (0.04)	0.92 (0.03)
FA1	1.00 (0.01)	0.98 (0.08)	1.00 (0.03)
A2	0.95 (0.01)	1.03 (0.06)	0.82 (0.06)
M4A1	0.97 (0.04)	0.95 (0.07)	1.00 (0.04)
FA2	0.99 (0.02)	0.99 (0.08)	0.81 (0.02)
M5	0.96 (0.04)	0.97 (0.08)	0.88 (0.06)
FA1G1	0.97 (0.01)	1.00 (0.04)	0.80 (0.01)
M5A1/M4A1G1	0.96 (0.01)	1.02 (0.06)	1.00 (0.07)
FA2G1 [6]	0.98 (0.01)	0.98 (0.06)	0.59 (0.07)
FA2G1[3]	1.00 (0.01)	0.96 (0.08)	0.69 (0.02)
FM5A1/FM4A1G1	0.99 (0.00)	0.98 (0.05)	0.99 (0.01)
FA1G1Gal1	0.95 (0.02)	0.97 (0.09)	0.64 (0.01)
M5A1G1/M4A1G1Gal1	0.96 (0.02)	1.02 (0.13)	0.75 (0.05)
FA2G2	0.94 (0.04)	1.00 (0.05)	0.44 (0.03)
FM5A1G1	0.99 (0.06)	1.02 (0.09)	0.72 (0.04)
FA1G1Sg1	1.03 (0.03)	1.01 (0.08)	0.72 (0.03)
FA2G1Sg1	1.02 (0.01)	1.05 (0.11)	0.54 (0.01)
FA2G2Gal1	0.95 (0.08)	0.94 (0.13)	0.39 (0.01)
FA2G1Sg1	1.02 (0.06)	0.97 (0.16)	0.63 (0.08)
FM4A1G1Sg1	1.04 (0.02)	0.98 (0.10)	0.66 (0.02)
FA2G2Sg1	0.96 (0.02)	0.99 (0.06)	0.41 (0.02)
M5A1G1Sg1	0.94 (0.02)	0.96 (0.10)	0.65 (0.04)
FM5A1G1Sg1	0.99 (0.03)	0.98 (0.09)	0.66 (0.02)
FA2G2Sg1Gal1	0.99 (0.01)	1.02 (0.04)	0.41 (0.02)

As seen from Table 1, the replicate analysis of individual lots (lot 1 *versus* lot 1 and lot 2 *versus* lot 2) indicated excellent and precise quantitative performance of the developed platform, with ratios near unity, as expected, for each *N*-glycan annotated. Interestingly, when the two lots were compared against each other distinct differences in the levels of certain glycans present were easily identified. The relative ratios of the majority of high mannose and hybrid oligosaccharides present on both lots were highly comparable with relative light heavy ratios of unity observed for many of these structures. Interestingly, the relative light heavy ratios for the complex bi-antennary glycans, particularly sialylated glycans and those containing a galactose α1-3 linked galactose residue, showed a dramatic difference between the two lots, Fig. 2 (B) and Table 1. Glycosylation site occupancy was also determined using LC-MS^E by subjecting aliquots of both lots of mAb to trypsin (Sequence coverage: Lot 1; 69.9% light chain, 66.0% heavy chain, Lot 2; 66.2% light chain, 56.2% heavy chain) and PNGase F followed by trypsin (Sequence coverage: Lot 1; 50.9% light chain, 52.0% heavy chain, Lot 2; 44.4% light chain, 39.1% heavy chain) and evaluating the relative peak area of the unoccupied glycopeptide (EEQTNSTYR) and the deamidated glycopeptide (EEQTDSTYR) generated following enzymatic deglycosylation. The *N*-glycosylation site occupancy was determined to be 99.92 and 99.90% for lot 1 and lot 2, respectively. The reason for the difference in *N*-glycosylation between the two commercial lots is unclear but may be process related, for example, cellular age at time of harvest has been previously demonstrated to exhibit a similar effect of the glycosylation of an IgG4 mAb produced in CHO cells.[30] Process manufacturing changes have also been shown to be detectable through the analysis of different lots of drug product.[31] It is acknowledged that many more drug product lots would need to be analysed to deduce such a conclusion; however, the presented twoplex $^{12/13}C_6$ 2-AA labelling method represents a simple solution for glycosylation comparability analysis, capable of detecting differences between products due to the minimisation of technical variability and operator subjectivity from the analysis.

