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Research Article

A Fit-for-Purpose Method for the Detection of Human Antibodies to Surface-Exposed Components of BMS-986263, a Lipid Nanoparticle-Based Drug Product Containing a siRNA Drug Substance

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Abstract. ND-L02-s0201/BMS-986263 is a lipid nanoparticle (LNP) drug product containing a heat shock protein 47 (HSP47)–specific small interfering ribonucleic acid (siRNA) and being developed for the treatment of liver and idiopathic pulmonary fibrosis. To address immunogenicity-related issues, we developed a robust, fit-for-purpose (FFP) three-tier electrochemiluminescent (ECL) anti-drug antibody (ADA) assay for the detection of antibodies (Abs) generated to surface-exposed components of BMS-986263. The drug was coated directly on plates, and several Abs specific for polyethylene glycol (PEG) and other surface components were tested for use as positive quality controls (QCs). Following selection of a rabbit monoclonal anti-PEG Ab, the assay was optimized, and various method development challenges specific to the modality and pseudo surrogate rabbit control were addressed. Screening, confirmatory, and titer cut points were validated following a statistical evaluation of 41 individual K_2EDTA human plasma samples at a minimum required dilution (MRD) of 100. Assay precision, sensitivity, selectivity, drug tolerance, and hook effect were determined for the rabbit Ab prepared in human K_2EDTA plasma matrix. The assay was used to interrogate anti-drug Ab (ADA) responses in normal human subjects who were administered 90 mg of the drug intravenously (IV) once every week for 3 weeks in phase I clinical trials. All pre- and post-dose samples were found to be negative for ADA. Based on these results, we concluded that BMS-986263 is not immunogenic. To the best of our knowledge, this work represents the first ADA method developed and reported for an LNP-based drug product.

KEY WORDS: ADA assay; BMS-986263; clinical analysis; HSP47 siRNA; lipid nanoparticle.

INTRODUCTION

Ribonucleic acid (RNA) interference (RNAi)–based therapies are becoming increasingly important in abrogating the expression of genes contributing to disease (1–3). These highly specific therapies utilize short double-stranded RNA (ds RNA) fragments that are delivered to target cells where one of the two strands engages the complementary, target disease-inducing messenger RNA (mRNA) in an RNA-induced silencing complex (RISC). This results in the degradation of the target mRNA and a decrease in protein expression levels (4). Since naked dsRNA is susceptible to nuclease-mediated degradation, the therapeutic is often contained within LNP formulations for delivery (5–7).

ND-L02-s0201/BMS-986263 drug product (hereafter referred to as BMS-986263 or drug product or drug) is an intravenously administered LNP containing a small interfering RNA (siRNA) that reversibly inhibits the production of HSP47, a collagen-specific chaperone protein that is necessary for hepatic collagen deposition and collagen fibril formation (8). Hepatic stellate cells (HSCs) are the primary cellular mediators of fibrosis. With the help of HSP47, activated HSCs or myofibroblasts synthesize and secrete procollagen which is cleaved and accumulates as insoluble collagen, causing fibrosis (9). This novel formulation uses retinoid moieties conjugated to the nanoparticle surface to target drug delivery to HSCs.

Due to the complexity of LNP formulations, which include novel cationic and anionic lipids, target cell receptor ligands, and PEG polymers, there is potential for an immunogenic response that could have safety and efficacy implications for the RNAi therapeutic in question. The immune response may involve the innate as well as the adaptive arms of the immune system where the lipid, nucleotide, and protein components of the RNAi therapeutic

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may induce the production of Abs, cytokines, and complement, potentially resulting in systemic reactions and drug clearance (10–13).

One of the immunogenicity assessments that we employed in our phase 1 trials in healthy participants was a FFP ADA assay designed to detect Abs generated to surface-exposed components of BMS-986263. This assay, which we term the whole LNP ADA assay, is an ECL ELISA involving the direct coating of BMS-986263 on 96-well polystyrene ECL plates followed by detection with diluted human K_2EDTA plasma samples and pan-isotype anti-human κ and λ detection Abs. In this paper, we describe the development of this method beginning with an evaluation of various positive control Abs, identification of a rabbit monoclonal anti-PEG Ab control, comparisons of chemiluminescent (CL) and ECL methods of ADA detection, and ECL assay optimization. Statistical assessment of cut points for a three-tier screen, confirmatory and titer assay, and other assay parameters such as sensitivity, drug tolerance, selectivity, and hook effect are described. Finally, the application of the FFP assay to clinical ADA sample analysis and resulting outcomes as far as ADA responses to BMS-986263 are presented.

METHODS

Buffers, Supplies, and Chemicals

Dulbecco's phosphate-buffered saline (1× DPBS) without calcium and magnesium was purchased from Lonzawhitaker (Walkersville, MD) and used to dilute BMS-986263 for ELISA plate coating. The same buffer was used to wash the plates at all steps in the methods described below. Tween-free StartingBlock™ blocking buffer was purchased from ThermoFisher Scientific (Rockford, IL). Corning® 96-well black flat polystyrene plates for the chemiluminescent ELISA were purchased from Corning Inc. (Corning, NY). L15XA (standard) and L15XB (high-binding) 96-well black polystyrene plates were purchased from MSD (Rockville, MD) for initial comparison. L15XB was selected for the final assay format. Ninety-six-well polypropylene plates used for sample or reagent dilutions were bought from Eppendorf (Hauppauge, NY). Whatman microplate sealers were used to cover the plates during incubation and were purchased from Sigma (St. Louis, MO). The SuperSignal™ ELISA Pico chemiluminescent substrate was bought from ThermoFisher Scientific (Rockford, IL), and the 4× MSD Read Buffer T was purchased from MSD (Rockville, MD). The 4× MSD buffer was diluted two times using double-distilled water prior to use.

Equipment and Instruments

The ELx405 model plate washer from Biotek (Winooski, VT) was used for all washes. Chemiluminescence ELISA plates were read in a SpectraMax M5 from Molecular Devices (Sunnyvale, CA), and MSD ELISA plates were read using the Sector Imager 2400 from MSD (Rockville, MD).

Antibodies and Reagents

Several mouse, mouse-human chimeric, or rabbit primary Abs with respective horse radish peroxidase (HRP)–labeled detection Abs were initially tested in a chemiluminescent ELISA and are shown in Table I. Anti-PEG:PEG.1 and anti-PEG:PEG.2 Abs were acquired from Bristol Myers Squibb (BMS) Biologics Discovery (Sunnyvale, CA) and represent two different mouse F_{ab} region clones with a human IgG_1 Fc. Anti-PEG clone 6.3 and clone AGP4 were bought from Academia Sinica Institute of Biomedical Sciences (Nankang, Taipei). The anti-vitamin A pAb was purchased from Creative Diagnostics (Shirley, NY), and anti-PEG-B-47 Abs were bought from Abcam (Cambridge, MA) and Epitomics (Burlingame, CA). HRP-conjugated anti-human κ and λ Abs were bought from Southern Biotech (Birmingham, Al), HRP-anti-human IgG and HRP-anti-mouse IgM from Sigma (St. Louis, MO), HRP-anti-mouse IgG from Abcam (Cambridge, MA), and HRP-anti-rabbit IgG from Jackson ImmunoResearch Labs (Westgrove, PA).

