

Analytical characterization of ch14.18

A mouse-human chimeric disialoganglioside-specific therapeutic antibody

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Key words: monoclonal antibodies, chimeric antibody, characterization assays, SEC-MALS, imaged cIEF, N-glycoprofiling, N-glycan analysis, FcγRIIIA:ch14.18 interaction, surface plasmon resonance, complement-dependent cytotoxicity

Abbreviations: ADCC, antigen-dependent cellular cytotoxicity; APTS, 8-Aminopyrene-1,3,6-trisulfonate; BSA, bovine serum albumin; CbELISA, cell based ELISA; Cat #, catalog number; CE, capillary electrophoresis; CDC, complement-dependent cytotoxicity; cIEF, capillary isoelectric focusing; CV, coefficient of variation; DAD, diode array detector; DLS, dynamic light scattering; ELISA, enzyme-linked immune-sorbent assay; FACS, fluorescence-activated cell sorter; FDA, Food and Drug Administration; FITC, fluorescein isothiocyanate; GD2, disialoganglioside; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; IND, investigational new drug; LIF, laser induced fluorescent; LS, light scattering; mAb, monoclonal antibody; MALS, multi-angle light scattering; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; MW, weight average molecular weight; NCI, National Cancer Institute; PBS, phosphate buffered saline; PI, propidium iodide; PNGase F, peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase; RH, hydrodynamic radius; RI, refractive index; RU, response unit; SD, standard deviation; SEC, size exclusion chromatography; SPR, surface plasmon resonance; UV, ultra violet

Ch14.18 is a mouse-human chimeric monoclonal antibody to the disialoganglioside (GD2) glycolipid. In the clinic, this antibody has been shown to be effective in the treatment of children with high-risk neuroblastoma, either alone or in combination therapy. Extensive product characterization is a prerequisite to addressing the potential issues of product variability associated with process changes and manufacturing scale-up. Charge heterogeneity, glycosylation profile, molecular state and aggregation, interaction (affinity) with Fcγ receptors and functional or biological activities are a few of the critical characterization assays for assessing product comparability for this antibody. In this article, we describe the in-house development and qualification of imaged capillary isoelectric focusing to assess charge heterogeneity, analytical size exclusion chromatography with online static and dynamic light scattering (DLS), batch mode DLS for aggregate detection, biosensor (surface plasmon resonance)-based Fcγ receptor antibody interaction kinetics, N-glycoprofiling with PNGase F digestion, 2-aminobenzoic acid labeling and high performance liquid chromatography and N-glycan analysis using capillary electrophoresis. In addition, we studied selected biological activity assays, such as complement-dependent cytotoxicity. The consistency and reproducibility of the assays are established by comparing the intra-day and inter-day assay results. Applications of the methodologies to address stability or changes in product characteristics are also reported. The study results reveal that the ch14.18 clinical product formulated in phosphate-buffered saline at a concentration of 5 mg/ml and stored at 2–8°C is stable for more than five years.

Introduction

Monoclonal antibodies (mAbs) and related molecules (e.g., Fc-fusion proteins, antibody fragments) comprise the majority of recombinant protein therapeutics under clinical study¹ with many being evaluated in the clinic for oncology indications.² The recent success of therapeutic mAbs is due to the development of

chimeric and humanized mAbs, which are less immunogenic, exhibit longer half-lives and efficiently promote effector functions in humans compared with the murine precursors.^{3,4}

General biological product standards are described in the US Code of Federal Regulations, 21 CFR Part 610, which includes guidelines on potency, purity and identity. Appropriate control of critical quality attributes is a common review issue for

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both US Food and Drug Administration (FDA) investigational new drug (IND) and license applications. Adequate characterization of mAb product quality attributes is also important to facilitate product development. For example, scale-up and other manufacturing changes are common during the development of biotechnology products under IND, and the FDA encourages continuous improvement throughout the product life cycle. It is critical that sufficient comparability is demonstrated to ensure that the manufacturing changes have not affected the safety or efficacy of the product. The ability to sufficiently compare product attributes is dependent on a variety of methods needed to characterize different attributes or the orthogonal methods used to characterize the same attribute.^{5,6}

Second, a thorough understanding of product attributes can also facilitate a “quality by design” (QbD) approach,⁶ which is a more systematic approach to product development than conventional methods. Woodcock et al. recently published a historical perspective on the FDA’s evaluation of comparability, describing examples of the FDA’s assessments of product manufacturing changes, second-generation products and follow-on protein products. An important factor in these assessments is the degree to which structural similarity may be evaluated and the extent to which the mechanism of action is understood.

mAbs, mainly the immunoglobulin G1 (IgG1), have been successfully employed as pharmaceuticals and diagnostics. Like other protein therapeutics, mAbs are prone to undergo a variety of physical and chemical changes.⁸ Currently, protein aggregation is considered a potential cause of immunogenicity of protein therapeutics in patients.^{9,10} Assessing pharmaceutical quality and understanding the immunogenic effects of mAbs thus involves quantification of the level of aggregates and determination their physicochemical and structural properties.

Therapies based on mAbs that specifically target disialoganglioside (GD2) on tumor cells may improve treatment results for high-risk neuroblastoma. A mouse-human chimeric form of the 14.18 murine anti-GD2 mAb, designated ch14.18, was created to reduce the immunogenicity associated with the murine antibody. This chimeric antibody was less immunogenic and more effective than the murine parent antibody 14G2a in mediating lysis of neuroblastoma cells with natural killer (NK) cells.¹¹ The ch14.18 antibody has undergone clinical testing as a single-agent therapy, or in combination with cytokines. Simon et al.¹² have published their results using standard induction treatment (chemotherapy with autologous stem cell rescue) for children and infants with stage 4 neuroblastoma, followed by consolidation with ch14.18 antibody for 5 d every 2 mo vs. 12 mo of oral maintenance chemotherapy or no further therapy. In patients <1 y old, there was no significant difference in event-free survival or overall survival in the three consolidation groups, with an overall survival of >90%. In patients >1 y old, the 3-y overall survival of ch14.18 treatment was superior to maintenance therapy or no additional therapy ($p = 0.018$), although there was no difference in event-free survival.¹³

During the early clinical feasibility evaluation stages, the ch14.18 clinical lots were released with the standard characterization assays, including SDS-PAGE and high-performance liquid

chromatography-size-exclusion chromatography (HPLC-SEC), which assess product purity; UV (UV) absorbance at 280 nm to assess protein quantification and a capture ELISA on a GD2-coated plate to define specificity and biological functional activity. Additional characterization assays were introduced as clinical development progressed. Initial isoelectric profiling studies revealed that the ch14.18 has isoforms with $pI > 8.0$ (alkaline pH range). An imaged capillary isoelectric focusing (cIEF) assay was developed using the Convergent iCE₂₈₀ system, and this assay was then introduced as a product release and characterization assay. Following initial clinical trials, the National Cancer Institute (NCI) supported the continued production of ch14.18, necessitating process changes and potential manufacturing scale-up. To address the comparability during the process changes and manufacturing scale-up, it was essential to include additional characterization assays and biological or functional activity assays.

Here, we describe the stepwise expansion of analytical characterization assays to meet the requirement for manufacturing scale-up and associated process changes. Although most of the characterization assays are not fully validated to the extent that is required for product release tests with assigned acceptance criteria set, the assays should be qualified for their intended use, which is to establish product comparability. The characterization assays described here include imaged cIEF profiling, N-glycoprofiling, SEC with online-light scattering, dynamic light scattering (DLS), Fcγ receptor interaction studies using BIAcore affinity measurements and MTS dye-based colorimetric cell proliferation inhibition assay to measure complement-dependent cytotoxicity (CDC).

Results

Imaged cIEF. A typical cIEF profile of ch14.18 used in early clinical development studies is shown in **Figure 1**. The profiles of four different lots of ch14.18 manufactured using the same process are overlaid. Component peaks at equilibration are captured by digital photographic imaging at 280 nm UV light. The integration of component peaks was feasible, and computation of relative peak areas of the component peaks gave much better reproducibility compared with the computation of absolute peak areas from intra- and inter-day runs. **Table 1** shows the pI values and relative peak areas of the individual component peaks of different lots compared with the total peak areas of the individual run. As evident from **Table 1**, the relative peak areas were consistent between the lots (~30% for each of the two major components peaks 4 and 5, ~18% for peak 3 and between 0 and 9% for the other four component peaks). The percentage coefficient of variation (CV%) of the relative peak areas between lots for the major peaks was <5%, but >10% for the minor component peaks due to the low signals. **Table 2** shows the pI values and relative peak areas of a reference standard lot over a 2-y period (inter-day variation/stability). The data reveal the consistency, reproducibility and applicability of this technology for the characterization and stability monitoring of ch14.18 for charge heterogeneity.

This methodology was successfully applied to demonstrate lot-to-lot consistency. Different lots of ch14.18 manufactured at different times using the same process showed the identical profile, and the profile was consistent during storage in 2–8°C for extended time periods (>5 y). The profile and percentage of relative peak areas of the reference lot of ch14.18 over a one-year period is shown in Table 2.

We also utilized the procedure for monitoring the stability of ch14.18 during shipping to clinical sites and storage at different clinical sites by analyzing a returned sample. Table 3 shows the consistency in the pI profile of retains from post-use vials from three different clinical sites and the in-house stability control sample.

Size exclusion chromatography with multi-angle light scattering system (SEC-MALS). SEC-MALS is the technology that emerged during the last decade for analysis of size, molecular weight (MW), size distribution heterogeneity and detection of aggregates in biotechnology products, especially antibody and antibody conjugates. We previously reported the applicability of this methodology using the online quadruple detection system from Viscotek (presently Malvern, MA).¹⁴ Currently, we use the Wyatt Technology DAWN HELEOS light scattering (LS) and refractive index (RI) detectors coupled with Agilent 1100 HPLC-SEC with pump and UV detector to determine size and size heterogeneity. A typical SEC-MALS chromatographic profile of ch14.18 is shown in Figure 2A and B. Because the intended use is primarily focused on consistency and reproducibility of methodology in determining the molecular parameters like MW, polydispersity of molecular components, hydrodynamic radius (R_H) as well as molecular aggregation, attention was focused on assay reproducibility. The reproducibility of the methodology for MW estimates is demonstrated in Table 4A, in which MW values of the system suitability marker bovine serum albumin (BSA) from three dozen independent runs conducted over a 3-y period are tabulated. The consistency in the molecular parameter estimates for the same lot of ch14.18 from ten independent runs over a 2-y period is shown in Table 4B.