Considering the importance of the *N*-glycans on the structure functional relationship of mAbs it is surprising that many stability studies focus on critical quality attributes (CQAs) related to the primary sequence, such as aggregation, differences in the levels of charge variants and oxidised residues, whilst overlooking the importance of the stability of the oligosaccharides. Using the developed twoplex $^{12/13}C_6$ 2-AA labelling method we investigated the stability of the *N*-glycans present on the chimeric IgG1 mAb (lot 2 above) under forced degradation conditions. Exposure to elevated temperatures, temporal stress, UV light at two different exposure wavelengths and acidic and basic pH shocks were undertaken, with comparison performed relative to a replicate sample held under the recommended storage conditions for the same duration.[32-35] Replicate samples of the IgG1 were also stressed but retained for the determination of alterations in intact protein level CQAs, i.e. aggregation profiling by size exclusion chromatography with UV detection (SEC-UV), charge variant analysis by cation exchange chromatography (CEX) and determination of oxidised species using C_4 reversed-phase chromatography, to examine whether the protein structure was also altered following stress exposure.

For temporal stress analysis, aliquots containing an equal concentration (500 µg) of the chimeric IgG1 mAb in individual sample tubes were held at 35°C for up to 15 days. Samples were collected for analysis on days 0, 1, 2, 3, 5, 7, 10, 12 and finally day 15 by removing a sample tube from the incubator and immediately freezing it to -80°C until the time of analysis. For each time point comparative analysis was performed relative to an aliquot of the mAb that was held at 4°C for the equivalent duration. The *N*-glycans released from the mAb stored under recommended conditions labelled with $^{12}C_6$ 2-AA and those released from the stressed mAb labelled with $^{13}C_6$ 2-AA. Resulting data for the temporal stress analysis is depicted in Fig. 3 (A) which demonstrates excellent stability of the *N*-linked oligosaccharides present on the mAb over time. CQA evaluation using replicate non-deglycosylated mAb aliquots revealed a slight increase in aggregation over the same time course with an increase in the percentage of the peak corresponding to the mAb dimer in the SEC chromatograms from 0.2% on day 0 to 0.4% on day 15, Supplementary Fig. 2 (A). No significant alterations in the distribution of charged variants or oxidised species were determined over the duration tested using CEX and reversed-phase C_4 chromatography, respectively, Supplementary Fig. 2 (B&C).

Subjection of the mAbs to heat stress was performed by exposure of aliquots of the IgG1 to increasing temperature, in 10°C intervals for 48 hours. Again, *N*-glycans released from the mAb stored under recommended conditions were labelled with $^{12}C_6$ 2-AA and those released from the stressed mAb labelled with $^{13}C_6$ 2-AA. Data generated from the comparative *N*-glycan analysis is shown in Fig. 3 (B), wherein excellent stability of the oligosaccharides present was observed up to and including exposure to 45°C. Further increases in temperature above 55°C resulted in a difference in the relative light to heavy ratio, suggesting hydrolysis or degradation of the *N*-glycans on the mAb as a result of exposure to elevated temperature. Alterations were also observed for the non-deglycosylated mAb following heat stress, aggregation was observed to increase (percentage dimer present increased from 0.2 – 0.9%), higher levels of acidic variants were detected using CEX with increased heat exposure (15.1-19.9% for the most acidic CEX peak). An increase in

the levels of oxidised species was also observed (11.3-15.2%), Supplementary Fig. 3. Decreased stability of the protein and also the glycans with increasing temperature is expected. IgG1 is known to begin to unfold at ≥60°C resulting in increased interaction and aggregation of the partially unfolded protein.[35, 36] Increased levels of acidic variants and other post translational modifications (PTMs) were also noted with exposure of mAbs to elevated temperatures.[35]