Upon a final selection of the B-47 rabbit monoclonal Ab (mAb) as the positive control in the human ADA assay and transition to the ECL detection method, a SULFO-TAG™ Goat anti-rabbit Ab from MSD (Rockville, MD) was used for detecting the B-47 control at a final concentration of 800 ng/mL. Anti-human Abs in the plasma samples or the human IgG control (Det QC) were detected with ruthenylated goat anti-human κ and anti-human λ Abs (40 ng/mL each). Human IgG control was purchased from Sigma (St. Louis, MO). The ruthenylated anti-human Abs were provided by the Reagent Center of Excellence at BMS (Princeton, NJ).

Matrix Pool

Human K_2EDTA plasma samples were purchased from BioreclamationIVT (Westbury, NY, USA). The screening of individual plasma samples and identification of samples for the matrix pool are explained in "Results" section. The pool was created by combining 30–40 mL each of the selected plasma samples in polypropylene containers, divided into 50 mL aliquots in polypropylene tubes, and stored at −80°C until use.

Sample Preparation for ELISA

For the final FFP whole LNP ADA assay, pre-MRD rabbit B-47 mAb positive high- and low-quality controls (HQC and LQC) were prepared in the human plasma pool and stored in 50 μL aliquots at −80°C until use. Similar aliquots of the plasma pool alone were also made and stored at −80°C to be used as negative quality controls (NQCs). Prior to use, all quality controls (QCs) and clinical samples were diluted two times, 10-fold each time with block buffer for a final MRD of 100 and stored covered at 4–8°C until use. In the confirmatory tier, block buffer with and without drug was prepared and used to dilute the QCs and samples to the final MRD. Titer QCs (TQCs) were prepared fresh.

In method development (MD) experiments comparing the CL and ECL detection methods, 1× DPBS instead of matrix was used to create a B-47 Ab dilution series before further diluting to an MRD of 100 using block buffer. In other

Table I. List of Primary Antibodies and Respective HRP-Conjugated Detection Abs Tested as Positive Controls in the Whole LNP ADA Assay

Primary test antibodies			HRP-conjugated detection antibodies		Test outcome
Primary test Ab	Isotype	Final concentration	Detection Ab	Final dilution	
Anti-PEG:PEG.2 chimeric mouse	Human IgG_1	1 μg/mL	Goat anti-human κ and λ	1:5000	Negative
Anti-PEG:PEG.1 chimeric mouse	Human IgG_1	1 μg/mL	Goat anti-human κ and λ	1:5000	Negative
Anti-PEG:PEG.2 chimeric mouse	Human IgG_1	1 μg/mL	Goat anti-human IgG	1:5000	Negative
Anti-PEG:PEG.1 chimeric mouse	Human IgG_1	1 μg/mL	Goat anti-human IgG	1:5000	Negative
Anti-PEG clone 6.3	Mouse IgG_1	1 μg/mL	Goat anti-mouse IgG	1:5000	Positive
Anti-PEG clone AGP4	Mouse IgM	1 μg/mL	Goat anti-mouse IgM	1:5000	Positive
Anti-vitamin A polyclonal	Rabbit IgG	8.7 μg/mL	Donkey anti-rabbit IgG	1:5000	Negative
Anti-PEG monoclonal clone B-47*	Rabbit IgG	1 μg/mL	Donkey anti-rabbit IgG*	1:5000	Positive*

Manufacturer-recommended final concentrations of the primary and detection Abs were used *Ab pairs used in the final assay format

MD experiments, an MRD was not applied to the B-47 control, and Abs were diluted directly in block buffer. In specificity testing experiments for B-47 Ab and plasma samples, as well as in drug tolerance experiments, the controls and samples and the competing drug were prepared at two times higher than the intended final concentration using plasma matrix as the diluent, mixed together in equal volumes in polypropylene plates to one time concentration, sealed, and incubated at 25°C with moderate shaking for at least an hour prior to use. Such samples were either used directly in the ELISA or diluted to an MRD of 100 prior to use. Specific details of experiments are indicated in figure legends.

Selectivity LQC samples were prepared by initially creating an intermediate dilution of the B-47 Ab in 98% matrix for each of the ten individual K_2EDTA plasma lots prior to diluting each intermediate to 4000 ng/mL using 100% of the same plasma lots.

Chemiluminescent and MSD ELISA Methods

BMS-986263 (drug product or DP) was diluted using 1× DPBS to a coating concentration of 15 μg/mL (siRNA weight/volume) in the final method. Various coating concentrations were initially tested as indicated below. When applicable, human IgG was prepared at 1 μg/mL in 1× DPBS and was added separately to at least three wells of the same plate to serve as a control for the anti-human κ and λ or anti-human IgG detection Abs. This control was referred to as detection QC (Det QC). Ninety-six-well plates were coated with 100 μL/well of the diluted drug or human IgG, covered with plate sealers and incubated for 16–18 h at 4–8°C. Plates were washed three times with 1× DPBS; 300 μL blocking buffer was added to each well; plates were sealed and incubated stationary for 2.5–3 h at 25°C in an incubator. During this time, QCs and clinical samples were prepared as described earlier. Following another round of three times wash, 100 μL of prepared samples, QCs, or blocking buffer (to Det QC wells) was added to the plates and incubated sealed for 2 h ± 15 min at 25°C on a shaker set to moderate speed. Detection Abs were diluted to the indicated concentrations using blocking buffer and stored at 4–8°C until use. Plates were washed three times, and 100 μL of the relevant detection Abs was added to all wells; plates were sealed and incubated for 1 h ± 15 min at 25°C on a shaker. Plates were washed three times, and either 100 μL of the Pico substrate (CL ELISA plates) or 150 μL of the 2× MSD buffer (ECL plates) was added to all wells. Plates were read within 10–15 min on respective plate readers.

Calculations and Data Analysis

Percent inhibition of signal was calculated as previously described (14). All graphing and statistical analyses were done using GraphPad Prism v 7.03.

RESULTS

Evaluation of Antibodies and Identification of the Anti-PEG-B-47 as a Positive Control for the Whole LNP ADA Assay

At the minimum, the intact LNP drug has the following surface-exposed components: PEG-vitamin A conjugate and the hydrophilic heads of lipids constituting the outer shell of the drug. We evaluated several in-house and commercially available purified anti-PEG Abs and a semi-pure, anti-vitamin A Ab for use as a positive Ab control in the assay. The Abs included two mouse-human chimeras (15), as well as rabbit and mouse monoclonal Abs, and are listed with clone identities, isotype, and the corresponding peroxidase-conjugated detection Abs that were used for detection (Table I). The mouse-human chimeric anti-PEG:PEG1 and PEG 2 Abs were tested separately using anti-human IgG as well as anti-human kappa and lambda constant region detection Abs. Abs for lipids were unavailable and not included in the panel of tested Abs.