Having established the consistency in the molecular parameter estimate, we used the methodology to evaluate potential changes in molecular properties and the aggregation of ch14.18 under the conditions of the infusion used in clinical setup. A mock infusion study was conducted, and samples collected at different times were analyzed. An infusion modeling study was conducted at a two-dose concentration. While the method worked very well in the high-concentration infusion model study, there were issues related to method sensitivity at the low-concentration of the mock infusion study (data not shown). The results, which are summarized in Table 4C, showed that the molecular properties, protein concentration (total UV 280 nm peak area), total LS intensity peak area, the monomer and dimer contents, as well as the estimated MW of the product peak, remained the same,

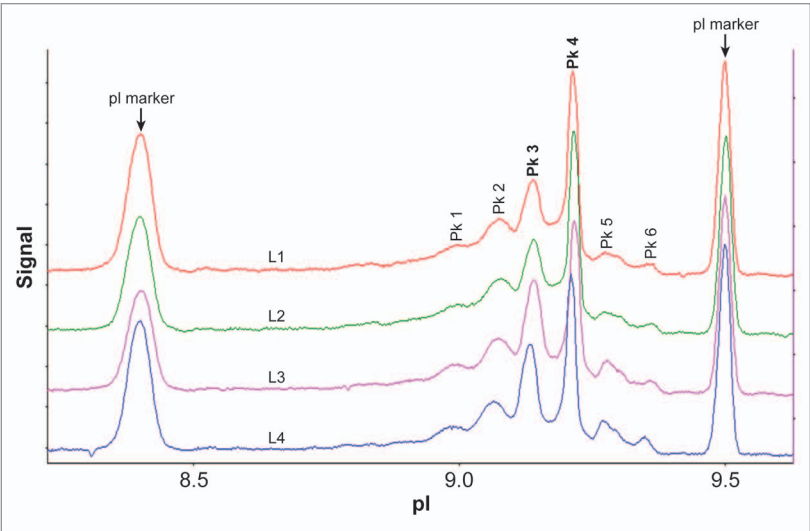


Figure 1. Imaged cIEF profiling of ch14.18. Imaged cIEF profiling was performed as described in Materials and Methods. Four different lots of ch14.18 (labeled L1, L2, L3 and L4) manufactured at different times using the same manufacturing procedure were analyzed. Overlay of the profiles is shown.

Table 1. pI and relative % peak area for different clinical lots of ch14.18							
Ch14.18 Lot #	A: pI of Peaks						
	Pk 1	Pk 2	Pk 3	Pk 4	Pk 5	Pk 6	Pk 7
Lot 1	8.87	8.94	9.00	9.08	9.14	9.20	9.29
Lot 2	8.88	8.95	9.00	9.08	9.15	9.21	9.27
Lot 3	8.87	8.94	9.02	9.08	9.14	9.20	9.28
Lot 4	8.87	8.95	9.03	9.08	9.14	9.20	9.29
B: Relative Peak Area (% Total Area)							
Lot 1	3.0	7.9	19.2	29.5	29.2	9.2	1.9
Lot 2	3.0	7.3	16.7	30.5	30.9	9.3	2.3
Lot 3	3.0	10.5	18.8	31.4	30.7	4.5	1.1
Lot 4	3.7	9.4	18.0	31.2	30.1	6.3	1.3
Average	3.18	8.78	18.18	30.65	30.23	7.33	1.65
SD	0.35	1.45	1.10	0.86	0.76	2.34	0.55

and were within the limits of anticipated assay variations. We also monitored the stability and molecular properties of the samples retained post-administration (hereafter referred to as post-use samples) and shipped back from three clinical sites. The post-use samples showed no differences from the in-house control samples from the same lots. There were no changes in molecular properties or aggregation of the samples (data not shown).

Batch-mode dynamic light scattering (DLS). While SEC with an online UV/LS/RI detector provides valuable information on the molecular state and molecular aggregation, the methodology is limited because the sample may become diluted as it goes through the SEC column. In addition, very high MW aggregates or particulates may potentially be removed before column entry, resulting in a sample that is not representative of the material administered to patients. Batch-mode DLS has the advantage of enabling the researcher to analyze the sample in the

Table 2. Inter-day variations in the relative peak areas of the components of ch14.18 reference standard lot

Date of experiment	Pk 1 (8.85–8.87)	Pk 2 (8.93–8.97)	Pk 3 (8.97–9.05)	Pk 4 (9.08–9.10)	Pk 5 (9.14–9.21)	Pk 6 (9.18–9.35)	Pk 7 (9.26–9.35)	Pk 8 (9.31–9.34)
10-22-09	*nd	10.1	18.0	27.6	31.8	9.5	3.0	nd
01-14-10	3.0	7.9	19.2	29.5	29.2	9.3	1.9	nd
01-28-10	3.2	7.4	19.4	30.3	29.5	8.5	1.7	nd
03-10-10	3.4	9.1	18.6	28.9	27.9	9.4	2.0	nd
03-18-10	4.1	7.5	17.7	30.3	28.9	9.0	2.2	0.2
04-06-10	2.0	8.6	20.1	28.9	29.4	8.3	2.7	nd
04-13-10	1.8	8.6	18.9	29.2	29.0	9.1	3.4	nd
04-16-10	1.9	8.5	20.1	29.0	29.5	8.3	2.7	nd
05-03-10	3.0	8.2	20.5	28.5	29.8	8.8	1.1	nd
06-08-10	4.1	9.6	19.9	27.7	26.7	10.0	2.1	nd
06-28-10	3.3	10.5	19.0	27.4	29.0	9.4	1.3	nd
06-30-10	2.4	9.4	18.9	28.4	29.1	8.6	3.2	nd
07-07-10	3.2	10.6	18.8	28.3	27.9	9.8	1.5	nd
10-12-10	3.5	8.7	18.2	29.1	28.0	9.4	2.5	0.7
10-13-10	3.1	9.3	18.5	29.6	29.2	8.8	1.4	nd
10-15-10	4.4	9.7	18.6	26.8	29.2	9.4	1.8	nd
10-20-10	4.0	9.3	18.3	27.4	28.3	9.5	2.2	1.0
10-20-10	4.2	9.4	18.5	27.4	28.4	9.3	2.0	0.8
11-04-10	2.85	8.4	19.1	29.5	29.9	8.5	1.8	nd
11-10-10	3.3	7.9	20.1	28.3	28.8	8.5	2.4	0.7
11-23-10	3.8	8.7	18.4	28.0	28.4	8.5	2.4	0.7
02-03-11	3.0	8.8	19.3	27.6	29.5	9.9	2.0	nd
03-16-11	3.3	9.4	19.2	28.7	29.0	8.9	1.5	nd

*nd, not detected; Pk, component peak or signal. The observed pl range of the Pk is shown in parentheses.

cuvette under conditions that mimic those of the actual material in the vial.

Typical DLS histograms for ch14.18 are shown in **Figure 2C**. Eight different sample signals are overlaid in the figure. These eight samples include the reference standard, two other lots retained in-house under controlled stability monitoring, as well as three samples retained after clinical use and returned from the clinical site. Also included are two 0.2 μ m filtered samples. As evident from the figure, the DLS signals are superimposable. The weighted average MW estimated from the intensity-weighted plots ranged from 168–170 kDa (CV = 0.52%) for the eight samples run, with (R_h) values from 5.3–5.4 nm (CV = 0.66%). The mass-weighted plots showed MWs ranging from 144–149 kDa (CV = 1.18%), with an estimated R_h value of 5.0–5.1 nm (CV = 0.7%). The variations in MW and R_h values were well within the anticipated assay variations. DLS analysis of post-use samples retained from different clinical sites also revealed no sign of molecular aggregation or any differences in the DLS histograms compared with the stability storage vials kept in-house under controlled conditions.

N-glycoprofiling. The normal phase HPLC method of N-glycoprofiling by 2-aminobenzoic acid labeling of the carbohydrates¹⁵ was adapted in-house and qualified to meet the requirement for establishing product consistency or differences.

Figure 3A and B shows the HPLC N-glycoprofile of the ch14.18 reference standard and another lot of ch14.18. The results illustrate the run-to-run and lot-to-lot consistency of the profile of ch14.18 lots that were manufactured using the same process.

N-glycan analysis by CE. **Figure 4A** shows the N-glycan analysis profile for the ch14.18 reference standard, a human IgG1(κ) control and a murine IgG1(κ). Recombinant human IgG1s such as ch14.18 are generally glycosylated at the heavy chain Asn297 residue by a core-fucosylated, biantennary glycan with variable galactose content (G0F, G1F and G2F).¹⁶ There are four N-glycan component species detected in the ch14.18 core corresponding to G0F, G1F (two components that correspond to G1F.3 and G1F.6) and G2F. The glycan profile of ch14.18 is comparable to that of the control hIgG1(κ), and both have a relatively higher abundance of G1F compared with murine IgG1(κ), while murine IgG1(κ) has a relatively higher G0F. This reference lot of ch14.18 was analyzed in 17 different experiments performed over an 18-mo period (42 individual injections total). The percentage relative contributions of these glycans in different injections are shown in **Figure 4B**. There is excellent day-to-day and run-to-run consistency.

Ch14.18:FcyIIIa interaction [surface plasmon resonance (SPR)]. The conditions for the kinetic runs were optimized after evaluating the data from different amounts of FcyIIIa receptor

immobilized on the sensor chip surface (data not shown); mass transport effects were ruled out using different flow rates (30 $\mu\text{L}/\text{min}$, 50 $\mu\text{L}/\text{min}$ and 75 $\mu\text{L}/\text{min}$) under optimized conditions. Highly reproducible sensorgrams were obtained for each concentration of the ch14.18 reference standard. The robustness of the Fc γ IIIa receptor capability to bind ch14.18 in a reproducible way was established for multiple cycles of antibody binding and regeneration (>100 cycles) by including a 5 μM sample of the reference standard at the beginning and end of the kinetic runs (each of which consisted of 19 sample injections with 19 subsequent regenerations). The difference in the maximum binding obtained for these control samples at the beginning and end of the kinetic runs was <1%.

The equilibrium dissociation constants obtained under the optimized conditions (flow rate 30 $\mu\text{L}/\text{min}$; receptor density of ~ 300 RU; association time of 40 s and dissociation time of 300 s; volume of regeneration buffer of 15 μL) are shown in Table 5. Figure 5A shows a typical dose-dependent binding of ch14.18 (reference standard) and a control hIgG1 (Fig. 5B) to the Fc γ IIIa receptor as monitored by SPR technology. The study was performed under optimized conditions, which show monovalent interaction kinetics. Table 5 shows the kinetic parameters from different independent experiments performed on different days using a reference standard lot of ch14.18. For comparison and as a positive control, kinetic runs were performed using a commercial IgG1(κ). The sensorgrams obtained for the kinetic runs with IgG1(κ) are shown in Figure 5B. The equilibrium dissociation constant obtained for the ch14.18 reference standard and the control IgG1(κ) were very similar (~ 0.5 μM).

Complement-dependent cytotoxicity (CDC). The ch14.18 exhibits CDC as well as antigen-dependent cellular cytotoxicity (ADCC) on GD2-expressing neuroectodermal cells.¹⁷⁻²⁰ While ADCC is speculated to be better correlated with in vivo function, the development and qualification of ADCC as a product release assay is difficult. Therefore, CDC may be considered as an alternate in vitro potency marker assay for product release. The ^{51}Cr release assay using ^{51}Cr -labeled target cells¹⁸ is the most commonly used method to assess CDC and ADCC.

We developed a MTS-based cell proliferation inhibition assay to monitor CDC effects of ch14.18. Figure 6A shows the effect of ch14.18 on the cell proliferation of three different cell lines in the presence of human complement sera. Figure 7B shows the GD2 expression on the three different cell lines as probed by fluorescence-activated cell sorting (FACS) using fluorescein isothiocyanate (FITC)-labeled ch14.18; Figure 7A shows the GD2 binding on these cells monitored by cell-based (Cb) ELISA. The NMB7 and IMR 32 neuroblastomas showed a high level of GD2 expression, and ch14.18 induced effective CDC, whereas ch14.18 had no CDC effect on U87MG cell line, which showed little or no GD2 expression. The CDC effect of ch14.18 was GD2-specific, as a control human IgG did not have a CDC effect on these cell lines (Fig. 6B). Rabbit complement sera also showed similar ch14.18-induced CDC (data not shown).

