Methods

Binding potency Assay for ^{131}I-labeled chTNT-1/B (Cotara®)

Note: all of the work described for the binding potency assays must be conducted in polypropylene tubes to prevent loss of proteins to the tubing through adsorption.

The antigenic target for Cotara® is adsorbed to polystyrene microparticle beads (0.6 μm diameter, 10% w/v suspension; from Seradyn, Indianapolis) and is prepared by first complexing 5 mg of calf thymus histone H1 to 25 mg of herring sperm DNA (both from Roche, Basel, Switzerland) in 50 mM MES, pH 6.1 (Sigma, St. Louis). Twenty milligrams (200 μl) of beads are incubated with the histone-DNA complex for at least 1 hour at room temperature prior to the addition of a formaldehyde crosslinking solution (final concentration: 1% Formaldehyde, 10 mM NaCl, 0.1 mM EDTA in 50 mM MES pH 6.1). Histone H1 and DNA are crosslinked to each other during an 8 minute incubation, followed by the pelleting of the beads in a micro-centrifuge and the removal of supernatant. The beads were twice resuspended with 1 ml of 50 mM MES pH 6.1 followed by micro-centrifugation in order to remove H1/DNA that has not adsorbed to the polystyrene beads. The beads were then resuspended in 50 mM MES pH 6.1 containing 1% Micro-O-protect (Roche, Basel, Switzerland) and brought to a final microparticle concentration of 1% w/v for long term storage at 4°C. The quantity of protein adsorbed to the microparticles was determined by a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL), which was modified according to the instructions provided by the bead manufacturer, Seradyn *(10)*.

Once coated with antigen, 100 µl aliquots of beads were used to collect each data point. Beads were pelleted in a microfuge, the 50 mM MES supernatant removed, and then resuspended in Blocking/Washing Buffer (4% Fish Gel, 0.5% Rabbit IgG, 0.05% Tween 20, 100mM NaCl, 1% Micro-O-protect in 50 mM MES pH 6.1; all from Sigma, except Micro-O-protect is from Roche) for 1-2 hours at room temperature. Upon completion of the blocking step, the beads are pelleted and the supernatant aspirated without contacting the beads at the bottom of the tube. The beads are then washed with Blocking/Washing buffer, pelleted, aspirated and suspended, once again, in Blocking/Washing buffer prior to the addition of antibody. The binding potency assay consists of incubating duplicate sets of beads. One set is first incubated with a "cold competitor" (i.e. unlabeled chTNT-1/B) before being exposed to the radiolabeled chTNT-1/B. This cold competitor sample is used to determine the level of non-specific "background" gamma counts from the samples. The set receiving the cold competitor had 0.5 ± 0.1 mg of cold chTNT-1/B antibody added to it and was incubated for at least 10 min. at room temperature. A similar volume of buffer was added to the tube not receiving cold competitor. Upon completing incubation with a cold competitor, both sets of tubes were incubated at room temperature, for at least 1 hour, with approximately 100,000 counts per minute (cpm) of radiolabeled chTNT-1/B. During this incubation period, total radiation activity was measured with a gamma counter (Cobra II Auto-Gamma Counter, Packard Bioscience Company, Meriden, CT). After incubation was completed, the samples were pelleted, the supernatant aspirated, and the beads washed twice with Blocking/Washing buffer. Washing involved vortexing the beads, pelleting them, aspirating and then resuspending them in buffer each time. Upon the final wash, the beads must be resuspended to the

same volume as existed during the incubation with radiolabeled chTNT-1/B, in order to ensure that the activity of the samples after washing will be measured under the same geometrical conditions as the earlier readings. The samples must now be measured again in the gamma counter.

These wash steps ensure that non-functional Cotara® is washed off of the beads before measuring again in a gamma counter, this time for the *functional* radiolabelled antibodies. The binding potency is determined by subtracting the background readings obtained with the cold competitor sample from the readings obtained with the non-competitor sample. This value represents the cpm from the *functional* antibody. To obtain the percentage of antibody that is potent, this value must be divided by the activity value for total radiolabeled antibody obtained from the gamma counter during the 1 hour incubation period.

Furthermore, the results are normalized for the effects of lost activity due to chloramine T oxidation by labeling chTNT-1/B both with ^{131}I, and then separately with ^{125}I (a radio-isotope with only a fraction of the energy emission). The labeling with ^{125}I allows us to separate effects on binding potency due to beta and strong gamma emission radiolysis from effects due to chloramine T. We would consider the damage from chloramine-T to be an unavoidable "background" level of damage that the antibodies would incur regardless of which isotope was being used. The ratio of binding potency obtained for the ^{131}I antibody versus the ^{125}I antibody provides a relative binding potency value which

is reported as a percentage. We have reported this relative binding potency value in the paper.

Binding potency Assay for ^{131}I-labeled Lym-1 antibody

Note: all of the work described for the binding potency assays must be conducted in polypropylene tubes to prevent loss of proteins to the tubing through adsorption.