A CL ELISA which is described in "Methods" section was used to test the positive control Ab candidates. The test Abs and corresponding peroxidase-conjugated detection Abs were diluted in starting block buffer prior to use. The rabbit anti-PEG-B-47 clones from two different sources gave the highest ELISA signals followed by anti-PEG clone 6.3 and anti-PEG clone AGP4 (Fig. 1). The B-47 clone is specific for the terminal methoxy group of the PEG molecule whereas clone 6.3 and clone AGP4 recognize the repeating subunits of the PEG backbone. The two mouse-human chimeric Abs did

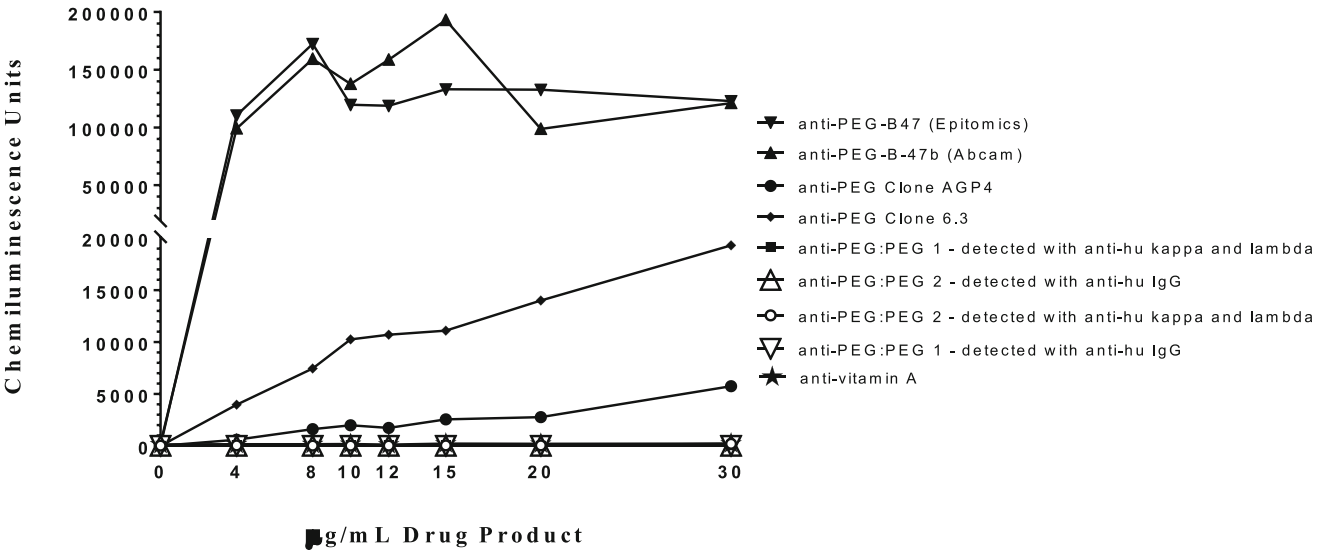


Fig. 1. Positive control Ab screening in the chemiluminescence ELISA. Drug product was coated on the plates at concentrations shown on the *x*-axis (siRNA μg/mL), probed with test Abs listed in Table I and detected with corresponding HRP-conjugated detection Abs. Raw data units corresponding to the ELISA signal are shown on the *y*-axis

not show a strong signal either with the anti-human IgG or anti-human light chain Abs (raw data units < 60 at 15 μg/mL coating). The anti-vitamin A Ab also failed to recognize the plate-bound DP (raw data units < 235 at 15 μg/mL coating). We selected the rabbit anti-PEG-B-47 clone from Abcam as the positive control for further assay development for the reason that in repeat experiments, the Abcam B-47 clone showed higher precision compared with the Epitomics B-47 Ab (data not shown).

Comparison of the ECL and CL Methods of Detection

The CL ELISA method of detection initially showed good precision between replicates (% CV < 20). However, precision dropped over time, and following initial unsuccessful attempts to resolve the issue, we decided to evaluate the ECL and CL methods side by side. The B-47 mAb was diluted to the concentrations shown using 1× DPBS as the diluent (Fig. 2). A further 100-fold dilution was done using block buffer and used in the two methods (100 μL for each method from a single dilution source). Both methods are similar at all major steps, the only differences being the type of plate used and the method of signal detection (see "Methods" section). Assay precision was ≤ 20% in the ECL method whereas in the CL method, precision was mostly above 30% (Fig. 2). In data shown in Table II, the ECL method gave a 3–11-fold higher S/N ratio in the mid-region of the curve between 1000 and 16,000 ng/mL of B47 mAb. The ECL method is more sensitive than the chemiluminescent method up to 32,000 ng/mL of the B47 mAb (Table II). Combined with the higher sensitivity and superior precision, we selected the ECL method for further optimization.

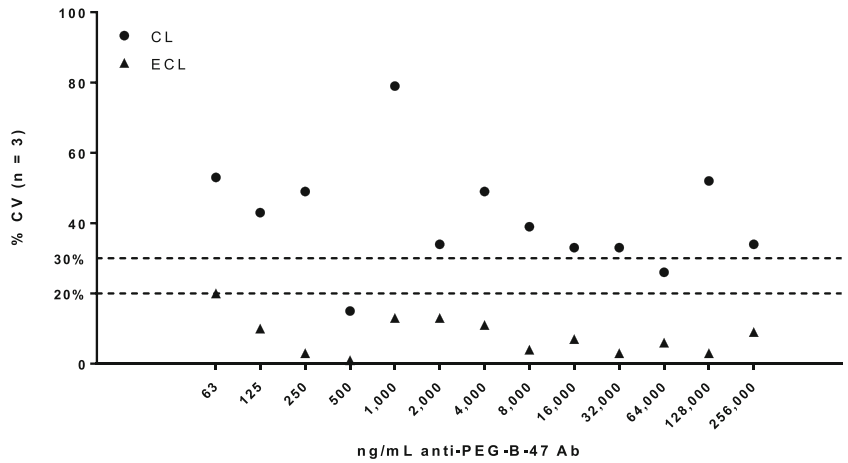


Fig. 2. Inter-replicate precision comparison in the CL and ECL detection methods. Drug product was coated on standard black polystyrene and MSD polystyrene plates at 15 μg/mL (siRNA-based concentration). B-47 mAb was diluted in DPBS and prepared to an MRD of 100 with block buffer prior to addition to each plate type and detected as described in "Methods" section. % CV was calculated using the mean and standard deviation of the raw data units obtained in each detection method for three replicates and is shown on the *y*-axis

Table II. Comparison of Chemiluminescent and Electrochemiluminescent Methods

ng/mL B-47 mAb	S/N-CL	S/N-ECL
125	1	1
250	1	1
500	2	2
1000	2	6
2000	4	17
4000	9	92
8000	34	212
16,000	58	338
32,000	156	333
64,000	358	331
128,000	1135	266
256,000	1242	262

The signal to noise (S/N) for B47 mAb binding to BMS-986263 in the chemiluminescent (CL) method was compared with the S/N obtained in the electrochemiluminescent method (ECL). Noise is determined by the signal obtained in each method when BMS-986263 is incubated with block buffer alone followed by the anti-rabbit detection antibody

ECL Method Optimization

Coating concentration of the DP and the anti-rabbit detection Ab concentration were optimized in the ECL method using the anti-PEG B-47 Ab. Briefly, DP was diluted in 1× DPBS and coated overnight on MSD plates at the siRNA concentrations indicated on the *x*-axis (Fig. 3a). It was probed the following day using several concentration combinations of the B-47 and anti-rabbit detection Abs. At 15 μg/mL coating of the DP, a concentration of 800 ng/mL of the detection Ab showed a 21.6-fold difference between 10 ng/mL and 500 ng/mL of B-47, whereas a concentration of 160 ng/mL showed only a 2.4-fold difference at the same two concentrations of B-47 (Fig. 3a). Control wells (blocking buffer and detection Ab) for noise showed similar low signals at all conditions tested (data not shown). Since we anticipated the S/N of the control QCs to drop when formulated in matrix, we selected a detection Ab concentration of 800 ng/mL to enable a wider window of separation between the low and high QCs and increase assay range as determined by the B-47 control Ab. A coating concentration of 15 μg/mL (based on siRNA weight/volume) was selected for further experiments since the signal stabilized in this region for most of the tested conditions (Fig. 3a).