After demonstrating the GD2 specificity of the CDC effect of ch14.18, experiments were performed to optimize the CDC assay conditions, and an assay procedure was developed using

Table 3. Relative peak area % of total area for each integrated peak of all the post-administration clinical vial samples shipped back from clinical sites

Sample #	Relative % peak area					
	Pk 1	Pk 2 + 2'	Pk 3	Pk 4	Pk 5	Pk 6 + 6'
1	1.7	26.7	29.4	34.1	7.2	0.8
2	1.5	26.8	28.2	35.7	7.1	0.7
3	*nd	23.5	30.9	37.0	7.5	1.2
4	1.4	27.5	25.9	37.1	6.9	1.1
5	1.6	26.2	28.7	34.6	7.5	1.4
6	1.8	27.0	27.8	34.5	7.7	1.2
7	1.4	25.6	29.0	33.1	9.6	1.3
8	1.5	27.0	29.1	33.2	7.6	1.5
9	1.7	28.0	27.7	33.5	7.7	1.4
10	2.2	23.8	30.3	32.0	9.5	2.3
11	1.4	26.1	29.9	32.1	9.2	1.3
12	1.7	26.6	29.0	32.8	7.9	2.1
13	2.4	25.1	30.0	32.9	7.5	2.0
14	2.1	25.8	29.5	33.2	7.6	1.8
15	2.5	26.0	29.3	33.5	7.7	0.9
16	1.6	27.9	29.5	33.0	6.2	1.7
17	1.9	25.9	30.1	34.1	6.8	1.1
18	1.5	25.9	30.4	33.7	6.5	2.0
19	1.7	25.8	30.8	33.3	6.8	1.6
20	0.7	26.7	30.3	34.9	6.1	1.3
21	1.3	28.0	27.3	36.1	6.4	1.0
22	1.8	27.7	27.6	35.0	6.9	1.1
23	1.6	26.4	30.3	33.9	6.4	1.4
24	2.0	27.2	29.2	32.2	7.7	1.7
25	2.6	27.7	28.5	32.3	7.6	1.4
26	2.2	27.7	22.6	37.8	8.3	1.5
27	2.1	27.8	26.9	33.8	7.7	1.7
28	2.2	27.7	26.8	34.0	7.8	1.6
29	2.1	27.9	28.2	32.1	8.0	1.7
30	2.7	27.7	26.1	33.9	8.2	1.4
31	2.6	26.4	28.9	32.6	7.7	1.9
32	3.2	24.8	28.4	31.2	10.7	1.7
Average	1.89	26.59	28.64	33.85	7.63	1.46
SD	0.51	1.18	1.72	1.57	1.02	0.38
**CV%	26.98	4.44	6.01	4.64	13.38	26.03

*nd, not detected. The % peak areas were calculated by EZChrom vs 6.8 software used in iCE₂₈₀ data analysis. The 32 samples include post-use return vials from clinical sites, control in house samples and also include samples after 0.2 micron filtration. Minor secondary peaks 2' and 6', integrated as separate peaks in some samples, have been included in peak 2 and 6, respectively, in the table. **The CV% of minor peaks (small peak areas) is high.

the optimized assay conditions with NMB7 cells. Experiments were performed to evaluate assay variations (intra-day within plate; intra-day inter-plate; and inter-day variations). Significant intra-day inter-plate and inter-day variations in the ED₅₀ values

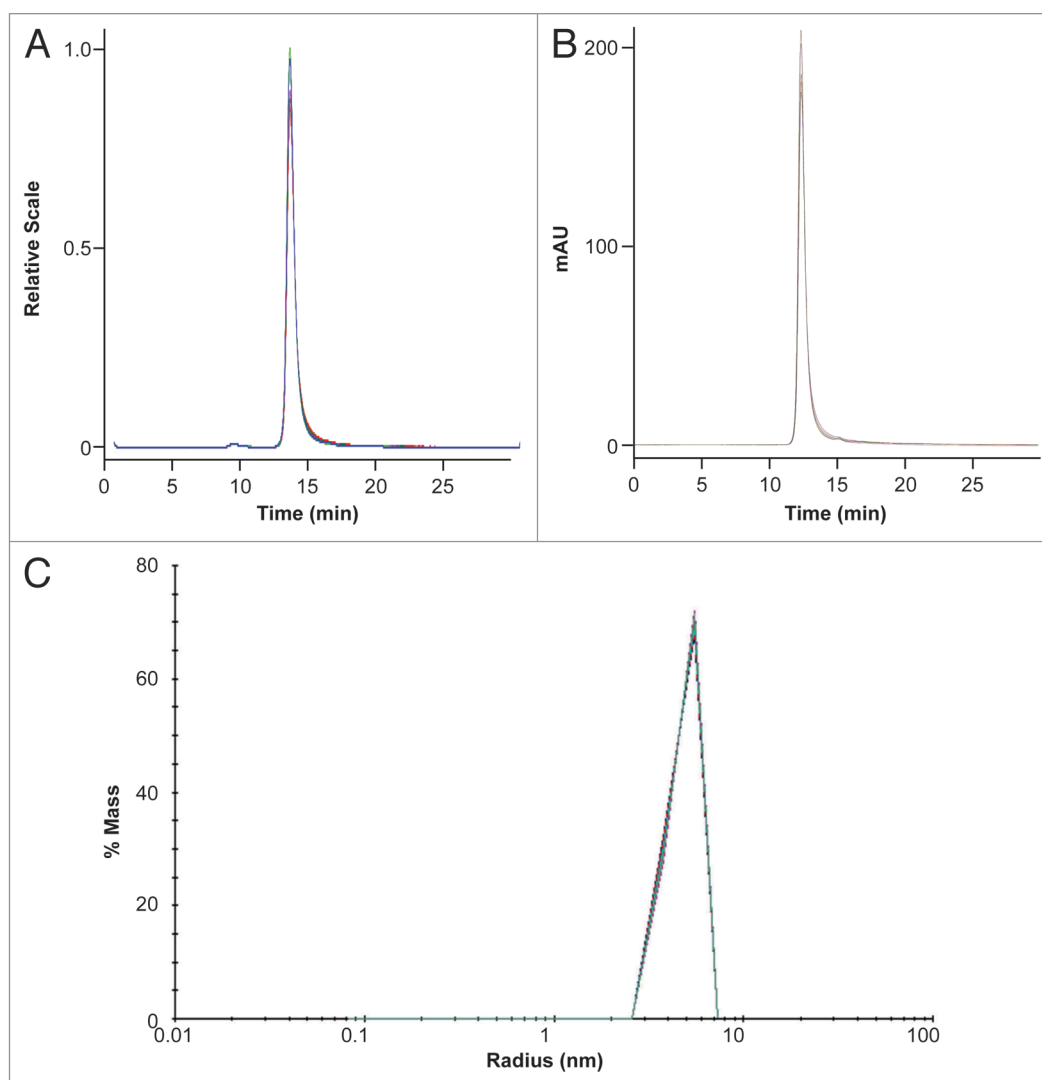


Figure 2. Analytical SEC-MALS profile of ch14.18. SEC-MALS and data analysis are performed as described in Materials and Methods. (A) is light-scattering signal overlay and (B) is UV280 signal overlay. (C) is batch mode DLS of ch14.18. Shown is a typical DLS overlay of the % mass vs. radius plot of ch14.18. DLS of eight sample vials (reference standard, samples control vials retained under stability control in-house and sample material retained after use in the clinic and returned for test). The overlay includes samples scanned directly and after filtration through 0.2 μ m filtration.

were noticed (Table 6A). These assay variations suggest that the CDC assay can be used for comparing the relative activity of a test article lot to that of a reference lot when both test article and reference lot are run on the same day within the same plate (Table 6B). The validity of the CDC assay to monitor stability was assessed by performing a ch14.18 stress study with low pH and high temperature treatment. Analytical SEC was performed side by side to evaluate changes in molecular state (Fig. 8A and Table 7). Under the stressed conditions, low pH (acid treatment) had minimal effect on the molecular state, and the CDC was within the assay variation limit, although there was a slight decrease in CDC of the acid-treated sample (Fig. 8B) consistent with the SEC result. With the heat-treated sample, there was a noticeable change in the molecular state and the total peak area in the SEC, suggesting soluble aggregates and potential insoluble aggregates were removed by centrifugation. The CDC

results showed significant reduction in the CDC, consistent with the SEC results (Fig. 8B). The CDC activity of ch14.18 was also verified using propidium iodide (PI) staining of cells before and after treatment with ch14.18 plus CDC, ch14.18 alone or CDC alone (Fig. 8C).

Discussion

During the last two decades, substantial changes have occurred in the development of biotherapeutics. Regarding regulatory requirements, the focus on the relevant properties of specified biologicals such as therapeutic mAbs has changed from manufacturing control to quality control. The International Harmonization Committee, QbD and biosimilar development guidelines focus on quality attributes of biomolecules of higher-order structures. Analytical methodologies for characterization of quality and

quality attributes of drugs that address the comparability of product before and after changes that may occur during clinical development and post-approval (e.g., process and manufacturing changes associated with scale-up, formulation and storage) and, most recently, comparability assessment of biosimilar drugs, are the topics of much discussion by pharmaceutical firms and regulatory agencies. Analytical characterization efforts are focused on product quality such as identity, purity, specificity and functional activity; changes in higher-order structures that contribute to micro-heterogeneity; conformation changes; changes in molecular states due to aggregation; and the effect of structural and conformation changes on the efficacy, toxicity and immunogenicity of the drug.^{5,21,22} Characterization assays used for product comparability should be qualified to prove the applicability for intended use and to address the issues of reliability, consistency and reproducibility.

The analytical methodologies discussed here include imaged cIEF for charge-based micro-heterogeneity; SEC-MALS for molecular weights and soluble molecular aggregates determination; DLS for molecular size homogeneity and aggregation; N-glycoprofiling and CE-based N-glycan analysis for glycosylation consistency or variation. BIAcore-based analyses of FcγRIIIA:ch14.18 interaction and CDC were used as in vitro methods to explore the potential biological or functional activity. A CbELISA utilizing the GD2-expressing melanoma cell line M21/P6 was used as the in vitro functional activity assay.²³

Imaged cIEF has emerged as the solution phase cartridge-based electrophoretic method in which the charge-based separation of heterologous molecular species is captured by a digital camera at UV 280 nm.^{24,25} We demonstrated the applicability of this methodology for isoelectric profiling of mAbs and the qualification and validation of the method in earlier publications.^{24,25} The method works very satisfactorily with ch14.18. This antibody displayed charge-based micro-heterogeneity with more than five components in the pI range of 8.8–9.4. The imaged cIEF profile showed excellent consistency from run to run, intra-day and inter-day, over an extended period of time (more than 7 y). Different lots of ch14.18 produced and purified using the same process showed a consistently comparable profile with respect to the component peaks and to relative peak areas of the individual component peaks compared with total peak area. The consistency and reproducibility of the reference standard lot demonstrated here suggest the applicability of the method for comparing product lots during changes in the production process. In fact, a change in the upstream production process involving a change in media components, without any other changes in the process, resulted in quantitative changes in the component peak distribution of the product (data not shown). SEC is one of the most widely used analytical technologies used for demonstrating the purity and stability of biopharmaceutical drugs. During the last decade, coupling of SEC online with LS and concentration detectors has proven to be a powerful technique for determining absolute MWs, size and polydispersity, as well as for detecting molecular aggregates. The dependence of the LS signal on molecular size or MW made the LS detector much more sensitive to detection of soluble molecular aggregates that can enter the

Table 4A. Inter-day variation in the molecular weight estimates of albumin standard

Experiment #	Date	MW (Da)
1	10-30-08	66,510
2	12-03-08	65,680
3	12-09-08	65,710
4	01-05-09	65,730
5	03-03-09	65,660
6	03-04-09	65,750
7	03-05-09	65,790
8	03-30-09	66,670
9	04-15-09	66,770
10	04-13-09	66,830
11	07-06-09	66,980
12	07-08-09	66,880
13	07-28-09	66,610
14	09-16-09	65,400
15	09-22-09	65,050
16	10-20-09	65,060
17	10-30-09	65,130
18	11-19-09	66,770
19	01-12-10	65,730
20	02-02-10	65,650
21	02-17-10	65,450
22	03-28-10	65,480
23	05-07-10	65,320
24	05-24-10	66,750
25	06-04-10	65,050
26	06-14-10	66,210
27	06-23-10	65,000
28	08-16-10	66,160
29	11-16-10	63,920
30	1-17-10	65,220
31	01-26-11	64,660
32	02-17-11	64,130
33	02-18-11	64,510
34	02-19-11	63,890
35	03-01-11	64,320
36	03-03-11	64,440
*Average MW		65,600
SD		888

*Average of the 36 estimates.

sizing column. Sharma and Kalonia²⁶ recently summarized the analytical methodologies currently in use for detection of aggregates in proteins. We previously demonstrated the application of this methodology using a triple-detector system from Viscotek (Malvern) with an Agilent HPLC system with diode array detector.¹⁴ Here, we used LS and RI detectors from Wyatt Technology coupled with an Agilent HPLC system with diode array detector.