The antigenic target for the Lym-1 antibody is HLA DR10, a cell surface antigen of B-cells. Rather than adsorb this difficult to isolate antigen to microparticle beads, we developed a method of fixing the HLA DR10 antigen naturally found on the surface of Raji cells. Raji cells (purchased from ATCC, Manassas, VA) are grown to 700,000 cells/mL, harvested, pelleted and the supernatant dumped. The cells are then washed twice with phosphate buffered saline (PBS), and on the final wash they are transferred to a beaker and gently stirred with a stir bar. Enough PBS is added for resuspension to a concentration of 5 million cells/mL and enough formaldehyde crosslinking solution is also added for a final concentration of 1% formaldehyde, 10 mM NaCl, 0.1 mM EDTA (all from Sigma-Aldrich, St. Louis). Formaldehyde crosslinking occurs for 8 minutes, followed by the pelleting of the Raji cells and the removal of the supernatant. The cells are rinsed twice with PBS and then adjusted to a concentration of 5 million cells/mL in PBS containing 0.02% sodium azide (Sigma-Aldrich, St. Louis) for long term storage at 4°C.

Fixed Raji cells are pelleted and then resuspended to a concentration of 5 million cells/mL in a 2% skim milk powder-PBS blocking buffer for 1-2 hours at room

temperature. Similar to the microparticles used in the Cotara binding potency assay, these Raji cells are washed with blocking buffer prior to a final suspension of 25 million cells/mL. Duplicate sets of Raji cell dilutions are made to 10, 5, 2.5, 1.25 and 0.625 million cells. A duplicate pair of controls are also prepared at 10 million cells each. These control cells are first incubated with a "cold competitor" (i.e. unlabeled Lym-1) before being exposed to the radiolabeled Lym-1. This cold competitor sample is used to determine the level of non-specific "background" gamma counts from the samples. The set receiving the cold competitor has 200 μg of cold Lym-1 added to it and is incubated for at least 30 min. at 4°C. Upon completing incubation with a cold competitor, both the controls and the remaining tubes are incubated at 4°C for 2 hours with 50,000-100,000 cpm of radiolabeled Lym-1. During the incubation period with ^{131}I-labeled Lym-1, total radiation activity is measured with a gamma counter (Cobra II Auto-Gamma Counter, Packard Bioscience Company, Meriden, CT). Following incubation, the cells are centrifuged out of solution, washed with blocking buffer and re-centrifuged, twice. The final cell pellet is counted in a gamma counter. Upon the final wash, the cells must be resuspended to the same volume as existed during the incubation with radiolabeled Lym-1, in order to ensure that the activity of the samples after washing will be measured under the same geometrical conditions as the earlier readings. The samples must now be measured again in the gamma counter.

The binding potency is determined by subtracting the background readings obtained with the cold competitor sample from the readings obtained with the non-competitor samples. This value represents the cpm from the *functional* antibody. The percentage of antibody

that is potent is derived from a Lindmo-Bunn Plot *(11)*. Briefly, the x-axis for such a plot represents 1/ [Raji cell concentrations]. The y-axis represents the total activity in each sample divided by the activity from the functional antibodies. Linear regression analysis through the data points reveals the binding potency of the antibody at the y-intercept on the graph. The inverse of the y-intercept represents the binding potency of the antibody under conditions of infinite antigen access and it is this value that is reported.

Free Iodide Content

The free iodide content of the final drug product is measured using one of two methods of instant thin layer chromatography. The first method uses an inorganic mobile phase where normal saline is added to a depth of not more that 0.5 cm in a clear glass chamber with a lid. One to three microliters of radiolabelled sample and bulk iodide (control) are dotted onto separate silica gel impregnated glass fiber sheets (Cat. # 61886, Pall Corporation, East Hills, NY) and allowed to dry completely. The dried sheets are added to separate chambers and the bulk iodide and antibody-bound and unbound iodide resolved by capillary action. Each sheet is sectioned into 14 fractions and individually counted with a gamma counter and the data manually recorded. The second TLC method uses an organic mobile phase that consists of methanol/water, (85/15, v/v). While the process is similar to the one described above, samples and controls are separated using Silica Gel 60 (Merck KGaA, Darmstadt, Germany) as the stationary phase. Once the antibody-bound and unbound ^{131}I are resolved, the silica gel strips are cut in two before each section is individually counted at the 364 keV energy level using an EG&G Ortec NR.3710 Si(Li) Detector combined with a Tenelec TC 245 Amplifier and Interwinner 5.0

software (all from Bächli Instruments, Affoltern am Albis, Switzerland). In each of the two methods the amount of unbound "free iodide" is reported as a percentage of the total radioactive iodine isotope in the sample.