Using several concentrations of B-47 and 800 ng/mL of detection Ab and a DP coating of 15 μg/mL, we further compared two different types of polystyrene plates, L15XA (standard binding) and L15XB (high binding). L15XB was superior for coating this liposomal DP (Fig. 3b).

Human K_2EDTA Plasma Screening and Creation of a Matrix Pool

We tested 22 different human plasma samples on DP-coated plates in the ECL ELISA. The purpose was to determine the level and specificity of background ECL signal in drug-naive individuals and to identify and pool samples having low background for use as a matrix for QC preparation and titer sample dilution. Plasma samples were diluted 1:10 and 2-fold in the 1:100–1:800 dilution range with block buffer, and 100 μL of the dilution was added to MSD plates coated with 15 μg/mL of the drug and detected with ruthenium-labeled anti-κ and anti-λ Abs in the ECL ELISA. There was a significant reduction in background signal at an 800-fold dilution; however, it did not reach background observed in the presence of block buffer alone (approximately 50 ECL units, Fig. 4). We selected a MRD of 100 for further analysis to avoid over dilution of ADA-containing samples. At this MRD, most of the samples showed background > 1000 ECL units and a minimum to maximum range of 737–2081 ECL units (Fig. 4).

Sixteen of the 22 samples that were available in bulk were diluted 100-fold and tested for specificity of binding by adding 8 μg/mL of the DP (siRNA *w/v*) to the block buffer used to dilute the plasma samples. A second set was diluted with block buffer lacking the competing drug. The percent inhibition of signal was calculated as described in "Methods" section. The anti-PEG-B-47 control showed 99% inhibition of signal, and the plasma samples showed < 20% inhibition, indicating that the background generated by the latter was likely non-specific in nature (Table III). However, given the lack of a confirmatory cut point at the time of this analysis, 12 of 15 samples that showed the lowest percent inhibition (≤ 10%) were selected for the pool.

Selection of Low and High QCs and Confirmation of Drug Concentration for Confirmatory Tier

Using the screened human plasma pool matrix, we proceeded to prepare and test a titration curve of the B-47 Ab at an MRD of 100 in the optimized ECL ELISA to enable the selection of low and high QCs for the ADA assay. To finalize the drug concentration used in the confirmatory tier, we diluted two replicate sets of the same titration curve with block buffer containing 7.5 μg/mL or 15 μg/mL of the DP (immune depletion buffer). Forty-five replicate wells of plasma matrix alone were also tested at an MRD of 100 with the anti-human detection Abs to give a statistically meaningful estimate of the background signal (NQC). Background signal in the matrix was 1667 ECL units, and starting at 2000 ng/mL, 5 of the 15 tested B-47 concentrations showed signals above this background (Fig. 5a). These signals were inhibited 96–99% when incubated in the immune depletion buffers (Fig. 5a, b). Three different low QCs and one high QC was identified for further work (Fig. 5a). Matrix components did not interfere with the detection of the B-47 Ab since titration in DPBS showed only a small expected difference relative to the titration in matrix (< 2.3-fold overall, Fig. 5c). Additionally, rabbit background in human matrix was low at 57 units and very similar to that observed in DPBS (Fig. 5d).

Method Qualification for Clinical Sample Analysis

A statistical assessment of the following parameters was done to qualify the ECL ELISA method for clinical sample analysis, screening, confirmatory, and titer cut points, and QC precision. Forty-one individual normal human K_2EDTA plasma samples were tested in the ELISA in the presence and absence of 8 μg/mL DP at an MRD of 100. QCs prepared in human plasma matrix were also tested at the same MRD. A single HQC control at 20,000 ng/mL and three LQC controls (2500 ng/mL, 3000 ng/mL, and 4000 ng/mL) were tested with the intent of selecting the lowest LQC level that passed the screening cut point resulting from the statistical evaluation. The rabbit QCs were detected with anti-

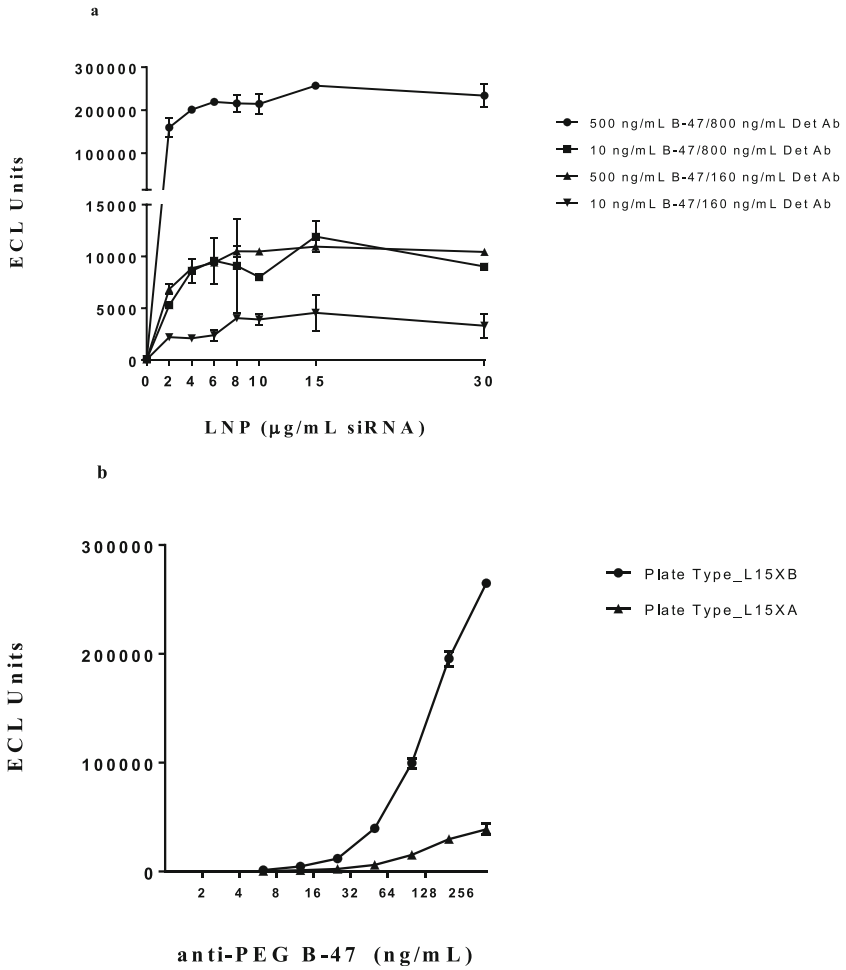


Fig. 3. ECL method optimization. **a** Drug product coating concentration and detection Ab optimization. Anti-PEG Ab (B-47) and anti-rabbit detection Ab (Det Ab) were diluted in block buffer and tested at the combination concentrations shown on L15XB plates coated with the drug concentrations indicated on the *x*-axis. Mean and standard deviation of the raw data units for two replicates are shown on the *y*-axis. **b** Comparison of standard and high binding ECL plates. B-47 Ab was diluted in block buffer to the concentrations indicated on the *x*-axis and tested on L15XA (standard) or L15XB (high-binding) plates coated with 15 µg/mL of the DP. Mean and standard deviation of the raw data units for three replicates are shown on the *y*-axis

rabbit detection Abs. The human plasma samples, NQC (matrix), and human IgG control (Det QC) were detected with anti-human κ and λ detection Abs. The plasma samples were run in two replicates using a total of five plates. Two analysts conducted the experiments for 2 days and generated ELISA data from a total of 20 plates for analysis.