Table 4B. Estimated molecular parameters of ch14.18 reference standard from SEC-MALS experiments

Date of Experiment	MW (Da)	RH (nm)	Pd (MW/Mn)
01-14-09	146,900	5.3	1.001
01-20-09	146,650	5.3	1.000
07-28-09	149,500	5.3	1.000
10-20-09	145,100	5.5	1.000
10-30-09	145,900	5.3	1.000
03-28-10	144,500	5.2	1.000
05-07-10	145,700	5.3	1.000
06-04-10	145,500	5.4	1.000
11-17-10	146,500	5.4	1.001
02-19-11	143,300	5.3	1.000

Table 4C. Molecular state of ch14.18 control and infusion model mock samples during 24 h mock infusion model study

Ch14.18	SEC-HPLC UV ₂₈₀ Peak Area			MALS	
Sample Info	Monomer (mAU)	Dimer (mAU)	Total (mAU)	LS Peak Area (Raleigh Ratio) x 10 ⁶	MW (Da)
Control (1.00 mg/ml)					
t = 0 h	7381	65	7562	2.87	145,500
t = 3 h	7441	69	7624	2.81	145,500
t = 6 h	7357	65	7558	2.74	146,600
t = 24 h	7464	65	7639	2.71	146,000
High Dose (Infusion mock, 0.68 mg/ml)					
t = 0 h	4225	30	4425	1.66	146,200
t = 3 h	4386	33	4995	1.64	145,600
t = 6 h	4376	33	5121	1.62	146,800
t = 24 h	4433	32	4813	1.70	145,500

UV₂₈₀/LS peak area of monomer and dimer peaks at different time points in control and high dose ch14.18. Sample injections are 100 µl for each run. The estimated MW is also shown in the table. The MW estimates for low-dose samples are less accurate due to the low sample concentration, with resultant low signal intensity. SEC-MALS and data analysis were performed as described in Materials and Methods.

Our results show that the ch14.18 is a monodisperse IgG monomer with a MW of 145–150 kDa, and the drug product is more than 95% pure monomer, with negligible molecular aggregates (dimer and higher forms). The results also show that different lots of ch14.18 produced and purified using the same process are comparable in purity and molecular parameters, and the drug product is stable to storage at 2–8°C when formulated in phosphate buffered saline (PBS) for an extended period.

The SEC-MALS and batch mode DLS results presented here revealed the long-term stability of the ch14.18 and retention of the integrity molecular state. The potential presence of microparticles in vial biotherapeutic drugs is an on-going concern. Microparticulates are particles >1 micron in size that generally form during interactions of drug molecules with the container or container closure matrix. Although the number of particles

and the loss of drug molecules due to aggregate formation may be negligible, these particulates are of concern because they may induce an immune response. Aggregates in general are highly immunogenic, and these large particulates could render a strong adverse effect due to immunogenic reactions and neutralization of drug activity. The SEC with online LS or DLS may not be useful in the detection and quantitation of these particulates. Alternate methods of particulate analysis are to be applied.^{22,26-28} During the clinical administration of ch14.18, the drug solution in the vial is filtered through a 0.2 micron filter to remove any large particulates. We have routinely analyzed the samples before and after filtration for any loss of protein content, purity and biological activity. Imaged cIEF, SEC-MALS, protein assay and biological activity using in vitro CbELISA and CDC are routinely performed on filtered and unfiltered vial products as part of product release and stability monitoring (data not shown). No quantitative or qualitative differences were detected between the pre-filtered and post-filtered drug products (results were comparable within the limits of assay variations).

There are conflicting reports on the impact of variations in the Fc glycan forms on the therapeutic mAbs. Goetz et al. recently reported on the glycan structure attached to the CH2 domain of the Fc region of immunoglobulin G (IgG) and its role in modulating the clearance. The authors concluded that high-mannose glycans may be cleared at a faster rate, but may be significant only with a very high level of high-mannose glycan. Chen et al.³⁰ showed that the relatively faster disappearance of high-mannose glycans in vivo and in vitro is consistent with the mannosidase activity level and suggested that N-glycan cleavage, rather than differential antibody clearance, is the mechanism for faster disappearance of high-mannose glycans. It has also been suggested that the N-glycan structure at Asn297 results in modulation of ADCC through FcγRIIIA receptor interactions and also through CDC.³¹⁻³⁴

Ch14.18 has been shown to induce ADCC as well as CDC in GD2-expressing cell lines, and ADCC is implicated as a potential mechanism associated with therapeutic effects in neuroblastomas and melanomas. In preparation for scale-up and associated process changes to meet the demands of the clinical evaluation and marketing, it was essential to characterize the glycosylation profile and glycan content both qualitatively and quantitatively. The N-glycoprofiling and N-glycan analysis procedures were adapted and qualified in-house to address the assay reproducibility as well as issues associated with process change and scale up issues. Both methods showed a reproducible and consistent profile when the same product is tested at different times or when different product lots are manufactured by the same process; the N-glycan analysis was also shown to be quantitatively reproducible.

Therapeutic antibodies may achieve clinical efficacy by target-specific binding that blocks a ligand or receptor, or via effector functions such as CDC or ADCC, which requires interaction of the antibody with Fc receptors.³⁵ The current knowledge of antibody-Fc functionality is summarized in a recent review article.³⁶ Antibodies that achieve efficacy wholly or predominantly through Fc-mediated mechanisms represent rich opportunities for future

therapeutics in oncology.³⁷ Fc glycosylation is necessary for therapeutic antibodies to elicit effector functions, but heterogeneity in Fc-glycan plays a substantial role in that function. The removal of core fucose is known to enhance the FcγRIIIA binding affinity of IgG1s and result in increased ADCC.³⁶ The mechanisms by which Fc:Fc-receptor interactions regulate immune responses to affect tumor cell death have been extensively reviewed in reference 38. There is significant variation in the affinities of IgG isotypes for individual Fc receptors, which is reflected by the capacity of active isotypes to recruit immune effector cells efficiently based on their Fcγ receptor expression profile. IgG1 and IgG3 are considered the principal active human isotypes based on their comparative affinities for activatory receptors. In the murine setting, IgG2a and IgG2b represent the most active functional isotypes. Our current study focused attention on the consistency and assay variability in the glycoprofile, N-glycan analysis and Fc:FcγRIIIA interaction affinity; the observations that a glycoform of FcγRIIIa influences binding and activation³⁹ suggests another interesting area of study for the future. NCI has a number of mAbs and recombinant immunoconjugates in early stage development within the Biopharmaceutical Development Program. Our laboratory is currently engaged in detailed glycosylation analysis by the methodologies described here, along with LC/MS analysis and the role of glycosyl structure and heterogeneity in Fc-mediated effector functions such as CDC and ADCC.

Although it is generally anticipated that physicochemical comparability may be correlated with biological or functional activity, there are several exemptions. We have noted a number of cases in which subtle changes in physicochemical characteristics were not reflected in changes in biological activity, but we have also observed cases in which there were significant changes in biological activity when no detectable changes in physicochemical properties could be detected (unpublished results). It is necessary to evaluate the biological activity to release product lots and to monitor the stability and comparability of product. For ch14.18, we use two in vitro cell-based assays to monitor the GD2 antigen-binding activity²³ and the CDC of the mAb. The development and qualification of an MTS dye-based cell proliferation inhibition assay for monitoring the CDC of ch14.18 was briefly described here. The assay is reproducible and is used for product release and stability monitoring. As with most of the bioassays, the inherent assay variations in the absolute ED₅₀ values limit the application of the methodology for comparing relative activity with the reference standard lot. The sensitivity of the assay to demonstrate variations in the CDC with changes in physicochemical characteristics was demonstrated using stressed samples (Fig. 8A and B). The intra-day, plate-to-plate variations in the ED₅₀ values highlights the importance of testing the reference standard and test articles side by side on the same plate for meaningful comparison of relative activity.

Ch14.18 and other anti-GD2 antibodies or conjugates are known to affect ADCC. ADCC is associated with the Fc region of the antibody, and Fc/FcγR interaction is believed to be the mechanism for ADCC. The ADCC effect has been correlated with FcγR interaction affinity for a number of antibodies. An Fc/

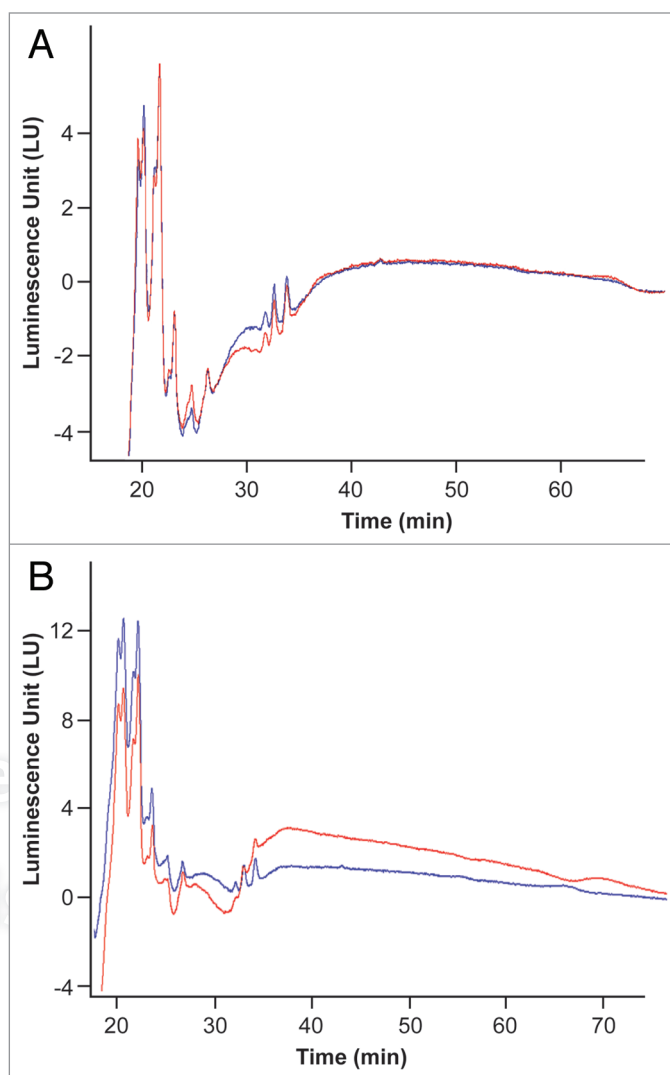


Figure 3. N-glycosyl profile of ch14.18. N-glycoprofiling was performed following the procedure described in Materials and Methods. (A) Overlay of the HPLC profile of the same lot from independent analysis. (B) Overlay of HPLC profile of two separate lots of ch14.18.