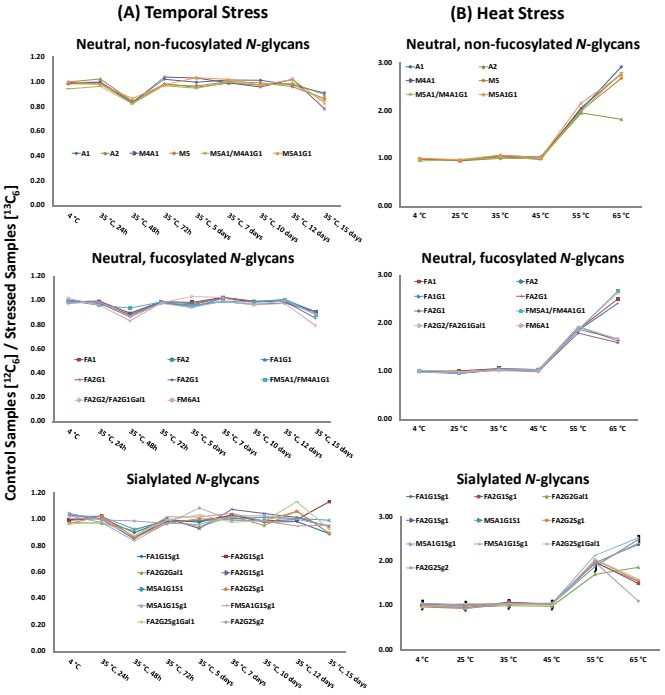

Fig.3: (A) Temporal stability plots for the *N*-glycans present on the chimeric IgG1 following incubation at 35°C for the times indicated. The oligosaccharides are separated into three individual plots based upon structural features for clearer visualisation. (B) Relative light to heavy ratios for the mAb samples that were subjected to thermal stress as indicated. No differences were detectable at temperatures <45°C, at temperatures >45°C reduced quantities of certain *N*-glycans were determined in the stressed samples, suggesting oligosaccharide hydrolysis at high temperatures. Stressed samples were labelled with $^{13}C_6$ 2-AA, non-stressed samples were labelled with $^{12}C_6$ 2-AA.

Irradiation of the IgG1 with UV and visible light (350 nm and 575 nm) was observed not to induce any significant alterations in the levels of N-glycans present on the mAb when compared to replicate aliquots that had been maintained in the dark, Supplementary Fig. 4 (A&B). Similarly, no significant changes in protein stability were observed either, with the relative areas of all peaks in the SEC and CEX data remaining unchanged, Supplementary Fig. 5 (A&B). A significant increase in the level of oxidised product was observed on the mAb following irradiation at 350 nm, Supplementary Fig. 5 (C). Increased oxidation of mAb drug candidates was previously reported following UV irradiation, the majority of methionine residues present in the primary sequence and a large proportion of tryptophan residues were oxidised following photosensitisation.[37] These modifications were thought to have arisen due to increased levels of reactive oxygen species present in solution resulting from initial photoactivation of tryptophan residues to yield *N*-formylkynurenine (NFK).[37] pH stress, both acidic and basic shocks induced through the introduction of 6 M HCl or 6 M NaOH solutions followed by incubation at room temperature for 48 hours resulted in complete denaturation of the mAb with a distinct white precipitate visible in the sample vials following incubation, thereby inhibiting all further evaluations of both the *N*-glycosylation and the protein level CQAs.

Conclusions

The development and application of a twoplex method for comparative glycomics of *N*-glycosylation present on mAbs is described based upon the differential labelling of samples for comparison with either 'light' $^{12}C_6$ 2-AA or 'heavy' $^{13}C_6$ 2-AA prior to hydrophilic interaction UPLC with sequential fluorescence and accurate mass QToF-MS/MS detection. The method was found to be linear and precise with expected light heavy relative ratios of approximately unity determined for six technical replicate analyses of the *N*-glycans present on polyclonal serum IgG, normalised at the protein concentration level. Application of the method for lot-to-lot comparability analysis of a commercial IgG1 mAb revealed altered levels of certain *N*-glycans between the two drug product lots. Excellent stability of the *N*-glycans present on the mAb, even following exposure to forced degradation conditions, was observed relative to samples stored under conditions recommended by product information sheet. The presented twoplex stable isotope labelling approach for quantitative glycomics is ideally suited for comparability analyses due to the minimisation of technical variation and operator subjectivity from the analysis, combined with the ability to perform full quantitative structural characterisation of all oligosaccharides, including isomeric species, present in the glycan pool. This method builds on widely used chemistry and concepts but adds an additional dimension through the application of the stable isotope labelling based quantitation. The described platform can be readily employed for physiochemical demonstration of molecular comparability following process or manufacturing changes, or for the assessment of similarity between innovator and biosimilar candidate molecules.

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Notes and references

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Electronic Supplementary Information (ESI) available: including detailed methods and experimental procedures and analytical data for the forced

degradation studies as mentioned in the main text. See DOI: 10.1039/c000000x/

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