The screening cut point factor (CPF) for the normal population was determined to be 1.29 and represented the 95th percentile above NQC background following outlier removal in the statistical analysis. The titer cut point factor was determined to be 1.59 and represented the 99th percentile above background. The confirmatory cut point (CCP) was determined to be 24.8% and was obtained by adding the mean percent change of signal or inhibition in the drug-spiked sample relative to the unspiked samples plus 2.33 times total standard deviation of the percent inhibition values after exclusion of outliers (1% false positive rate). When the screening cut point (SCP) was applied back to the samples after excluding biological outliers, the false positive rate was 5%. These criteria were in keeping with industry standards (16).

The upper bound of the 99% prediction interval (PI) was used to set the acceptance criteria (AC) for NQC and the lower bound PI for LQC and HQC. Of the three tested levels of LQC, only the highest at 4000 ng/mL gave a lower bound PI that was greater than the SCP (≥ 3071 and 2293, respectively). A tentative AC of ≥ 20,616 ECL units was set for the Det QC. Inter-replicate precision in the statistical analysis for HQC was 2.5 and for LQC was 20.3. In the final method, an AC of ≤ 25.0% was selected. Titer QCs, drug tolerance, sensitivity, and selectivity were assessed at a later time as described below.

Assay Sensitivity

Sensitivity data was collated from nine different experiments conducted by two analysts. Starting at a concentration of 32,000 ng/mL and ending at 31.3 ng/mL, the B-47 Ab was diluted 2-fold each time using human matrix as the diluent. The preparation was further diluted to an MRD of 100, and two replicates of each dilution and QCs were tested in the assay as described previously.

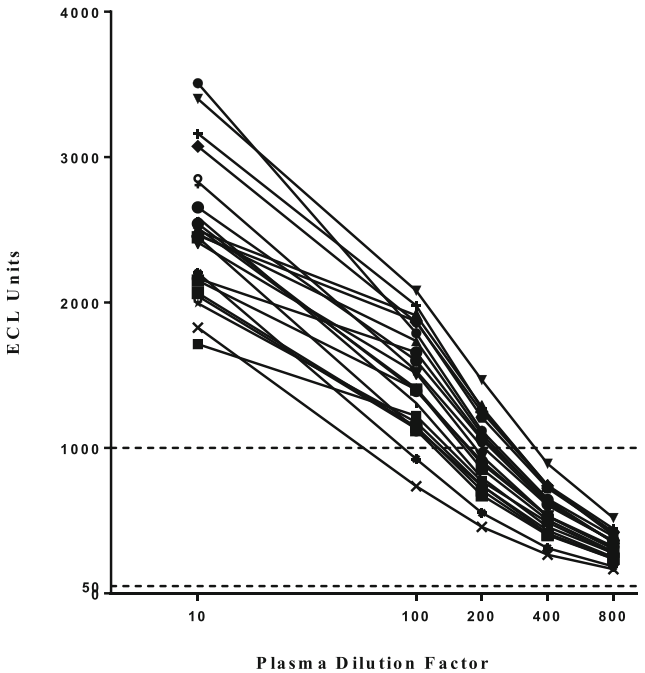


Fig. 4. Background ECL signal in diluted human K_2EDTA plasma samples. Twenty-two individual samples were diluted with block buffer as shown on the *x*-axis and incubated on DP-coated plates prior to detection with 40 ng/mL each ruthenium-labeled anti-human κ and λ Abs

After ensuring that the QCs and samples met AC, the statistically evaluated CPF and the mean NQC from each experiment were used to calculate individual SCPs. The minimum concentration of B-47 at which mean RLU of the replicates exceeded the SCP was determined in each experiment, and the median concentration and assay sensitivity were identified as 1000 ng/mL from these evaluations (Table IV).

Drug Tolerance, Selectivity, and Hook Effect

Assay drug tolerance was assessed by pre-incubating various concentrations of B-47 Ab in the linear range with two different concentrations of the DP and by comparing the results with B-47 Ab alone in the absence of added drug. The details of the sample preparation and ELISA method are described in "Methods" section. B-47 was detected above the SCP at the established sensitivity (1000 ng/mL) and at higher levels in the absence of drug (Fig. 6a). In the presence of 300 ng/mL and 3000 ng/mL of drug, assay sensitivity decreased to 2000 ng/mL and 4000 ng/mL, respectively. The drug concentration used was based on the siRNA *w*/*v* content of the formulated drug, and the implications of these results are discussed further in the last section.

Hook effect was determined by titrating the B-47 Ab in matrix to the concentrations indicated prior to an MRD of 100 and ELISA analysis (Fig. 6b). There is a downward trend in signal beginning at 128 μg/mL and continuing at the highest concentration tested. The Ab could not be tested at > 256 ng/mL owing to the limitation of the stock Ab concentration. The implications of these findings to clinical sample ADA concentration and the approach to address potential hook effect–related false negatives during sample analysis are described in the section below.

Selectivity was determined by adding B-47 Ab at 4000 ng/mL (LQC) into ten different lots of human plasma samples prior to dilution to an MRD of 100 and ELISA analysis. Each of the ten individual samples was also analyzed without added QC for an evaluation of potential specific or non-specific proteins that bound the plate-coated drug. All ten samples spiked with the LQC screened positive in the ELISA, and the un-spiked blank samples remained negative (Fig. 6c). These results indicate that the assay is refractory to the presence of individual components in different plasma samples in the detection of ADA to BMS-986263 as assessed using the pseudo surrogate rabbit control.

Clinical ADA Results

Using the fit-for-purpose method described so far, we evaluated ADA response to BMS-986263 in normal humans in a phase I clinical trial. Ninety milligrams of drug was administered in a placebo-controlled double-blind fashion in three weekly IV infusions on days 1, 8, and 15. The study consisted of three parts, part A (ten non-Japanese subjects), part B (six Japanese subjects), and part C (six Japanese and non-Japanese). Parts A and B had an infusion time of 2.5 h, and part C had a more rapid infusion time of 1 h. Samples that yielded mean ECL units ≥ 1.29 times the mean NQC ECL units were considered to be putative positives. Samples that showed a % inhibition ≥ 24.8 were to be considered confirmed positives. All 16 tested subjects in parts A and B screened negative for ADA at pre-treatment, days 1, 8, 15, and 28. In part C, one of the six subjects screened positive on days 15 and 28; however, these samples were negative in the confirmatory tier. All other samples in part C including pre-treatment samples were negative in the screening tier. The false positive percentage in the screening tier was 6.7% for part C. In-study inter-replicate precision for all QCs (LQC and HQC) across ten experiments (runs) needed to complete the ADA analysis was < 20%. Inter-run precision for these two QCs was also < 20%. Acceptance rate for the ten runs was 100%.