FcγR interaction study using BIAcore binding kinetics might be used as an in vitro physicochemical method to mimic the ADCC effect. With that goal in mind, we developed the Fc/FcγRIIIA binding affinity measurements to supplement the ADCC study performed in clinical laboratories for the ch14.18 drug. The assay is qualified to demonstrate reproducibility and consistency using a reference standard lot.

The results described in this report establish the validity and reproducibility of critical characterization assays for defining the charge and molecular state heterogeneity, glycosylation profile, FcγRIIIA interaction or binding kinetics, and CDC of ch14.18. Consistent and reproducible results were observed with ch14.18 produced using the same manufacturing process (lot-lot consistency), and the characteristics of the ch14.18 formulated in 20 mM sodium phosphate-150 mM sodium chloride, pH 7.4 (PBS) at 5 mg/ml were retained for more than 5 y.

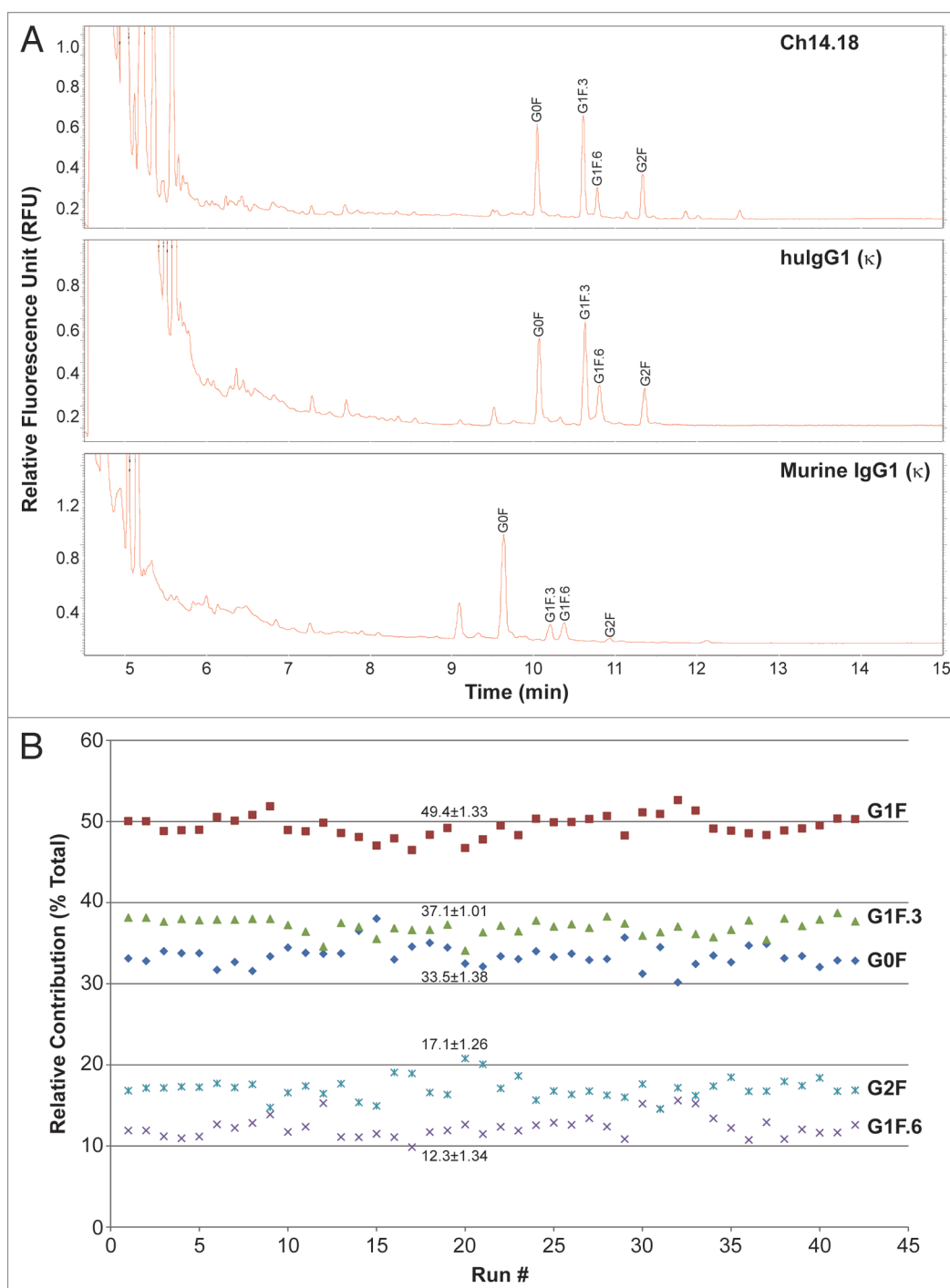


Figure 4. N-glycan analysis of ch14.18. (A) Comparison of N-glycan profile ch14.18, control human IgG1(κ) and murine IgG1(κ). Experimental details are described in Materials and Methods. (B) Intra-day and inter-day consistency in the N-glycan profile of ch14.18. Data were collected from analysis of a reference lot ch14.18 from different experiments performed within the same day or on different days and plotted. The mean of all the estimates and the standard deviation from average are also indicated.

After development and qualification of these analytical methodologies, the methodology has been applied to the monitoring of product comparability associated with changes in culture media. We have observed that changes in culture medium components

result in changes in the glycosylation and isoelectric profiles in terms of relative contribution of the different isoforms, although the profiles were qualitatively comparable and we have initiated studies of the effects of the changes on ch14.18.

Materials and Methods

Materials. Methyl cellulose and Pharmalyte carrier ampholytes were purchased from GE Healthcare Biosciences (Catalog numbers 17-0456-01 and 17-0455-01). The pI markers were purchased from Convergent Bioscience Ltd., (Cat # 102229 and 101996). The anolyte (80 mM H_3PO_4) and catholyte (100 mM NaOH) and 0.5% methyl cellulose were from the iCE Chemical Test Kit (Convergent Bioscience Ltd., Cat # 101801). Analytical size exclusion chromatographic columns, a G3000SWXL column and a TSKgel SWXL guard column were from Tosoh Bioscience LLC (Cat # 08541 and 08543). CM5 chips (Cat # BR-1000-12), amine coupling reagent kit (Cat # BR-1000-500 and other BIAcore reagents, acetate buffer pH 5.5, Cat # BR-1003-52 immobilization buffer) and HBS-EP (running buffer, HEPES buffered saline containing 3 mM EDTA and 0.005% P20), Cat # BR-1001-88 were obtained from GE Healthcare Life Science. Fc γ RIIIA (Murine myeloma cell line, NS0 derived Gly17 Gln208, with a C-terminal 6 His-tag Accession # AAH17865) was obtained from R&D Systems, Inc., (Cat. # 4325-FC-050). N-glyco profiling reagents [2-AA Labeling kit (Cat # LT-KAA-A2), disialylated core-fucosylated biantennary complex type N-glycan (A2F, Cat # SA-A2F), monosialylated biantennary complex-type N-glycan (A1, Cat # SA-A1) and Asialo, galactosylated, biantennary oligosaccharide (NA2, Cat # SA-NA2)] were obtained from QA-Bio, LLC. Fetuin was from Sigma (Cat # F2379-1G). Analytical HPLC column Asahipak-NH2P-50 2D column (Cat # CHO-5582) and Asahipak 5 μ NH2P-50 0A guard column (Cat # CHO-5583) were obtained from Phenomenex. CE-glycan analysis reagents and labeling kit were obtained from Beckman Coulter. The human IgG1kappa purified from human plasma and formulated in Tris buffered saline pH 8.0 was obtained from Sigma Aldrich (Cat # 15154). The mouse IgG1kappa purified from murine myeloma clone MOPC 31C was also obtained from Sigma Aldrich (Cat # M9035). Fluorescein isothiocyanate (FITC) and FITC protein-labeling kit was obtained from Molecular Probes, Inc., (Cat # F6434). Propidium iodide (PI) was from BD PharMingen (Cat # 556463).

Cell lines and cell culture. The melanoma cell line M21/P6, which expresses GD2 on the surface,⁴⁰ was a generous gift from Jean Surfus of the University of Wisconsin, Madison. NCI-H460 cells, a large cell lung cancer cell line expressing none or a very low level of GD2⁴¹ were obtained from Dr. William Kopp of the Applied and Developmental Research Directorate of SAIC-Frederick, Inc., NCI-Frederick. U87-MG, an astrocytoma cell line, was obtained from Dr. John H. Sampson of Duke University, Durham, North Carolina, and cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids solution. NMB7, a human neuroblastoma cell line, was obtained from Dr. Shuen-Kuei Liao of Chang Gung University, Taiwan, through Professor Alice Yu of the University of California, San Diego. NMB7 cells were cultured in RPMI-1640 with L-glutamine supplemented with 10% FBS. The cells were cultured in DMEM with L-glutamine supplemented with 10% FBS

Table 5. Intra-day and inter-day variations in the equilibrium dissociation constant of the Fc γ RIIIA receptor binding interaction of ch14.18

Date	K_D (μ M)				Intra-day Average	CV%
	Run 1	Run 2	Run 3	Run 4		
05172010	0.48	0.47	0.56	0.49	0.50	8.16
02252011	0.48	0.53	0.44	0.51	0.49	7.99
12072010	0.55	0.53	0.54	0.54	0.54	1.51
03292010	0.47	0.43	0.50	0.53	0.48	8.85
11022010	0.52	0.54	0.50	0.55	0.53	4.20
08122011	0.44	0.42	0.54	0.58	0.50	15.60
Average	0.49	0.49	0.51	0.53		
SD	0.04	0.05	0.04	0.03		
CV%	8.0	11.1	8.4	5.9		

Average of the 24 individual K_D estimates = 0.505 μ M; CV% = 8.74. Binding kinetics study and data analysis performed as described in Materials and Methods.

and 1% nonessential amino acids solution. IMR32 cells were obtained from ATCC and cultured in Eagle's minimum essential medium supplemented with 10% FBS. M21/P6 cells were routinely cultured and maintained in RPMI-1640 containing 10% FBS, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The NCI-H460 cell line was cultured in RPMI-1640, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Imaged cIEF. Imaged cIEF profiling was performed using the iCE₂₈₀-D₂ electrophoresis system from Convergent Bioscience Ltd., in conjunction with the Alcott 718 AL autosampler following the procedure previously described in reference 24. The ampholyte was 80 mM H_3PO_4 , and the catholyte was 100 mM NaOH. A hemoglobin standard was prepared in 8% pH 3–10 Pharmalyte with two internal pI markers, 4.22 and 9.46, from the iCE Chemical Test Kit, and samples were prepared in 0.5% methyl cellulose with 8% Pharmalyte, i.e., pH 3–10 (25%) and pH 8–10.5 (75%) in 200 μ l total volume. The system was calibrated and tested for suitability by running the hemoglobin standard. Focusing time for the hemoglobin standard was 4.5 min at 3 kV with pre-focusing at 1.5 kV for 1 min. Sample focusing was done for 7 min at 3 kV. pI markers used for sample runs were 8.4 and 9.5.