Percentage of Intact Antibody Monomer

Size-Exclusion Chromatography (SEC) is used to determine the amount of antibody monomer in the sample as opposed to the amount of aggregating and degrading protein. Briefly, radiolabelled Cotara® is fractionated on an isocratic gradient using a Toso Hass TSK G3000SWXL column (Phenomenex, Torrance, CA) at a flow rate of 1.0 mL/min over the course of 20 minutes. The mobile phase consists of 50 – 100 mM phosphate buffered saline (PBS) (Sodium Phosphate Dibasic and Sodium Chloride both from Sigma-Aldrich, St.Louis). Two different, but equivalent High Pressure Liquid Chromatography instruments were used in the assay: 1) The Beckman Gold System (Beckman Instruments, Fullerton, CA) equipped with a 168 NM Photo Diode Array Detector, 126NM Solvent Module, 32 Karat Software, and Rheodyne Manual Injector 7010, and 2) The Waters system (Waters Corporation, Milford, MA) with 515 Pump, 717 Autosampler, 2487 Dual Wavelength Detector, PCM Pump Control Module, SAT/IN A/D module, and Millenium 32 Software. System suitability was performed using BioRad Gel Filtration Standards (Cat. # 151-1901, Bio-Rad, Hercules, CA) diluted with 2 mL sterile water for injection using conditions listed above. Antibody monomer and aggregates are detected both by absorbance (at 280 nm) and radiometrically, using a gamma counter (Bioscan Flow Count B-FC-1000 and the FC-3100 Na/I PMT Photo Multiplier Tube Detector, both from Bioscan Inc., Washington DC). The percentage of

antibody existing as intact monomer is determined by measuring the areas under the curve (AUC) for the radiometric peaks. The AUC for the monomer is reported as a percentage of the total AUC values.

Statistical Analysis using Block Randomization

For each of three parameters measured (potency, % monomer, and free iodide) we addressed the question of whether the measures supported a finding of ***Not Lower*** (or ***Lower***) when comparing percentage values obtained with the 10mCi/mg radiolabelings using the Batch method to those produced at one of the two higher specific activities (13mCi/mg or 17mCi/mg) using the In-Line method. The measures from each parameter were subdivided into nine "Blocks of scores" by two conditions: 3 Elapsed Time points and 3 Dilution Levels and it was noted that the measures within these blocks were not paired.

In response to the stated objectives, and the independence of measures, we developed the following analytic procedure: we randomized the measured percentage values from the higher radiation level within blocks (Block-Randomization) and calculated the proportion of these percentage values from the 13 mCi/mg or 17 mCi/mg specific activities that were lower in value than the adjacent scores from the 10 mCi/mg radiolabelings. Next we computed the 2-sided lower 95% Confidence limit for the observed proportion of lower scores and if it was above the no-difference standard of 0.5, we classified the observed results as supporting the finding of ***Lower***. Then we executed this procedure 10,000 times using 10,000 distinct Block-Randomizations, each of which provided a finding (***Lower*** or

Not Lower) and collectively produced a summary table that gave us an estimate of the likelihood of the indicated finding.

Finally, for each of the three parameters measured and two specific activities (13 mCi/mg or 17 mCi/mg), we replicated this procedure three times (from 3 different initial seeds) to provide a best estimate of the likelihood of the indicated finding and provide information about how much our choice of initial seed affected the stability of our final result. It is this likelihood that is discussed in the section below.

Results

In-Line and batch radiolabeling are statistically comparable

By comparing the data from the In-Line process **(Fig. 3B, 3C)** to the batch process **(Fig. 3A)**, even at concentrations of 27-29 mCi/mL, we were able to obtain comparable results for binding potency and % monomer. We addressed this question statistically for each of the three parameters measured (potency, % monomer, and free iodide) by directly comparing the values obtained with the 10mCi/mg radiolabelings in a Block Randomization method to those values produced at 13mCi/mg, and separately with 17mCi/mg. After comparing 10,000 random pairings of the values obtained for free iodide from the 13 mCi/mg results to 10 mCi/mg, we estimated a 99% likelihood of obtaining a lower free iodide value using the In-Line process at 13 mCi/mg. Similarly, we estimated a 99% likelihood for a lower free iodide content when labeling at 17 mCi/mg with the In-Line method. Block randomization analysis at 10,000 iterations also finds a 99% likelihood that the % monomer will not be statistically lower at 13 mCi/mg

compared to 10 mCi/mg, and a 98% likelihood that it will not be worse when labeling at 17 mCi/mg. However, at 10,000 random pairings, potency was estimated to have a 42% likelihood that it would not be statistically lower in value at 13 mCi/mg compared to a 10 mCi/mg batch method. Such an estimate for potency may improve with the availability of a greater set of values for analysis. Particularly since a general trend for potency was not seen when our analysis estimated a 93% likelihood potency will not be statistically lower at 17 mCi/mg using the In-Line method. These values were obtained *without any optimization of the In-Line process*.

References

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