To ensure that there were no hook effect–related false negative samples, all day 28 samples (most likely to contain the highest titers) were diluted 2, 10, 50, and 250-fold using matrix and further diluted to an MRD of 100 prior to testing in the ELISA. Titer QCs at 250, 500, 1000, and 2000 ng/mL were included in addition to all other assay QCs. All plates passed AC. None of the diluted samples screened positive in the assay, making it unlikely that highly concentrated ADA samples were present at day 28 in the hook region. Day 7 and day 15 were not analyzed in the hook effect analysis.

DISCUSSION

In this paper, we have described the development, fit-for-purpose validation, and clinical application of an ECL ADA assay for the detection of human Abs to surface-exposed components of a lipid nanoparticle formulation containing a HSP47 siRNA drug substance. The assay employed a rabbit Ab (clone B-47) specific for the terminal methoxy group of the PEG molecule as the positive control and was detected

Table III. Specificity of ECL Signal in Human K_2EDTA Plasma Samples

Sample	Mean ECL units (non-competed)	Mean ECL units (competed)	% Inhibition
1	1988	1938	2
2	1561	1503	4
3	1858	1762	5
4	879	867	1
5	2254	2092	7
6	54	52	5
7	793	751	5
8	1254	1248	0
9	1138	1095	4
10	1779	1620	9
11	1584	1590	0
12	1132	1038	8
13	1912	1792	6
14	1294	1071	17
15	1521	1364	10
16	1347	1252	7
Human IgG	52242	50585	N/A
Anti-PEG B-47 (80 ng/mL)	24224	136	99
Anti-rabbit Ab blank	49	52	−5
Anti-human κ and λ Ab blank	52	50	4

Samples were diluted 100-fold and incubated on DP-coated plates in the presence (competed) or absence (non-competed) of 8 μg/mL drug to determine the specificity of background observed in the assay. Percent inhibition of signal in the presence of drug was calculated as described in "Methods" section. B-47 Ab was included as a positive control for inhibition, and human IgG-coated wells were included as controls for the anti-human κ and λ detection Abs. Two blanks shown below were included to assess background generated by the detection Abs alone. Mean of two replicates is shown (% CV was 0–3% for non-competed and 0–17% for competed)

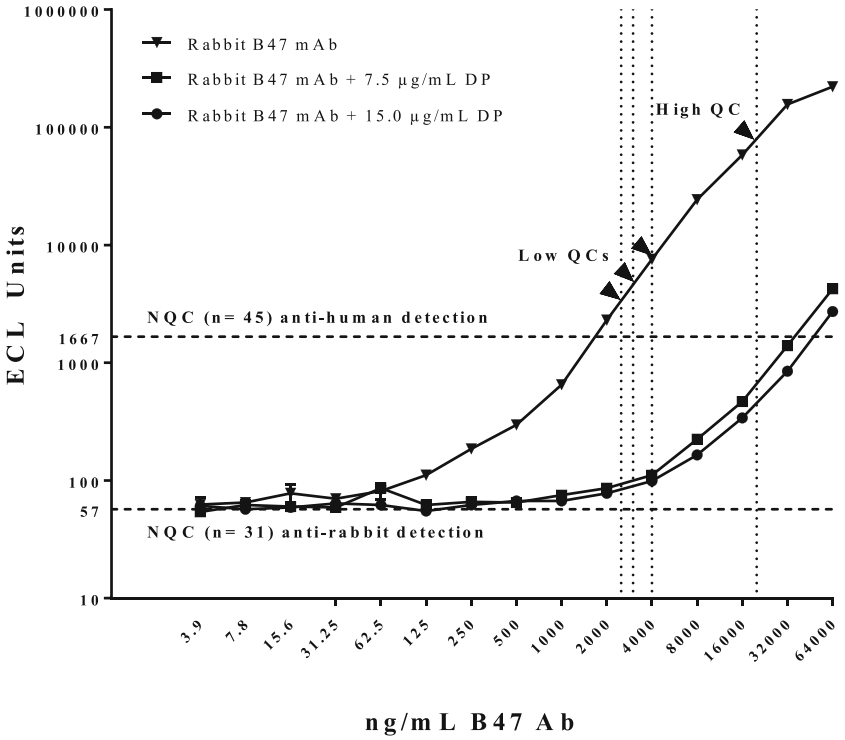
using an anti-rabbit detection Ab. Human samples were detected with pan-isotype anti-human κ and λ detection Abs and purified human IgG coated directly on plates served as a positive control for human detection. Although we tested several mouse-human Fc region chimeras, we were unable to identify a positive control with a human Fc region. Thus, the rabbit positive control is a pseudo surrogate control for the human ADA detection method. However, the rabbit QCs were prepared in human matrix, and the QC concentration, specifically LQC, was selected such that the anti-rabbit detection signal was greater than the human assay background resulting from the anti-human detection Abs.

The whole LNP ADA assay described here is designed to capture ADA specific for various surface-exposed components of the DP including PEG, PEG-vitamin A conjugate, and hydrophilic heads of the lipids constituting the outer surface. The anti-human κ and anti-human λ detection Abs in the assay were selected to enable the detection of Abs of all isotypes including IgG and IgM. This was important given that several groups have reported the generation of anti-PEG IgM Ab response to PEGylated liposomes or PEGylated drug products in clinical and non-clinical studies (11,13,17). We have also previously shown that compared with anti-human IgG detection Ab alone, the pan-isotype κ and λ detection Abs identify a higher number of Ab-positive individuals presumably due to the additional isotypes that are captured (14). Since we were unable to identify positive controls for lipids and vitamin A, we cannot be certain that current assay conditions are conducive to the detection of Abs of these other specificities. Ongoing efforts involve immunizing rabbits with BMS-986263 as well as individual components to identify and characterize Abs that are made in response to the immunogenic components. The resulting Ab controls will be used to explore various ELISA assay conditions, for example, those that have previously been reported in the literature for identification of anti-lipid Abs (18–20).

Using the fit-for-purpose assay described here, we demonstrated that all 22 subjects in the study were ADA negative at baseline and post-treatment on days 1, 8, 15, and 28 as assessed in the confirmatory tier. The false positive rate in part C corresponded approximately with the false positive rate in validation and that prescribed for ADA assays (16). The individual in part C who screened positive on days 15 and 28 showed a mean RLU of 1294 and 1528, respectively, and a SCP of 1203 RLU. Since these samples are drawn at consecutive time points after drug infusion from the same individual, we suspect that some of the signals for these samples may be masked under the higher than normal assay background.