Analytical SEC with online MALS. Online SEC coupled with multi-angle light scattering (MALS) with embedded quasi-elastic-light scattering (QELS), refractive index (RI) and UV-Vis detectors were used in the determination of molar mass, particle size and detection of aggregates in the product. Chromatographic separation was achieved under isocratic conditions using a G3000SWXL column and a TSKgel SWXL guard column from Tosoh Bioscience. The method used 20 mM potassium phosphate, 300 mM sodium chloride, pH 7.4, as the mobile phase at a flow rate of 0.75 ml/minute and a sample injection volume of 20 μ l. The duration of each run was 30 min. The column was connected to an Agilent 1100 HPLC system equipped sequentially with a diode array detector (DAD; Agilent 1100), a

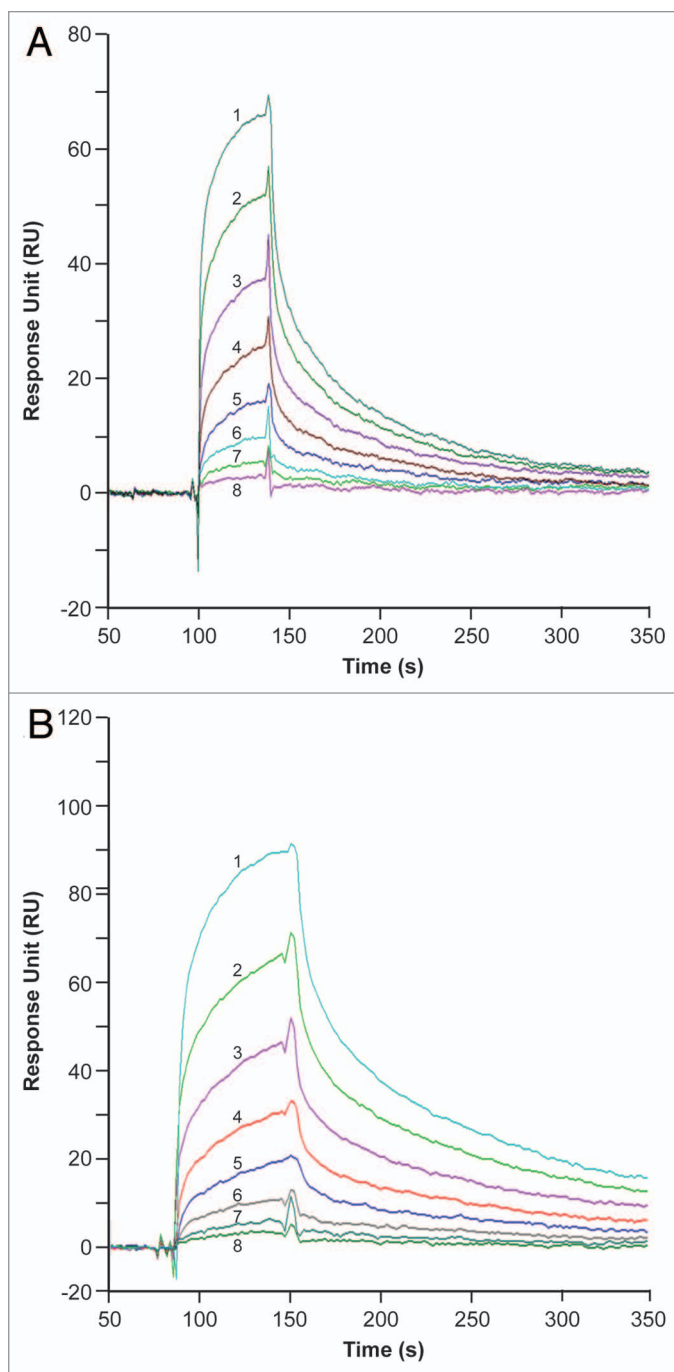


Figure 5. Sensorgrams showing dose-dependent binding of ch14.18 (A) and a control human IgG1(κ) (B) on Fc γ RIIIA coupled to a CM-5 chip. Fc γ RIIIA binding kinetics was performed as described in Materials and Methods. Serial 2-fold dilutions (eight) of ch14.18 or control IgG1(κ), starting from 5 μ M (curve 1) to 39.8 nM (curve 8), were injected.

MALS detector (Wyatt Technology Corporation, Santa Barbara, CA) and an RI detector (Wyatt Technology). The MALS detector employed a laser light at 658 nm and 18 detectors at angles evenly positioned between 22.5 and 147 degrees. The output signals from DAD, MALS and RI were imported into ASTRA V[®] software for data processing. A blank run using sample buffer injection was performed and subtracted from sample run

signals. HPLC (UV/VIS) data analysis was performed using HP Chemstation software, and Astra V 5.3.4 was used for analyzing the MALS data.

DLS. Batch mode DLS was performed using the DynaPro device from Proterion (now Wyatt Technology Corporation) Model DynaPro 99-E-15, s/n 99-404 connected to a laser beam of 779.9 nm. Data were analyzed with Dynamics V6, version 6.10.1.2, software.

N-glycoprofiling. N-linked carbohydrates on the glycoprotein were cleaved using a glycosidase, peptide-N4-(acetyl- β -glucosaminyl)-asparagine amidase (PNGase F). A PNGase F kit (contents: PNGase F, 5x reaction buffer, denaturation solution and 15% Triton X-100) was used for this procedure. The cleaved carbohydrates were separated from the protein and lyophilized, and the lyophilized samples were labeled using a fluorophore, 2-amino benzoic acid (2-AA). The labeled samples were dialyzed extensively, and the samples were lyophilized again. The dried, labeled samples were dissolved in 500 μ L water and analyzed on an HPLC fitted with a fluorescent detector using a Phenomenex Asahipak-NH2P-50 2D column and Phenomenex Asahipak 5 μ NH2P-50 0A guard column for the separation. The runs were performed using a linear gradient of mobile phase A (5% acetic acid, 4% diethylamine in Direct-Q water) and mobile phase B (2% acetic acid, 1% diethylamine in acetonitrile) with a flow rate of 0.2 mL/min. The column compartment was kept at 50°C. The fluorescence detector was set at an excitation wavelength of 320 nm and an emission wavelength of 420 nm. Carbohydrates released from fetuin were used for system suitability/assay control. Disialylated, core-fucosylated biantennary complex type N-glycan (A2F), monosialylated biantennary complex-type N-glycan (A1) and asialo-biantennary complex-type N-Glycan (NA2) were also used as controls for the HPLC runs.

N-glycan analysis by CE. N-glycan profiling of the samples was performed using capillary electrophoresis (CE) with laser induced fluorescent (LIF) detection to determine the relative quantity of the G0F, G1F.3, G1F.6 and G2F glycans. 8-aminopyrene-1,3,6-trisulfonate (APTS)-labeling and separation by CE were performed using the Beckman Coulter ProteomeLab Carbohydrate Labeling and Analysis Kit following the procedure provided from the supplier (Beckman Coulter, Cat# 477600). The kit contains the reagents, buffer and coated capillaries required for the labeling, separation and quantification of the oligosaccharides.

The N-linked glycans were released from ch14.18 using PNGase F enzyme. Approximately 50 μ g of glycoprotein sample in phosphate buffer was treated with 2 μ L of PNGase F (1,000 units; New England BioLabs, Cat# po705L), followed by incubation at 37°C overnight (for 5 to 24 h). The released carbohydrates were separated from protein using a Microcon-10 centrifugal filter device (Millipore, Cat# 42407) and centrifugation at 13,000 rpm for 30 min. The filtrate from each tube was dried using a centrifugal evaporator (Thermo Scientific) and labeled with the fluorophore APTS. To each of the dried samples, sodium cyanoborohydride/THF (Sigma Aldrich Cat# 296813) and APTS-labeling solution was added, followed by incubation at 55°C for 90 min. The labeling reaction was stopped by the addition of

water. Before CE analysis, samples were diluted 1:1 in 1/10th strength CE separation buffer.

The APTS-labeled glycans were separated on an N-CHO-coated CE capillary using a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with LIF excitation at 488 nm and using a 520 nm emission filter. Data analysis was performed using 32 Karat software provided by Beckman Coulter.

FcγRIIIA:ch14.18 interaction (SPR). BIAcore, a label-free detection platform utilizing the SPR phenomenon, was used to study the binding interaction between ch14.18 and Fcγ receptor. Initially, a 200 μg/mL stock solution of FcγRIIIA receptor was prepared using PBS and used within three months, as per vendor recommendations. The frozen stock solution of FcγRIIIA receptor was thawed and diluted 200x using the immobilization buffer (10 mM sodium acetate, pH 5.5). FcγRIIIA receptor (1 μg/mL) was coupled to a carboxymethyl dextran (CM5) sensor chip following the amine coupling procedure provided by the vendor (GE Healthcare). Amine coupling reagents were used to covalently attach the FcγRIIIA receptor to the sensor chip surface. Two flow cells of a CM5 sensor chip were immobilized with ~6 ng of FcγRIIIA. Another flow cell was used as a control surface and was prepared in the same manner as were the other two flow cells, but without the FcγRIIIA receptor. After the preparation of the sensor chip surface and before performing any kinetic runs, a 5 μM solution of ch14.18 reference standard was injected four times to confirm the reproducibility/robustness of the antigen/antibody binding and subsequent regeneration of the sensor chip surface. After each sample injection and before a new sample injection, the entire captured antibody was removed completely by the injection of 15 μL of a 0.05% sodium dodecylsulfate (SDS) solution prepared in water. All measurements were made at a flow rate of 30 μL/min. The running buffer used was HEPES buffered saline containing 3 mM EDTA and 0.005% P20 surfactant (HBS-EP). All data analysis was performed using the BIAEvaluation Software (BIAcore, Piscataway, NJ).

For kinetic analysis, different concentrations of the ch14.18 were injected onto the immobilized receptor surfaces on the sensor chip to determine the association and dissociation rate constants for the binding to the receptor. Injections in duplicate, at concentrations of 5, 2.5, 1.25, 625, 312.5, 156.25, 78.13 and 39.06 nM were used. HBS-EP buffer was used as the dilution buffer for preparing the different concentrations of ch14.18. It was also used as the running buffer. Blank injection were also made using HBS-EP buffer. To monitor the robustness of the receptor immobilized on the sensor chip surface, a 5 μM sample of the reference lot was also used at the beginning and end of each set of kinetic runs. Kinetic runs, identical to the runs mentioned above, were also performed using a human IgG1kappa (Sigma).

A MW of 150 kDa was used for the calculation of concentrations used in the kinetic runs. The samples were kept in the auto-sampler at room temperature (20°C). For each run, the injection volume was 20 μL and the dissociation time was 5 min. After each binding/dissociation of the analyte, the sensor surfaces were

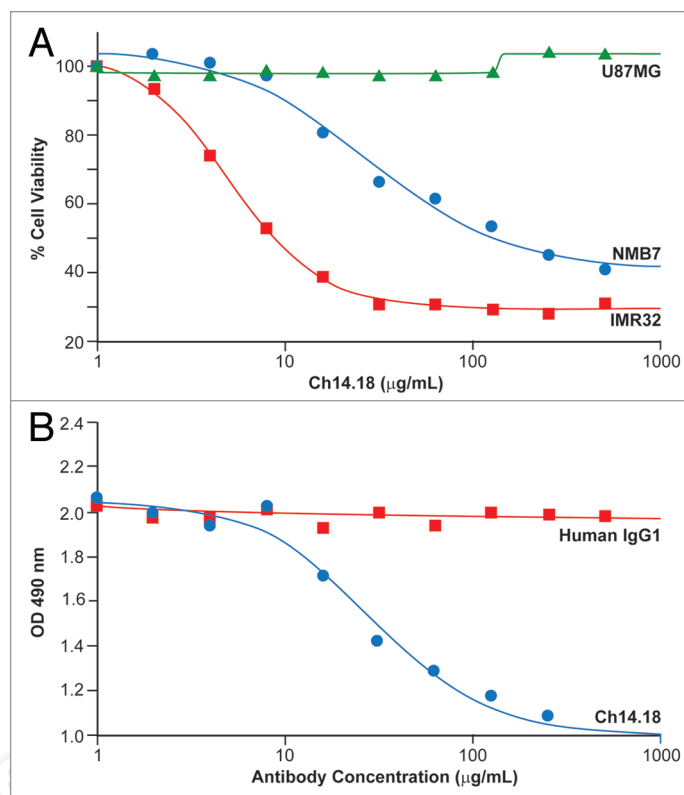


Figure 6. Ch14.18-mediated CDC determined by MTS colorimetric cell proliferation inhibition assay. (A) Cell specificity; (B) ch14.18 specificity. Assay was performed as described in Materials and Methods.

regenerated using 15 μL of 0.05% SDS. The data were analyzed using a 1:1 binding model with BIAEvaluation software.

CDC by MTS colorimetric cell proliferation assay. Cells were seeded into 96-well plates at a cell density of 2.5×10^4 cells per well in cell growth medium (100 μL/well) and cultured overnight at 37°C, 5% CO₂ incubator. The ch14.18 reference standard and test articles were run in serial dilutions. Ch14.18 samples at different concentrations (50 μL/well) were added to the plate containing cells, incubated at 37°C for 1 h and then washed in PBS by centrifugation at 1,000 rpm for 10 min. The cell growth medium (150 μL/well) and appropriately diluted complement (50 μL) were added to each well and incubated for 1.5 h at 37°C, 5% CO₂. Final concentration was 12.5% for human complement and 5% for rabbit complement. MTS reagent (20 μL/well) was added and incubation was continued for 4 h at 37°C, 5% CO₂. SDS was added to a final concentration of 1%. The optical density (OD) was recorded at 490 nm using a plate reader from Molecular Devices. The OD 490 read-out was plotted against ch14.18 concentration using a four-parameter curve fit. The C value (the concentration of ch14.18 that causes half-maximal killing) of the curve-fit was reported as ED₅₀. Relative activity (%) = (ED₅₀ ref.std ÷ ED₅₀ of test article) × 100%.

GD2 expression analysis by fluorescence-activated cell sorter (FACS). Ch14.18 was labeled with FITC following the protocol of FluoReporter FITC protein labeling kit provided by Molecular Probes, Inc. For immunofluorescent staining, cells

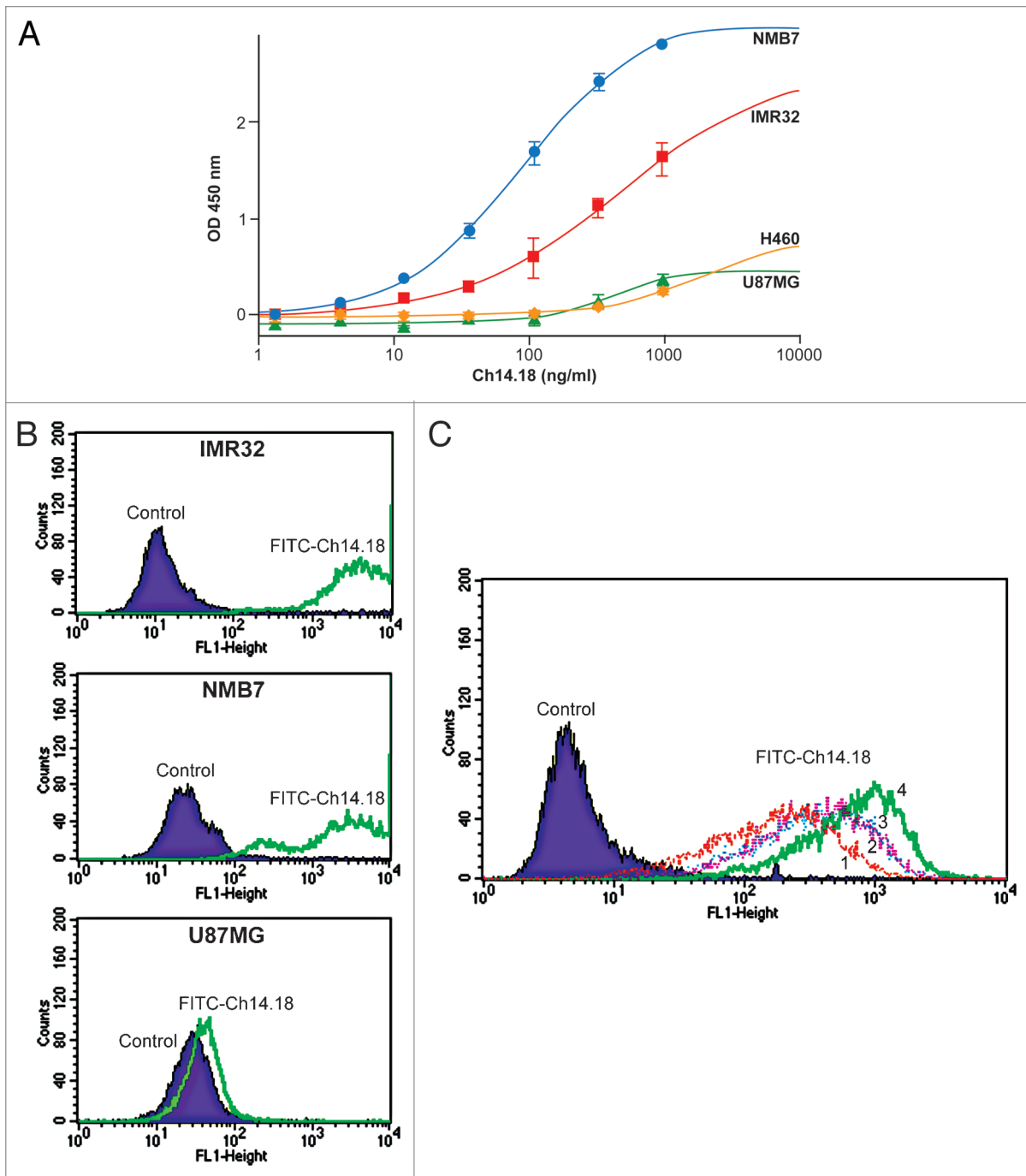


Figure 7. Binding specificity of ch14.18 probed by CbELISA. (A) CbELISA specificity of ch14.18 binding to GD2 positive cells. CbELISA was performed as described previously in reference 28. (B) GD2 specificity of FITC-labeled ch14.18 binding cellular specificity determined by cell sorting using FACS analysis. Cells were stained with FITC labeled ch14.18 (6 μ g/ml) and FACS analysis performed as described in Materials and Methods. (C) FITC-ch14.18 dose-dependent shift in FACS sorting of GD2-expressing IMR32 neuronal cell. Assays were performed as described in Materials and Methods. Control has no ch14.18 added. Histograms 1, 2, 3 and 4 have 1, 3, 9 and 10 μ g FITC-labeled ch14.18, respectively.

were harvested and suspended in PBS. Cells (10^6 /ml) were incubated with fluorescence-labeled antibodies for 45 min at 2–8°C and then centrifuged for 5 min at 1,000 rpm. The cell pellet was washed in PBS. The washed cells were re-suspended in 1 ml PBS. FACS analysis was performed using FACSCalibur from Beckton Dickinson as described earlier by Yang et al.⁴²

Propidium iodide (PI) staining of dead cells and FACS measurement. Cells (10^6 /ml) were incubated with ch14.18 at 37°C for 1 h. The human complement serum was added and incubated at 37°C for another 4 h. Following incubation, cells were transferred to falcon tubes. After washing two times with PBS, the cell pellet was suspended in 1 mL PBS. PI (1 µg/mL) was added to each tube (100 µl per tube) and mixed well. All samples were kept on ice in the dark for 10 min. FACS analysis was performed using FACSCalibur from Beckton Dickinson.

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Table 6A. Intra-day and inter-day variations in the ch14.18-mediated CDC of NMB7 cells with human and rabbit complement sera

Date	ED ₅₀ human complement (µg/ml)	ED ₅₀ rabbit complement (µg/ml)
052206	1.280	0.360
110909-1	0.133	**nd
110909-2	0.174	nd
111709	2.480	0.560
030910-1	1.602	nd
030910-2	1.030	nd
32910-1	1.658	0.600
32910-2	3.752	0.576
052010	0.549	1.229
111510-1	0.560	nd
111510-2	0.663	nd
111510-3	0.648	nd
*113010	6.646	0.843
*30111-1	1.579	nd
*30111-2	2.758	nd
*31010-1	4.255	1.244
*31010-2	5.468	nd
*31010-3	3.910	nd
*31010-4	3.997	nd
*31010-5	2.755	nd
*042511-1	4.178	nd
*042511-2	2.467	nd
*042511-3	1.731	nd
*042511-4	1.765	nd

*These experiments used a second lot of complement sera from the same source. Different plates run on the same day are denoted by -1, -2, -3, -4, -5. **nd, not detected.

Table 6B. Relative CDC of two different lots of ch14.18 compared with the CDC of a reference lot of ch14.18

Date	ED ₅₀ (µg/ml)				Rel. CDC (% of Ref Lot)	
	Ref Lot	Lot A	Ref Lot	Lot B	Lot A	Lot B
102909	0.133	0.167	0.174	0.153	80	114
030110	1.602	1.738	1.030	1.215	92	78
020711	1.579	2.041	2.758	2.968	77	93

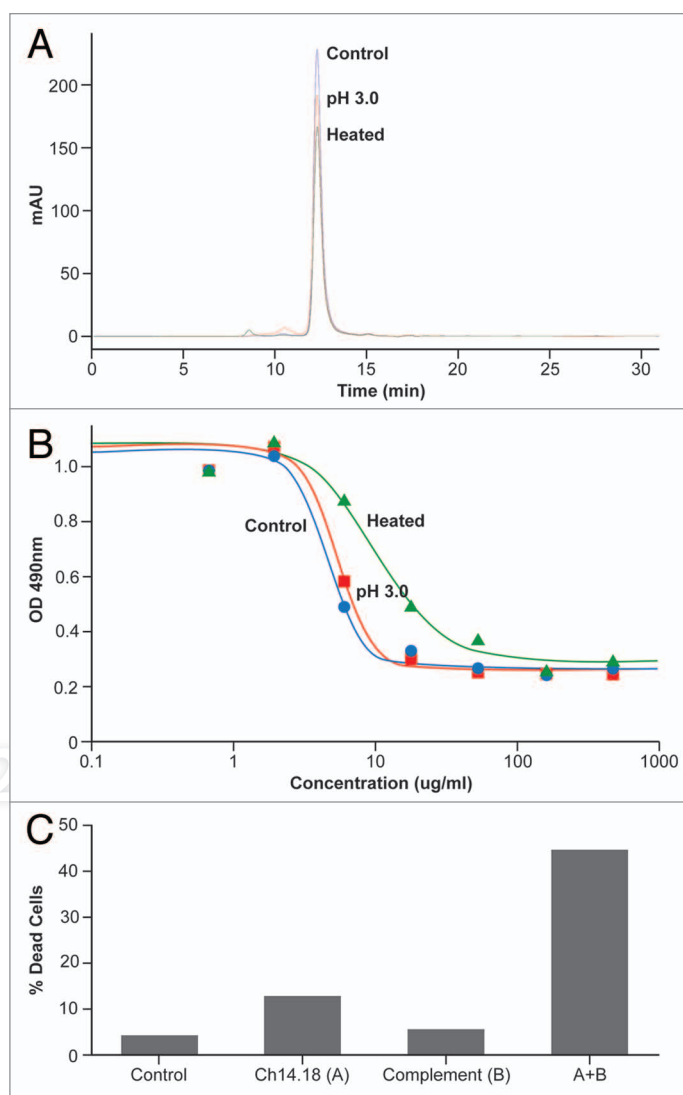


Figure 8. (A) Effect of acid (low pH) and high temperature on the molecular state of ch14.18. SEC-HPLC was performed as described in Materials and Methods. (B) Change in molecular state of ch14.18 on high temperature treatment parallels the CDC effect. (C) CDC effect of ch14.18 is demonstrated using cell death probed by FACS analysis using propidium iodide. Experimental details are described in Materials and Methods.