We normally observe about 500 RLU of background in a sandwich MSD ADA assay format. The relatively higher background in the whole LNP ADA assay is likely due to the exclusion of Tween-20 in the wash and block buffers. Tween-20 has historically been included in ELISA buffers as a surfactant to facilitate reagent spreading and to reduce background. However, initial use of 0.05% Tween-20 in the assay wash and block buffers showed that the B-47 Ab does not work in the ELISA, likely due to cross-reactivity with and sink effect from the methoxy groups in Tween. Abs specific for the PEG backbone that were tested initially as potential positive controls (e.g., Clone 6.3) ran into the same issues.

a



b

ng/mL B-47	% Inhibition of ECL Signal		Signal/Noise (ECL Units/1667)
	7.5 µg/mL Drug	15 µg/mL Drug	
2000	96	97	1.4
4000	99	99	4.5
8000	99	99	14.6
16000	99	99	35.0
32000	99	99	93.9
64000	98	99	132.6

Fig. 5. **a** The B-47 Ab was titrated in matrix and diluted 100-fold in the absence or presence of immune depletion buffer (7.5 µg/mL or 15 µg/mL added drug product). Diluted B-47 Ab in each case was added in duplicates to drug-coated plates, and the ELISA was conducted as described previously. Pre-MRD concentration of B-47 is shown on the *x*-axis and ECL units on the *y*-axis. Two NQCs were included: one was matrix, probed with anti-human κ and λ Abs, and the other was matrix probed with anti-rabbit detection Abs. Selected low and high QCs are depicted by the vertical broken lines and arrows. **b** Percent inhibition of ECL signal generated by the B-47 Ab in the presence of added drug was calculated as described previously, and the calculation uses signals that are detected in each case using anti-rabbit detection Abs. A surrogate human S/N range in the assay was calculated by dividing the signal obtained for the B-47 Ab (anti-rabbit detection) with the background signal in human plasma matrix (anti-human κ and λ detection)**. c** B-47 Ab titration curves were prepared in matrix and DPBS and diluted 100-fold to assess the impact of matrix on rabbit Ab detection. Pre-MRD concentration is shown on the *x*-axis. **d** Human plasma matrix ($n = 31$) or DPBS ($n = 14$) was diluted to an MRD of 100 and used to probe drug-coated plates in the ELISA with anti-rabbit detection Abs in both cases. Mean and standard deviation are shown for each population (matrix = 57, DPBS = 49)

The high background may also be due to non-specific IgG and other serum components that bind the nanoparticle coated on the plates and that subsequently get detected with generic anti-human κ and λ detection Abs. While the screening and confirmatory cut point takes this background into account, low or borderline positive samples run the risk

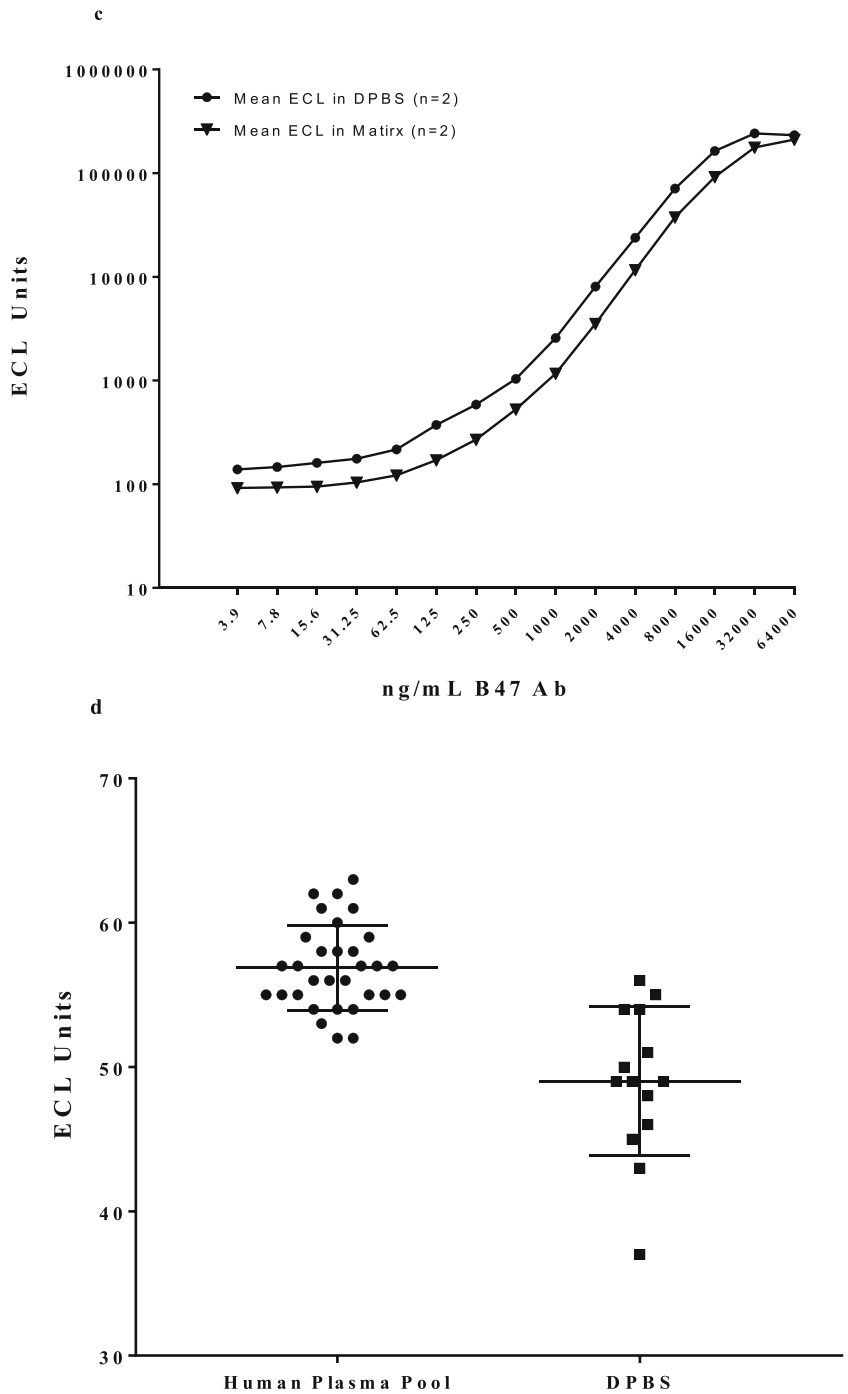


Fig. 5. (continued)

of going undetected. Future generations of the assay will explore alternate wash and block buffers, detection reagents, and assay formats to reduce background and lower the MRD to facilitate detection of low-titer ADA-positive subjects. In addition, human chimeric anti-PEG Ab controls with a human Fc will be produced and incorporated into the assay, and assay performance characteristics such as sensitivity, selectivity, and drug tolerance will be repeated to more closely reflect human ADA detected in clinical samples.

The drug design of BMS-986263 incorporates a retinoid moiety for targeted delivery of the HSP47 siRNA to hepatic stellate cells in the liver. The assumptions are that the nanoparticle stays intact from the time it is administered to the time that it reaches the liver and that during this time, ADA generated against surface components of the LNP may neutralize or clear the drug, resulting in a loss or reduction in drug efficacy. These assumptions form the basis of the whole LNP ADA assay format described here. Given the presence of PEG within the outer shell of the drug product and previous observations that anti-PEG Abs mediate the clearance of liposomes containing PEG molecules in non-clinical models (18) and lead to the clearance of PEGylated drugs in humans (17,21), it was a reasonable assay format to begin with.

Table IV. Whole LNP ADA Assay Sensitivity

Experiment number	Screening cut point (SCP)	ng/mL B-47 Ab above SCP*
1	1667	500
2	1562	500
3	1047	500
4	1465	1000
5	1548	1000**
6	1745	1000
7	1754	1000
8	2219	2000
9	2246	2000

B-47 Ab was titrated and tested in the ELISA using anti-rabbit detection Ab in nine experiments. The SCP for each experiment and the minimum Ab concentration resulting in mean RLU above the SCP are shown
*Assessed from a titration curve in each experiment
**Median assay sensitivity

While the aforementioned suppositions regarding the intactness of the drug product and Ab response to surface components dictated the assay format, it is important to note that they also form the basis for assay sensitivity and drug tolerance assessments reported here. Sensitivity is determined for an Ab control (anti-PEG B-47) that binds the surface PEG component in an intact particle. Drug tolerance assessment uses the same control with the intact drug product as a potential interferant. Sensitivity was determined to be 1000 ng/mL using the rabbit anti-PEG B-47 Ab control in the absence of added drug. However, the clinical relevance of this level of sensitivity is unknown and will have to await correlative findings between ADA concentration and decrease in drug exposure and efficacy. Current FDA guidelines recommend a sensitivity of 100 ng/mL for therapeutic protein products based on clinical relevance (22), but this may not be applicable to siRNA lipid nanoparticle-based drug products.

Assay sensitivity decreased in the presence of increasing concentrations of spiked drug product. Sensitivity was 4000 ng/mL at a spike of 3000 ng/mL drug (based on siRNA concentration and at approximate C_{max}) and increased by 2-fold to 2000 ng/mL at a spike of 300 ng/mL of drug. The highest observed geometric mean C_{trough} siRNA concentration on days 1 and 15 ranged from 37 to 40 ng/mL (PK data not shown, determined using a validated PK assay), approximately 10-fold lower than the lowest tested drug spike. Given a 2-fold reduction in sensitivity with a 10-fold lower spike, we expect assay sensitivity at C_{trough} drug levels to be close to that observed in the absence of drug, i.e., 1000 ng/mL. Thus, the C_{trough} drug concentrations are not expected to interfere with ADA measurement. Additionally in data not shown, the siRNA PK profiles after repeated infusions remained unaltered, suggesting similar C_{trough} drug level on day 8 of infusion. Thus, the drug tolerance established in this assay seems to suffice for detecting at least 1000 ng/mL of ADA. More importantly, consistent PK profiles after repeated drug infusion and the ADA negative status of the patients together suggest that a drug-clearing ADA response level was not induced by BMS-986263.

For continued relevance of assay sensitivity and drug tolerance as described here, it will be important to understand

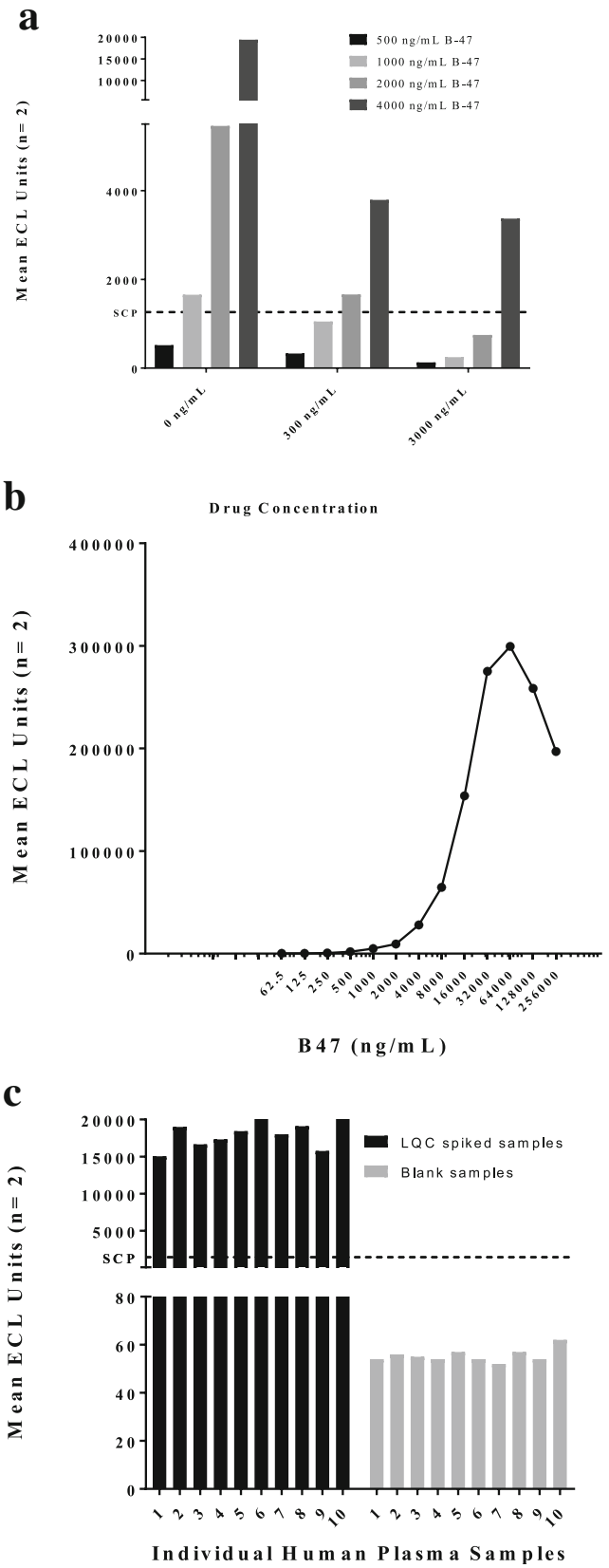


Fig. 6. Drug tolerance, hook effect, and selectivity assessment. An MRD of 100 was applied to all samples shown above. Mean ECL of two replicates is shown in each figure above. **a** Drug tolerance (% CV range 2–18%), **b** hook effect (% CV range 0–5%), and **c** selectivity (% CV range 0–18%)

the extent and duration of the intactness of the lipid nanoparticle as well as siRNA release and stability in plasma following administration before reaching the target organ. Such information along with a knowledge of various ADA specificities (e.g., to several different internal or surface-exposed components of the formulated DP) will enable a more detailed understanding of the impact of ADA on drug exposure and safety. In the event that multiple PK analytes are monitored for lipid nanoparticle DPs, information on the intactness of the DP or released siRNA will enable the selection of the most relevant analyte and respective PK parameters such as C_{max} and C_{trough} concentrations when considering the level of drug tolerance that is built into a particular type of ADA assay (e.g., whole LNP ADA assay or siRNA ADA assay).

The use of lipid nanoparticles is becoming increasingly important in the delivery of siRNA and other DNA and RNA-based drug substances. In comparison with protein and monoclonal Ab therapeutics where a wealth of industry-wide experience and knowledge guides immunogenicity determination, very little is known about the immune response to lipid nanoparticle-based drug products and ways of monitoring and understanding the impact on toxicity, exposure, and drug efficacy. In an initial effort in this direction, we have developed and validated a fit-for-purpose three-tier screening, confirmatory, and titer ECL assay for the detection of ADA generated in response to BMS-986263, a lipid nanoparticle-based drug product containing an HSP47 siRNA. The assay was shown to be precise, selective, specific, and reproducible and was used to determine the ADA response in phase I clinical trials. All subjects were shown to be negative for ADA to surface components of BMS-986263. To the best of our knowledge, this report constitutes the first ADA assay developed for a lipid nanoparticle-based drug product. Our future efforts will focus on understanding the stability of the DP in human plasma including siRNA release kinetics and stability and adaptation of the current assay to determine ADA specificity (for example by using individual components of the DP as immunodepletory competitors in the confirmatory tier).

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest Uma Kavita, Wendy Miller, Qin Ji, and Renuka Pillutla are current employees of Bristol-Myers Squibb Company (BMS). All financial support for the studies reported herein was provided by BMS. The authors have no further relevant affiliations or financial involvement with any other organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Ethical Conduct of Research The authors state that they have followed the principles outlined in Good Clinical Practice for all human experimental investigations reported here. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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