Table 7. Effect of acid and heat treatment on the SEC profile of ch14.18

Sample Details	Aggregate (mAU)	Dimer (mAU)	Monomer (mAU)	Total (mAU)	Monomer (% Control)	Total (% Control)
Control	*nd	98	7286	7600	100	100
pH 3.0 (Acid)	nd	467	6369	7063	87.4	92.9
Heat 60°C x 4 h	143	63	5380	5918	73.8	77.9

*nd, not detected.

References

- Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. *Nat Biotechnol* 2005; 23:1073-8; PMID:16151394; <http://dx.doi.org/10.1038/nbt0905-1073>.
- Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov* 2007; 6:349-56; PMID:17431406; <http://dx.doi.org/10.1038/nrd2241>.
- Glennie MJ, Johnson PW. Clinical trials of antibody therapy. *Immunol Today* 2000; 21:403-10; PMID:10916144; [http://dx.doi.org/10.1016/S0167-5699\(00\)01669-8](http://dx.doi.org/10.1016/S0167-5699(00)01669-8).
- Shapiro MA, Swann PG, Hartsough M. Regulatory considerations in the development of monoclonal antibodies for diagnosis and therapy. In: Dubel S, Editor. *Handbook of Therapeutic Antibodies*. Weinham, Germany: Wiley VCH 2005.
- Chirino AJ, Mire-Sluis A. Characterizing biological products and assessing comparability following manufacturing changes. *Nat Biotechnol* 2004; 22:1383-91; PMID:15529163; <http://dx.doi.org/10.1038/nbt1030>.
- Kozlowski S, Swann PG. Current and future issues in the manufacturing and development of monoclonal antibodies. *Adv Drug Deliv Rev* 2006; 58:707-22; PMID:16828921; <http://dx.doi.org/10.1016/j.addr.2006.05.002>.
- Woodcock J, Griffin J, Behrman R, Cherney B, Crescenzi T, Fraser B, et al. The FDA's assessment of follow-on protein products: a historical perspective. *Nat Rev Drug Discov* 2007; 6:437-42; PMID:17633790; <http://dx.doi.org/10.1038/nrd2307>.
- Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability and formulation. *J Pharm Sci* 2007; 96:1-26; PMID:16998873; <http://dx.doi.org/10.1002/jps.20727>.
- Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. *Pharm Res* 2004; 21:897-903; PMID:15212151; <http://dx.doi.org/10.1023/B:PHAM.0000029275.41323.a6>.
- Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS J* 2006; 8:501-7; PMID:17025268; <http://dx.doi.org/10.1208/aapsj080359>.
- Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res* 1991; 51:144-9; PMID:1988079.
- Simon T, Hero B, Faldum A, Handgretinger R, Schrappe M, Niethammer D, et al. Consolidation treatment with chimeric anti-GD2-antibody ch14.18 in children older than 1 year with metastatic neuroblastoma. *J Clin Oncol* 2004; 22:3549-57; PMID:15337804; <http://dx.doi.org/10.1200/JCO.2004.08.143>.
- Simon T, Hero B, Faldum A, Handgretinger R, Schrappe M, Niethammer D, et al. Infants with stage 4 neuroblastoma: the impact of the chimeric anti-GD2-antibody ch14.18 consolidation therapy. *Klin Padiatr* 2005; 217:147-52; PMID:15858706; <http://dx.doi.org/10.1055/s-2005-836518>.
- Hartmann WK, Sapharishi N, Yang XY, Mitra G, Soman G. Characterization and analysis of thermal denaturation of antibodies by size exclusion high-performance liquid chromatography with quadrupole detection. *Anal Biochem* 2004; 325:227-39; PMID:14751257; <http://dx.doi.org/10.1016/j.ab.2003.10.031>.
- Anumula KR, Dhume ST. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology* 1998; 8:685-94; PMID:9621109; <http://dx.doi.org/10.1093/glycob/8.7.685>.
- Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends Pharmacol Sci* 2009; 30:356-62; PMID:19552968; <http://dx.doi.org/10.1016/j.tips.2009.04.007>.
- Mujoo K, Kipps TJ, Yang HM, Cheresch DA, Wargalla U, Sander DJ, et al. Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal antiganglioside GD2 antibody 14.18. *Cancer Res* 1989; 49:2857-61; PMID:2720646.
- Mueller BM, Romerdahl CA, Gillies SD, Reisfeld RA. Enhancement of antibody-dependent cytotoxicity with a chimeric anti-GD2 antibody. *J Immunol* 1990; 144:1382-6; PMID:2303711.
- Honsik CJ, Jung G, Reisfeld RA. Lymphokine-activated killer cells targeted by monoclonal antibodies to the disialogangliosides GD2 and GD3 specifically lyse human tumor cells of neuroectodermal origin. *Proc Natl Acad Sci USA* 1986; 83:7893-7; PMID:3094017; <http://dx.doi.org/10.1073/pnas.83.20.7893>.
- Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res* 1991; 51:144-9; PMID:1988079.
- Robinson CJ, Jones C. Quality control and analytical techniques for biopharmaceuticals. *Bioanalysis* 2011; 3:81-95; PMID:21175369; <http://dx.doi.org/10.4155/bio.10.161>.
- Vázquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. *Biotechnol Bioeng* 2011; 108:1494-508; PMID:21480193; <http://dx.doi.org/10.1002/bit.23155>.
- Soman G, Yang XY, Jiang H, Giardina S, Mitra G. Comparison of GD2 binding capture ELISA assays for anti-GD2-antibodies using GD2-coated plates and a GD2-expressing cell-based ELISA. *J Immunol Methods* 2011; 373:181-91; PMID:21893062; <http://dx.doi.org/10.1016/j.jim.2011.08.016>.
- Janini G, Sapharishi N, Waselus M, Soman G. Element of a validation method for MU-B3 monoclonal antibody using an imaging capillary isoelectric focusing system. *Electrophoresis* 2002; 23:1605-11; PMID:12179978; [http://dx.doi.org/10.1002/1522-2683\(200206\)23:11<1605::AID-ELPS1605>3.0.CO;2-O](http://dx.doi.org/10.1002/1522-2683(200206)23:11<1605::AID-ELPS1605>3.0.CO;2-O).
- Soman G, Sapharishi N, Janini GM, Mitra G. Imaged capillary isoelectric focusing of proteins. *Bioforum Int* 2002; 6:263-7.
- Sharma VK, Kalonia DS. Experimental detection and characterization of protein aggregates. In: Wang W, Robert CJ, Eds. *Aggregation of Therapeutic Proteins*. John Wiley and Sons Publishing, NJ USA 2010; 205-56.
- Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, et al. An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. *J Pharm Sci* 2010; 99:3302-21; PMID:20310025; <http://dx.doi.org/10.1002/jps.22097>.
- Narhi LO, Jiang Y, Cao S, Benedek K, Shnek D. A critical review of analytical methods for subvisible and visible particles. *Curr Pharm Biotechnol* 2009; 10:373-81; PMID:19519412; <http://dx.doi.org/10.2174/138920109788488905>.
- Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, et al. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology* 2011; 21:949-59; PMID:21421994; <http://dx.doi.org/10.1093/glycob/cwr027>.
- Chen X, Liu YD, Flynn GC. The effect of Fc glycan forms on human IgG2 antibody clearance in humans. *Glycobiology* 2009; 19:240-9; PMID:18974198; <http://dx.doi.org/10.1093/glycob/cwn120>.
- Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* 2003; 278:3466-73; PMID:12427744; <http://dx.doi.org/10.1074/jbc.M210665200>.
- Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002; 277:26733-40; PMID:11986321; <http://dx.doi.org/10.1074/jbc.M202069200>.
- Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FcγRIII. *Biotechnol Bioeng* 2001; 74:288-94; PMID:11410853; <http://dx.doi.org/10.1002/bit.1119>.
- Hodoniczky J, Zheng YZ, James DC. Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. *Biotechnol Prog* 2005; 21:1644-52; PMID:16321047; <http://dx.doi.org/10.1021/bp050228w>.
- Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* 2010; 10:301-16; PMID:20414204; <http://dx.doi.org/10.1038/nri2761>.
- Jiang XR, Song A, Bergelson S, Arroll T, Parekh B, May K, et al. Advances in the assessment and control of the effector functions of therapeutic antibodies. *Nat Rev Drug Discov* 2011; 10:101-11; PMID:21283105; <http://dx.doi.org/10.1038/nrd3365>.
- Griggs J, Zinkewich-Peotti K. The state of the art: immune-mediated mechanisms of monoclonal antibodies in cancer therapy. *Br J Cancer* 2009; 101:1807-12; PMID:19809433; <http://dx.doi.org/10.1038/sj.bjc.6605349>.
- Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. *Nat Rev Immunol* 2008; 8:34-47; PMID:18064051; <http://dx.doi.org/10.1038/nri2206>.
- Zeck A, Pohlentz G, Schlothauer T, Peter-Katalinić J, Regula JT. Cell type-specific and site directed N-glycosylation pattern of FcγRIIIa. *J Proteome Res* 2011; 10:3031-9; PMID:21561106; <http://dx.doi.org/10.1021/pr1012653>.
- Sen G, Chakraborty M, Foon KA, Reisfeld RA, Bhattacharya-Chatterjee M. Preclinical evaluation in nonhuman primates of murine monoclonal anti-idiotypic antibody that mimics the disialoganglioside GD2. *Clin Cancer Res* 1997; 3:1969-76; PMID:9815586.
- Yoshida S, Fukumoto S, Kawaguchi H, Sato S, Ueda R, Furukawa K. Ganglioside G(D2) in small cell lung cancer cell lines: enhancement of cell proliferation and mediation of apoptosis. *Cancer Res* 2001; 61:4244-52; PMID:11358851.
- Yang X, Chen E, Jiang H, Muszynski K, Harris RD, Giardina SL, et al. Evaluation of IRES-mediated, cell-type-specific cytotoxicity of poliovirus using a colorimetric cell proliferation assay. *J Virol Methods* 2009; 155:44-54; PMID:18951922; <http://dx.doi.org/10.1016/j.jviromet.2008.09.020>.